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MAPKKK-independent Regulation of the Hog1 Stress-activated Protein Kinase in *Candida albicans*[*] [S]

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Jill Cheetham[‡], Donna M. MacCallum[§], Kathryn S. Doris[‡], Alessandra da Silva Dantas[‡], Susan Scorfield[‡], Frank Odds[§], Deborah A. Smith[‡], and Janet Quinn[‡1]

From the [‡]Institute for Cell and Molecular Biosciences, Faculty of Medicine, Newcastle University, Newcastle upon Tyne, NE2 4HH and the [§]Aberdeen Fungal Group, Institute of Medical Sciences, University of Aberdeen, Aberdeen AB25 2ZD, United Kingdom

The Hog1 stress-activated protein kinase regulates both stress responses and morphogenesis in *Candida albicans* and is essential for the virulence of this major human pathogen. Stress-induced Hog1 phosphorylation is regulated by the upstream MAPKK, Pbs2, which in turn is regulated by the MAPKKK, Ssk2. Here, we have investigated the role of phosphorylation of Hog1 and Pbs2 in Hog1-mediated processes in *C. albicans*. Mutation of the consensus regulatory phosphorylation sites of Hog1 (Thr-174/Tyr-176) and Pbs2 (Ser-355/Thr-359), to non-phosphorylatable residues, resulted in strains that phenocopied *hog1*Δ and *pbs2*Δ cells. Consistent with this, stress-induced phosphorylation of Hog1 was abolished in cells expressing non-phosphorylatable Pbs2 ($Pbs2^{AA}$). However, mutation of the consensus sites of Pbs2 to phosphomimetic residues ($Pbs2^{DD}$) failed to constitutively activate Hog1. Furthermore, Ssk2-independent stress-induced Hog1 activation was observed in $Pbs2^{DD}$ cells. Collectively, these data reveal a previously uncharacterized MAPKKK-independent mechanism of Hog1 activation in response to stress. Although $Pbs2^{DD}$ cells did not exhibit high basal levels of Hog1 phosphorylation, overexpression of an N-terminal truncated form of Ssk2 did result in constitutive Hog1 activation, which was further increased upon stress. Significantly, both $Pbs2^{AA}$ and $Pbs2^{DD}$ cells displayed impaired stress resistance and attenuated virulence in a mouse model of disease, whereas only $Pbs2^{AA}$ cells exhibited the morphological defects associated with loss of Hog1 function. This indicates that Hog1 mediates *C. albicans* virulence by conferring stress resistance rather than regulating morphogenesis.

Stress responses are intimately linked with the pathogenicity of the dimorphic fungus *Candida albicans*, the major systemic fungal pathogen of humans. *C. albicans* colonizes numerous niches within humans, and thus, its success as a pathogen is dependent on its ability to adapt to fluctuations in the growth environment. This is exemplified by a number of studies that demonstrated that the inactivation of stress-protective enzymes or stress-responsive signaling proteins significantly attenuates the virulence of *C. albicans* (1–3). In addition, an examination of the genome-wide responses of *C. albicans* in various infection models revealed that distinct stress responses are induced at different stages of infection (4–7). Interestingly, there is mounting evidence that although key stress-signaling proteins are conserved in *C. albicans*, their role and regulation in mediating stress responses have diverged from that documented in the model yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (8, 9). These data indicate that *C. albicans* has developed specialized stress responses that have adapted to promote survival in the human host.

The Hog1 stress-activated protein kinase (SAPK)[2] in *C. albicans* plays a central role in stress responses and is activated in response to many stress stimuli likely to be encountered in the host such as osmotic stress, reactive oxygen species, and antimicrobial peptides (10–13). Following activation, Hog1 accumulates in the nucleus and promotes the induction of stress-responsive genes (12, 14). Hog1 also functions to repress morphogenetic switching in *C. albicans*, as cells lacking *HOG1* form filaments under non hyphae inducing conditions (14) and display enhanced hyphae formation in the presence of serum (1). Consistent with Hog1 regulating such well established virulence determinants, *hog1*Δ cells display significantly attenuated virulence in a mouse model of systemic candidiasis (1) and are much more sensitive to killing by phagocytes (15). However, despite the importance of Hog1 in stress signaling, morphogenesis, and virulence in *C. albicans*, little is known about how stress signals are relayed to the Hog1 SAPK module or the precise role of Hog1 signaling in establishing the virulence of this fungal pathogen.

SAPKs are evolutionarily conserved signaling molecules found in all eukaryotes. Activation of the SAPK is mediated by phosphorylation, which is tightly regulated by a three-tiered cascade of protein kinases that comprise a SAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK). The MAPKKK activates the MAPKK by phosphorylation of conserved serine and/or threonine residues located within kinase domain subdomain VIII. This then subsequently phosphorylates the SAPK on conserved threonine and tyrosine residues located in the activation loop of the catalytic domain. This three-tiered cascade of protein kinases is evident in the *C. albicans*, *S. cerevisiae*, and *S. pombe* SAPK modules. For example, each contains a single SAPK and MAPKK, namely Hog1 and Pbs2 in *S. cerevisiae* (16) and *C. albicans* (17, 18) and Sty1 (also

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.
⌘ *Author's Choice*—Final version full access.
[1] To whom correspondence should be addressed. Tel.: 44-191-2227434; Fax: 44-191-2227424; E-mail: janet.quinn@ncl.ac.uk.

[2] The abbreviations used are: SAPK, stress-activated protein kinase; Ni^{2+}-NTA, Ni^{2+}-nitrilotriacetic acid.

TABLE 1
Strains used in this study

	Relevant genotype	Source
C. albicans		
RM1000	*ura3*::λ *imm434/ura3*::λ*imm434, his1*::*hisG/his1*::*hisG*	54
BWP17	*ura3*::λ *imm434/ura3*::λ*imm434, his1*::*hisG/his1*::*hisG, arg4*::*hisG/arg4*::*hisG*	46
JC21	RM1000 CIp20	12
JC64	BWP17 CIp30	This work
JC50	RM1000 *hog1*::*loxP-ura3-loxP/hog1*::*loxP-HIS1-loxP* CIp20-*HOG1*	12
JC52	RM1000 *hog1*::*loxP-ura3-loxP/hog1*::*loxP-HIS1-loxP* Cip20	12
JC36	BWP17 *hog1*::*loxP-ARG4-loxP/HOG1*	14
JC154	BWP17 *hog1*::*loxP-ARG4-ura3-loxP/hog1*::*loxP-HIS1-loxP* CIp20	14
JC76	BWP17 *hog1*Δ::*loxP-ARG4-loxP/ HOG1^{AF}-ProtA*:*URA3*	This work
JC80	BWP17 *hog1*Δ::*loxP-ARG4-loxP/HOG1 -ProtA*:*URA3*	This work
JC72	BWP17 *pbs2*::*loxP-HIS1-loxP/PBS2*	20
JC74	BWP17 *pbs2*::*loxP-ARG4-loxP/pbs2*::*loxP-HIS1-loxP*	20
JC112	BWP17 *pbs2*::*loxP-HIS1-loxP/ PBS2-HM*:*URA3*	20
JC124	BWP17 *pbs2*::*loxP-HIS1-loxP/ PBS2^{DD}-HM*:*URA3*	This work
JC126	BWP17 *pbs2*::*loxP-HIS1-loxP/ PBS2^{AA}-HM*:*URA3*	This work
JC167	BWP17 *pbs2*::*loxP-ARG4-ura3-loxP/pbs2*::*loxP-HIS1-loxP* CaEXP-*PBS2-HM* (*URA3*)	20
JC174	BWP17 *pbs2*::*loxP-ARG4-ura3-loxP/pbs2*::*loxP-HIS1-loxP* CaEXP-*PBS2^{AA}-HM* (*URA3*)	This work
JC181	BWP17 *pbs2*::*loxP-ARG4-ura3-loxP/pbs2*::*loxP-HIS1-loxP,* CaEXP-*PBS2^{DD}-HM* (*URA3*)	This work
JC482	BWP17 *ssk2*::*loxP-ARG4-loxP/ssk2*::*loxP-HIS1-loxP*	20
JC507	BWP17 *ssk2*::*loxP-ARG4-loxP/ssk2*::*loxP-HIS1-loxP, PBS2^{DD}-HM*:*URA3*	20
JC517	BWP17 *ssk2*::*loxP-ARG4-loxP/ssk2*::*loxP-HIS1-loxP, PBS–HM*:*URA3*	20
JC1614	BWP17 *ssk2*::*loxP-ARG4-loxP/ssk2*::*loxP-HIS1-loxP* pACT1 (*URA3*)	This work
JC1616	BWP17 *ssk2*::*loxP-ARG4-loxP/ssk2*::*loxP-HIS1-loxP* pACT1-*SSK2*Δ*N* (*URA3*)	This work
JC1618	BWP17 *ssk2*::*loxP-ARG4-loxP/ssk2*::*loxP-HIS1-loxP* pPGK1 (*URA3*)	This work
JC1620	BWP17 *ssk2*::*loxP-ARG4-loxP/ssk2*::*loxP-HIS1-loxP* pPGK1- *SSK2*Δ*N* (*URA3*)	This work
S. pombe		
CHP428	*h$^+$ his7–366 ade6-210 leu1-32 ura4-D18*	Gift from C. Hoffman
JM1521	*h$^+$ sty1*(*HA6His*):*ura4$^+$*	24
GD1682	*h$^-$ wis1*::*ura4$^+$ sty*(*HA6His*):*ura4$^+$*	28
KS2088	*h$^-$ wis1^{DD}*(*12myc*):*ura4$^+$ sty1*(*HA6His*):*ura4$^+$*	28

known as Spc1 or Phh1) and Wis1 in *S. pombe* (19). Interestingly, however, whereas the *C. albicans* Pbs2 MAPKK is regulated by a single MAPKKK, Ssk2 (20), the analogous MAPKKs in *S. cerevisiae* and *S. pombe* are regulated by three (21, 22) or two (23, 24) MAPKKKs, respectively.

Previous analyses of SAPK and MAPKK phosphorylation site mutants in the model yeasts *S. cerevisiae* and *S. pombe* have provided significant insight into eukaryotic stress-signaling mechanisms. For example, analysis of *S. cerevisiae* Hog1 and *S. pombe* Sty1 phosphorylation site mutants provided the first evidence that phosphorylation is essential for the stress-induced nuclear accumulation of SAPKs in eukaryotes (25, 26) and that a basal level of SAPK activation is necessary to restrict cross-talk to other MAPK pathways (27). With regard to MAPKK phosphorylation, mutation of the conserved serine and threonine phosphorylation sites of both *S. cerevisiae* Pbs2 and *S. pombe* Wis1 to nonphosphorylatable alanine residues abolished stress signaling to the respective SAPKs (21, 28). Conversely, expression of *S. cerevisiae* Pbs2 or *S. pombe* Wis1 mutants, in which the predicted phosphorylation sites were mutated to the phosphomimetic aspartic acid residue, resulted in constitutive activation of the Hog1 and Sty1 SAPKs, respectively (28, 29). Although this proved lethal in *S. cerevisiae*, subsequent analysis of *S. pombe wis1-DD* cells revealed a novel MAPKKK-independent mechanism of SAPK activation in response to heat stress (28, 30). However, despite this wealth of information obtained from studying SAPK and MAPKK phosphorylation mutants in *S. cerevisiae* and *S. pombe*, a similar analysis of analogous *C. albicans* mutants has not been reported.

Here. we investigated the potential roles of Hog1 and Pbs2 phosphorylation in Hog1-mediated processes in *C. albicans*. Not only do our data uncover mechanisms of SAPK regulation in *C. albicans* not previously documented in *S. cerevisiae* and *S. pombe*, but they also demonstrate that the attenuated virulence exhibited by *hog1*Δ cells is likely attributed to impaired stress responses rather than defects in morphogenesis.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—The strains used in this study are listed in Table 1. *C. albicans* strains were grown in either YPD media (2% yeast extract, 1% bactopeptone, 2% glucose) or SD media (6.79 g/liter yeast nitrogen base without amino acids, 2% glucose) supplemented with the required nutrients for auxotrophic mutants (31). *S. pombe* strains were grown in EMM synthetic minimal medium as described previously (32, 33). All strains were grown at 30 °C.

