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 (Thr-174/Tyr-176) and Pbs2 (Ser-355/Thr-359), to non-

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

Received for publication, May 26, 2011, and in revised form, October 4, 2011 Published, JBC Papers in Press, October 12, 2011, DOI 10.1074/jbc.M111.265231

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

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 hog1Δ and pbs2Δ cells. Consistent with this, stress-induced phosphorylation of Hog1 was abolished in cells expressing non-phosphorylatable Pbs2 (Pbs2AA). However, mutation of the consensus sites of Pbs2 to phosphomimetic residues (Pbs2DD) failed to constitutively activate Hog1. Furthermore, Ssk2-independent stress-induced Hog1 activation was observed in Pbs2DD cells. Collectively, these data reveal a previously uncharacterized MAPKKK-independent mechanism of Hog1 activation in response to stress. Although Pbs2DD 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 Pbs2AA and Pbs2DD cells displayed impaired stress resistance and attenuated virulence in a mouse model of disease, whereas only Pbs2AA 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, hog1Δ 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 MAPKKK 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

---

*This work was supported by the Biotechnology and Biological Sciences Research Council and the Wellcome Trust.

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; Ni2+-NTA, Ni2+-nitrilotriacetic acid.
known as Spc1 or Phh1) and Wts1 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 (28, 29). Although this proved lethal in S. cerevisiae, sub- sequent analysis of S. pombe wts1-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 hog1Δ 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 previ- ously (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 Clp-C-ProtA-CyC (34). The resulting Clp-C-HOG1ProtA plasmid was linearized by digestion with Sty1 to target chromosomal integration at the remaining HOG1 locus in hog1/HOG1 cells (IC36) to subsequently generate strain IC80. Chromosomal insertion of the protein A tag was confirmed by PCR and DNA sequencing. Mutagenesis of HOG1 to create HOG1TT74A,Y176F was performed by PCR using oligonucleotides HOG1TAYF and HOG1SalIF. The resulting PCR fragment was digested with Sall and cloned into Clp-C- HOG1ProtA previously digested with Sall to remove the wild-

---

**TABLE 1**

| Strains used in this study | Relevant genotype | Source |
|---------------------------|------------------|--------|
| **C. albicans**           |                  |        |
| RM1000                    | ura3::λimm434/ura3::λimm434, his1::hisG/his1::hisG | This work |
| BWP17                     | ura3::λimm434/ura3::λimm434, his1::hisG/his1::hisG, arg4::arg4::hisG | This work |
| IC21                      | RM1000 Clp20     | 12     |
| IC64                      | BWP17 Clp30      | 12     |
| IC50                      | RM1000 hog1::loxP-ura3-loxP/hog1::loxP-HIS1-loxP Clp20-HOG1 | 12     |
| IC52                      | RM1000 hog1::loxP-ura3-loxP/hog1::loxP-HIS1-loxP Clp20 | 12     |
| IC36                      | BWP17 hog1::loxP-ARG4-loxP/HOG1 | 46     |
| IC154                     | BWP17 hog1::loxP-ARG4-ura3-loxP/hog1::loxP-HIS1-loxP Clp20 | 14     |
| IC76                      | BWP17 hog1::loxP-ARG4-loxP/HOG1AF-ProtA-URA3 | This work |
| IC80                      | BWP17 hog1::loxP-ARG4-loxP/HOG1AF-ProtA-URA3 | This work |
| IC72                      | BWP17 PBS2::loxP-HIS1-loxP/PBS2 | 20     |
| IC74                      | BWP17 PBS2::loxP-ARG4-loxP/PBS2::loxP-HIS1-loxP | 20     |
| IC112                     | BWP17 PBS2::loxP-HIS1-loxP/PBS2::HM-URA3 | 20     |
| IC124                     | BWP17 PBS2::loxP-HIS1-loxP/PBS2::HM-URA3 | This work |
| IC126                     | BWP17 PBS2::loxP-HIS1-loxP/PBS2::HM-URA3 | This work |
| IC167                     | BWP17 PBS2::loxP-ARG4-ura3-loxP/PBS2::HM-URA3 | This work |
| IC174                     | BWP17 PBS2::loxP-ARG4-ura3-loxP/PBS2::HM-URA3 | This work |
| IC181                     | BWP17 PBS2::loxP-ARG4-ura3-loxP/PBS2::HM-URA3 | This work |
| IC482                     | BWP17 ssk2::loxP-ARG4-loxP/skk2::loxP-HIS1-loxP | 20     |
| IC507                     | BWP17 ssk2::loxP-ARG4-loxP/skk2::loxP-HIS1-loxP, PBS2::HM-URA3 | 20     |
| IC517                     | BWP17 ssk2::loxP-ARG4-loxP/skk2::loxP-HIS1-loxP, PBS::HM-URA3 | 20     |
| IC1614                    | BWP17 ssk2::loxP-ARG4-loxP/skk2::loxP-HIS1-loxP, PBS::HM-URA3 | This work |
| IC1616                    | BWP17 ssk2::loxP-ARG4-loxP/skk2::loxP-HIS1-loxP, PBS::HM-URA3 | This work |
| IC1618                    | BWP17 ssk2::loxP-ARG4-loxP/skk2::loxP-HIS1-loxP, PBS::HM-URA3 | This work |
| IC1620                    | BWP17 ssk2::loxP-ARG4-loxP/skk2::loxP-HIS1-loxP, PBS::HM-URA3 | This work |

