A Single MAPKKK Regulates the Hog1 MAPK Pathway in the Pathogenic Fungus *Candida albicans*[□]

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The Hog1 mitogen-activated protein kinase (MAPK) plays a central role in stress responses in the human pathogen *Candida albicans*. Here, we have investigated the MAPK kinase kinase (MAPKKK)-dependent regulation of the pathway. In contrast to the Hog1 pathway in *Saccharomyces cerevisiae*, which is regulated by three MAPKKKs (Ssk2, Ssk22, and Ste11), our results demonstrate that Hog1 in *C. albicans* is regulated by a single MAPKKK Ssk2. Deletion of *SSK2* results in comparable stress and morphological phenotypes exhibited by *hog1Δ* cells, and Ssk2 is required for the stress-induced phosphorylation and nuclear accumulation of Hog1, and for Hog1-dependent gene expression. Furthermore, phenotypes associated with deletion of *SSK2* can be circumvented by expression of a phosphomimetic mutant of the MAPKK Pbs2, indicating that Ssk2 regulates Hog1 via activation of Pbs2. In *S. cerevisiae*, the Hog1 pathway is also regulated by the MAPKKK Ste11. However, we can find no connection between Ste11 and the regulation of Hog1 in *C. albicans*. Furthermore, expression of a chimeric Pbs2 protein containing the Ste11-dependent regulatory region of *S. cerevisiae* Pbs2, fails to stimulate Ste11-dependent stress signaling in *C. albicans*. Collectively, our data show that Ssk2 is the sole MAPKKK to relay stress signals to Hog1 in *C. albicans* and that the MAPK signaling network in *C. albicans* has diverged significantly from the corresponding network in *S. cerevisiae*.

INTRODUCTION

Mitogen-activated protein kinase (MAPK) pathways are important signaling modules found in all eukaryotes (for review, see Pearson *et al.*, 2001). Each pathway comprises of three tiers of protein kinases, a MAP kinase, a MAP kinase kinase (MAPKK), and a MAPKK kinase (MAPKKK). The MAPKKK phosphorylates and thereby activates the MAPKK, which subsequently phosphorylates the MAPK on conserved threonine and tyrosine residues located in the activation loop of the catalytic domain. This increases the kinase activity and the nuclear translocation of the MAPK, which results in the MAPK-dependent phosphorylation of cellular substrates, thereby triggering an appropriate cellular response to the stimulus.

One of the best-studied MAPK modules is the high osmolarity glycerol (HOG) pathway in the model yeast *Saccharomyces cerevisiae* that responds to changes in external osmolarity. Activation of the HOG pathway culminates in the phosphorylation, activation, and nuclear translocation of the Hog1 MAPK. Activated Hog1 mounts a multifaceted response to osmotic stress, which includes the induction or repression of the expression of various genes, regulation of protein translation, adjustments to cell cycle progression, and synthesis of the

osmolyte glycerol (for review, see Hohmann, 2002; Saito and Tatebayashi, 2004).

Extracellular hyperosmolarity in *S. cerevisiae* is largely detected by two independent transmembrane osmosensors, Sln1 and Sho1. Signals from both these sensors converge at the MAPKK Pbs2, which activates the Hog1 MAPK by phosphorylation of conserved threonine and tyrosine residues. The Sln1 osmosensor is a membrane-located histidine kinase that regulates phosphotransfer to a response regulator, Ssk1, via the phosphorelay protein Ypd1 (Maeda *et al.*, 1995; Posas *et al.*, 1996). Sln1 is active under conditions of low osmolarity that results in the phosphorylation and inactivation of Ssk1. However, upon increases in osmolarity, the Sln1 histidine kinase domain is inactivated, resulting in a cessation of the Sln1–Ypd1–Ssk1 phosphorelay and an accumulation of unphosphorylated Ssk1 that can now bind and activate the two redundant MAPKKKs Ssk2 and Ssk22 (Posas and Saito, 1998). When activated the Ssk2/Ssk22 MAPKKKs specifically phosphorylate and activate the Pbs2 MAPKK (Maeda *et al.*, 1995), by binding to an Ssk2/Ssk22-specific docking site in the N-terminal region of Pbs2 (Tatebayashi *et al.*, 2003). The Sho1 osmosensor contains four transmembrane segments and a cytoplasmic Src homology (SH)3 domain that interacts with a proline-rich region on Pbs2. Under conditions of high osmolarity, signal transduction from Sho1 to Pbs2 also requires Cdc42, Ste20, Ste50, and Ste11 (Posas and Saito, 1997; O'Rourke and Herskowitz 1998; Posas *et al.*, 1998; Raitt *et al.*, 2000; Reiser *et al.*, 2000). Cdc42 is a Rho-type small G protein that binds and activates Ste20, a p21-activated protein kinase (PAK) homologue. Ste20 in turn phosphorylates and activates the MAPKKK Ste11, which is present in a complex with the SAM-domain containing protein Ste50. Activated Ste11, in turn phosphorylates the Pbs2

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MAPKK. However, in contrast to the specificity of the Ssk2/Ssk22 MAPKKs for Pbs2, Ste11 can also phosphorylate the MAPKK Ste7 of the pheromone response and the pseudohyphal/invasive growth MAPK pathways. Significantly, there is no nonphysiological cross-talk between these MAPK pathways as the Pbs2 MAPKK and the Sho1 osmosensor act as coscaffolds to direct osmotic stress signals from Ste11 to the Hog1 MAPK (Posas and Saito, 1997; Zarrinpar *et al.*, 2004; Tatebayashi *et al.*, 2006).

The Sln1 and Sho1 osmosensing pathways have been shown to be largely functionally redundant in that both pathways can stimulate the phosphorylation of Pbs2, stimulate the phosphorylation and nuclear translocation of Hog1, and allow growth on high osmolarity media (Maeda *et al.*, 1995; Posas *et al.*, 1998). Consequently, osmotic-stress signaling to Hog1 is prevented only when both pathways are inactivated; for example, only when the Ssk2 and Ssk22 MAPKKs are deleted in combination with the Ste11 MAPKK are osmosensitive phenotypes and defects in osmotic stress-induced activation of Pbs2 and Hog1 observed (Posas and Saito, 1997). However, some differences between the two pathways have been identified; for example, the kinetics of Hog1 phosphorylation are different in *sln1* and *sho1* pathway mutants and only the Sln1 pathway responds to moderate levels of osmotic stress (Maeda *et al.*, 1995; Van Wuytswinkel *et al.*, 2000; O'Rourke and Herskowitz, 2004).

Homologues of *S. cerevisiae* Hog1 have been studied in the human fungal pathogens *Cryptococcus neoformans* (Bahn *et al.*, 2005, 2006), *Aspergillus fumigatus* (Xue *et al.*, 2004; Du *et al.*, 2006), and *Candida albicans* (San-Jose *et al.*, 1996). Such studies have uncovered interesting differences in the role and regulation of Hog1 in pathogenic fungi compared with that in *S. cerevisiae*. In *C. neoformans*, Hog1 responds to diverse stress conditions, and it also has important roles in drug sensitivity, sexual reproduction, and virulence (Bahn *et al.*, 2005). Intriguingly, Hog1 is constitutively phosphorylated under normal growth conditions in the more virulent serotype A, but not the less virulent serotype D, strains. This unusual constitutive phosphorylation of Hog1 is thought to result in cross-talk with signaling cascades regulating *C. neoformans* virulence factors (Bahn *et al.*, 2005). With regard to the relay of stress signals to Hog1, the response regulator Ssk1 plays a major role (Bahn *et al.*, 2006), and although *C. neoformans* does not contain a homologue of the *S. cerevisiae* Sln1 histidine kinase, seven other histidine kinases (Tco1-7) have been identified. Recently, Tco1 and Tco2 have been demonstrated to have overlapping and distinct roles in the regulation of Hog1 (Bahn *et al.*, 2006). In *A. fumigatus*, the Hog1 homologue SakA is activated in response to oxidative and osmotic stress, and it also negatively regulates conidial germination under nitrogen-limiting conditions (Xue *et al.*, 2004; Du *et al.*, 2006). In contrast to *S. cerevisiae*, deletion of the Sln1 histidine kinase homologue *tscB*, in *A. fumigatus*, is viable, and does not impair stress signaling to Hog1 (Du *et al.*, 2006).