Strain Construction—All of the oligonucleotide primers used for generating the constructs described below are listed in Table 2.

Tagging and Mutagenesis of Hog1—Initially *HOG1*, expressed from its native chromosomal locus, was tagged at the C terminus with protein A. *HOG1* was amplified by PCR using the oligonucleotide primers CaHOG1PstF and CaHOG1PstR and ligated into the vector CIp-C-ProtA-CyC (34). The resulting CIp-C-*HOG1*ProtA plasmid was linearized by digestion with StyI to target chromosomal integration at the remaining *HOG1* locus in *hog1/HOG1* cells (JC36) to subsequently generate strain JC80. Chromosomal insertion of the protein A tag was confirmed by PCR and DNA sequencing. Mutagenesis of *HOG1* to create *HOG1^{T174A,Y176F}* was performed by PCR using oligonucleotides HOG1TAYF and HOG1Sal1F. The resulting PCR fragment was digested with SalI and cloned into CIp-C-*HOG1*ProtA previously digested with SalI to remove the wild-

TABLE 2
Oligonucleotides used in this study

Oligonucleotide	Sequence 5′–3′
CaHOG1PstF	cttggcttccgccaacggagctgcctgcagaaaagaacctaaggcggttgcctcgacggacgtctttt
CaHOG1PstR	ttttctgcaggcagctccgttggcggactccaag
HOG1TAYF	caccacaagtcgacttcagtgtcgtatttttgccaagttaacatgatttctggtgctcgataatatctggttgacacaaaacggccatttgtggact
HOG1SalF	gcacgcgtcgacgaaacatgagaatttgattaccttgg
PBS2TagR	gaggatcccgggggacacgagaaaatattatcatatgtttcagca
PBS2AA	ggtaatttagttgccgcattagccaaagcaaatattggttgt
PBS2PstB	cgtcatctgcagctatatcagcttcaacatcgg
CaPBS2FLF	gcgcggatccatggttgaagataaagattagac
CaPBS2FLR	gcgcggatccttaatggtgatgatggtctaagtcctcc
SSK2NACTF	aggcgggtcgacatgtcgttattgatttctcatttcg
SSK2NACTR	aggcgggtcgacttattctgaaaccggacccgcaacc
SSK2NPGKF	gcgcggatccatgtcgttattgatttctcatttcg
SSK2NPGKR	gcgcggatccctagttcaacttaataaataaggggag
PGKpromF	ccagatgagcgcgacattaatacg
PGKpromR	gcgcggatcctttgatagttattcttctgcaattg
STL1F	gtggttggtagagtcatcactggt
STL1R	ataacaaccccaaccactagcaga
RHR2F	gacaaagactcaacaaccag
RHR2R	ccttgaattcgtcagtttcc
GPD2F	tgtattgtcggttccggtaactgg
GPD2R	ccttaacatttctagcacctgagc
ACT1F	gatgaagcccaatccaaaag
ACT1R	ggagttgaaagtggtttggt

type *HOG1* sequence. The resulting CIp-C-*HOG1*^{AF}ProtA was linearized with *PshA*1 to target chromosomal integration at the remaining *HOG1* locus in *hog1/HOG1* cells (JC36) to generate strain JC76. Levels of Hog1-ProtA and Hog1^{AF}-ProtA were detected by Western blot analysis of cell extracts prepared from JC80 and JC76 respectively, using an anti-Hog1 antibody (Santa Cruz Biotechnology).

Tagging and Mutagenesis of PBS2—The construction of a strain in which *PBS2* was expressed from its native chromosomal locus and tagged at the C terminus with His_6 residues and two copies of the Myc epitope, using the plasmid CIp-C-*PBS2HM*, was described previously (20). Mutagenesis of *PBS2* to create *PBS2*^{S355A,T359A} was performed by overlapping PCR using the oligonucleotides PBS2TagR, PBS2^{AA}, and PBS2PstB. The resulting PCR fragment was digested with PstI and NdeI and cloned into CIp-C-*PBS2HM* (20), previously digested with the same enzymes to remove the wild-type sequence. Construction of the plasmid CIp-C-*PBS2*^{DD}*HM* was described previously (20). To generate strains expressing His-Myc-tagged wild-type and mutant *PBS2* alleles, the CIp-C-*PBS2HM*, CIp-C-*PBS2*^{AA}*HM*, and CIp-C-*PBS2*^{DD}*HM* plasmids were linearized by digestion with SgrAI to target integration at the remaining *PBS2* locus in *PBS2/pbs2*Δ (JC72) cells to generate strains JC112, JC126, and JC124, respectively. The DNA sequences of the integrated open reading frames and the correct chromosomal insertion of the tagged derivatives of *PBS2* were confirmed by PCR and DNA sequencing. Levels of His_6-Myc-tagged Pbs2 proteins were detected by Western blot analysis of cell extracts prepared from JC112, JC124, and JC126, following enrichment on Ni^{2+}-NTA-agarose, using an anti-Myc antibody (9E10, Sigma).

Regulatable Expression of PBS2 Alleles—To achieve regulatable expression of wild-type and mutant *PBS2* alleles in *C. albicans*, *PBS2*, *PBS2*^{AA}, and *PBS2*^{DD} were first amplified by PCR using the primer pairs CaPBS2FLF and CaPBS2FLR and genomic template DNA from JC112, JC126, and JC124, respectively. The resulting PCR products were digested with BamHI and ligated into the BamHI site of the plasmid CaEXP (35) to generate plasmids CaEXP-*CaPBS2* (20), CaEXP-Ca*PBS2*^{AA}, and CaEXP-*CaPBS2*^{DD}. Each plasmid was linearized at the StuI restriction site in the *RPS10* locus and introduced into *pbs2*Δ cells (JC74) to create strains JC167, JC174, and JC181, respectively. Correct integration at the *RPS10* locus, and the DNA sequences of the integrated open reading frames, was confirmed by PCR and DNA sequencing. CaEXP directs the expression of genes under the control of the *MET3* promoter, which is repressed in the presence of methionine and cysteine (35). Hence, strains carrying CaEXP derivatives were grown in minimal SD media in the presence or absence of methionine (2.5 mM) and cysteine (2 mM). Levels of His_6-Myc-tagged Pbs2 proteins were detected as described above.

Expression of PBS2 and PBS2^{DD} *in S. pombe*—The *C. albicans PBS2* and *PBS2*^{DD} genes were amplified by PCR from genomic DNA extracted from strains JC112 and JC124, respectively, using the oligonucleotide primers CaPBS2FLF and CaPBS2FLR. The resulting PCR products were digested with BamHI and cloned into the BamHI site of pREP41HM (36), which directs the expression of genes in *S. pombe* from the thiamine-repressible medium strength *nmt41*$^{+}$ promoter (37). The resulting plasmids pREP41HM-*PBS2* and pREP41HM-*PBS2*^{DD} were transformed into *wis1*$^{-}$ cells (GD1682), and the resulting transformants were grown in EMM media without thiamine to allow expression of the *C. albicans PBS2* alleles.

Expression of Truncated SSK2, Lacking the N-terminal Noncatalytic Domain Coding Sequence, from the ACT1 and PGK1 Promoters—The *C. albicans SSK2* gene lacking the N-terminal noncatalytic domain was amplified from genomic DNA using the oligonucleotide primer pairs SSK2NACTF/SSK2NACTR. The resulting PCR product was digested with SalI and ligated into the SalI site between the *C. albicans ACT1* promoter and *S. cerevisiae CYC1* terminator in pACT1 (38) to generate plasmid pACT1-*SSK2*Δ*N*. Both pACT1 and pACT1-*SSK2*Δ*N* were linearized at the StuI restriction site in the *RPS10* locus and introduced into *ssk2*Δ cells (JC482) (20) to create strains JC1614 and JC1616, respectively. In addition, the truncated *SSK2* gene plus terminator was amplified from genomic DNA using the oligo-

nucleotide primer pairs SSK2NPGKF/SSK2NPGKR. The resulting PCR product was digested with BamHI and ligated into the BamHI site adjacent to the *PGK1* promoter in pPGK1 to generate pPGK1-*SSK2*Δ*N*. pPGK1 was previously generated by amplifying the *PGK1* promoter from genomic DNA using the oligonucleotides PGKpromF and PGKpromR. The resulting PCR product was digested with BglII and BamHI and ligated into the BamHI site of CIp10 (39). Both pPGK1 and pPGK1-*SSK2*Δ*N* were linearized at the StuI restriction site in the *RPS10* locus and introduced into *ssk2*Δ cells (JC482) to create strains JC1618 and JC1620, respectively. Correct integration at the *RPS10* locus, and the DNA sequences of the integrated open reading frames, was confirmed by PCR and DNA sequencing.

Stress Sensitivity Tests—C. albicans strains to be tested were grown in liquid culture to mid-log phase and then spotted onto YPD or SD plates containing the indicated compounds, using a 48-well replica plater (Sigma). *S. pombe* strains were streaked onto EMM plates containing the indicated compounds. Plates were incubated at 30 °C for the times indicated.

Co-precipitation of Hog1 with Pbs2—Co-precipitation of Hog1 with wild-type and mutant Pbs2 proteins was performed using extracts isolated from strains JC112, JC124, and JC126, as described previously (20).

Protein Phosphorylation Assays—Protein extracts were prepared, and phosphorylated Hog1 or Sty1 was detected by Western blot with an anti-phospho-p38 antibody (New England Biolabs) as described previously (12). Blots were stripped, and total levels of Hog1 were determined by probing with an anti-Hog1 antibody (Santa Cruz Biotechnology) or in the case of Sty1 with an anti-HA antibody (Sigma). Cek1 phosphorylation was detected using an anti-phospho-p42/p44 antibody (New England Biolabs, Beverly, MA) as described previously (40).

Microscopy—Cells were fixed in 3.7% paraformaldehyde, washed in PEM (100 mM PIPES, pH 7.6, 1 mM EGTA, 1 mM $MgSO_4$), and spread onto poly-L-lysine-coated slides. Coverslips were mounted onto slides using Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Differential interference contrast images were captured using a Zeiss Axioscope, with a ×63 oil immersion objective, and Axiovision imaging system.

RNA Analysis—RNA preparation and Northern blot analyses were performed as described previously (14). Gene-specific probes were amplified by PCR from genomic DNA using oligonucleotide primers specific for *STL1, RHR2*, *GPD2,* and *ACT1* (Table 2).

Virulence Analysis—The standard 28-day survival method was employed to examine the role of Hog1 in mediating *C. albicans* virulence. Female BALB/c mice (6–8 weeks; Harlan, UK) were housed in groups of six with food and water provided *ad libitum*. *C. albicans* strains RM1000 + CIp20 (JC21), *hog1*Δ + CIp20 (JC50), and *hog1*Δ + CIp20-*HOG1* (JC52) were grown in NGY (0.1% neopeptone, 0.4% glucose, 0.1% yeast extract) medium supplemented with 50 μg/ml histidine for 18 h at 30 °C with constant agitation. Cells were harvested in sterile saline and washed twice, and cell counts were adjusted by hemocytometer to provide a cell suspension required to deliver a challenge dose of 2.5×10^4 CFU/g body weight in a 100-μl volume. Mice were infected intravenously via a lateral tail vein. The actual challenge doses of strains were determined from viable counts read 24 h later and were $\sim 2.6 \times 10^4$ CFU/g. Mouse condition and weight was monitored daily, with mice culled when they showed signs of severe infection or if mouse weight decreased by more than 20% from the initial body weight. Mice were culled by cervical dislocation. For all culled mice, death was recorded as occurring on the following day. Kidney fungal burdens were also determined when mice were sampled. Mouse survival was plotted and compared by Kaplan-Meier survival plots, and kidney counts were compared by Mann-Whitney *U* test.