**S. pombe**

| Strains | Genotype | Source |
|---------|----------|--------|
| CHP428  | his7Δ::366 adel6-210 lex1-32 ura4::D18 | Gift from C. Hoffman |
| ML1221  | sty1(HA6His):ura4+ | 24     |
| GD1682  | wts1::ura4+ sty(HA6His):ura4+ | 28     |
| KS2088  | wts1::loxP(12myc):ura4+ sty1(HA6His):ura4+ | 28     |
TABLE 2

| Oligonucleotide   | Sequence 5’–3’                                                                 |
|-------------------|--------------------------------------------------------------------------------|
| CaHOG1PrF         | ctgtgttcgccccaacagggactgcctgcagaaagacctaacggagtctctgagctgcagagactttt        |
| CaHOG1PrR         | ttcttcgcagagcctccggcggcgcagcagaaagacctaacggagtctctgagctgcagagactttt        |
| HOG1TAYF          | ccaccactgtcatgaggttttgatttctgctcttcgagatctacttggtgctgcttcgagctgcagagactttt|
| HOG1SalF          | gcacgcgtcgacgaaacatgagaatttgattaccttgg                                      |
| PBS2TagR          | gcggacgatcactaaatgtctaggtagcgagctaatgctacttcc                                |
| PBS2AA            | gcggacgatcactaaatgtctaggtagcgagctaatgctacttcc                                |
| PBS2PstB          | gcacgcgtcgacgaaacatgagaatttgattaccttgg                                      |
| CaPBS2FLF         | gcacgcgtcgacgaaacatgagaatttgattaccttgg                                      |
| CaPBS2FLR         | gcacgcgtcgacgaaacatgagaatttgattaccttgg                                      |
| SSK2NACTF         | gcgcggatcccttagttgatatgtttcttgctctcc                                      |
| SSK2NACTR         | gcgcggatcccttagttgatatgtttcttgctctcc                                      |
| CaPBS2DD         | gcacgcgtcgacgaaacatgagaatttgattaccttgg                                      |
| PGKpromm          | gcacgcgtcgacgaaacatgagaatttgattaccttgg                                      |
| PGKpromR          | gcacgcgtcgacgaaacatgagaatttgattaccttgg                                      |
| STL1F             | gtgtgttgtagatcatcagg                                                        |
| STL1R             | ataacaccccccaacactagcag                                                    |
| RHR2F             | gacaagactacaaacacag                                                      |
| RHR2R             | cttggaatctgtagtcc                                                      |
| GP2F              | cttggaatctgtagtcc                                                      |
| GP2R              | cttggaatctgtagtcc                                                      |
| ACT1F             | cttggaatctgtagtcc                                                      |
| ACT1R             | cttggaatctgtagtcc                                                      |

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 His6 residues and two copies of the Myc epitope, using the plasmid Clp-C-PBS2, was described previously (20). Mutagenesis of PBS2 to create PBS2DD, was performed by overlapping PCR using the oligonucleotides PBS2TagR, PBS2AA, and PBS2PstB. The resulting PCR fragment was digested with PstI and NdeI and cloned into Clp-C-PBS2HM (20), previously digested with the same enzymes to remove the wild-type sequence. Construction of the plasmid Clp-C-PBS2DDHM was described previously (20). To generate strains expressing His6-Myc-tagged wild-type and mutant PBS2 alleles, the Clp-C-PBS2HM, Clp-C-PBS2DDHM, and Clp-C-PBS2DDDDHM plasmids were linearized by digestion with SgrAI to target integration at the remaining PBS2 locus in PBS29/pbs2Δ (IC72) 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 His6-Myc-tagged Pbs2 proteins were detected as described above.

Expression of PBS2 and PBS2DD in S. pombe—The C. albicans PBS2 and PBS2DD 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 BglII and cloned into the BamHI site of pREP41HM. The resulting plasmids pREP41HM-PBS2 and pREP41HM-PBS2DD 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 Sall sites between the C. albicans ACT1 promoter and S. cerevisiae CYC1 terminator in pACT1 (38) to generate plasmid pACT1-SSK2AN. Both pACT1 and pACT1-SSK2AN were linearized at the Stul restriction site in the RPS10 locus and introduced into ssk2Δ cells (JC482) to create strains JC1614 and JC1616, respectively. In addition, the truncated SSK2 gene plus terminator was amplified from genomic DNA using the oligo...