C. albicans HOG1 was originally cloned by functional complementation of the osmosensitive phenotype associated with *S. cerevisiae* hog1 mutant cells (San-Jose *et al.*, 1996), and it is essential for the virulence of this pathogen (Alonso-Monge *et al.*, 1999; Arana *et al.*, 2007). Subsequent studies revealed that the *C. albicans* Hog1 MAPK responds to a diverse range of environmental signals, in addition to osmotic stress, including various oxidative stress agents (Alonso-Monge *et al.*, 2003; Smith *et al.*, 2004). Recently, homologues of the *S. cerevisiae* PBS2, SSK1, and SHO1 genes have been identified in *C. albicans*, and their potential role in the relay of stress signals to Hog1 examined. Deletion of PBS2 in *C. albicans* prevents the

relay of both osmotic and oxidative stress signals to Hog1, indicating this to be the sole MAPKK that phosphorylates Hog1 in response to these stresses (Arana *et al.*, 2005). In contrast, deletion of SSK1 in *C. albicans* prevents activation of Hog1 in response to oxidative stress, whereas wild-type levels of Hog1 phosphorylation are induced in *ssk1Δ* cells after osmotic stress (Chauhan *et al.*, 2003). Furthermore, osmotic stress-induced activation of Hog1 is not impaired in an *ssk1sho1* double mutant in *C. albicans* (Roman *et al.*, 2005). This contrasts with the observation in *S. cerevisiae* where inactivation of both the Sho1- and Sln1-osmosensing branches prevents the relay of osmotic stress signals to Hog1 (Maeda *et al.*, 1995).

Here, we describe our studies seeking to identify the MAPKKs that relay stress signals to the Pbs2 MAPKK in *C. albicans*. We have identified putative homologues of the *S. cerevisiae* Ssk2,22 and Ste11 MAPKKs and constructed deletions in these genes. Importantly, our results reveal that the Ssk2 MAPKK functions alone to relay stress signals to Hog1 in *C. albicans*. This is in stark contrast with the situation in *S. cerevisiae* in which the Ssk2, Ssk22, and Ste11 MAPKKs function in overlapping pathways to relay osmotic stress signals to Hog1. The potential significance of this rewiring of Hog1 signaling components in *C. albicans* is discussed.

MATERIALS AND METHODS

Strains and Growth Conditions

The strains used in this study are given in Table 1. *C. albicans* strains were grown in either rich YPD medium or SD minimal medium (Sherman, 1991). All strains were grown at 30°C. Cell morphology was analyzed using a Zeiss axioscope (Carl Zeiss, Jena, Germany).

Strain Construction

Oligonucleotide primers used in this study are listed in Table 2.

Deletion of SSK2, STE11, and PBS2

To delete SSK2, disruption cassettes comprising either the ARG4 or HIS1 gene flanked by *loxP* sites and 80 base pairs of DNA sequence corresponding to regions 5' and 3' of the SSK2 open reading frame (ORF) were generated by polymerase chain reaction (PCR) using the oligonucleotide primers SSK2delF and SSK2delR and the plasmid templates pLAL2 or pLHL2, respectively (Dennison *et al.*, 2005). Disruption cassettes were transformed into *C. albicans* BWP17 (Wilson *et al.*, 1999) to sequentially disrupt both alleles of SSK2 and generate strain JC482. The same strategy was used to delete STE11 and PBS2 by using the oligonucleotide primers STE11delF and STE11delR, or PBS2delF and PBS2delR, to generate strains JC524 or JC74, respectively. The SSK2 disruption cassettes deleted codons 1-1344 of the 1483 codon ORF of SSK2, the STE11 disruption cassettes deleted codons 6-820 of the 823 codon STE11 ORF, and the PBS2 disruption cassettes deleted codons 28-539 of the 545 codon PBS2 ORF. Gene disruptions were confirmed by PCR. To construct reintegrant control strains, the SSK2 gene plus 550 base pairs of upstream and 200 base pairs of downstream sequences (total 5.2 kb), and the STE11 gene plus 999 base pairs of upstream and 232 base pairs of downstream sequences (total 3.7 kb), were amplified by PCR by using the oligonucleotide primers SSK2BgIF and SSK2BgIR or STE11BamF and STE11BamR, respectively, and cloned into the BamHI site of Clp20, a derivative of Clp10 (Murad *et al.*, 2000). The resulting plasmids were digested with StuI and introduced into the Ura⁻ null strains.

Tagging and Mutagenesis of PBS2

To chromosomally tag Pbs2 at the C terminus with 6-His residues and two copies of the myc-epitope, the PBS2 gene was amplified by PCR by using the oligonucleotide primers PBS2MHR and PBS2PstA and ligated into Clp-C-ZZ (Blackwell *et al.*, 2003), which had been digested with PstI and NheI to remove the TEV-protein A sequence. The resulting Clp-C-PBS2HM plasmid was linearized by digestion with SgrAI to target integration at the PBS2 locus in *C. albicans* *ssk2Δ* cells to generate strain JC517. Chromosomal insertion of the C-terminal 6His-myc tag was confirmed by PCR and DNA sequencing. Mutagenesis of PBS2 to create PBS2^{S355D,T359D} was performed by overlapping PCR using the oligonucleotides PBS2TagR, PBS2DD, and PBS2PstB, and the resulting PCR fragment was digested with PstI and NdeI and cloned into

Table 1. Strains used in this study

Strain	Genotype	Reference
BWP17	<i>ura3::λ imm434/ura3::λimm434, his1::hisG/his1::hisG, arg4::hisG/arg4::hisG</i>	Wilson <i>et al.</i> (1999)
JC47	<i>hog1::loxP-ARG4-loxP/hog1::loxP-HIS1-loxP</i>	Enjalbert <i>et al.</i> (2006)
JC74	<i>pbs2::loxP-ARG4-loxP/pbs2::loxP-HIS1-loxP</i>	This work
JC482	<i>ssk2::loxP-ARG4-loxP/ssk2::loxP-HIS1-loxP</i>	This work
JC524	<i>ste11::loxP-ARG4-loxP/ste11::loxP-HIS1-loxP</i>	This work
JC620	<i>ssk2::loxP-ARG4-loxP/ssk2::loxP-HIS1-loxP, Cip20-SSK2 (URA3,HIS1)</i>	This work
JC638	<i>ste11::loxP-ARG4-loxP/ste11::loxP-HIS1-loxP, Cip20-STE11 (URA3,HIS1)</i>	This work
JC112	<i>PBS2-HM:URA3</i>	This work
JC517	<i>ssk2::loxP-ARG4-loxP/ssk2::loxP-HIS1-loxP, PBS2-HM:URA3</i>	This work
JC507	<i>ssk2::loxP-ARG4-loxP/ssk2::loxP-HIS1-loxP, PBS2^{DD}-HM:URA3</i>	This work
JC167	<i>pbs2::loxP-ARG4-ura3-loxP/pbs2::loxP-HIS1-loxP, CaEXP-CaPBS2 (URA3)</i>	This work
JC559	<i>pbs2::loxP-ARG4-ura3-loxP/pbs2::loxP-HIS1-loxP, CaEXP-ScCaPBS2 (URA3)</i>	This work
JC613	<i>ssk2::loxP-ARG4-loxP/ssk2::loxP-HIS1-loxP, CaEXP-ScCaPBS2 (URA3)</i>	This work
JC54	<i>HOG1-YFP-URA3</i>	This work
JC522	<i>ssk2::loxP-ARG4-loxP/ssk2::loxP-HIS1-loxP, HOG1-YFP-URA3</i>	This work
JC545	<i>ste11::loxP-ARG4-loxP/ste11::loxP-HIS1-loxP, HOG1-YFP-URA3</i>	This work

All strains were constructed in the BWP17 background (*ura3::λ imm434/ura3::λimm434, his1::hisG/his1::hisG, arg4::hisG/arg4::hisG*).