The 3-day murine intravenous challenge model of *C. albicans* infection (41, 42) was also employed to determine the impact of deleting or mutating Hog1, or mutating Pbs2, on virulence. Female BALB/c mice (6–8 weeks; Harlan, UK) were housed in groups of six with food and water provided *ad libitum*. *hog1*Δ + CIp20 (JC50), *hog1*Δ + CIp20-*HOG1* (JC52), *HOG1*ProtA (JC80), $HOG1^{AF}$ProtA (JC76), *PBS2HM* (JC112), $PBS2^{AA}HM$ (JC124), and $PBS2^{DD}HM$ (JC126) cells were grown overnight in NGY medium, with constant agitation, at 30 °C. Cells were harvested in sterile saline, and cell counts were adjusted by hemocytometer to provide a cell suspension estimated to deliver a challenge dose of 3×10^4 CFU/g body weight. Actual challenge doses were determined from viable counts read 24 h later and were $\sim 2.2 \times 10^4$ CFU/g. Mice were infected intravenously via a lateral tail vein. Weight change and kidney fungal burdens were determined at 72 h post-challenge. Fungal burdens were measured by viable counts for two half-kidneys per animal; the other half-kidneys were fixed, embedded, and stained for histopathological examination. The virulence of challenge strains was assessed by fungal kidney burdens and by percent weight change at 72 h, from which an outcome score was calculated (41, 42). Differences between mean body weight changes and mean kidney burdens were tested statistically by the Mann-Whitney *U* test. Statistical tests were performed using PASW (version 18). All animal experimentation conformed to the requirements of United Kingdom Home Office legislation and the Ethical Review Committee of the University of Aberdeen.

RESULTS

*Mutation of the Phosphorylation Sites of Hog1 Results in Comparable Phenotypes to hog1*Δ *Cells*—Sequence analysis of the *C. albicans* Hog1 SAPK illustrated that the dual phosphorylation sites (Thr-174 and Tyr-176) at the activation site of the catalytic domain are highly conserved (Fig. 1*A*). To confirm that phosphorylation of these sites was essential for function, a strain was generated in which one allele of *HOG1* was deleted and the second wild-type allele was mutated such that the codons encoding Thr-174 and Tyr-176 were replaced with codons encoding the nonphosphorylatable alanine and phenylalanine residues ($HOG1^{AF}$), respectively. Tyr-176 was replaced with phenylalanine rather than alanine to maintain the presence of the aromatic side chain. The $HOG1^{AF}$ allele was also tagged at the C terminus with a sequence encoding protein A, and as a control, a strain expressing wild-type *HOG1* tagged in the same way (*HOG1*-ProtA) was also constructed. Such protein A-tagged Hog1 strains were generated to allow subsequent

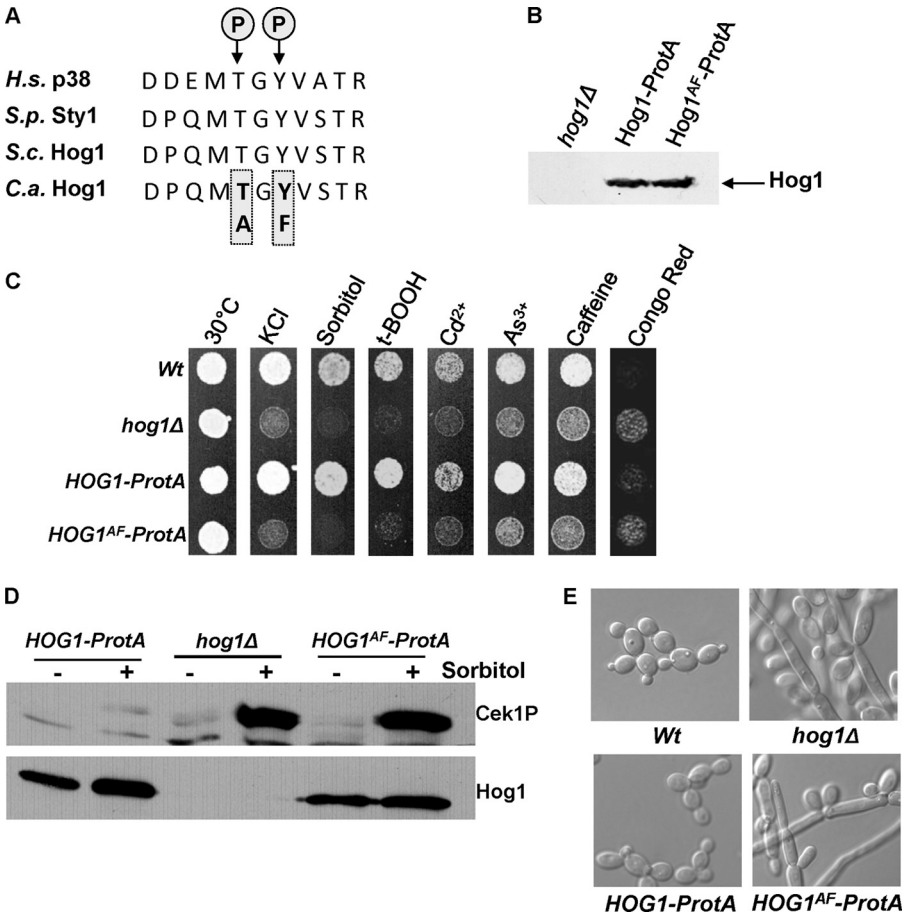

FIGURE 1. ***HOG1^{AF}* cells have comparable phenotypes to *hog1*Δ cells.** *A,* alignment of sequences from *H. sapiens* (*H.s.*) p38, *S. pombe* (*S.p.*) Sty1, *S. cerevisiae* (*S.c.*) Hog1, and *C. albicans* (*C.a.*) Hog1 SAPKs illustrates that the sequence surrounding and including the Thr and Tyr phosphorylation sites is highly conserved. The Thr-174 and Tyr-176 residues of *C. albicans* Hog1 (*boxed*) were substituted with alanine and phenylalanine, respectively, to generate Hog1^{AF}. *B,* mutation of Hog1 phosphorylation sites does not impact on protein stability. Western blot analysis of whole cell extracts isolated from *hog1*Δ cells or cells expressing Hog1-ProtA (JC80) or Hog1^{AF}-ProtA (JC76). Hog1 levels were detected using an anti-Hog1 antibody. *C,* cells expressing *HOG1^{AF}-ProtA* display equivalent stress-sensitive or -resistant phenotypes as *hog1*Δ cells. Approximately 10^3 cells of exponentially growing wild-type (*Wt*, BWP17), *hog1*Δ (JC154), *HOG1-ProtA* (JC80), and *HOG1^{AF}-ProtA* (JC76) strains were spotted onto YPD plates containing the indicated additives: KCl (0.6 M), sorbitol (1.2 M), *t*-BOOH (1.5 mM), $CdSO_4$ (0.4 mM), $AsNaO_2$ (2 mM), caffeine (8 mM), and Congo red (300 μg/ml). Plates were incubated at 30 °C for 24 h. *D,* cells expressing Hog1AF result in a high basal level and osmotic stress induction of Cek1 phosphorylation. *E,* both *hog1*Δ and *HOG1^{AF}-ProtA* cells form filaments under noninducing conditions. Micrographs illustrating the morphology of wild-type (*Wt*, BWP17), *hog1*Δ (JC154), *HOG1-ProtA* (JC80), and *HOG1^{AF}-ProtA* (JC76) strains grown in YPD at 30 °C.

purification of Hog1 and associated proteins.[3] Importantly, Western blot analysis confirmed that mutation of the conserved phosphorylation sites did not result in protein destabilization, as Hog1 levels were found to be very similar in Hog1-ProtA- and Hog1^{AF}-ProtA-expressing strains (Fig. 1*B*).

Previous studies revealed that *C. albicans hog1*Δ cells display increased sensitivity to a diverse range of stress stimuli (10, 12, 18). To investigate the role of the conserved Thr-174 and Tyr-176 phosphorylation sites in Hog1-mediated stress responses, exponentially growing wild-type, *hog1*Δ, *HOG1*-ProtA, and *HOG1^{AF}*-ProtA cells were exposed to various stress conditions. Cells expressing *HOG1*-ProtA displayed equivalent stress-resistant phenotypes compared with wild-type cells, indicating that the protein A tag did not affect the function of Hog1. In contrast, *C. albicans* cells expressing *HOG1^{AF}*-ProtA displayed equivalent stress-sensitive phenotypes to *hog1*Δ cells in response to the oxidative stress agent (*t*-BOOH), osmotic stress agents (sorbitol and KCl), heavy metals ($Cd^{2+}$and As^{3+}), and the purine analog caffeine (Fig. 1*C*). Such findings indicate that Thr-174 and Tyr-176 are important for the stress-response functions of the Hog1 SAPK. Deletion of *HOG1* also results in resistance to cell wall inhibitors such as calcofluor white and Congo red (1, 17). Indeed, cells expressing *HOG1^{AF}*-ProtA displayed similar increased resistance to Congo red as *hog1*Δ cells (Fig. 1*C*). Such resistance is attributed to activation of the cell integrity/filamentation Cek1 MAPK pathway, as deletion of *HOG1* results in constitutive Cek1 phosphorylation (17, 43). To analyze the effects of mutating the activating phosphorylation sites of Hog1 on the Cek1 MAPK, Cek1 phosphorylation was monitored using a p42/p44 antibody that recognizes the active form of Cek1 (40). As illustrated in Fig. 1*D*, *HOG1^{AF}* cells, like *hog1*Δ cells, display a high basal level of phosphorylated Cek1 before stress. Significant levels of Cek1 phosphorylation have also been reported in *hog1*Δ cells in response to osmotic stress (44). Similarly, Cek1 phosphorylation was also strongly induced in *HOG1^{AF}-ProtA*, but not *HOG1-ProtA*, cells following exposure to sorbitol (Fig. 1*D*). Collectively, these results indicate that a basal level of Hog1 phosphorylation is sufficient to

[3] D. Smith and J. Quinn, unpublished data.

