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# A Conserved Stress-activated Protein Kinase Regulates a Core Stress Response in the Human Pathogen *Candida albicans*

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**Previous work has implicated the Hog1 stress-activated protein kinase (SAPK) in osmotic and oxidative stress responses in the human pathogen *Candida albicans*. In this study, we have characterized the role of Hog1 in mediating these and other stress responses in *C. albicans*. We provide evidence that a SAPK-dependent core stress response exists in this pathogen. The Hog1 SAPK is phosphorylated and it accumulates in the nucleus in response to diverse stress conditions. In addition, we have identified Hog1-regulated genes that are induced in response to stress conditions that activate Hog1. These analyses reveal both activator and repressor functions for the Hog1 SAPK. Our results also demonstrate that stress cross-protection, a classical hallmark of the core stress response, occurs in *C. albicans* between stresses that activate the Hog1 SAPK. Importantly, we find that the core stress response in *C. albicans* has adapted to the environmental niche of this human pathogen. This niche specificity is reflected by the specific environmental conditions that drive the Hog1-regulated core stress response in *C. albicans* and by differences in the molecular circuitry that control this response.**

## INTRODUCTION

The ability of cells to sense and respond rapidly to changes in their environment is critical for survival. Pathogenic microbes adapt to changing microenvironments during disease progression and have the added challenge of contending with host defenses. *Candida albicans* is the major systemic fungal pathogen of humans (Odds, 1988). This yeast frequently causes superficial infections in relatively immunocompetent individuals, and life-threatening systemic infections in immunocompromised patients. Its success as a pathogen is based partly upon its resistance to oxidative stresses and other environmental insults. For example, *C. albicans* can evade oxidative killing by macrophages (Lo *et al*., 1997), and inactivating stress responses attenuates virulence (Wysong *et al*., 1998; Alonso-Monge *et al*., 1999). Although these observations suggest that *C. albicans* has evolved specific responses to protect it in the diverse microenvironments it encounters during disease progression in its human host, the molecular mechanisms underlying such responses are poorly understood.

Global approaches have been employed to elucidate the molecular responses of yeast cells to a diverse range of environmental stresses. For example, transcript profiling of the budding yeast *Saccharomyces cerevisiae* (Gasch *et al*., 2000; Causton *et al*., 2001) and the distantly related fission yeast *Schizosaccharomyces pombe* (Chen *et al*., 2003) revealed that a large fraction of their genomes respond in a stereotypical manner to a range of diverse stress conditions. These stereotypical responses have been termed the Environmental Stress Response (ESR) or the Common Environmental Response (CER) in budding yeast (Gasch *et al*., 2000; Causton *et al*., 2001), and the Core Environmental Stress Response (CESR) in fission yeast (Chen *et al*., 2003). These responses are thought to underlie the previously identified general stress response and the phenomenon of stress cross-protection (reviewed in Siderius and Mager, 1997), in which exposure to one type of stress can protect the cell against subsequent exposure to an apparently unrelated stress.

*S. pombe* and *S. cerevisiae* use different strategies to regulate their core stress response genes and this partly involves differential regulation of their stress-activated protein kinase (SAPK) pathways. In *S. pombe*, the CESR is controlled predominantly by the Sty1 SAPK (Chen *et al*., 2003), which is activated in response to diverse stress conditions such as oxidative stress, osmotic stress, temperature upshift, nutrient limitation, DNA damaging agents, and heavy metals (Millar *et al*., 1995; Shiozaki and Russell, 1995, 1996; Degols *et al*., 1996; Degols and Russell, 1997; Shieh *et al*., 1997, 1998; Shiozaki *et al*., 1998; Buck *et al*., 2001). In contrast, the ESR/CER in *S. cerevisiae* is not governed by a single regulatory pathway. Instead, different stresses control a common set of genes via different signaling pathways and transcription factors (Gasch *et al*., 2000; Causton *et al*., 2001). For example, the *S. cerevisiae* Hog1 SAPK, unlike the homologous fission yeast Sty1 SAPK, responds mainly to changes in osmolarity. Hence, in budding yeast, Hog1 plays a role in the regulation of core stress genes in response to this stress (Posas *et al*., 2000; Rep *et al*., 2000; O'Rourke *et al*., 2002; O'Rourke and Herskowitz, 2004), but not other stresses (Gasch *et al*., 2001).

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**Table 1.** Strains used in this study