Cip-C-PBS2HM previously digested with the same enzymes to remove the wild-type sequence. The resulting Cip-C-PBS2^{DD}HM plasmid was introduced into *ssk2Δ* cells as described above to generate strain JC507.

To express a chimera of PBS2 in *C. albicans* made up of the kinase domain of *C. albicans* PBS2 fused to the N-terminal region of *S. cerevisiae* PBS2, initially the *C. albicans* PBS2 kinase domain was amplified by PCR by using the oligonucleotide primers PBS2KDF and PBS2KDR and Cip-C-PBS2HM as a template. The resulting PCR product was digested with BglII and BamHI and ligated into the BamHI site of CaEXP (Care *et al.*, 1999), to generate plasmid CaEXP-CaPBS2kin. The N-terminal region of *S. cerevisiae* PBS2 contains two CUG (Ser) codons at positions 127 and 180, which would be read as Leu codons in *C. albicans*. Hence, these were mutated to CUC (Ser) codons by overlapping PCR by using the oligonucleotides 127MUTF, 180MUTR, ScPBS2F, and ScPBS2R. The resulting 950-base pair fragment was digested with BamHI and ligated into CaEXP-CaPBS2kin to generate CaEXP-ScCaPBS2. As a control, the full-length *C. albicans* PBS2 sequence was amplified by PCR using the oligonucleotide primers CaPBS2FLF and CaPBS2FLR and Cip-C-PBS2HM as a template, and ligated into the BamHI site of CaEXP to generate plasmid CaEXP-CaPBS2. The CaEXP-ScCaPBS2 and CaEXP-CaPBS2 plasmids

were linearized at the StuI restriction site in the *RSP10* locus and introduced into *pbs2Δ* and *ssk2Δ* cells. CaEXP directs the expression of genes under the control of the *MET3* promoter, which is repressed in the presence of methionine and cysteine (Care *et al.*, 1999). Hence, strains carrying CaEXP derivatives were grown in minimal SD media lacking methionine and cysteine.

Chromosomal Tagging of HOG1

To chromosomally tag Hog1 with yellow fluorescent protein (YFP) in *ssk2Δ* and *ste11Δ* cells, *HOG1*-specific sequences were added to the universal primer sequences described previously (Gerami-Nejad *et al.*, 2001) to generate the oligonucleotide primers HOG1YFPF and HOG1YFPR. These primers were used in combination with the plasmid template pYFP-URA3 to generate a *HOG1-YFP* cassette by PCR (Smith *et al.*, 2004). This cassette was introduced into JC482 (*ssk2Δ*) and JC524 (*ste11Δ*) cells to create strains JC522 and JC545, respectively. Integration at the *HOG1* locus was confirmed by PCR and DNA sequencing.

Table 2. Oligonucleotides used in this study

Oligonucleotide	Sequence 5'–3'
PBS2delF	atgtgttgagataaagatatagacttgatattaataatttgaattcagcatgcgccaacacgaacaccaccggtgaagccagggtttccagtcacg
PBS2delR	ctgtttctatataatatactgtttataataacagcccaataaactgggtctcatatcatcaatgattattaagaaagcttctcactaaagggaacaaaagc
SSK2delF	gaatacatcttcacactactactgtctaatagttcaattggaaaaggcccaataattacaattataattatcttataaccagggtttccagtcacg
SSK2delR	gtagaggctcctgttaattggcttggtgacatgaacatcagtgatccgtctctggaattcaattattatggccatcacgcctcactaaagggaacaaaagc
STE11delF	tcatatagaaacctaatacatagttgctgtgtataggtactcaaatcaacaacagtcagacagagattaatgatccagggtttccagtcacg
STE11delR	aaacatctctctagattgttctactactgcatacaacaatcacgtgtgtacagtataactttatattatctgtttcctcactaaagggaacaaaagc
SSK2BglF	aagggaagatctgtcaattaaattcttactctcc
SSK2BglR	aagggaagatcttaataaaaaaaggaggagaatagagtc
STE11BamF	gcgcggatccaaaaccccgaaagtacaccagg
STE11BamR	gcgcggatccgattcaaaaagcttagccctccc
PBS2MHR	gaattcgtagcttaattgatggtgatgctctctctctctcgatcaattttgttcttcagccatggacaaactcttctcagaataaactttgtcctcatgattattaagaagctctt
PBS2PstA	cgtcatctgcagcagcatgcgccaacacgaacacc
PBS2PstB	cgtcatctgcagctatatcagcttcaacatcgg
PBS2TagR	gaggatccccgggacacgagaaaatattatcatatgtttcagca
PBS2DD	ggtaatttagttgcccatttagccaaagataattggtgtgtaa
PBS2KDF	gcgcggatccggtgtgttcttaattattctgat
PBS2KDR	aagggaagatctttaattgatggtgatggtgtaagtctcc
PBS2FLF	gcgcggatccatggtgaagataaagatatagac
PBS2FLR	gcgcggatcccttaattggtgatggtgtaagtctcc
127MUTF	agcactcttaagaacgttctcgacaatc
180MUTR	ccttctgttagattgagcaattgcgc
ScPBS2F	gcgcggatccatggaagacaagttgctaaccctcag
ScPBS2R	gcgcggatccaccactactaccagaatttgaagag

Stress Sensitivity Tests

For stress sensitivity assays, strains to be tested were grown in liquid culture and spotted onto YPD or SD plates containing the indicated compounds. Plates were incubated at 30°C for 24 h, unless indicated otherwise.

Hog1 Phosphorylation Assays

Cells were grown to mid-exponential phase and exposed to a range of stress conditions for the indicated times. Protein extracts were prepared and phosphorylated Hog1 was detected by Western blot with an anti-phospho-p38 antibody (New England Biolabs, Ipswich, MA) as described previously (Smith *et al.*, 2004). Blots were stripped and total levels of Hog1 were determined by probing with an anti-Hog1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Fluorescence Microscopy

Localization of chromosomally YFP-tagged Hog1 was determined by fluorescence microscopy as described previously (Smith *et al.*, 2004).

RNA Analysis

Preparation and Northern blot analysis of RNA isolated from *C. albicans* were performed as described previously (Enjalbert *et al.*, 2006). Gene-specific probes were amplified by PCR from genomic DNA by using oligonucleotide primers specific for *GPD2*, *RHR2*, and *ACT1* (Enjalbert *et al.*, 2006).

Coprecipitation Assays

Exponentially growing wild-type (BWP17) and *ssk2Δ* (JC482) cells, or wild-type (JC112) and *ssk2Δ* (JC517) cells expressing chromosomally tagged *PBS2-6His-myc*, were harvested and snap frozen in liquid nitrogen. Pelleted cells were lysed into lysis buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 20 mM imidazole, 0.1% NP-40, 50 mM NaF, 2 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.07 trypsin inhibition unit/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin). *Pbs2-6His-myc* was precipitated from extracts by using nickel-nitrilotriacetic acid (Ni-NTA) agarose (QIAGEN, Dorking, Surrey, United Kingdom). The agarose beads were washed five times with lysis buffer and resuspended in SDS-loading buffer. Proteins were resolved on SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting was performed using an anti-myc antibody (Sigma Chemical, Poole, Dorset, United Kingdom) to detect precipitation of *Pbs2-6His-myc*, and coprecipitation of Hog1 was monitored using an anti-Hog1 antibody (Santa Cruz Biotechnology).