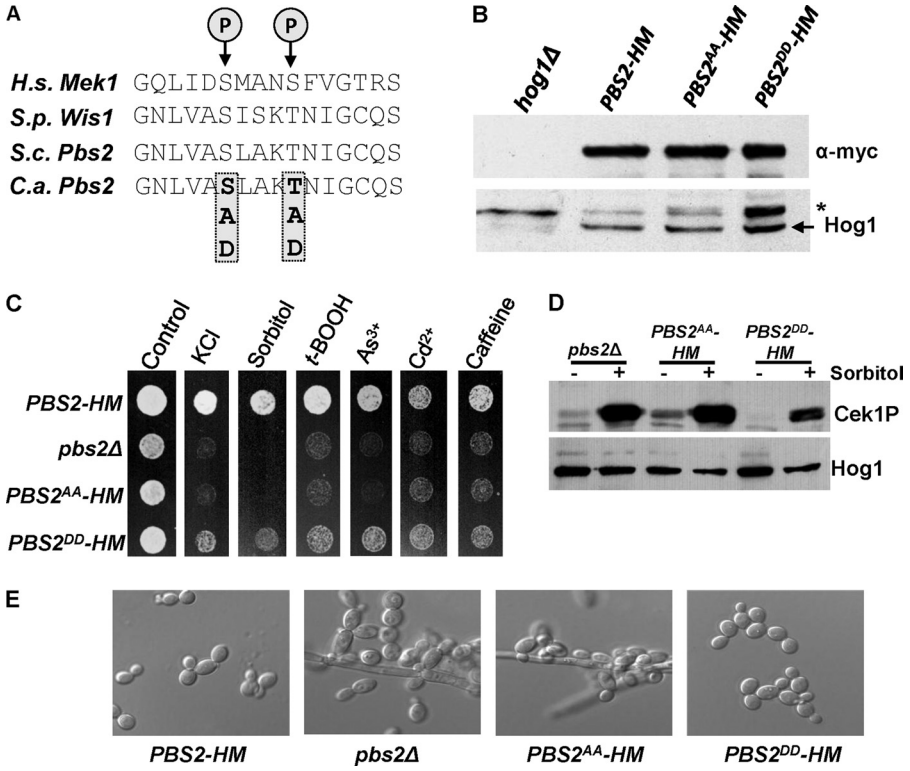

FIGURE 2. **Cells expressing *PBS2^{AA}*, but not *PBS2^{DD}*, phenocopy *pbs2*Δ cells.** *A,* alignment of sequences from *H. sapiens* (*H.s.*) Mek1, *S. pombe* (*S.p.*) Wis1, *S. cerevisiae* (*S.c.*) Pbs2, and *C. albicans* (*C.a.*) Pbs2 MAPKKs illustrates that the sequence surrounding and including the Ser/Thr phosphorylation sites is highly conserved. The Ser-355 and Thr-359 sites of *C. albicans* Pbs2 (*boxed*) were mutated to either alanine (Pbs2^{AA}) or aspartic acid (Pbs2^{DD}) residues. *B,* Pbs2 phosphorylation is not required for interaction with Hog1. Extracts were prepared from mid-log cultures of *hog1*Δ cells (JC152) and cells expressing His$_6$-Myc-tagged Pbs2 (*PBS2-HM*, JC112), Pbs2^{AA} (*PBS2^{AA}-HM*, JC126), and Pbs2^{DD} (*PBS2^{DD}-HM*, JC124). The His$_6$-Myc-tagged Pbs2 proteins were precipitated using Ni^{2+}-NTA-agarose, and co-precipitation of Hog1 was analyzed by Western blotting using an anti-Hog1 antibody (Hog1). A nonspecific band that runs above the Hog1 band is marked with an *asterisk*. Blots were reprobed with an anti-Myc antibody (α-*myc*) to allow comparison of the levels of wild-type and mutant Pbs2-His$_6$-Myc-tagged proteins. *C,* cells expressing *PBS2^{AA}-HM* are equally stress-sensitive as *pbs2*Δ cells, whereas *PBS2^{DD}-HM* cells display an intermediate stress-resistant phenotype. Approximately 10^3 cells of exponentially growing *PBS2-HM* (JC112), *pbs2*Δ (JC74), *PBS2^{AA}-HM* (JC126), and *PBS2^{DD}-HM* (JC124) strains were spotted onto YPD plates containing the additives as detailed in Fig. 1*C* legend. Plates were incubated at 30 °C for 24 h. *D,* prevention of Hog1 phosphorylation stimulates Cek1 activation. Western blot analysis of whole cell extracts isolated from *pbs2*Δ (JC74), *PBS2^{AA}-HM* (JC126), and *PBS2^{DD}-HM* (JC124) cells after treatment with 1 M sorbitol for the specified times. Western blots were probed with an anti-phospho-p42/p44 antibody that recognizes the phosphorylated, active form of *C. albicans* Cek1. Levels of Hog1 protein, which provided a loading control, were determined by stripping and reprobing the blot with an anti-Hog1 antibody. *E, PBS2^{AA}* cells but not *PBS2^{DD}* cells form filaments under nonhyphae-inducing conditions. Micrographs illustrate the morphology of *PBS2-HM* (*JC112*), *pbs2*Δ (JC74), *PBS2^{AA}-HM* (JC126), and *PBS2^{DD}-HM* (JC124) strains grown in YPD at 30 °C.

repress inappropriate activation of the Cek1 MAPK pathway and that *HOG1^{AF}* cells behave like the *hog1*Δ mutant.

Previous work also indicated a role for the Hog1 SAPK in the regulation of morphogenetic switching in *C. albicans*. Hog1 appears to function as a repressor of morphogenesis, as cells lacking Hog1 form filaments under non hyphae-inducing conditions (14) and display accelerated filamentation in the presence of serum (1, 17, 43). Notably, *HOG1^{AF}* cells displayed a similar level of filamentation to that exhibited by *hog1*Δ cells when grown under the non hyphae-inducing conditions of liquid YPD at 30 °C (Fig. 1*E*). This indicates that a basal level of Hog1 phosphorylation is also essential to repress the morphogenetic switch under noninducing conditions.

*Cells Expressing PBS2^{AA}, but Not PBS2^{DD}, Phenocopy pbs2*Δ *Cells*—The activating phosphorylation sites of the MAPKK, which functions upstream of the SAPK, are also highly conserved (Fig. 2*A*). To investigate the role of the equivalent Ser-355- and Thr-359-activating phosphorylation sites of Pbs2 in *C. albicans*, strains were constructed expressing either Pbs2, where these sites were replaced with alanine residues to create a nonphosphorylatable Pbs2 protein (Pbs2^{AA}), or with phosphomimetic aspartic acid residues (Pbs2^{DD}) to create a constitutively active Hog1 pathway (Fig. 2*A*). The wild-type and mutant *PBS2* alleles were also tagged with sequences encoding six histidine residues and two copies of the Myc epitope (HM) at the C terminus. We had previously demonstrated that Pbs2 interacts with Hog1 *in vivo* (20). Hence, co-precipitation experiments were performed to investigate whether mutation of the phosphorylation sites of Pbs2 abrogated the interaction with Hog1. Wild-type and mutant Pbs2-HM proteins were precipitated from cell extracts, and co-precipitation of Hog1 was determined. As illustrated in Fig. 2*B*, Hog1 association with Pbs2 was maintained in cells expressing either *PBS2^{AA}* or *PBS2^{DD}*. This indicates that the conserved Ser-355 and Thr-359 phosphorylation sites of Pbs2 are dispensable for the association of Hog1 with Pbs2 *in vivo*.

To investigate the role of phosphorylation of Pbs2 in stress responses in *C. albicans*, the resistance of cells expressing *PBS2-HM*, *PBS2^{AA}-HM,* and *PBS2^{DD}-HM* was compared with *pbs2*Δ cells to a range of stress agents. As illustrated in Fig. 2*C*, the increased stress sensitivity exhibited by *PBS2^{AA}* cells phenocopied that of *pbs2*Δ cells. This indicates that the *PBS2^{AA}*

mutant is nonfunctional and that the Ser-355 and Thr-359 phosphorylation sites are essential for stress signaling to Hog1. In contrast to *S. cerevisiae*, where overexpression of $PBS2^{DD}$ is lethal due to hyperactivation of the Hog1 MAPK (29), *C. albicans* cells expressing $PBS2^{DD}$ from its native promoter are viable. Furthermore, cells expressing $PBS2^{DD}$-*HM* exhibited an intermediate stress-sensitive phenotype; cells were more sensitive than the wild-type *PBS2-HM* strain, but more resistant than $PBS2^{AA}$-*HM* cells, to a range of stress-inducing compounds (Fig. 2*C*). The increased stress sensitivity exhibited by $PBS2^{AA}$ and $PBS2^{DD}$ cells was not due to protein destabilization as the wild-type and mutant Pbs2 proteins were present at similar levels (Fig. 2*B*, and data not shown).

Mutation of Hog1 phosphorylation sites resulted in high basal levels and osmotic stress induction of Cek1 phosphorylation. Hence, the effects of mutating the consensus phosphorylation sites of Pbs2 on Cek1 activation were also examined. Similar to $HOG1^{AF}$ cells, $PBS2^{AA}$ cells displayed a high basal level of phosphorylated Cek1, and Cek1 phosphorylation was further induced in $PBS2^{AA}$ cells in response to osmotic stress. In contrast to these findings, cells expressing $PBS2^{DD}$ did not display a high basal level of Cek1 activation, and the osmotic stress-induced activation of Cek1 was significantly attenuated (Fig. 2*D*). These results suggest that $Pbs2^{AA}$ cannot phosphorylate Hog1 and that a basal level of Hog1 phosphorylation was necessary to repress cross-talk to the Cek1 MAPK pathway. Furthermore, the activity of Hog1 present in $PBS2^{DD}$ cells was sufficient to partially inhibit inappropriate activation of Cek1.

Cells lacking *PBS2*, similar to *hog1*Δ cells, form filaments in the absence of a morphogenetic stimulus (Fig. 2*E*). Furthermore, cells expressing $PBS2^{AA}$ showed the same level of filamentation as observed in *pbs2*Δ cells when grown in liquid YPD at 30 °C. As previous results demonstrated that basal levels of Hog1 phosphorylation are essential for the repression of filamentation under noninducing conditions (Fig. 1*E*), the filamentation phenotype exhibited by $PBS2^{AA}$ cells is consistent with the conclusion that $Pbs2^{AA}$ cannot phosphorylate Hog1. In contrast, no filamentation was observed in $PBS2^{DD}$ cells, and cell morphology was indistinguishable to that of wild-type cells (Fig. 2*E*).

Expression of $PBS2^{DD}$ Results in Stress Induction, but Not High Basal Levels, of Hog1 Phosphorylation in C. albicans—To directly investigate whether the consensus phosphorylation sites, Ser-355 and Thr-359, of Pbs2 are important for stress-induced phosphorylation of Hog1, cells expressing *PBS2-HM*, $PBS2^{AA}$-*HM*, and $PBS2^{DD}$-*HM* were exposed to osmotic stress (0.6 M KCl), and Hog1 activation was determined (12). No osmotic stress-induced Hog1 phosphorylation was detected in $PBS2^{AA}$-*HM* cells, which is consistent with the increased sensitivity to osmotic stress exhibited by this strain. It was predicted that Hog1 would be constitutively phosphorylated in $PBS2^{DD}$-*HM* cells, as induced expression of $PBS2^{DD}$ from the *GAL* promoter in *S. cerevisiae* or expression of $wis1^{DD}$ in *S. pombe* results in a high basal level of SAPK phosphorylation (28, 29). However, in contrast, the basal level of Hog1 phosphorylation was not increased in *C. albicans* cells expressing $PBS2^{DD}$-*HM* (Fig. 3*A*). Furthermore, an unexpected osmotic stress-induced increase in Hog1 phosphorylation was observed

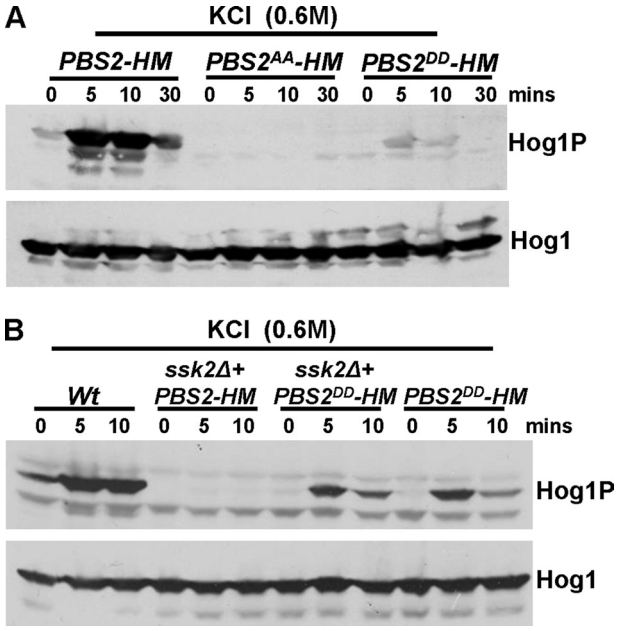

FIGURE 3. **Osmotic stress-induced activation of Hog1 is observed in $PBS2^{DD}$ cells, and this is independent of Ssk2.** *A,* expression of $PBS2^{AA}$ but not $PBS2^{DD}$ abolishes osmotic stress-induced Hog1 phosphorylation. Western blot analysis of whole cell extracts isolated from *PBS2-HM* (JC112), $PBS2^{AA}$-*HM* (JC126), and $PBS2^{DD}$-*HM* (JC124) cells after treatment with 0.6 M KCl 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 (Hog1P). Total levels of Hog1 protein were determined by stripping and reprobing the blot with an anti-Hog1 antibody, which recognizes both phosphorylated and unphosphorylated forms of Hog1 (Hog1). *B,* $Pbs2^{DD}$-mediated Hog1 activation is independent of Ssk2. Western blot analysis of whole cell extracts isolated from wild-type (JC112) and $PBS2^{DD}$-*HM* (JC124) cells and *ssk2*Δ cells expressing either *PBS2-HM* (JC517) or $PBS2^{DD}$-*HM* (JC507) after treatment with 0.6 M KCl for the specified times. Phosphorylated Hog1 (Hog1P) and total Hog1 (Hog1) levels were determined as described above.

in *C. albicans* $PBS2^{DD}$ cells (Fig. 3*A*). The level of Hog1 phosphorylation was significantly lower than that of wild-type cells, which probably underlies the intermediate stress-sensitive phenotype exhibited by $PBS2^{DD}$ cells (Fig. 2*C*). However, this contrasts to studies of $Wis1^{DD}$ in *S. pombe*, as no stress-induced Sty1 phosphorylation was observed in response to osmotic stress in $wis1^{DD+}$ cells (28). These data are indicative of a mechanism of Hog1 activation that is independent of MAPKKK-mediated phosphorylation of Pbs2 in *C. albicans*. To directly test this, *PBS2-HM* and $PBS2^{DD}$-*HM* were expressed in *C. albicans* cells lacking the Ssk2 MAPKKK. As illustrated in Fig. 3*B*, expression of $PBS2^{DD}$-*HM*, but not *PBS2-HM*, stimulated osmotic stress-induced phosphorylation of Hog1 in *ssk2*Δ cells. Furthermore, the level of $Pbs2^{DD}$-dependent stress-induced Hog1 activation was indistinguishable between wild-type and *ssk2*Δ cells. Collectively, these experiments confirm the presence of a MAPKKK-independent mechanism of stress-induced Hog1 activation in *C. albicans*.