Type HOG1 sequence. The resulting Clp-C-HOG1AFProtA was linearized with PshA1 to target chromosomal integration at the remaining HOG1 locus in hog1/HOG1 cells (JC36) to generate strain JC76. Levels of Hog1-ProtA and Hog1AFProtA were detected by Western blot analysis of cell extracts prepared from JC76, respectively. Correct integration at the Hog1 locus and introduction into each of the strains JC76, JC124, and JC126, following digestion with SgrAI to target integration at the remaining HOG1 locus in hog1/HOG1 cells (JC36), was described previously (20). To generate strains expressing His-Myc-tagged wild-type and mutant HOG1 alleles, the CIp-C-HOG1AFProtA was transformed into JC112, JC124, and JC126, respectively. To achieve regulatable expression of PBS2 alleles, the CIp-C-PBS2 was linearized at the StuI restriction site in the PBS2 locus and introduced into each of the strains JC76, JC124, and JC126, respectively. The resulting plasmids were digested with BamHI and cloned into the BamHI site of pREP41HM (36), which directs the expression of genes in the thiamine-repressible medium strength RPS10 promoter (37). Hence, strains carrying CiEXP derivatives were grown in minimal SD media in the presence or absence of methionine (2.5 mM) and cysteine (2 mM). Levels of His6-Myc-tagged Pbs2 proteins were detected as described above.

Expression of PBS2 and PBS2DD in S. pombe—The C. albicans PBS2 and PBS2DD 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 BglII and cloned into the BamHI site of pREP41HM. The resulting plasmids pREP41HM-PBS2 and pREP41HM-PBS2DD 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.
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-SSK2ΔN. 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 oligonucleotides PGKpromF and PGKpromR. The result was linearized at the Stul restriction site in the RPS10 locus and introduced into ssk2Δ 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.

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 MgSO4), 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 Axiosvison 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 + Clp20 (JC21), hog1Δ + Clp20 (JC50), and hog1Δ + Clp20-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 × 10^5 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 ~2.6 × 10^5 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. hog1Δ + Clp20 (JC50), hog1Δ + Clp20-HOG1 (JC52), HOG1ProtA (JC80), HOG1^AFProtA (JC76), PBS2HM (JC112), PBS2^AAHM (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 × 10^5 CFU/g body weight. Actual challenge doses were determined from viable counts read 24 h later and were ~2.2 × 10^5 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 hog1Δ 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. 1A). 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
purification of Hog1 and associated proteins. 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 Hog1AF-ProtA-expressing strains (Fig. 1B).

Previous studies revealed that C. albicans hog1/H9004 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/H9004, HOG1-ProtA, and HOG1AF-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 HOG1AF-ProtA displayed equivalent stress-sensitive phenotypes to hog1Δ cells in response to the oxidative stress agent (t-BOOH), osmotic stress agents (sorbitol and KCl), heavy metals (Cd²⁺ and As³⁺), and the purine analog caffeine (Fig. 1C). 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 HOG1AF-ProtA displayed similar increased resistance to Congo red as hog1/H9004 cells (Fig. 1C). 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. 1D, HOG1AF-ProtA cells form filaments under noninducing conditions. Micrographs illustrating the morphology of wild-type (Wt, BWP17), hog1Δ (JC154), HOG1-ProtA (JC80), and HOG1AF-ProtA (JC76) strains grown in YPD at 30°C.

FIGURE 1. HOG1AF cells have comparable phenotypes to hog1Δ 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 Hog1AF. B, mutation of Hog1 phosphorylation sites does not impact on protein stability. Western blot analysis of whole cell extracts isolated from hog1/H9004 cells or cells expressing Hog1-ProtA (JC80) or Hog1AF-ProtA (JC76). Hog1 levels were detected using an anti-Hog1 antibody. C, cells expressing HOG1AF-ProtA display equivalent stress-sensitive or -resistant phenotypes as hog1Δ cells. Approximately 10⁵ cells of exponentially growing wild-type (Wt, BWP17), hog1Δ (JC154), HOG1-ProtA (JC80), and HOG1AF-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), CdSO₄ (0.4 mM), AsNaO₂ (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 hog1Δ and HOG1AF-ProtA cells form filaments under noninducing conditions. Micrographs illustrating the morphology of wild-type (Wt, BWP17), hog1Δ (JC154), HOG1-ProtA (JC80), and HOG1AF-ProtA (JC76) strains grown in YPD at 30°C.
repress inappropriate activation of the Cek1 MAPK pathway and that HOG1AF cells behave like the hog1Δ mutant.

Preceding work also indicated a role for the Hog1 MAPK 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, HOG1AF cells displayed a similar level of filamentation to that exhibited by hog1Δ cells when grown under the non-hyphae-inducing conditions of liquid YPD at 30 °C (Fig. 1E). This indicates that a basal level of Hog1 phosphorylation is also essential to repress the morphogenetic switch under noninducing conditions.