RESULTS

C. albicans ssk2Δ Cells Are Phenotypically Comparable to *hog1Δ* Cells

In *S. cerevisiae*, *Ssk2/22* and *Ste11* function in overlapping pathways to relay osmotic stress signals to the *Pbs2-Hog1* module (Posas and Saito, 1997). A search of the *C. albicans* genome database (<http://www.candidagenome.org/genome>) revealed one locus designated *SSK2* (orf19.3775), encoding an 1344-codon open reading frame, with significant similarity to *S. cerevisiae SSK2* and *SSK22*, and one locus designated *STE11* (orf19.844), encoding an 823-codon open reading frame, with significant similarity to *S. cerevisiae STE11*. To avoid confusion, we use the prefixes *Ca* and *Sc* to distinguish between the *C. albicans* and *S. cerevisiae SSK2* and *STE11* orthologues in this article.

The primary amino acid sequences of *ScSte11* and *ScSsk2* were aligned with the putative *C. albicans* orthologues using ClustalW (Thompson *et al.*, 1994). Alignment of the *ScSte11* and the *CaSte11* sequences revealed significant similarity (65%) across the entire length of the protein (our unpublished data). However, whilst alignment of *ScSsk1* with *CaSsk2* revealed a high degree of similarity, the C-terminal region of *ScSsk2*, containing the kinase activation loop and subdomains VIII to XI, was absent in the *CaSsk2* sequence. To clarify this, we sequenced the *CaSSK2* allele in the laboratory strain BWP17 (Wilson *et al.*, 1999), and we discovered that codon 1345, annotated as a STOP codon in the *C. albicans* genome database, encodes a lysine residue and that the *CaSSK2* ORF in the BWP17 background extends to 1483 codons (Supplemental Figure 1). We also found that the stop codon at position 430, which is annotated as sepa-

rating two exons of *CaSSK2* (1-1287 and 1291-4038; <http://www.candidagenome.org/genome>), encodes a lysine residue.

The extended *CaSsk2* sequence in BWP17 contains the kinase activation loop and subdomains VIII to XI. Importantly, Thr1460 in the *ScSsk2* kinase activation loop, which has been shown to be important for the activation of the kinase (Posas and Saito, 1998), is conserved in the extended *CaSsk2* sequence (Thr1359). Furthermore, a region at the N terminus of *ScSsk2* (residues 294-413), which binds to the upstream response regulator *ScSsk1* (Posas and Saito, 1998), is also conserved in the *CaSsk2* sequence (Supplemental Figure 1).

To examine the function of *CaSsk2* and *CaSte11* in *C. albicans*, homozygous null mutants were generated. Each copy of the *CaSSK2* allele in this diploid fungus was inactivated by replacing 1344 codons of the 1483-codon ORF with either the *HIS1* or *ARG4* disruption cassette. Similarly, each *CaSTE11* allele was inactivated by replacing the majority of the 823 codon ORF with either the *HIS1* or *ARG4* disruption cassette (see *Materials and Methods*). Previous studies have shown that deletion of *C. albicans HOG1* results in cells with pleiotropic stress phenotypes (San-Jose *et al.*, 1996; Alonso-Monge *et al.*, 2003; Smith *et al.*, 2004). Hence, to examine the potential role of the *CaSsk2* and *CaSte11* MAPKKs in relaying stress signals to Hog1, we compared the sensitivity of *C. albicans ssk2Δ*, *ste11Δ*, and *hog1Δ* cells to a range of stress conditions. Due to the functional redundancy of *ScSsk2* and *ScSte11* in *S. cerevisiae*, we were surprised to find that deletion of *CaSSK2* resulted in comparable stress-sensitive phenotypes to those exhibited by *C. albicans hog1Δ* cells. Both *hog1Δ* and *ssk2Δ* mutants displayed impaired growth in response to the oxidative stress agents H_2O_2 , *tert*-butyl hydroperoxide (*t*-BOOH), and menadione, the osmotic stress agents sorbitol and NaCl, the heavy metals Cd^{2+} and As^{3+} , the purine analogue caffeine, and the drug staurosporine (Figure 1A). Furthermore, *hog1Δ* and *ssk2Δ* cells were equally sensitive to a range of concentrations of osmotic and oxidative stress agents (Supplemental Figure 2), verifying that these mutants display equivalent stress phenotypes. *C. albicans hog1Δ* and *ssk2Δ* cells also displayed similar increased resistance, compared with wild-type cells, to the cell wall biogenesis inhibitor calcofluor white (Figure 1A). Hyperactivation of the MAPK *Cek1* in *C. albicans hog1Δ* cells is thought to underlie the resistance to cell wall inhibitors such as calcofluor white (Eisman *et al.*, 2006). Consistent with this, we found that *ssk2Δ* cells, like *hog1Δ* cells, display a high basal level of phosphorylation of the *Cek1* MAPK (our unpublished data). Importantly, all of the stress-phenotypes exhibited by *ssk2Δ* cells were reversed upon reintroduction of the wild-type *SSK2* gene into the *ssk2Δ* strain (Figure 1A).

In contrast, *C. albicans ste11Δ* cells did not demonstrate the stress-sensitive phenotypes exhibited by *ssk2Δ* and *hog1Δ* cells, and overall displayed wild-type levels of stress resistance to the compounds tested (Figure 1A). However, we did find that *ste11Δ* cells are more sensitive than wild-type cells to the cell wall-damaging agent calcofluor white, and this sensitivity can be reversed by reintegration of the wild-type gene (Figure 1A). This phenotype contrasts with that seen with *ssk2Δ* and *hog1Δ* cells, which display significant resistance to this agent (Figure 1A).

Previous work has shown that Hog1 also functions as a repressor of morphogenetic switching in *C. albicans* (Alonso-Monge *et al.*, 1999; Arana *et al.*, 2005; Eisman *et al.*, 2006; Enjalbert *et al.*, 2006). For example, *hog1Δ* mutant cells form filaments in the absence of any morphogenetic signal (Enjalbert *et al.*, 2006). Significantly, such morphogenetic defects were also observed in *ssk2Δ* cells but not *ste11Δ* cells;

Figure 1. *ssk2Δ* and *hog1Δ* cells display similar phenotypes. (A) Approximately 10³ cells, and 10-fold dilutions thereof, of exponentially growing wild-type (*Wt*-BWP17), *hog1Δ* (JC47), *ssk2Δ* (JC482), *ssk2Δ+SSK2* (JC620), *ste11Δ* (JC524) and *ste11Δ+STE11* (JC637) strains were spotted onto YPD plates containing the indicated additives: NaCl (0.6 M), sorbitol (1.0 M), *t*-BOOH (2.0 mM), menadione (0.35 mM), H₂O₂ (3 mM), CdSO₄ (0.4 mM), Na₂As₃ (2 mM), staurosporine (2.0 μM), caffeine (10 mM), and calcofluor white (CFW; 20 μg/ml). Plates were incubated at 30°C for 24 h. (B) Morphology of wild-type (*Wt*), *hog1Δ*, *ssk2Δ*, and *ssk2Δ+SSK2* strains grown in YPD at 30°C. (C) Northern analysis of hyphae-associated transcripts (*HWP1* and *ECE1*) in wild-type (*Wt*), *hog1Δ*, and *ssk2Δ* strains grown in YPD at 30°C. *ACT1* was analyzed as a loading control.

similar levels of filamentation were observed in *hog1Δ* and *ssk1Δ* cells when cultured in YPD, and reintroduction of *SSK2* into *ssk2Δ* cells reversed this morphogenetic defect

(Figure 1B). Furthermore, consistent with Hog1 and Ssk2 functioning as negative regulators of filamentation, we found that the hypha-specific genes *HWP1* and *ECE1* were

up-regulated in *hog1Δ* and *ssk2Δ* cells, compared with wild-type cells, under noninducing conditions (Figure 1C).