*Induction of $PBS2^{DD}$ in pbs2*Δ *Cells Allows for Stress-induced Phosphorylation of Hog1 and Expression of Hog1-dependent Genes*—One possibility underlying the lack of Hog1 phosphorylation in *C. albicans* cells expressing $PBS2^{DD}$ is that an adaptation mechanism is induced to prevent constitutive activation of Hog1. To investigate this, *pbs2*Δ strains were constructed expressing *PBS2-HM*, $PBS2^{AA}$-*HM*, or $PBS2^{DD}$-*HM*

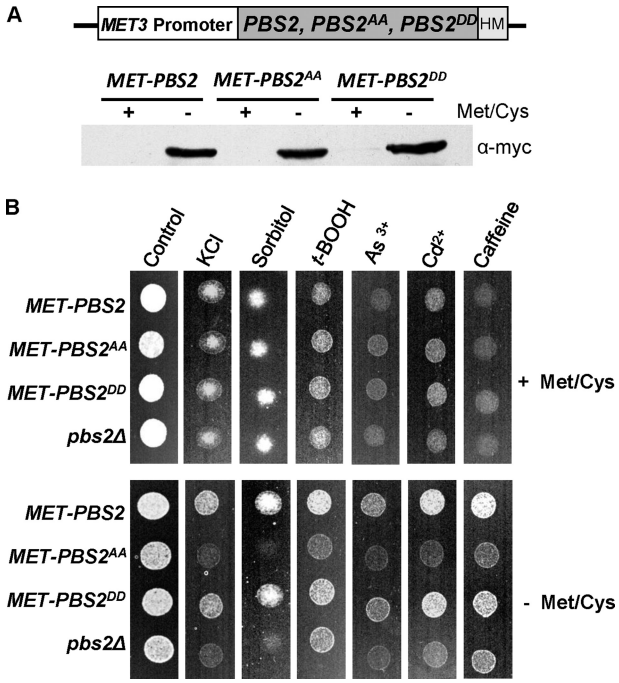

FIGURE 4. **Inducible expression of *PBS2* alleles from the *MET3* promoter results in similar stress-related phenotypes as cells expressing native promoter-driven *PBS2* alleles.** *A*, schematic diagram of the *MET3* promoter-driven His_6-Myc-tagged *PBS2*, *PBS2*^{AA}, and *PBS2*^{DD} alleles (*top panel*). *pbs2*Δ cells expressing *MET3*-driven *PBS2-HM* (*MET-PBS2*, JC167), *PBS2*^{AA}*-HM* (*MET-PBS2*^{AA}, JC174), and *PBS2*^{DD}*-HM* (*MET-PBS2*^{DD}, JC181) were grown in SD minimal media containing methionine and cysteine (+), or lacking these amino acids (−), for 7 h and harvested. The His_6-Myc-tagged Pbs2 proteins were precipitated from cell extracts using Ni^{2+}-NTA-agarose and detected by Western blot using an anti-Myc antibody. *B,* stress-resistant phenotypes of cells expressing *MET3*-driven *PBS2* alleles. Approximately 10^3 cells of exponentially growing *pbs2*Δ cells (JC74) and *pbs2*Δ cells expressing *MET-PBS2* (JC167), *MET-PBS2*^{AA} (JC174), and *MET-PBS2*^{DD} (JC181) were spotted onto SD plates supplemented or not with methionine and cysteine and containing the indicated compounds: KCl (0.6 M), sorbitol (1.2 M), *t*-BOOH (0.75 mM), $AsNaO_2$ (1 mM), $CdSO_4$ (0.4 mM), and caffeine (8 mM). Plates were incubated at 30 °C for 36 h.

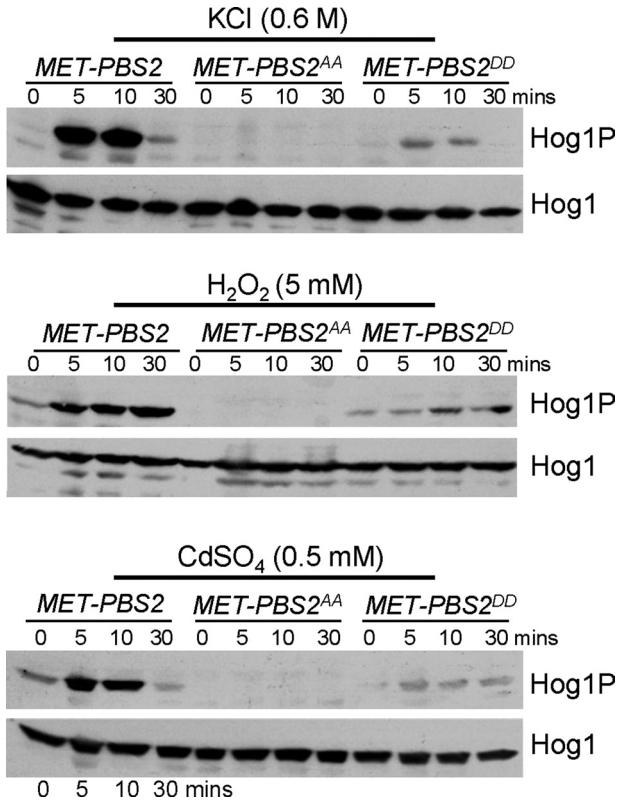

FIGURE 5. **Inducible expression of *PBS2*^{DD} from the *MET3* promoter results in stress-induced activation of Hog1.** Western blot analysis of whole cell extracts from *pbs2*Δ cells expressing *MET-PBS2* (JC167), *MET-PBS2*^{AA} (JC174), and *MET-PBS2*^{DD} (JC181), grown in SD media minus methionine and cysteine for 11 h, and subsequently treated with either KCl (0.6 M), H_2O_2 (5 mM), or $CdSO_4$ (0.5 mM), for the indicated times. Phosphorylated Hog1 (Hog1P) and total Hog1 (Hog1) levels were determined as described in Fig. 3*A* legend.

alleles from the regulatable *MET3* promoter (Fig. 4*A*), which is inhibited by the presence of methionine (Met) and cysteine (Cys) in the growth media (35). This resulted in a collection of strains in which the expression of wild-type and mutant *PBS2* alleles could be controlled. As expected, expression of wild-type or mutant Pbs2 kinases was repressed in media supplemented with Met and Cys, whereas similar expression levels of Pbs2, Pbs2^{AA}, and Pbs2^{DD} were observed in media lacking Met and Cys (Fig. 4*A*). Subsequently, the sensitivity of cells expressing *pMET3*-driven *PBS2* alleles to a range of stress-inducing compounds was examined on media supplemented or not with Met and Cys. When the *MET3* promoter was repressed, all strains were phenotypically identical to *pbs2*Δ cells (Fig. 4*B*). Moreover, when the *MET3* promoter was induced, cells expressing *PBS2*^{AA} maintained an identical phenotype to that of *pbs2*Δ cells, similar to that seen upon expressing *PBS2*^{AA} from its endogenous promoter (Fig. 2*C*). In contrast, cells expressing *PBS2*^{DD} displayed wild-type levels of stress resistance (Fig. 4*B*). As induced expression of *PBS2*^{DD} from the *MET3* promoter resulted in similar phenotypes to cells expressing native-promoter driven *PBS2*^{DD}, this indicates that the viability of *PBS2*^{DD} cells is not a result of adaptation mechanisms preventing the constitutive activation of Hog1.

However, as adaptation mechanisms were possibly evoked during growth on plates, cells containing the p*MET3*-driven *PBS2* alleles were freshly diluted into media lacking Met and Cys, grown until *PBS2* expression was evident, and then treated with either osmotic stress (0.6 M KCl), oxidative stress (5 mM H_2O_2), or heavy metal stress (0.5 mM $CdSO_4$). Hog1 phosphorylation was stimulated in cells expressing p*MET3-PBS2* in response to osmotic, oxidative, and heavy metal stress, and this was abolished in cells expressing p*MET3-PBS2*^{AA}. Increased basal levels of Hog1 phosphorylation were not observed at any time following induction of p*MET3*-driven *PBS2*^{DD} (Fig. 5 and data not shown), although low levels of stress-induced Hog1 phosphorylation were clearly evident in response to osmotic stress and heavy metal stress and, albeit to a lesser extent, oxidative stress (Fig. 5). Thus, the distinct MAPKKK-independent mechanism of osmotic stress-induced Hog1 activation observed in cells expressing *PBS2*^{DD} from its native promoter (Fig. 3) is reproduced in cells expressing *pMET3*-driven *PBS2*^{DD}. Significantly, such a mechanism is also evoked in response to heavy metal stress and oxidative stress (Fig. 5).

To determine the impact of mutating the consensus phosphorylation sites of Pbs2 on Hog1-dependent gene expression, mRNA levels of the Hog1 target genes, *GPD2*, *RHR2*, and *STL1* (14), were examined in *pbs2*Δ cells and cells expressing the p*MET3*-driven *PBS2* alleles. Consistent with the Hog1 phos-

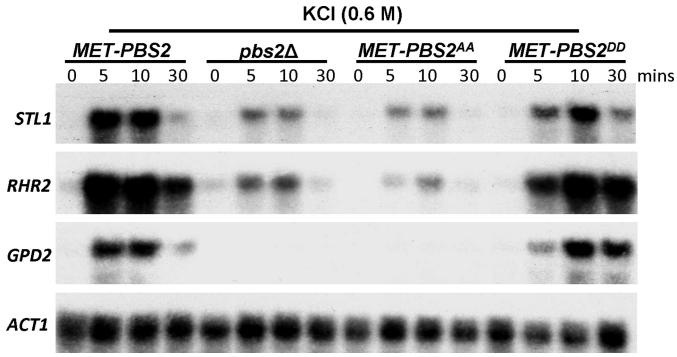

FIGURE 6. **Stress-induced activation of Hog1-dependent genes is observed in *PBS2*^{DD} cells.** Northern blot analysis of RNA isolated from *pbs2*Δ cells (JC74) and *pbs2*Δ cells expressing *MET-PBS2* (JC167), *MET-PBS2*^{AA} (JC174), and *MET-PBS2*^{DD} (JC181), grown in SD media minus methionine and cysteine for 11 h, and subsequently treated with KCl (0.6 M) for the indicated times. Probes specific for the Hog1-dependent genes *GPD2*, *RHR2,* and *STL1* were used, with *ACT1* as a loading control.

phorylation profiles, osmotic stress induction of *GPD2*, *RHR2*, and *STL1* mRNA levels was significantly impaired in *pbs2*Δ cells or in cells expressing p*MET3-PBS2*^{AA} (Fig. 6). In contrast, significant levels of stress-induced gene expression were observed in cells expressing p*MET3-PBS2*^{DD}, although the kinetics of induction was slightly delayed compared with cells expressing wild-type *PBS2* (Fig. 6). This significant induction of *GPD2*, *RHR2,* and *STL1* mRNA is consistent with wild-type levels of stress resistance seen in cells expressing p*MET3-PBS2*^{DD}. However, p*MET3*-driven expression of *PBS2*^{DD} does not result in a detectable higher basal level of these Hog1-dependent genes, which is consistent with the inability to detect constitutive Hog1 phosphorylation in this strain background.

Taken together, these results demonstrate that p*MET3*-driven *PBS2* can restore Hog1 phosphorylation in *pbs2*Δ cells and, moreover, that mutation of the predicted phosphorylation sites of Pbs2 (Ser-355/Thr-359) to nonphosphorylatable alanine residues abolishes stress signaling to Hog1. However, the fact that high basal levels of Hog1 activation were not observed in cells upon induction of p*MET3*-driven *PBS2*^{DD} indicates that the lack of Hog1 phosphorylation seen in cells constitutively expressing *PBS2*^{DD} is not due to adaptation mechanisms that circumvent constitutive activation of Hog1. Nonetheless, the stress-induced phosphorylation of Hog1 in cells expressing p*MET3*-driven *PBS2*^{DD} substantiates and extends earlier findings (Fig. 3) that a MAPKKK-independent mechanism of Hog1 activation operates in response to osmotic and other stress conditions.