**Cells Expressing PBS2AA, but Not PBS2DD, Phenocopy pbs2Δ Cells**—The activating phosphorylation sites of the MAPKK, which functions upstream of the SAPK, are also highly conserved (Fig. 2A). 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 (Pbs2AA), or with phosphomimetic aspartic acid residues (Pbs2DD) to create a constitutively active Hog1 pathway (Fig. 2A). The wild-type and mutant PBS2-AF 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 using 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-His6-His6-Myc-tagged proteins. C, cells expressing PBS2AA-HM are equally stress-sensitive as pbs2Δ cells, whereas PBS2DD-HM cells display an intermediate stress-resistant phenotype. Approximately 10^4 cells of exponentially growing PBS2-HM (JC112), pbs2Δ (JC74), PBS2AA-HM (JC126), and PBS2DD-HM (JC124) strains were spotted onto YPD plates containing the additives as detailed in Fig. 1 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 pbs2Δ (JC74), PBS2AA-HM (JC126), and PBS2DD-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 Hog1. Levels of Hog1 protein, which provided a loading control, were determined by stripping and reprobing the blot with an anti-Hog1 antibody (Hog1).

**FIGURE 2.** Cells expressing PBS2AA, but not PBS2DD, phenocopy pbs2Δ 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 (Pbs2AA) or aspartic acid (Pbs2DD) residues. B, Pbs2 phosphorylation is not required for interaction with Hog1. Extracts were prepared from mid-log cultures of hog1Δ cells (JC152) and cells expressing His6-Myc-tagged Pbs2 (PBS2-HM, JC112), PBS2AA-HM, JC126), and PBS2DD-PBS2DD-HM, JC124). The His6-Myc-tagged Pbs2 proteins were precipitated using Ni²⁺-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-His6-Myc-tagged proteins. C, cells expressing PBS2AA-HM are equally stress-sensitive as pbs2Δ cells, whereas PBS2DD-HM cells display an intermediate stress-resistant phenotype. Approximately 10^4 cells of exponentially growing PBS2-HM (JC112), pbs2Δ (JC74), PBS2AA-HM (JC126), and PBS2DD-HM (JC124) strains were grown in YPD at 30 °C.
MAPKKK-independent Hog1 Activation in C. albicans

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 PBS2DD is lethal due to hyperactivation of the Hog1 MAPK (29), C. albicans cells expressing PBS2DD from its native promoter are viable. Furthermore, cells expressing PBS2DD-HM exhibited an intermediate stress-sensitive phenotype; cells were more sensitive than the wild-type PBS2-HM strain, but more resistant than PBS2AA-HM cells, to a range of stress-inducing compounds (Fig. 2C). The increased stress sensitivity exhibited by PBS2AA and PBS2DD cells was not due to protein destabilization as the wild-type and mutant Pbs2 proteins were present at similar levels (Fig. 2B, 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 HOGL1 cells, PBS2AA cells displayed a high basal level of phosphorylated Cek1, and Cek1 phosphorylation was further induced in PBS2AA cells in response to osmotic stress. In contrast to these findings, cells expressing PBS2DD did not display a high basal level of Cek1 activation, and the osmotic stress-induced activation of Cek1 was significantly attenuated (Fig. 2D). These results suggest that Pbs2AA 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 PBS2DD cells was sufficient to partially inhibit inappropriate activation of Cek1.

Cells lacking Pbs2, similar to hog1Δ cells, form filaments in the absence of a morphogenetic stimulus (Fig. 2E). Furthermore, cells expressing PBS2AA showed the same level of filamentation as observed in pbs2Δ 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. 1E), the filamentation phenotype exhibited by PBS2AA cells is consistent with the conclusion that Pbs2AA cannot phosphorylate Hog1. In contrast, no filamentation was observed in PBS2DD cells, and cell morphology was indistinguishable to that of wild-type cells (Fig. 2E).

Expression of PBS2DD 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, PBS2AA-HM, and PBS2DD-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 PBS2AA-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 PBS2DD-HM cells, as induced expression of PBS2DD from the GAL promoter in S. cerevisiae or expression of wis1DD 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 PBS2DD-HM (Fig. 3A). Furthermore, an unexpected osmotic stress-induced increase in Hog1 phosphorylation was observed in C. albicans PBS2DD cells (Fig. 3A). The level of Hog1 phosphorylation was significantly lower than that of wild-type cells, which probably underlies the intermediate stress-sensitive phenotype exhibited by PBS2DD cells (Fig. 2C). However, this contrasts to studies of Wis1DD in S. pombe, as no stress-induced Sty1 phosphorylation was observed in response to osmotic stress in wis1DD 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 PBS2DD-HM were expressed in C. albicans cells lacking the Ssk2 MAPKK. As illustrated in Fig. 3B, expression of PBS2DD-HM, but not PBS2-HM, stimulated osmotic stress-induced phosphorylation of Hog1 in ssk2Δ cells. Furthermore, the level of Pbs2DD-dependent stress-induced Hog1 activation was indistinguishable between wild-type and ssk2Δ cells. Collectively, these experiments confirm the presence of a MAPKKK-independent mechanism of stress-induced Hog1 activation in C. albicans.