These data, which collectively illustrate that *C. albicans* *hog1Δ* and *ssk2Δ* cells display equivalent phenotypes, indicate that CaSsk2 functions independently of CaSte11 to relay signals to Hog1 in *C. albicans*. This contrasts with the situation in *S. cerevisiae* in which ScSsk2 and ScSsk22 function redundantly with the ScSte11 MAPKKK to regulate the Hog1 MAPK (Posas and Saito, 1997).

CaSsk2, but Not CaSte11, Is Required for the Activation of Hog1 in C. albicans

Exposure of *C. albicans* to a range of stress conditions results in the phosphorylation and nuclear translocation of Hog1 (Alonso-Monge *et al.*, 2003; Smith *et al.*, 2004; Enjalbert *et al.*, 2006). We explored the effect of deleting *CaSSK2* and *CaSTE11* on each of these processes. To investigate whether CaSsk2 or CaSte11 regulates Hog1 phosphorylation in response to stress, wild-type, *ssk2Δ* and *ste11Δ* cells were treated with osmotic stress (0.3 M NaCl), or oxidative stress (5 mM H₂O₂), and Hog1 phosphorylation was monitored by Western blotting with an antibody that recognizes only the active phosphorylated form of Hog1 (Millar *et al.*, 1995; Smith *et al.*, 2004). Blots were subsequently reprobed with an anti-Hog1 antibody to determine total levels of Hog1. As illustrated in Figure 2A, Hog1 was rapidly phosphorylated in extracts isolated from wild-type cells and *ste11Δ* cells after exposure to osmotic or oxidative stress. In contrast, phosphorylated Hog1 could not be detected in extracts isolated from *ssk2Δ* cells after equivalent stress treatments. However, Hog1 phosphorylation was restored in *ssk2Δ* upon reintegration of the wild-type *CaSSK2* gene (Figure 2A). Increased exposure of the Western blots also revealed that CaSsk2 is required for the low basal level of Hog1 phosphorylation seen in wild-type cells under nonstressed conditions (Figure 2B). Importantly, deletion of *CaSSK2* does not, however, affect the total cellular levels of Hog1, because equivalent amounts of Hog1 are present in both wild-type and *ssk2Δ* cells (Figure 2).

Studies in *S. cerevisiae* have uncovered concentration-dependent differences in the activation of the two osmosensing

branches (Maeda *et al.*, 1995; Van Wuytswinkel *et al.*, 2000; O'Rourke and Herskowitz, 2004); hence, we also compared the levels of Hog1 phosphorylation in wild-type, *ssk2Δ*, and *ste11Δ* cells in response to increased levels of osmotic stress (1 M NaCl). However, even at elevated levels of osmotic stress, no significant activation of Hog1 was observed in extracts isolated from *ssk2Δ* cells and wild-type levels of Hog1 phosphorylation were detected in extracts isolated from *ste11Δ* cells (Supplemental Figure 3).

To determine the effect of deleting *CaSSK2* or *CaSTE11* on the stress-induced nuclear accumulation of Hog1, one copy of the *HOG1* gene was chromosomally tagged with YFP in *ssk2Δ* and *ste11Δ* cells, and the cellular localization of Hog1-YFP was examined by fluorescence microscopy. Our earlier work validated the use of Hog1-YFP in examining the cellular localization of Hog1 (Smith *et al.*, 2004). As observed previously, Hog1-YFP rapidly accumulated in the nucleus in wild-type cells after osmotic (0.3 M NaCl) and oxidative (5 mM H₂O₂) stress treatments (Smith *et al.*, 2004). Osmotic and oxidative stress-induced nuclear accumulation of Hog1 was also evident in *ste11Δ* cells. However, in contrast, no stress-induced nuclear accumulation of Hog1-YFP was observed in *ssk2Δ* cells (Figure 3).

Because the above-mentioned data implicate CaSsk2, but not CaSte11, in the activation of Hog1, we examined the role of CaSsk2 in the regulation of Hog1-dependent gene expression. Northern analyses were performed to compare *RHR2* and *GPD2* expression in wild-type, *hog1Δ*, and *ssk2Δ* cells after exposure to oxidative (5 mM H₂O₂) and osmotic (0.3 M NaCl) stress. Consistent with previous studies (Smith *et al.*, 2004; Enjalbert *et al.*, 2006), *RHR2* and *GPD2* were found to be induced in response to both osmotic and oxidative stress in a Hog1-dependent manner (Figure 4). However, deletion of *CaSSK2* impaired the osmotic and oxidative stress-induced expression of *RHR2* and *GPD2* compared with wild-type cells. Furthermore, quantification of mRNA levels illustrated that the expression profiles of *RHR2* and *GPD2* were almost identical in *hog1Δ* and *ssk2Δ* cells (Figure 4).

Collectively, these experiments show that CaSsk2 is required for phosphorylation of Hog1, the nuclear accumulation of Hog1, and the induction of Hog1-dependent gene

Figure 2. CaSsk2 but not CaSte11 is required for Hog1 phosphorylation. (A) Western blot analysis of whole cell extracts isolated from wild-type (Wt-BWP17), *ssk2Δ* (JC482), *ssk2Δ* + *SSK2* (JC620), and *ste11Δ* (JC524) cells after treatment with either 5 mM H₂O₂ or 0.3 M NaCl for the specified times. Western blots were probed with an anti-phospho-p38 antibody, which only recognizes the phosphorylated, active form of *C. albicans* Hog1 (Hog1-P). Total levels of Hog1 protein was determined by stripping and reprobing the blot with an anti-Hog1 antibody that recognizes both phosphorylated and unphosphorylated forms of Hog1 (Hog1). (B) Western blot analysis of whole cell extracts isolated from unstressed Wt and *ssk2Δ* cells.

Figure 3. CaSsk2 but not CaSte11 is required for Hog1 nuclear localization. The localization of YFP-tagged Hog1 was determined by fluorescence microscopy in wild-type (*Wt*-JC54), *ssk2Δ* (JC522), and *ste11Δ* (JC545) cells before stress and after treatment with either 5 mM H₂O₂ or 0.3 M NaCl for 10 min (Hog1-YFP). The position of the nuclei in these cells was visualized with 4,6-diamidino-2-phenylindole (DAPI). An autofluorescence control is shown (control).

expression in response to osmotic and oxidative stress in *C. albicans*.

Hog1 Forms a Complex with the MAPKK Pbs2 Independently of CaSsk2 in *C. albicans*

Pbs2 has recently been identified as the sole MAPKK that relays stress signals to Hog1 in *C. albicans* (Arana *et al.*, 2005).