Expression of PBS2^{DD}*, but Not wis1*^{DD}*, in S. pombe Allows Osmotic Stress-induced Phosphorylation of Sty1*—In contrast to *C. albicans PBS2*^{DD} cells, the analogous *S. pombe wis1*^{DD} mutant exhibits a high basal level of SAPK phosphorylation that is not further induced in response to osmotic stress (28). Hence, to investigate the basis for these differences in signaling, we examined whether expression of *C. albicans PBS2*^{DD} in *S. pombe* would behave similarly to *wis1*^{DD} cells. *C. albicans PBS2* and *PBS2*^{DD} were expressed in *wis1*$^{-}$ cells from the thiamine-repressible medium strength *nmt41*$^{+}$ promoter (37). In the absence of thiamine, ectopic expression of either *PBS2* or *PBS2*^{DD} rescued the increased sensitivities of *wis1*$^{-}$ cells to osmotic and oxidative stresses (Fig. 7*A*) and also the *wis1*$^{-}$-associated cell cycle defect (Fig. 7*B*). Interestingly, however, expression of both *C. albicans PBS2* and *PBS2*^{DD} stimulated high basal levels of Sty1 phosphorylation, even more so than in cells expressing *wis1*^{DD} (Fig. 7*C*). Although the reason for this is unknown, a likely cause could be due to elevated levels of expression of the *nmt*-driven *PBS2* alleles compared with endogenous *wis1*$^{+}$ levels. Alternatively, the *PBS2* allele contains four CUG codons, which are translated as leucine residues in *C. albicans* but as serine residues in *S. pombe,* and these substitutions may impact on the activity of the kinase. Strikingly, however, in contrast to expression of *wis1*^{DD}, a small but reproducible osmotic stress-induced increase in Sty1 phosphorylation was observed upon expression of *C. albicans PBS2*^{DD} (Fig. 7*C*). These data are consistent with a model where the underlying *PBS2*-dependent MAPKKK-independent mechanism of osmotic stress-induced SAPK activation is present in both *C. albicans* and *S. pombe* but that Wis1 has not retained the ability to respond to this control.

Overexpression of a Truncated SSK2 Allele, Lacking the N-terminal Noncatalytic Domain, Results in Constitutively Active Hog1—As expression of *PBS2*^{DD} did not result in constitutive activation of Hog1 in *C. albicans*, we explored other strategies to constitutively activate the Hog1 SAPK. MAPKKKs have previously been demonstrated to be constitutively active upon removing the N-terminal noncatalytic domain. For example, overexpression of an N-terminally truncated *S. cerevisiae* Ssk2 MAPKKK from the *GAL* promoter resulted in a high basal level of Hog1 phosphorylation and consequent lethality (22). Likewise, overexpression from the *nmt1*$^{+}$ promoter of a similarly truncated Wik1 MAPKKK in *S. pombe* resulted in high levels of Sty1 phosphorylation in the absence of stress and lethality (45). In this regard, it is interesting that although expression of *wis1*^{DD} from its native promoter and *wik1*Δ*N* from the strong *nmt1*$^{+}$ promoter resulted in increases in the basal level of Sty1 phosphorylation, only *S. pombe* cells overexpressing *wik1*Δ*N* exhibited a lethal phenotype. Hence, we created *C. albicans ssk2*Δ null strains in which the Ssk2 MAPKKK lacking the N-terminal domain (Met-1079 to the C terminus of Ssk2) was expressed from the weak *ACT1* promoter (38) or the strong *PGK1* promoter.[4] Strikingly, only when *SSK2*Δ*N* was expressed from the strong *PGK1* promoter was a high level of Hog1 phosphorylation in the absence of stress observed (Fig. 8*A*). This indicates that although the *SSK2*Δ*N* construct is active, high levels of expression are necessary to drive increases in the basal levels of Hog1 phosphorylation. Notably, although the Ssk2ΔN kinase lacks the N-terminal regulatory domain, osmotic stress-induced activation of Hog1 occurred in cells expressing both the *ACT1*- and *PGK1*-promoter driven *SSK2*Δ*N* constructs, albeit to a greater extent in cells expressing *PGK1*-promoter driven *SSK2*Δ*N* (Fig. 8*A*). A similar pattern of stress induction was seen following oxidative stress imposed by 5 mM H_2O_2 (data not shown). The differences in the magnitude of Hog1 phosphorylation observed in cells expressing either pACT1- or pPGK1-*SSK2*Δ*N* impacted on both stress resistance and cell

[4] J. Quinn, unpublished data.

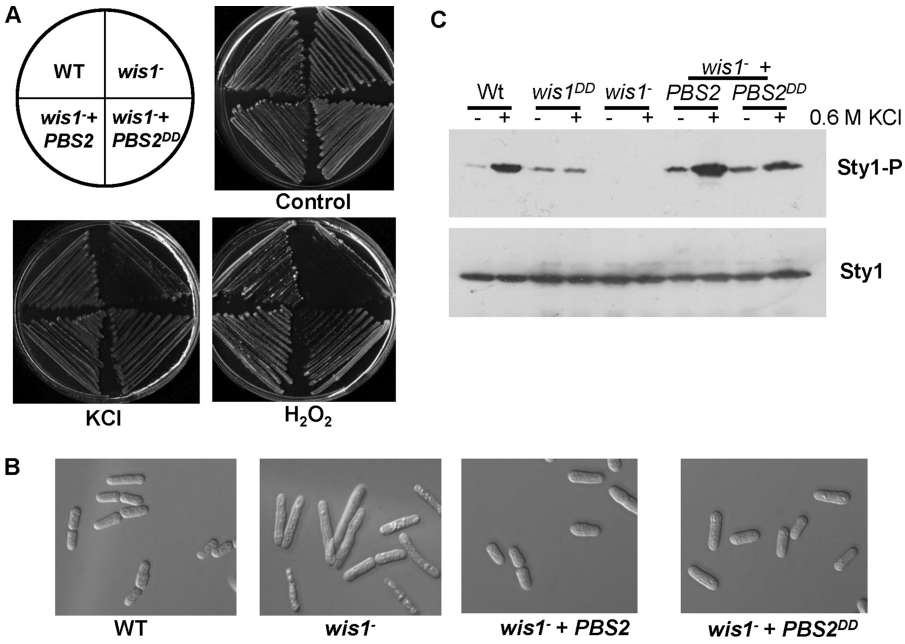

FIGURE 7. **Expression of *C. albicans PBS2* or *PBS2^{DD}* in *S. pombe* rescues *wis1*$^{-}$-associated phenotypes.** *wis1*$^{-}$ cells were transformed with either pREP41HM-*PBS2* or pREP41HM-*PBS2^{DD}* in which the expression of the *C. albicans PBS2* alleles is under the control of the fission yeast thiamine-repressible *nmt41* promoter. *A,* expression of either *PBS2* or *PBS2^{DD}* complements *wis1*$^{-}$-associated stress phenotypes. Wild-type cells (CHP428), *wis1*$^{-}$ cells (GD1682), and *wis1*$^{-}$ cells transformed with pREP41HM-*PBS2* and pREP41HM-*PBS2^{DD}* were streaked onto EMM plates containing 0.6 M KCl or 1 mM H_2O_2. Plates were incubated at 30 °C for 5 days. *B,* expression of *PBS2* or *PBS2^{DD}* rescues the *wis1*$^{-}$-associated cell cycle defect. *wis1*$^{-}$ cells (GD1682), transformed with pREP41HM-*PBS2* or pREP41HM-*PBS2^{DD}*, were grown in the absence of thiamine, and differential interference contrast images were captured. *C,* Western blot analysis of whole cell extracts isolated from wild-type (CHP428), *wis1^{DD}* (KS2088), and *wis1*$^{-}$ (GD1682) cells, all of which express Sty1 tagged with an HA epitope, and *wis1*$^{-}$ (GD1682) cells transformed with pREP41HM-*PBS2* and pREP41HM-*PBS2^{DD}* before and after treatment with 0.6 M KCl for 10 min. Western blots were probed with an anti-phospho-p38 antibody, which only recognizes the phosphorylated active form of *S. pombe* Sty1 (Sty1-P). Total levels of Sty1 protein was determined by stripping and reprobing the blot with an anti-HA antibody that recognizes both phosphorylated and unphosphorylated forms of Sty1 (Sty1).

morphology. For example, expression of *SSK2*Δ*N* from the *PGK1* promoter rescued the osmotic and oxidative stress phenotypes of *ssk2*Δ cells (Fig. 8*B*) and reversed the filamentous phenotype associated with loss of *SSK2* (Fig. 8*C*). In contrast, expression of *SSK2*Δ*N* from the *ACT1* promoter only partially rescued the stress sensitivities and morphological defects exhibited by *ssk2*Δ cells (Fig. 8, *B* and *C*). Taken together, these results illustrate that *C. albicans* Hog1 can be constitutively activated but only following overexpression of the C-terminal catalytic domain of the Ssk2 MAPKKK. Moreover, stress induction of Hog1 occurs independently of the N-terminal noncatalytic domain of the Ssk2 kinase.

Hog1 and Pbs2 Phosphorylation Mutants Display Attenuated Virulence in a Mouse Model of Systemic Disease—A previous study demonstrated that cells lacking *HOG1* displayed significantly attenuated virulence in the standard murine model of systemic candidiasis (1). However, since this study was published, it has been established that the genomic location of the *URA3* disruption marker can influence expression levels and subsequent *URA3*-encoded OMP decarboxylase activity, which impacts on virulence (47). Although care was taken in the original Hog1 virulence analysis to avoid *ura3* auxotrophs, the genomic location of *URA3* within the *hog1*Δ mutant and control strains differed (1). Hence, to confirm that the *hog1*Δ-associated virulence defect reported previously was due to loss of Hog1 and not levels of *URA3* expression, isogenic *hog1*Δ (JC50) and *hog1*Δ+*HOG1* (JC52) strains, which both expressed *URA3* from the *RPS10* locus (12), were retested in the murine model of systemic candidiasis. Consistent with the previous finding, deletion of *HOG1* significantly attenuated virulence as illustrated by the 100% survival rate of mice infected with *hog1*Δ cells over the 28-day experiment (Fig. 9). Conversely, a 100% mortality rate was seen 15 days post-infection with the reintegrant *hog1*Δ+*HOG1* strain (Fig. 9). Similar mortality was seen with the parental wild-type strain RM1000, also expressing *URA3* from the *RPS10* locus. Subsequently, the *hog1*Δ and *hog1*Δ+*HOG1* strains were also assessed using the 3-day murine intravenous challenge model of *C. albicans* infection (41, 42). This model eliminates the need for standard 28-day survival experiments, and it combines weight loss and kidney fungal burden measurements following 3 days of infection to give an "outcome score." A higher outcome score is indicative of greater weight loss and higher fungal burdens and thus virulence. Three days after intravenous challenge, mice infected with the *hog1*Δ (JC50) cells had gained weight (1.4 ± 1.3%) and had a kidney fungal burden of 2.6 ± 0.3 $\log_{10}$ CFU/g, although the group infected with *hog1*Δ+*HOG1* (JC52) reintegrant cells had lost weight (−1.8 ± 2.9%) and had a much higher kidney fungal burden of 4.3 ± 0.5 $\log_{10}$ CFU/g. This resulted in outcome scores for the *hog1*Δ mutant and the *hog1*Δ+*HOG1* reintegrant of 1.9 ± 0.7 and 5.2 ± 1.7, respectively (Table 3). This corresponds to mean survival times between 9.5 ± 5.8 days (reintegrant) and 28.0 ± 0.0 days (*hog1*Δ mutant) (41, 42). Statistical analysis revealed that for all parameters, weight loss, kidney fungal burden, and outcome score, the difference between *hog1*Δ cells and reintegrant was significant ($p < 0.05$). Taken together, these data illustrate that the *hog1*Δ mutant is clearly attenuated in virulence and that the 3-day infection