Induction of PBS2DD in pbs2Δ 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 PBS2DD is that an adaptation mechanism is induced to prevent constitutive activation of Hog1. To investigate this, pbs2Δ strains were constructed expressing PBS2-HM, PBS2AA-HM, or PBS2DD-HM cells.
alleles from the regulatable MET3 promoter (Fig. 4A), 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, Pbs2AA, and Pbs2DD were observed in media lacking Met and Cys (Fig. 4A). 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 pbs2Δ cells (Fig. 4B). Moreover, when the MET3 promoter was induced, cells expressing PBS2AA maintained an identical phenotype to that of pbs2Δ cells, similar to that seen upon expressing PBS2AA from its endogenous promoter (Fig. 2C). In contrast, cells expressing PBS2DD displayed wild-type levels of stress resistance (Fig. 4B).

As induced expression of PBS2DD from the MET3 promoter resulted in similar phenotypes to cells expressing native promoter-driven PBS2DD, this indicates that the viability of PBS2DD 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 pMET3-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 H2O2), or heavy metal stress (0.5 mM CdSO4). Hog1 phosphorylation was stimulated in cells expressing pMET3-PBS2 in response to osmotic, oxidative, and heavy metal stress, and this was abolished in cells expressing pMET3-PBS2AA. Increased basal levels of Hog1 phosphorylation were not observed at any time following induction of pMET3-PBS2 (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 PBS2DD from its native promoter (Fig. 3) is reproduced in cells expressing pMET3-driven PBS2DD. 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, GDP2, RHR2, and STL1 (14), were examined in pbs2Δ cells and cells expressing the pMET3-driven PBS2 alleles. Consistent with the Hog1 phos-
MAPKK-independent Hog1 Activation in C. albicans

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

phorylation profiles, osmotic stress induction of GPD2, RHR2, and STLI mRNA levels was significantly impaired in PBS2Δ cells or in cells expressing pMET3-PBS2^AA (Fig. 6). In contrast, significant levels of stress-induced gene expression were observed in cells expressing pMET3-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 STLI mRNA is consistent with wild-type levels of stress resistance seen in cells expressing pMET3-PBS2^DD. However, pMET3-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 pMET3-driven PBS2 can restore Hog1 phosphorylation in PBS2Δ 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 pMET3-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 pMET3-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^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^DD rescued the increased sensitivities of wis1^− cells to osmotic and oxidative stresses (Fig. 7A) and also the wis1^−-associated cell cycle defect (Fig. 7B). Interestingly, however, expression of both C. albicans PBS2^DD and PBS2^DD stimulated high basal levels of Sty1 phosphorylation, even more so than in cells expressing wis1^DD (Fig. 7C). Although the reason for this is unknown, a likely cause could be due to elevated levels of expression of the nmt-driven PBS2^DD alleles compared with endogenous wis1^+ levels. Alternatively, the PBS2^DD 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. 7C). 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 wik1ΔN from the strong nmt1^+ promoter resulted in increases in the basal level of Sty1 phosphorylation, only S. pombe cells overexpressing wik1ΔN exhibited a lethal phenotype. Hence, we created C. albicans ssk2Δ 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. Strikingly, only when SSK2ΔN was expressed from the strong PGK1 promoter was a high level of Hog1 phosphorylation in the absence of stress observed (Fig. 8A). This indicates that although the SSK2ΔN 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 SSK2ΔN constructs, albeit to a greater extent in cells expressing PGK1-promoter driven SSK2ΔN (Fig. 8A). A similar pattern of stress induction was seen following oxidative stress imposed by 5 mM H2O2 (data not shown). The differences in the magnitude of Hog1 phosphorylation observed in cells expressing either pACT1- or pPGK1-SSK2ΔN impacted on both stress resistance and cell

---

4 J. Quinn, unpublished data.
JOURNAL OF BIOLOGICAL CHEMISTRY

FIGURE 7. Expression of C. albicans PBS2 or PBS2DD in S. pombe rescues wis1- associated phenotypes. wis1- cells were transformed with either pREP41HM-PBS2 or pREP41HM-PBS2DD 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 PBS2DD complements wis1- associated stress phenotypes. Wild-type cells (CHP428), wis1- cells (GD1682), and wis1- cells transformed with pREP41HM-PBS2 and pREP41HM-PBS2DD were streaked onto EMM plates containing 0.6 M KCl or 1 mM H2O2. Plates were incubated at 30 °C for 5 days. B, expression of PBS2 or PBS2DD rescues the wis1- associated cell cycle defect. wis1- cells (GD1682), transformed with pREP41HM-PBS2 or pREP41HM-PBS2DD, 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), wis1DD (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-PBS2DD 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).