Similarly, as with Hog1, the levels of Pbs2 were not affected upon deletion of *CaSsk2* (Figure 5). We had previously found that Pbs2 forms a complex with Hog1 in *C. albicans* (our unpublished data); hence, we investigated whether CaSsk2 was necessary for this interaction. Such experiments were performed using wild-type and *ssk2Δ* strains in which Pbs2 was C-terminally tagged with six histidine residues and two copies of the myc epitope (Pbs2-6His-myc). Pbs2-6His-myc was precipitated from cell extracts using Ni-NTA-agarose, and coprecipitation of Hog1 was assayed by Western blotting using an anti-Hog1 antibody (α -Hog1). As illustrated in Figure 5, Hog1 coprecipitated with Pbs2-6His-myc purified from both wild-type and *ssk2Δ* cell extracts. Collectively, these results indicate that the loss of Hog1 signaling seen in *ssk2Δ* cells (Figures 2–4) is not due to a decrease in the stability of the downstream kinases Pbs2 and Hog1, nor is it due to Ssk2 functioning as a scaffold protein to promote interaction of Pbs2 with Hog1 in vivo.

Phenotypes Associated with Deletion of CaSsk2 Can Be Overcome by Mutating the Pbs2 Phosphorylation Sites to Phosphomimetic Residues

Several protein kinases, including MAPKKs and MAPKs, are activated by phosphorylation between the kinase subdomains VII and VIII (Johnson *et al.*, 1996). Two such phosphorylation sites have been identified in the *S. cerevisiae* Pbs2 (Maeda *et al.*, 1995) and *S. pombe* Wis1 MAPKKs (Shiozaki *et al.*, 1998), and significantly both of these sites (Ser355 and Thr359) are conserved in *C. albicans* Pbs2 (Figure 7). Because the above-mentioned data indicate that CaSsk2 is the MAPKKK that activates the Hog1 pathway in *C. albicans*, we predicted that mutation of the conserved phosphorylation sites of Pbs2 to phosphomimetic residues (aspartic acid residues) would bypass the requirement for CaSsk2. To test this prediction, *ssk2Δ* strains were created which expressed either a wild-type *PBS2* allele or a *PBS2^{DD}* allele in which the putative phosphorylation sites, Ser355 and Thr359, were mutated to

Figure 4. CaSsk2 is required for the activation of Hog1-dependent gene expression. (A) Northern blot analysis of RNA isolated from mid-log cultures of *Wt* (BWP17), *hog1Δ* (JC47), and *ssk2Δ* (JC482) cells treated with either 5 mM H₂O₂ or 0.3 M NaCl for 0, 10, 30 and 60 min by using probes specific for *RHR2*, *GPD2*, and *ACT1* (loading control). Fold-induction of the ratios of *RHR2* and *GPD2* to *ACT1* were calculated relative to time 0 for the wild-type strain.

Figure 5. CaSsk2 is not essential for the interaction of Hog1 with Pbs2. Coprecipitation experiments to examine the association of Hog1 with Pbs2 in wild-type and *ssk2Δ* cells. Extracts were prepared from mid-log cultures of Wt (BWP17) and *ssk2Δ* (JC482) cells, and wild-type (JC112) and *ssk2Δ* (JC517) cells expressing Pbs2-6His-myc. The 6His-myc-tagged Pbs2 protein was purified with Ni-NTA agarose, and coprecipitation of Hog1 was analyzed by Western blotting with an anti-Hog1 antibody (α -Hog1). Blots were reprobed with an anti-myc antibody (α -myc) to allow comparison of the levels of Pbs2-6His-myc precipitated from Wt and *ssk2Δ* cells.

aspartic acid residues. Both the *PBS2* and *PBS2^{DD}* alleles were expressed from the native *PBS2* locus and tagged with a sequence encoding six histidine residues and two copies of the *myc* epitope located just before the termination codon, to allow expressed mutant proteins to be detected by anti-myc epitope antibodies (see Materials and Methods). Western blotting experiments with lysates isolated from the Pbs2-tagged and Pbs2^{DD}-tagged strains after Ni-NTA enrichment showed that proteins of the expected molecular weight were expressed and that the wild-type and mutant proteins were present at similar levels (our unpublished data). Significantly, expression of the *PBS2^{DD}* allele, but not the wild-type allele, rescued the stress-sensitive phenotypes and the hyperfilamentation defect associated with deletion of *CaSSK2* (Figure 6). These results are consistent with the hypothesis that CaSsk2 functions upstream of Pbs2 in the Hog1 pathway, and, moreover, that it phosphorylates Pbs2 to relay stress signals to Hog1 in *C. albicans*.

Figure 6. Expression of a phosphomimetic mutant of Pbs2 rescues phenotypes associated with deletion of *CaSSK2*. (A) Approximately 10^3 cells of exponentially growing Wt (BWP17), *ssk2Δ* (JC482), or *ssk2Δ* strains expressing either Pbs2-6His-myc (JC517) or Pbs2^{DD}-6His-myc (JC507) were spotted onto YPD plates containing 1.2 M sorbitol, 0.6 M KCl, 300 mM menadione, 3.5 mM H₂O₂, 0.5 mM CdSO₄, and 2 mM Na₂As₃. Plates were incubated at 30°C for 24 h. (B) Morphology of *ssk2Δ* cells expressing either Pbs2-6His-myc or Pbs2^{DD}-6His-myc grown in YPD liquid media at 30°C.

A Pbs2 Chimera Containing the *S. cerevisiae* Pbs2 N-Terminal Region Complements *C. albicans* *pbs2Δ*- but not *ssk2Δ*-associated Phenotypes

In *S. cerevisiae*, the ScSsk2 and ScSte11 MAPKKs both relay osmotic stress signals to the Pbs2 MAPKK. However, our data indicate that CaSsk2 alone, and not CaSte11, relays stress signals to Pbs2 in *C. albicans*. Extensive analysis of the *S. cerevisiae* Pbs2 MAPKK has identified distinct regions in the N terminus of the protein that are required for the relay of signals via the Ssk2 or Ste11 MAPKKs (Tatebayashi *et al.*, 2003). Notably, as illustrated by the schematic diagram in Figure 7A, the N-terminal region of *C. albicans* Pbs2 is significantly shorter than that of *S. cerevisiae* Pbs2. In addition, strong homology between the two proteins is restricted to the C terminus, which contains the kinase domain. The lack of homology between *S. cerevisiae* and *C. albicans* Pbs2 proteins in the N-terminal regulatory region may underlie the differences in MAPKKK regulation of these kinases. To investigate this, we examined whether expression of ScPbs2 in *C. albicans* would permit CaSte11-dependent signaling to Hog1. Unfortunately, however, expression of ScPbs2 in *C. albicans* did not complement the osmotic stress phenotypes associated with deletion of *CaPbs2* (our unpublished observations). Although the reasons for this are unknown, ScPbs2 contains five CUG (Ser) codons that would be translated as Leu codons in *C. albicans*. Subsequently, we tested whether the N-terminal domain of ScPbs2 could function in *C. albicans*. To this end, we created a construct that expresses a chimera of the *S. cerevisiae* and *C. albicans* Pbs2 proteins, composed of residues 1–315 of ScPbs2 fused to residues 160–546 of CaPbs2, which essentially replaces the N-terminal region of CaPbs2 with the equivalent region from ScPbs2. In addition, the two CUG (Ser) codons present in the N-terminal region of *S. cerevisiae* Pbs2 were mutated to CUC (Ser) codons to ensure the correct sequence was maintained upon expression in *C. albicans* (see Materials and Methods). This construct was expressed from the *MET3* promoter (Care *et al.*, 1999) and tagged with a sequence encoding 2-myc epitopes and 6-His residues to allow detection (*MET-ScCaPbs2*). As a control a similar construct expressing the full-length *CaPbs2* sequence was created (*MET-CaPbs2*). These constructs were targeted to the *RPS10* locus in *C. albicans* *pbs2Δ* cells and expression induced by growing in defined medium lacking methionine and cysteine (Care *et al.*, 1999).