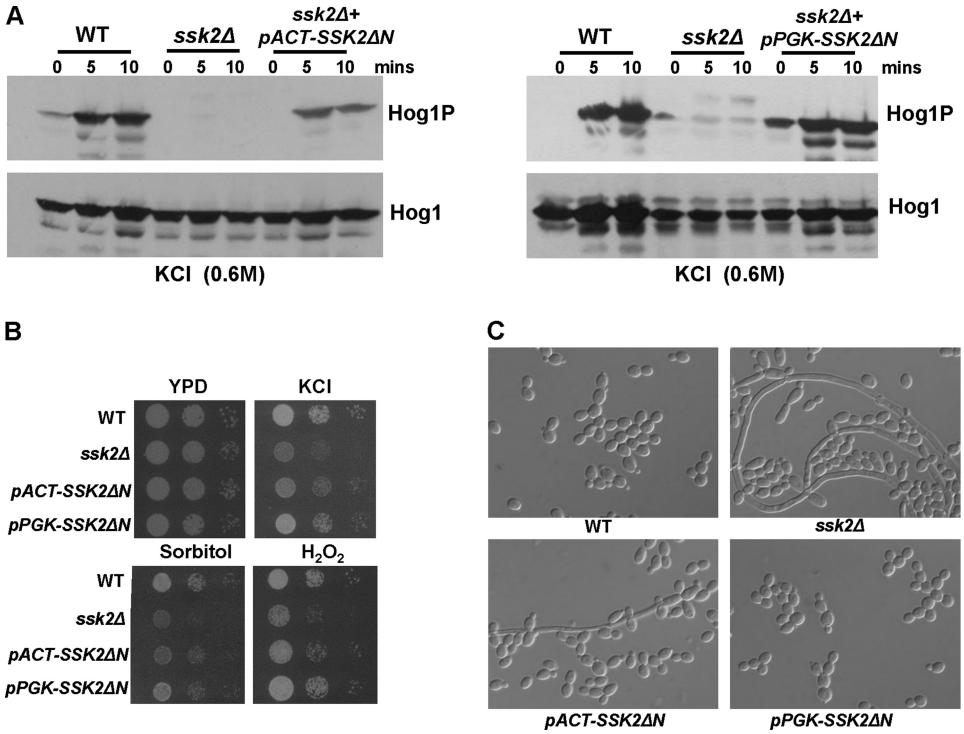

FIGURE 8. **Activation of Hog1 by overexpressing an N-terminal truncated form of Ssk2.** *A, PGK1* promoter-driven *SSK2ΔN* expression, but not *ACT1* promoter-driven *SSK2ΔN* expression, results in a high basal level of Hog1 phosphorylation. Western blot analysis of whole cell extracts from WT cells (JC64), *ssk2*Δ cells (JC1614 and JC1618), and *ssk2*Δ cells expressing pACT1-*SSK2ΔN* (JC1616) or pPGK1-*SSK2ΔN* (JC1620), before and following treatment with KCl (0.6 M) for the indicated times. Phosphorylated Hog1 (Hog1P) and total Hog1 (Hog1) levels were determined as described in Fig. 3A legend. *B,* stress-resistant phenotypes of *ssk2*Δ cells expressing ACT1 and PGK1 promoter-driven *SSK2ΔN* alleles. Approximately 10^4 cells, and 10-fold dilutions thereof, of exponentially growing WT (JC64), *ssk2*Δ cells (JC1614), and *ssk2*Δ cells expressing pACT1-*SSK2ΔN* (JC1616) or pPGK1-*SSK2ΔN* (JC1620) were spotted onto YPD plates containing the indicated compounds and incubated at 30 °C for 24 h. *C, PGK1* promoter-driven *SSK2ΔN* but not *ACT1*-driven *SSK2ΔN* expression rescues the filamentous phenotype exhibited by *ssk2*Δ cells under non hyphae-inducing conditions. Micrographs illustrate the morphology of wild-type (JC64), *ssk2*Δ (JC1614), and *ssk2*Δ cells expressing pACT1-*SSK2ΔN* (JC1616) or pPGK1-*SSK2ΔN* (JC1620) grown in YPD at 30 °C.

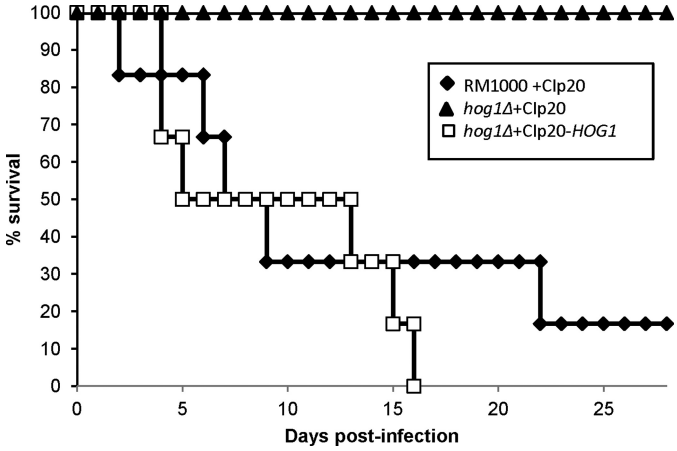

FIGURE 9. **Hog1 is essential for virulence in a mouse model of systemic candidiasis.** Survival rates of mice after intravenous challenge with wild-type (*Wt*, JC21), *hog1*Δ (JC50), and *hog1*Δ+*HOG1* (JC52) cells are shown. All strains express *URA3* from the *RPS10* locus.

model recapitulates findings with the standard 28-day infection model.

Next, the 3-day infection model was employed to assess the role of phosphorylation of Hog1 and Pbs2 in mediating *C. albicans* virulence. To assess the role of phosphorylation of Hog1, cells expressing *HOG1-ProtA* (JC80) and *HOG1^{AF}-ProtA* (JC76), in which the genomic location of *URA3* is identical (Table 1), were tested. As illustrated in Table 3, infection with cells expressing *HOG1^{AF}-ProtA* resulted in a lower outcome score (1.3 ± 0.7) compared with cells expressing *HOG1-ProtA* (3.5 ± 1.7). Statistical analysis showed that the kidney burdens were statistically different ($p < 0.01$) and that the outcome score was also statistically different ($p < 0.05$). This indicates that phosphorylation of Hog1 on the conserved activating Thr-174 and Tyr-176 sites is important in mediating *C. albicans* virulence, and it is consistent with data generated in this study demonstrating that *HOG1^{AF}* cells are phenotypically identical to *hog1*Δ cells with regard to stress sensitivity and morphology (Fig. 1). To test the role of phosphorylation of Pbs2, cells expressing *PBS2-HM* (JC112), *PBS2^{AA}-HM* (JC124), and *PBS2^{DD}-HM* (JC126) were also assessed in the 3-day infection model. As before, the genomic location of *URA3* is identical (Table 1). Notably, mutation of the conserved phosphorylation sites of Pbs2 to either the nonphosphorylatable alanine (*PBS2^{AA}-HM*) or the phosphomimetic aspartic acid (*PBS2^{DD}-HM*) resulted in similar weight changes and kidney fungal burdens and thus had similar outcome scores of 1.2 ± 1.4 and 1.5 ± 1.5, respectively (Table 4). Indeed, data analysis revealed no statistical difference for all three parameters as follows: weight loss, kidney fungal burden, and outcome score. In contrast, injection of mice with *C. albicans* expressing wild-type *PBS2-HM* resulted in greater weight loss and increased kidney fungal burdens (Table 4) and thus a higher outcome score (5.7 ± 0.6) to that observed with either the *PBS2^{AA}* or *PBS2^{DD}* mutants. Statistical analysis revealed that the weight change,

TABLE 3
Effect of deleting Hog1, or mutating Hog1 phosphorylation sites, on experimental infection outcome

Strain	Kidney burden (log 10 CFU/g)	% Weight change	Outcome score
*hog1*Δ(JC50)	2.6 ± 0.3	1.4 ± 1.3	1.9 ± 0.7
*hog1*Δ +*HOG1* (JC52)	4.3 ± 0.5	−1.8 ± 2.9	5.2 ± 1.7
HOG1^{AF}-*ProtA* (JC76)	2.4 ± 0.7	2.2 ± 1.1	1.3 ± 0.7
HOG1-ProtA (JC80)	3.8 ± 0.5	0.6 ± 3.0	3.5 ± 1.7

TABLE 4
Effect of mutating Pbs2 phosphorylation sites on experimental infection outcome

Strain	Kidney burden (log^{10} CFU/g)	% Weight change	Outcome score
PBS2-HM (JC112)	4.5 ± 0.5	−2.2 ± 1.2	5.7 ± 0.6
PBS2^{DD}-*HM* (JC124)	2.4 ± 0.7	2.4 ± 1.4	1.2 ± 1.4
PBS2^{AA}-*HM* (JC126)	2.5 ± 0.5	2.1 ± 2.9	1.5 ± 1.5

kidney burdens, and outcome score recorded for *PBS2-HM* cells were significantly different than those obtained for *PBS2*^{AA}-*HM* cells ($p < 0.01$) and *PBS2*^{DD}-*HM* cells (kidney burdens and outcome score $p < 0.01$; weight change $p < 0.05$). By comparison with the infection outcome scores and mean survival times found for the *hog1*Δ mutant, these data suggest that mice infected with either the *PBS2*^{AA} or *PBS2*^{DD} mutant would have significantly longer survival times compared with mice infected with cells expressing *PBS2*. The impaired virulence exhibited by *PBS2*^{AA}-*HM* cells is entirely consistent with the observation that stress signaling to Hog1 is abolished in this strain background (Figs. 2 and 3). The equally attenuated virulence exhibited by *PBS2*^{DD}-*HM* cells, which exhibit impaired stress-induced activation of Hog1 and stress resistance, but normal morphology (Figs. 2 and 3), is particularly significant for two reasons. First, this indicates that robust activation of Hog1 and wild-type levels of stress resistance are required for *C. albicans* survival in the host. Second, the Hog1-associated virulence defect is likely due to impaired stress responses rather than defects in morphogenesis.

DISCUSSION

Here, we have examined the roles of Hog1 and Pbs2 phosphorylation in Hog1-mediated processes in the major fungal pathogen of humans, *C. albicans*. This analysis has uncovered a previously uncharacterized MAPKKK-independent mechanism of osmotic stress-induced SAPK activation and has provided evidence that Hog1-mediated stress resistance, rather than repression of morphogenesis, underlies the role of Hog1 in mediating *C. albicans* virulence.

Mutation of the consensus phosphorylation sites of the *C. albicans* Hog1 SAPK and the Pbs2 MAPKK to nonphosphorylatable residues resulted in strains that displayed equivalent stress-sensitive phenotypes to those exhibited by *hog1*Δ and *pbs2*Δ null mutants (Figs. 1 and 2). Furthermore, basal and stress-induced phosphorylation of Hog1 was abolished in cells expressing the nonphosphorylatable *PBS2*^{AA} allele (Figs. 3 and 5). These results indicate that phosphorylation of these sites is essential for SAPK-mediated signaling in *C. albicans* and is consistent with previous studies in *S. cerevisiae* (21, 48) and *S. pombe* (26, 28). Moreover, *C. albicans* cells expressing the nonphosphorylatable Pbs2^{AA} and Hog1^{AF} kinases resulted in high basal levels and osmotic stress-induced phosphorylation of the Cek1 MAPK. This extends similar findings reported with *hog1*Δ and *pbs2*Δ mutants (17, 43, 44), by revealing that Hog1 phosphorylation is required to prevent inappropriate activation of the Cek1 pathway. This is reminiscent of studies in *S. cerevisiae* in which it was shown that Hog1 requires the conserved phosphorylation sites to prevent cross-talk to the Cek1-related Kss1/Fus3 MAPKs (27). In addition to inappropriate Cek1 phosphorylation, further analysis of the *C. albicans HOG1*^{AF} and *PBS2*^{AA} mutants revealed that a basal level of Hog1 phosphorylation is also essential to prevent the filamentation that occurs in *hog1*Δ and *pbs2*Δ strains under noninducing conditions (Figs. 1 and 2). The mechanism underlying this is unclear but would appear to be independent of cross-talk to the Cek1 pathway (43). Taken together, these results demonstrate that phosphorylation of the consensus Thr-174 and Tyr-176 phosphorylation sites of the *C. albicans* Hog1 SAPK is essential for stress signaling, prevention of cross-talk to the Cek1 MAPK pathway, and repression of filamentation under noninducing conditions.