Morality. For example, expression of SSK2ΔN from the PGK1 promoter rescued the osmotic and oxidative stress phenotypes of ssk2Δ cells (Fig. 8B) and reversed the filamentous phenotype associated with loss of SSK2 (Fig. 8C). In contrast, expression of SSK2ΔN from the ACT1 promoter only partially rescued the stress sensitivities and morphological defects exhibited by ssk2Δ 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 hog1Δ mutant and control strains differed (1). Hence, to confirm that the hog1Δ- associated virulence defect reported previously was due to loss of Hog1 and not levels of URA3 expression, isogenic hog1Δ (JC50) and hog1Δ + 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 hog1Δ cells over the 28-day experiment (Fig. 9). Conversely, a 100% mortality rate was seen 15 days post-infection with the reintegrant hog1Δ + HOG1 strain (Fig. 9). Similar mortality was seen with the parental wild-type strain RM1000, also expressing URA3 from the RPS10 locus. Subsequently, the hog1Δ and hog1Δ + 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 hog1Δ (JC50) cells had gained weight (1.4 ± 1.3%) and had a kidney fungal burden of 2.6 ± 0.3 log10 CFU/g, although the group infected with hog1Δ + HOG1 (JC52) reintegrant cells had lost weight (−1.8 ± 2.9%) and had a much higher kidney fungal burden of 4.3 ± 0.5 log10 CFU/g. This resulted in outcome scores for the hog1Δ mutant and the hog1Δ + 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 (hog1Δ mutant) (41, 42). Statistical analysis revealed that for all parameters, weight loss, kidney fungal burden, and outcome score, the difference between hog1Δ cells and reintegrant was significant (p < 0.05). Taken together, these data illustrate that the hog1Δ mutant is clearly attenuated in virulence and that the 3-day infection
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 HOG1AF-ProtA (JC76), in which the genomic location of URA3 is identical (Table 1), were tested. As illustrated in Table 3, infection with cells expressing HOG1AF-ProtA resulted in a lower outcome score (1.3/1.1) 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 HOG1AF cells are phenotypically identical to hog1 cells with regard to stress sensitivity and morphology (Fig. 1). To test the role of phosphorylation of Pbs2, cells expressing PBS2-HM (JC112), PBS2AA-HM (JC124), and PBS2DD-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 (PBS2AA-HM) or the phosphomimetic aspartic acid (PBS2DD-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 PBS2AA or PBS2DD mutants. Statistical analysis revealed that the weight change,

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), ssk2Δ cells (JC1614 and JC1618), and ssk2Δ 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 ssk2Δ cells expressing ACT1 and PGK1 promoter-driven SSK2ΔN alleles. Approximately 10⁶ cells, and 10-fold dilutions thereof, of exponentially growing WT (JC64), ssk2Δ cells (JC1614), and ssk2Δ 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 ssk2Δ cells under non hyphae-inducing conditions. Micrographs illustrate the morphology of wild-type (JC64), ssk2Δ (JC1614), and ssk2Δ 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), hog1Δ (JC50), and hog1Δ+HOG1 (JC52) cells are shown. All strains express URA3 from the RPS10 locus.
MAPKKK-independent Hog1 Activation in C. albicans

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 |
|-------------------------|-----------------------------|-----------------|---------------|
| hog1Δ/JC50              | 2.6 ± 0.3                   | 1.4 ± 1.3       | 1.9 ± 0.7     |
| hog1Δ + Hog1/JC52       | 4.3 ± 0.5                   | -1.8 ± 2.9      | 5.2 ± 1.7     |
| HOG1+/+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 (log10 CFU/g) | % Weight change | Outcome score |
|-------------------------|-----------------------------|-----------------|---------------|
| PBS2-HM/JC12            | 4.5 ± 0.5                   | -2.2 ± 1.2      | 5.7 ± 0.6     |
| PBS2DD-HM/JC12          | 2.4 ± 0.7                   | 2.4 ± 1.4       | 1.2 ± 1.4     |
| PBS2AAD-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 PBS2AAD-HM cells (p < 0.01) and PBS2DD-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 hog1Δ mutant, these data suggest that mice injected with either the PBS2AAD or PBS2DD mutant would have significantly longer survival times compared with mice infected with cells expressing PBS2. The impaired virulence exhibited by PBS2AAD-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 PBS2DD-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 hog1Δ and pbs2Δ null mutants (Figs. 1 and 2). Furthermore, basal and stress-induced phosphorylation of Hog1 was abolished in cells expressing the nonphosphorylatable PBS2AAD 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 PBS2AAD and Hog1AAD kinases resulted in high basal levels and osmotic stress-induced phosphorylation of the Cek1 MAPK. This extends similar findings reported with hog1Δ and pbs2Δ 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 HOG1AF and PBS2AAD mutants revealed that a basal level of Hog1 phosphorylation is also essential to prevent the filamentation that occurs in hog1Δ and pbs2Δ 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 (Pbs2DD) failed to activate the Hog1 SAPK under basal conditions. In S. cerevisiae, expression of the equivalent PBS2DD mutant is lethal due to hyperactivation of Hog1 under nonstress conditions (29), although it should be noted that PBS2DD 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 Wis1DD mutant MAPKK from its native promoter (28). Why C. albicans Hog1 is not hyperactivated upon expressing PBS2DD 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 Pbs2DD 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 PBS2DD 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 Pbs2DD protein retains some activity. For
MAPKKK-independent Hog1 Activation in *C. albicans*