Western blotting analysis revealed that proteins of the expected molecular weight were expressed from the *MET-ScCaPbs2* and the *MET-CaPbs2* constructs (Figure 7B). Although the levels of wild-type Pbs2 expressed from the *MET3* promoter were similar to those from the endogenous promoter, the ScCaPbs2 chimera was expressed at significantly lower levels (Figure 7B). Nonetheless, expression of *MET-ScCaPbs2* largely rescued the stress sensitive phenotypes associated with deletion of *PBS2* in *C. albicans* (Figure 7C). This was dependent on the presence of the ScPbs2 N-terminal regulatory domain, as expression of the CaPbs2 kinase domain-encoding fragment (residues 160–546) did not rescue the osmosensitive phenotype of *pbs2Δ* cells (our unpublished observations). Furthermore, Hog1 phosphorylation in response to osmotic and oxidative stress was restored in *pbs2Δ* cells upon expression of *MET-ScCaPbs2* (Figure 7D), although, consistent with the sensitivity data, not quite to wild-type levels. Collectively, these results illustrate that the ScCaPbs2 chimera can complement deletion of wild-type Pbs2 in *C. albicans* to relay osmotic and oxidative stress signals to Hog1. This suggests that ScCaPbs2 can

Figure 7. A Pbs2 chimera containing the *S. cerevisiae* Pbs2 N-terminal region complements *C. albicans pbs2Δ*, but not *ssk2Δ*, phenotypes. (A) Schematic illustration comparing the *S. cerevisiae* (S.c.) and *C. albicans* (C.a.) Pbs2 kinase sequences. The phosphorylation sites (P) and the *S. cerevisiae* Ssk2/22 and Sho1 binding domains are highlighted, and the kinase domain is depicted by a gray box. In the experiments below, full-length CaPbs2 (MET3-CaPbs2) or a chimera in which codons 1-315 of ScPbs2 were fused to codons 160-546 of CaPbs2 (MET3-ScCaPbs2) were tagged with a sequence encoding 2-myc epitopes and 6-His residues and expressed from the MET3 promoter. The junction at which the ScPbs2 sequence was fused to the CaPbs2 sequence to form a ScCaPbs2 chimera is indicated by an arrow. (B) Western blot analysis of Ni-NTA agarose-purified protein extracts isolated from cells expressing Pbs2-6His-myc from the native promoter (JC112), *pbs2Δ* cells (JC74), or *pbs2Δ* cells expressing either MET3-CaPbs2 (JC167) or MET3-ScCaPbs2 (JC559). Pbs2 levels were detected using an anti-myc antibody. (C) Approximately 10³ cells from exponentially growing *pbs2Δ* cells or *pbs2Δ* cells expressing MET3-CaPbs2 or MET3-ScCaPbs2 were spotted onto SD plates lacking methionine and cysteine and containing either 0.6 M KCl, 3.5 mM H₂O₂, 0.5 mM CdSO₄, 8 mM caffeine, and 2 mM Na₂As₃. In addition, *ssk2Δ* cells and *ssk2Δ* cells expressing MET3-ScCaPbs2 were spotted onto the same plates containing 0.6 M KCl. (D) Western blot analysis of whole-cell extracts isolated from *pbs2Δ* cells, *pbs2Δ* cells expressing MET3-CaPbs2 or MET3-ScCaPbs2, and *ssk2Δ* cells expressing MET3-ScCaPbs2 after the indicated treatments. Phosphorylated Hog1 (Hog1-P) and total levels of Hog1 were detected as described in Figure 2.

be phosphorylated by CaSsk2, because this MAPKKK regulates both osmotic and oxidative stress signal transmission to Hog1 in *C. albicans* (Figures 2–4). However, because ScCaPbs2 also contains the sequences necessary for Ste11 regulation of Pbs2 in *S. cerevisiae*, we investigated whether expression of the ScCaPbs2 chimera in *C. albicans ssk2Δ* cells would allow for CaSsk2-independent activation of Hog1 in response to osmotic stress. As illustrated in Figure 7, C and D, this did not occur, because expression of ScCaPbs2 in *ssk2Δ* cells failed to restore osmotic stress-induced Hog1 phosphorylation or resistance to osmotic stress. This was not due to increased instability of the chimera in *ssk2Δ* cells (our unpublished data). These results indicate that although the ScCaPbs2 chimera contains both *S. cerevisiae* Ssk2- and Ste11-dependent regulatory regions, the chimeric protein can only function in the presence of CaSsk2. Thus, these data suggest that the Sho1–Ste11 pathway in *C. albicans*, unlike the analogous pathway in *S. cerevisiae*, may not respond to changes in osmolarity.

DISCUSSION

In this study, we present several lines of evidence that Hog1 is regulated by a single MAPKKK, Ssk2, in the human fungal pathogen *C. albicans*. This is strikingly different from the situation in the model yeast *S. cerevisiae* in which the Hog1 MAPK is regulated by three functionally redundant MAPKKKs

Ste11, Ssk2, and Ssk22 (Posas and Saito, 1997). However, in *C. albicans* we find that deletion of the homologue of *S. cerevisiae* SSK2 and SSK22 results in 1) equivalent stress-related phenotypes as exhibited by *hog1Δ* cells (Figure 1 and Supplemental Figure 2); 2) the hyperfilamentous growth defect seen in *hog1Δ* mutants (Figure 1); 3) loss of detectable Hog1 phosphorylation, or nuclear accumulation of Hog1, in response to osmotic or oxidative stress (Figures 2 and 3); and 4) loss of activation of Hog1-dependent gene expression (Figure 4). Significantly, the stress sensitivities and morphological defects exhibited by *C. albicans ssk2Δ* cells can be rescued by expression of a mutant version of the Pbs2 MAPKK, in which the conserved MAPKKK phosphorylation sites (Ser355 and Thr359) have been mutated to phosphomimetic aspartic acid residues (Figure 6), strongly supporting the proposal that the function of CaSsk2 is to phosphorylate Pbs2. In contrast to the phenotypes exhibited by *ssk2Δ* cells, wild-type levels of stress-induced phosphorylation and nuclear accumulation of Hog1 are seen in *C. albicans ste11Δ* cells (Figures 2 and 3), and *ste11Δ* cells do not display any overlapping stress-sensitive phenotypes exhibited by *hog1Δ* cells (Figure 1).

What is the basis for this rewiring of MAPK signaling components in *C. albicans* compared with *S. cerevisiae*? In *S. cerevisiae*, the molecular mechanisms underlying the regulation of Hog1 by the different MAPKKKs are well characterized. The Ssk2 MAPKKK constitutively binds to a novel

docking site on the Pbs2 MAPK, which directs Ssk2 specificity (Tatebayashi *et al.*, 2003). This docking site is found between residues 46 and 56 of *S. cerevisiae* Pbs2, and it is largely conserved in the *C. albicans* Pbs2 sequence (Figure 7A). Four amino acids were identified in ScPbs2 to be essential for activation by ScSsk2 (Tatebayashi *et al.*, 2003), two of which (Ala52 and Ala56) are identical in the CaPbs2 sequence, and two of which (Arg53 and Val54) are substituted with functionally conserved amino acids. However, the regions of *S. cerevisiae* Pbs2 previously shown to be required for signaling from the Sho1–Ste11 branch (residues 55–107, which includes the proline-rich motif that binds to the Sho1-SH3 domain; Tatebayashi *et al.*, 2003), or the binding of Ste11 (residues 2–162; Zarrinpar *et al.*, 2004) are less conserved in the *C. albicans* Pbs2 sequence. It was possible that the lack of sequence homology between the N-terminal regulatory regions of the *S. cerevisiae* and *C. albicans* Pbs2 MAPKKs may underlie the differences in MAPKKK regulation of Hog1 in these fungi. However, expression of a chimeric Pbs2 protein containing the *S. cerevisiae* N-terminal regulatory domain of Pbs2 facilitated Ssk2-dependent, but not Ste11-dependent, signaling to Hog1 in *C. albicans* (Figure 7). Although we cannot rule out the possibility that the chimera may influence the function of the N-terminal regulatory domain, this result suggests that the Sho1–Ste11 pathway in *C. albicans* is not involved in osmosensing and signaling.