In contrast to findings in model yeast systems, mutation of the consensus phosphorylation sites of the *C. albicans* Pbs2 MAPKK to phosphomimetic aspartic acid residues (Pbs2^{DD}) failed to activate the Hog1 SAPK under basal conditions. In *S. cerevisiae*, expression of the equivalent *PBS2*^{DD} mutant is lethal due to hyperactivation of Hog1 under nonstress conditions (29), although it should be noted that *PBS2*^{DD} was expressed from the strong inducible *GAL* promoter in this study. However, in *S. pombe*, the Sty1 SAPK is activated in the absence of stress in cells expressing the analogous Wis1^{DD} mutant MAPKK from its native promoter (28). Why *C. albicans* Hog1 is not hyperactivated upon expressing *PBS2*^{DD} is unclear. It is possible that substitution of the conserved phosphorylation sites of *C. albicans* Pbs2 to aspartic acid residues simply does not mimic the phosphoserine and phosphothreonine residues necessary to activate the MAPKK or that the Pbs2^{DD} kinase is not expressed at sufficiently high levels from its native promoter to trigger Hog1 phosphorylation under basal conditions (see below). Alternatively, such mutations may affect scaffolding functions of the Pbs2 kinase, although in this regard it is noteworthy that the Hog1-Pbs2 interaction is maintained in *C. albicans* cells expressing *PBS2*^{DD} and that the Sho1 and Ste11 proteins (which interact with Pbs2 in *S. cerevisiae*) do not relay osmotic stress signals to Pbs2 in *C. albicans* (44). Nonetheless, it is evident that the Pbs2^{DD} protein retains some activity. For

example, in contrast to *C. albicans* cells expressing *PBS2*^{AA}, *PBS2*^{DD} cells are more resistant to stress than *pbs2*Δ cells and do not form filaments under basal conditions (Fig. 2). In addition, expression of *PBS2*^{DD} rescues the impaired stress resistance and morphological defects exhibited by *C. albicans* cells lacking the upstream Ssk2 MAPKKK (20), thus confirming that Ssk2 phosphorylates the consensus sites on Pbs2 to mediate Hog1 signaling. Based on the lethal phenotype exhibited by *S. cerevisiae PBS2*^{DD} cells (29), we reasoned that *C. albicans* may trigger adaptation mechanisms to prevent constitutive activation of Hog1 upon expression of *PBS2*^{DD}. However, induction of *PBS2*^{DD} from a regulatable promoter still failed to stimulate Hog1 phosphorylation under nonstressed conditions (Fig. 5). Hence, the lack of Hog1 phosphorylation seen in *PBS2*^{DD} cells is unlikely to be a result of adaptation of the strain to inhibit hyperactivation of Hog1. Taken together, these data support a mechanism in which phosphorylation of Pbs2 on the consensus sites Ser-355 and Thr-359, although essential, is not sufficient in this organism, when expressed from its native promoter or the *MET3* promoter, to result in significant levels of Hog1 activation.

Because of the inability of Pbs2^{DD} to activate Hog1 under basal conditions in *C. albicans*, we employed a second strategy previously shown in both *S. cerevisiae* and *S. pombe* to result in constitutive SAPK activation. This involved expressing the C-terminal catalytic domain of the Ssk2 MAPKKK, lacking the N-terminal noncatalytic domain (Ssk2ΔN). In both *S. cerevisiae* and *S. pombe*, expression of such MAPKKK catalytic domains from regulatable strong promoters resulted in high basal levels of SAPK activation and lethality (22, 45). Significantly, in *C. albicans,* expression of Ssk2ΔN only resulted in high basal levels of Hog1 phosphorylation when expressed from the strong *PGK1* promoter, which is often used to overexpress genes in yeast (49). In contrast, expression from the weak constitutive *ACT1* promoter failed to induce Hog1 phosphorylation under nonstressed conditions (Fig. 8). Thus, these data, taken together with that from overexpression studies of *PBS2*^{DD} and *SSK2ΔN* in *S. cerevisiae* and *wik1ΔN* in *S. pombe,* strongly indicate that the level of the "constitutively active" kinase impacts on downstream signaling to the respective SAPK. Hence, the levels of Pbs2^{DD} present in the heterozygous *C. albicans pbs2*Δ/*PBS2*^{DD} mutant generated in this study may simply not have been sufficient to trigger constitutive Hog1 phosphorylation in *C. albicans*.

Nonetheless, despite the lack of basal Hog1 phosphorylation in Pbs2^{DD} cells, stress-induced increases in Hog1 phosphorylation were observed (Figs. 3 and 5). This is indicative of a MAPKKK-independent mechanism of Hog1 activation as mutation of the Ssk2 target phosphorylation sites on Pbs2 to phosphomimetic residues did not abolish stress-induced phosphorylation of Hog1. Significantly, the osmotic stress-induced Hog1 activation observed upon expressing *PBS2*^{DD} was maintained in cells lacking *SSK2*, thus confirming that such a mechanism of Hog1 activation was MAPKKK-independent. Furthermore, stress-induced activation of Hog1 in *PBS2*^{DD} cells was observed in response to osmotic, oxidative, and heavy metal stress and thus may constitute a general MAPKKK-independent mechanism of Hog1 activation. It should be noted, however, that this mechanism still requires a basal level of Pbs2 activity, as Hog1 is not activated at all in *pbs2*Δ or *PBS2*^{AA} cells after osmotic, oxidative, or heavy metal stress. It is also clear that the level of stress-induced Hog1 phosphorylation observed in *C. albicans PBS2*^{DD} cells is greatly diminished compared with that in wild-type cells (Figs. 3 and 5). Nonetheless, this is sufficient to induce significant levels of Hog1-dependent gene expression (Fig. 6) and stress resistance (Figs. 2 and 4). Such findings are noteworthy as MAPKKK-independent activation of fungal SAPKs in response to osmotic, oxidative, and heavy metal stress has not previously been documented. Indeed, the observation that expression of *C. albicans* Pbs2^{DD}, but not the analogous *S. pombe* Wis1^{DD} kinase, in *S. pombe* triggers osmotic stress-induced activation of Sty1 (Fig. 7*C*) indicates that Wis1 has lost the ability to respond to this MAPKKK-independent mechanism of osmotic stress-induced SAPK activation. Instead, heat stress, but not oxidative or osmotic stress, has been shown previously to induce Sty1 activation in *wis1*^{DD} cells (28). Moreover, the major mechanism of Sty1 activation upon heat shock was found to be mediated by heat stress-induced inhibition of the interaction between Sty1 and the Pyp1 tyrosine phosphatase, which dephosphorylates and inactivates Sty1 (30). Although heat stress inhibits rather than activates the Hog1 SAPK in *C. albicans* (12), it was feasible that other stresses may inhibit the action of phosphotyrosine phosphatases on Hog1 resulting in a MAPKKK-independent mechanism of activation. In *S. pombe*, heat stress results in a significant and sustained increase in the amount of Tyr-173-phosphorylated Sty1 (30). Upon measuring Hog1 activation in *C. albicans,* we routinely use an anti-phospho-P38 antibody, which only recognizes the dually phosphorylated SAPK in yeast (30). Hence, to address whether stress-induced inhibition of the tyrosine phosphatases that dephosphorylate Hog1 occurs in *C. albicans*, we examined Tyr-176 phosphorylation of Hog1 using an anti-phosphotyrosine antibody. However, stress-induced increases in Tyr-phosphorylated Hog1 were only detected in wild-type cells and not in Pbs2^{DD} cells following osmotic stress (supplemental Fig. 1). This indicates that stress-induced inhibition of tyrosine phosphatases is unlikely to be the mechanism underlying MAPKKK-independent Hog1 regulation in *C. albicans.* Nonetheless, it remains possible that stress-induced inhibition of the type 2C protein phosphatases (PP2Cs), predicted to regulate dephosphorylation of Hog1 Thr-174, underlies the observed MAPKKK-independent Hog1 activation. However, although seven genes encoding PP2Cs have been identified in the *C. albicans* genome (50), those implicated in Hog1 regulation have yet to be characterized, and thus investigating this possibility was deemed outside the scope of this study. In an alternative mechanism, independent of phosphatase inhibition, exposure to stress may increase the activity of the Pbs2^{DD} kinase via a MAPKKK-independent mechanism. Unfortunately, extensive attempts to establish an *in vitro* kinase assay to measure the activity of Pbs2 and Pbs2^{DD} kinases precipitated from cells were unsuccessful. Furthermore, although a slight decrease in the mobility of Pbs2 on SDS-PAGE was observed following stress treatment, no stress-induced effects on the mobility of Pbs2^{DD} were observed (data not shown). Hence, the mechanism underlying the MAPKKK-independent activation of the

C. albicans Hog1 SAPK remains to be determined, but it is unlikely to involve inhibition of the phosphotyrosine phosphatases as previously characterized in *S. pombe*.

It is noteworthy that, similar to $Pbs2^{DD}$ cells, cells expressing a truncated version of the Ssk2 MAPKKK lacking the N-terminal noncatalytic domain also resulted in stress-inducible Hog1 phosphorylation in *C. albicans* (Fig. 8). In *S. cerevisiae*, binding of the response regulator Ssk1 to a region of Ssk2 within the N-terminal catalytic domain was found necessary to disrupt the autoinhibitory action of this domain (51). Furthermore, osmotic stress-induced phosphorylation of Ssk2 was inhibited if Ssk1 binding was prevented (51). In *C. albicans,* Ssk2 is the sole MAPKKK that phosphorylates the Pbs2 MAPKK (20). Ssk1 is required for oxidative stress-induced activation of Hog1 (52), and although Ssk1 likely regulates osmotic stress-induced regulation of Hog1 in *C. albicans* (the Ssk1-binding site identified in *S. cerevisiae* Ssk2 is conserved), it does so in parallel with an as yet undefined pathway (44). Hence, the fact that stress-induced activation of Hog1 is retained in *C. albicans ssk2*Δ cells expressing only the catalytic domain of Ssk2 may reflect activation of Ssk2 in a novel Ssk1-independent mechanism, or alternatively could be due to the MAPKKK-independent regulation of Hog1 characterized in this paper. Further experimentation is needed to dissect these possibilities.

Previous work illustrated that Hog1 is essential for the virulence of *C. albicans,* and this was confirmed in this study (Fig. 9). Furthermore, cells expressing the nonphosphorylatable and inactive $HOG1^{AF}$ and $PBS2^{AA}$ alleles displayed attenuated virulence, which is consistent with loss of function of the pathway. However, like the *hog1*Δ and *pbs2*Δ null mutants, cells expressing $HOG1^{AF}$ and $PBS2^{AA}$ display deregulated hyphae formation and inappropriate activation of the Cek1 MAPK, in addition to impaired stress responses. These pleiotropic phenotypes make it impossible to dissect which of the Hog1-mediated processes are essential for virulence. Strikingly, however, $PBS2^{DD}$ cells that display impaired stress-induced activation of Hog1 and stress resistance, but not morphological defects or high basal levels of Cek1 phosphorylation, demonstrated equally impaired virulence as that exhibited by $PBS2^{AA}$ cells. Significantly, therefore, this analysis provides the first evidence that the role of Pbs2-Hog1 signaling in virulence is likely related to its role in regulating stress resistance rather than morphogenesis. Moreover, as Hog1 activation is attenuated but not abolished in cells expressing $PBS2^{DD}$, this analysis also suggests that robust activation of Hog1 is necessary for survival of *C. albicans* within the host.

During evolution, *C. albicans* has retained many of the stress regulatory molecules that have been elucidated experimentally in *S. cerevisiae* and *S. pombe,* and yet it is more resistant to stresses such as osmotic and oxidative stress compared with these benign cousins (53). This study provides evidence that one such conserved stress-signaling module, the Hog1 SAPK pathway, is regulated by a MAPKKK-independent mechanism in *C. albicans* in a manner that has not been reported in *S. cerevisiae* and *S. pombe,* and moreover, it demonstrates that the full virulence of *C. albicans* depends upon its ability to mount robust responses to environmental stresses. As robust stress responses are central to the virulence of this major fungal pathogen of humans, further investigation into *C. albicans* stress-signaling mechanisms is clearly warranted.

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