example, in contrast to *C. albicans* cells expressing *PBS2*Δ*Δ*, *PBS2*DD cells are more resistant to stress than *pbs2Δ* 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* *pbs2Δ/*PBS2DD 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 phosphoamimetic 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 *pbs2Δ* or *PBS2*DD 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. 7C) 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<sup>DD</sup> 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* ssk2Δ 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 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* ssk2Δ 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 in *C. albicans* 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<sup>AF</sup>* and *PBS2<sup>AA</sup>* alleles displayed attenuated virulence, which is consistent with loss of function of the pathway. However, like the * hog1Δ* and *pbs2Δ* null mutants, cells expressing *HOG1<sup>AF</sup>* and *PBS2<sup>AA</sup>* display deregulated hyphal 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<sup>DD</sup>* 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<sup>AA</sup>* 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<sup>DD</sup>*, 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.

Acknowledgments—We thank Brian Morgan, Al Brown, and Ken Haynes for stimulating discussions and comments on the manuscript and Kazuhiro Shiozaki for the kind gift of *S. pombe* strains.

REFERENCES

1. Alonso-Monge, R., Navarro-García, F., Molero, G., Diez-Orejas, R., Gustin, M., Pla, J., Sánchez, M., and Nombela, C. (1999) J. Bacteriol. 181, 3058–3068
2. da Silva Dantas, A., Patterson, M. J., Smith, D. A., Maccallum, D. M., Erwig, L. P., Morgan, B. A., and Quinn, J. (2010) Mol. Cell. Biol. 30, 4530–4543
3. Wysong, D. R., Christin, L., Sugar, A. M., Robbins, P. W., and Diamond, R. D. (1998) Infect. Immun. 66, 1953–1961
4. Fradin, C., De Grooth, P., MacCallum, D., Schaller, M., Klis, F., Odds, F. C., and Hube, B. (2005) Mol. Microbiol. 56, 397–415
5. Thewes, S., Kretschmar, M., Park, H., Schaller, M., Filler, S. G., and Hube, B. (2007) Mol. Microbiol. 63, 1606–1628
6. Walker, L. A., Maccallum, D. M., Bertram, G., Gow, N. A., Odds, F. C., and Brown, A. J. (2009) Fungal Genet. Biol. 46, 210–219
7. Zakikhany, K., Naglik, J. R., Schmidt-Westhausen, A., Holland, G., Schaller, M., and Hube, B. (2007) Cell. Microbiol. 9, 2938–2945
8. Brown, A. J., Haynes, K., and Quinn, J. (2009) Curr. Opin. Microbiol. 12, 384–391
9. Smith, D. A., Morgan, B. A., and Quinn, J. (2010) FEMS Microbiol. Lett. 306, 1–8
10. Alonso-Monge, R., Navarro-García, F., Román, E., Negredo, A. I., Eisman, B., Nombela, C., and Pla, J. (2003) Eukaryot. Cell 2, 351–361
11. Rodaki, A., Bohovych, I. M., Enjalbert, B., Young, T., Odds, F. C., Gow, N. A., and Brown, A. J. (2009) Mol. Biol. Cell 20, 4845–4855
12. Smith, D. A., Nicholls, S., Morgan, B. A., Brown, A. J., and Quinn, J. (2006) Mol. Biol. Cell 17, 1018–1032
13. Arana, D. M., Alonso-Monge, R., Du, C., Calderone, R., and Pla, J. (2007) Cell. Microbiol. 9, 1647–1659
14. Enjalbert, B., Smith, D. A., Cornell, M. J., Alam, I., Nicholls, S., Brown, A. J., and Quinn, J. (2006) Mol. Biol. Cell 17, 1018–1032
15. Arana, D. M., Nombela, C., Alonso-Monge, R., and Pla, J. (2005) Microbiology 151, 1033–1049
16. San José, C., Monge, R. A., Pérez-Díaz, R., Pla, J., and Nombela, C. (1996) J. Bacteriol. 178, 5850–5852
17. Millar, J. B., Buck, V., and Wilkinson, M. G. (1995) Genes Dev. 9, 2117–2130
18. Cheetham, J., Smith, D. A., da Silva Dantas, A., Doris, K. S., Patterson, M. J., Bruce, C. R., and Quinn, J. (2007) Mol. Biol. Cell 18, 4603–4614
19. Maeda, T., Takekawa, M., and Saito, H. (1995) Science 269, 554–558
20. Posas, F., and Saito, H. (1997) Science 276, 1702–1705
21. Samejima, I., Mackie, S., Warbrick, E., Weisman, R., and Fantes, P. A. (1998) Mol. Cell. Biol. 18, 2325–2335
22. Shiek, J. C., Wilkinson, M. G., and Millar, J. B. (1998) Mol. Cell. Biol. 18, 311–322
23. Ferrigno, P., Posas, F., Koepp, D., Saito, H., and Silver, P. A. (1998) EMBO J. 17, 5606–5614
24. Gaitis, F., Degols, G., Shiozaki, K., and Russell, P. (1998) Genes Dev. 12, 1464–1473
25. O’Rourke, S. M., and Herskowitz, I. (1998) Genes Dev. 12, 2874–2886
26. Shiozaki, K., Shiozaki, M., and Russell, P. (1998) Mol. Cell. Biol. 19, 1339–1349
27. Wurgler-Murphy, S. M., Maeda, T., Witten, E. A., and Saito, H. (1997) Mol. Cell. Biol. 17, 1289–1297
28. Nguyen, A. N., and Shiozaki, K. (1999) Genes Dev. 13, 1653–1663
29. Sherman, F. (1991) Methods Enzymol. 194, 3–21
MAPKKK-independent Hog1 Activation in C. albicans