Consistent with these conclusions, a recent study revealed that deletion of *SHO1* in *C. albicans* does not impair the relay of osmotic stress signals to the Hog1 MAPK (Roman *et al.*, 2005). This is not due to functional redundancy of the Sho1 and two-component osmosensing pathways, as seen in *S. cerevisiae*, because osmotic stress-induced activation of Hog1 was also seen in *sho1Δssk1Δ* cells (Roman *et al.*, 2005). Instead, this study revealed Sho1 to play a major role in the control of the Cek1 MAPK and to regulate cell wall biogenesis and morphogenesis (Roman *et al.*, 2005). In this study, we find that *ste11Δ* cells, similar to *sho1Δ* cells, are sensitive to the cell wall inhibitor calcofluor white. This is consistent with a model in which Sho1 relays signals to the Cek1 MAPK via the Ste11 MAPKKK in *C. albicans*. Similar findings have been previously documented in *S. cerevisiae* in which Sho1 regulates the filamentous growth MAPK Kss1 (the homologue of *C. albicans* Cek1) in combination with the signaling mucin Msb2 (O'Rourke and Herskowitz, 1998; Cullen *et al.*, 2000, 2004). Thus, these data suggest that although Sho1–Ste11 regulation of the Kss1/Cek1 MAPKs is conserved between *S. cerevisiae* and *C. albicans*, the osmosensing function of Sho1 in the regulation of Hog1 seems to be restricted to *S. cerevisiae*. In this regard, it is also relevant that the Sho1 homologue in the filamentous fungus *Aspergillus nidulans* seems not to respond to changes in osmolarity (Furukawa *et al.*, 2005), and the fission yeast *Schizosaccharomyces pombe* does not contain an apparent Sho1 homologue.

What relays stress signals to the CaSsk2 MAPKKK in *C. albicans*? Figure 8 illustrates the known stress signaling components of the *C. albicans* Hog1 pathway. Intriguingly, deletion or mutation of the two-component response regulator Ssk1 impairs activation of Hog1 in response to oxidative stress but not osmotic stress (Chauhan *et al.*, 2003; Menon *et al.*, 2006). Hence the relay of oxidative stress signals to the Ssk2 MAPKKK in *C. albicans* seems to be two-component dependent. This begs the question as to which of the three histidine kinases in *C. albicans*, Chk1, Sln1, or Nik1, functions as a peroxide sensor? The most likely candidate would seem to be Chk1, because this kinase shows significant similarity to the peroxide-sensing histidine kinases Mak2 and Mak3 in *S. pombe* (Buck *et al.*, 2001; Quinn *et al.*, 2002). Indeed, *chk1*

Figure 8. Model depicting the relay of osmotic and oxidative stress signals to the Hog1 MAPK in *C. albicans*. Oxidative stress signaling to Hog1 requires the Ssk1 response regulator (Chauhan *et al.*, 2003), the Ssk2 MAPKKK (this study), and the Pbs2 MAPKK (Arana *et al.*, 2005). However, which histidine kinase(s) regulates Ssk1 in response to oxidative stress is unclear (Li *et al.*, 2004; Roman *et al.*, 2005). Although the relay of osmotic stress signals to Hog1 also requires Ssk2 (this study) and Pbs2 (Arana *et al.*, 2005), Hog1 is activated in response to osmotic stress in *ssk1* mutant cells (Chauhan *et al.*, 2003). This, together with the finding that the Sho1–Ste11 pathway does not transmit osmotic stress signals to the Pbs2 MAPKK (Roman *et al.*, 2005; this study) indicates the presence of a novel osmotic-stress signaling pathway in *C. albicans*. Such a pathway may function in parallel with the Ssk1 response regulator to relay osmotic stress signals to Hog1.

cells do show increased sensitivity to oxidative stress (Li *et al.*, 2004) and to killing by human neutrophils *in vitro* (Torosantucci *et al.*, 2002). Nonetheless, deletion of *CHK1* does not impair oxidative stress-induced activation of Hog1 (Li *et al.*, 2004). Furthermore, wild-type levels of Hog1 phosphorylation in response to oxidative stress are seen in double *chk1sln1* and *chk1nik1* histidine kinase mutants (Roman *et al.*, 2005). Hence, it remains unclear how oxidative stress signals are relayed to CaSsk2 via the Ssk1 response regulator. Similarly, it is not known how osmotic stress signals are relayed to the Hog1 pathway. As discussed above, osmotic stress regulation of Hog1 still occurs in cells in which the Sho1 and two-component pathways have been inactivated (Roman *et al.*, 2005). It was suggested that a homologue of the *S. cerevisiae* Msb2 mucin, which has been proposed to function in parallel with Sho1 to relay osmotic stress signals to Pbs2 via the MAPKKK3 Ste11 (O'Rourke and Herskowitz, 2002), may be responsible for the osmotic stress activation of Hog1 seen in *sho1Δssk1Δ* *C. albicans* cells (Roman *et al.*, 2005). However, based on our work, we think this unlikely as we find that the Ssk2 MAPKKK, and not Ste11, regulates Hog1 in response to osmotic stress. Moreover, recent studies indicate that the primary function of *S. cerevisiae* Msb2 may not

be in the regulation of the Hog1 MAPK pathway but rather in the regulation of the Kss1 invasive growth and cell wall integrity pathway (Cullen *et al.*, 2004). Instead, the integral membrane protein Opy2 seems to function with Sho1 for Hog1 pathway activation (Wu *et al.*, 2006). In the fission yeast, *S. pombe*, two-component signal transduction is used to specifically relay oxidative signals to the stress responsive MAPK, Sty1, and no Sho1 pathway has been described in this organism. Hence, it is possible that *C. albicans* and *S. pombe* may use a similar, but as yet, uncharacterized osmosensor (Figure 8).

A growing body of evidence is demonstrating that the role and regulation of the Hog1 stress-responsive MAPK pathway in the pathogenic yeast *C. albicans* has diverged significantly from analogous pathways in the model yeasts *S. cerevisiae* and *S. pombe*. For example, *C. albicans* Hog1 has a distinct activation profile that has likely evolved to survive the particular environmental niches found in the human host (Smith *et al.*, 2004). Furthermore, the role of the stress-responsive MAPK pathways in mediating core transcriptional responses to stress is different in *C. albicans* (Enjalbert *et al.*, 2006), *S. cerevisiae* (Gasch *et al.*, 2000; Causton *et al.*, 2001) and *S. pombe* (Chen *et al.*, 2003). Indeed, a systematic analysis of the Hog1/Sty1-regulated orthologues that exist in *C. albicans*, *S. cerevisiae*, and *S. pombe* revealed only one gene (*C. albicans* IPF5981, *S. cerevisiae* YML128C, and *S. pombe* SPBC365.12) to be commonly regulated by these pathways in all three yeast (Enjalbert *et al.*, 2006). In addition, work from this study and others has also uncovered significant differences in the regulation of the *C. albicans* Hog1 MAPK compared with analogous pathways in *S. cerevisiae* and *S. pombe* (Chauhan *et al.*, 2003; Arana *et al.*, 2005; Roman *et al.*, 2005; Menon *et al.*, 2006). The Hog1 MAPK pathway plays an important role in *C. albicans* virulence (Alonso-Monge *et al.*, 1999; Arana *et al.*, 2007) thus an important goal is to delineate how the specialization of the role and regulation of Hog1 promotes survival in the human host.

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