32. Alfa, C. E., Gallagher, I. M., and Hyams, J. S. (1993) Methods Cell Biol. 37, 201–222
33. Moreno, S., Klar, A., and Nurse, P. (1991) Methods Enzymol. 194, 795–823
34. Blackwell, C., Russell, C. L., Argimon, S., Brown, A. J., and Brown, J. D. (2003) Yeast 20, 1235–1241
35. Care, R. S., Trevethick, J., Binley, K. M., and Sudbery, P. E. (1999) Mol. Microbiol. 34, 792–798
36. Craven, R. A., Griffiths, D. J., Sheldrick, K. S., Randall, R. E., Hagan, I. M., and Carr, A. M. (1998) Gene 221, 59–68
37. Basi, G., Schmid, E., and Maundrell, K. (1993) Gene 123, 131–136
38. Tripathi, G., Wiltshire, C., Macaskill, S., Tournu, H., Budge, S., and Brown, A. J. (2002) EMBO J. 21, 5448–5456
39. Murad, A. M., Lee, P. R., Broadbent, I. D., Barelle, C. J., and Brown, A. J. (2000) Yeast 16, 325–327
40. Navarro-García, F., Eisman, B., Fiuza, S. M., Nombela, C., and Pla, J. (2005) Microbiology 151, 2737–2749
41. MacCallum, D. M., Castillo, L., Nather, K., Munro, C. A., Brown, A. J., Gow, N. A., and Odds, F. C. (2009) Eukaryot. Cell 8, 373–387
42. MacCallum, D. M., Coste, A., Ischer, F., Jacobsen, M. D., Odds, F. C., and Sanglard, D. (2010) Antimicrob. Agents Chemother. 54, 1476–1483
43. Eisman, B., Alonso-Monge, R., Román, E., Arana, D., Nombela, C., and Pla, J. (2006) Eukaryot. Cell 5, 347–358
44. Román, E., Nombela, C., and Pla, J. (2005) Mol. Cell. Biol. 25, 10611–10627
45. Shiozaki, K., Shiozaki, M., and Russell, P. (1997) Mol. Biol. Cell 8, 409–419
46. Wilson, R. B., Davis, D., and Mitchell, A. P. (1999) J. Bacteriol. 181, 1868–1874
47. Brand, A., MacCallum, D. M., Brown, A. J., Gow, N. A., and Odds, F. C. (2004) Eukaryot. Cell 3, 900–909
48. Schülter, C., Brewster, J. L., Alexander, M. R., Gustin, M. C., and Ruis, H. (1994) EMBO J. 13, 4382–4389
49. Piper, P. W., Curran, B., Davies, M. W., Hirst, K., Lockheart, A., Ogden, J. E., Stanway, C. A., Kingsman, A. J., and Kingsman, S. M. (1988) Nucleic Acids Res. 16, 1333–1348
50. Arñó, J., Casamayor, A., and González, A. (2011) Eukaryot. Cell 10, 21–33
51. Posas, F., and Saito, H. (1998) EMBO J. 17, 1385–1394
52. Chauhan, N., Inglis, D., Roman, E., Pla, J., Li, D., Calera, J. A., and Calderone, R. (2003) Eukaryot. Cell 2, 1018–1024
53. Nikolou, E., Agafioti, I., Stumpf, M., Quinn, J., Stansfield, I., and Brown, A. J. (2009) BMC Evol. Biol. 9, 44
54. Negredo, A., Monteoliva, L., Gil, C., Pla, J., and Nombela, C. (1997) Microbiology 143, 297–302