| Strain | Genotype | Source |
|---|---|---|
| RM1000 | *ura3*::λ*imm434/ura3*::λ*imm434, his1*::*hisG/his1*::*hisG* | Negredo *et al*., 1997 |
| WT | *ura3*::λ*imm434/ura3*::λ*imm434, his1*::*hisG/his1*::*hisG* CIp20 *(URA3, HIS1)* | This study |
| JC4 | *ura3*::λ*imm434/ura3*::λ*imm434, his1*::*hisG/his1*::*hisG, HOG1-HM:URA3 ura3*::λ*imm434/ura3*::λ*imm434, his1*::*hisG/his1*::*hisG, hog1*::*LoxP-URA3-LoxP, hog1*::*LoxP-HIS1-LoxP* | This study |
| JC10 | *ura3*::λ*imm434/ura3*::λ*imm434, his1*::*hisG/his1*::*hisG, hog1*::*LoxP-URA3-LoxP, hog1*::*LoxP-HIS1-LoxP* | This study |
| JC50 | *ura3*::λ*imm434/ura3*::λ*imm434, his1*::*hisG/his1*::*hisG, hog1*::*LoxP-ura3-LoxP, hog1*::*LoxP-HIS1-LoxP* CIp20 *(URA3, HIS1)* | This study |
| JC52 | *ura3*::λ*imm434/ura3*::λ*imm434, his1*::*hisG/his1*::*hisG, hog1*::*LoxP-ura3-LoxP, hog1*::*LoxP-HIS1-LoxP* CIp20-*HOG1(URA3, HIS1)* | This study |
| JC63 | *ura3*::λ*imm434/ura3*::λ*imm434, his1*::*hisG/his1*::*hisG, HOG1-YFP:URA3 HOG1-YFP:HIS1* | This study |
| CHP428 | *h*$^+$ *his7-366 ade6-210 leu1-32 ura4-D18* | Gift from Charlie Hoffman |
| JM1160 | *h*$^-$ *sty1*::*ura4*$^+$ *leu1-32 ura4-D18* | Millar *et al*., 1995 |

Although less is known about stress responses in *C. albicans*, these responses are clearly important for the survival of this pathogen in its mammalian host, because deletion of the *C. albicans* Hog1 SAPK results in impaired virulence (Alonso-Monge *et al*., 1999). Recent studies have shown that there are clear differences between the stress responses of this yeast and those of *S. cerevisiae* and *S. pombe*. For example, transcript profiling revealed that *C. albicans* does not mount a common transcriptional response after exposure to environmental conditions that stimulate general stress responses in *S. cerevisiae* and *S. pombe* (Enjalbert *et al*., 2003). Furthermore, homologues of the *S. cerevisiae* Msn2/4 transcription factors, which play a key role in regulating the ESR/CER in *S. cerevisiae*, have no obvious roles in stress responses in *C. albicans* (Nicholls *et al*., 2004).

In this study, we demonstrate for the first time, that *C. albicans* mounts a specialized core stress response that has evolved to fit the environmental niches occupied by this fungal pathogen. Significantly, we find that the *C. albicans* Hog1 SAPK plays a pivotal role in the regulation of this core stress response.

## MATERIALS AND METHODS

### *Strains and Growth Conditions*

The strains used in this study are given in Table 1. *C. albicans* strains were grown in either rich YPD medium or SD minimal medium (Sherman, 1991), and *S. pombe* strains were grown in either rich YE5S medium or EMM synthetic minimal medium as described previously (Moreno *et al*., 1991; Alfa *et al*., 1993). All strains were grown at 30°C unless indicated otherwise.

### *Strain Construction*

Oligonucleotide primers used in this study are listed in Table 2. To delete *HOG1*, disruption cassettes comprising of either the *URA3* or *HIS1* gene flanked by *loxP* sites and 80 base pairs of DNA sequence corresponding to regions of the *HOG1* open reading frame (ORF) were generated by polymerase chain reaction (PCR) by using the oligonucleotide primers HOGdelF and HOGdelR and the plasmid templates pLUL2 or pLHL2, respectively (Dennison, Ramsdale, Manson, and Brown, unpublished data). Disruption cassettes were transformed into *C. albicans* RM1000 to sequentially disrupt both alleles of *HOG1* and generate strain JC10. Accurate gene disruption was confirmed by PCR and DNA sequencing. Introduction of these cassettes resulted in the deletion of codons 45–359 of the 411 codon *HOG1* ORF. To reintroduce *HOG1* into JC10, the *HOG1* locus was PCR amplified using the oligonucleotide primers HOG1PromF and HOG1TermR and cloned into the *URA3*- and *HIS1*-containing integrating plasmid CIp20, a derivative of CIp10 (Murad *et al*., 2000). CIp20 and CIp20-HOG1 were integrated at the *RPS10* locus in a 5-fluoroorotic acid-resistant derivative of JC10, to create strains JC50 and JC52, respectively. To tag Hog1 at the C terminus with 6-His residues and two

**Table 2.** Oligonucleotide primers used in this study

| Oligonucleotide | Sequence 5′–3′ |
|---|---|
| HOGdelF | caccaatagatacactgagctaaatcccgtgggaatgggagcatttggtttggtgtgctcagccgttgatagattaactgccagggttttcccagtcacg |
| HOGdelR | gccacaccaacagtttgatgaaagtctaaaatttcactgtacatcataactctccaagtatccactggcaagtctgcgtcctcactaaagggaacaaaagc |
| HOG1PstF | aatgtctgcagatggagaatttacaagaacc |
| HOG1PromF | gcgcggatccgacatttccgttaaagtgtccac |
| HOG1TermR | gcgcggatccggggaaacagtgaatatgtaaaatgtg |
| HOG1HMR | gaattcgctagcttaatgatggtgatgatggtgtaagtcctcctcgctgatcaaatttttgttcttcagccatggacaaatcttcttcagaaattaacttttgctcctcagctccgttggcggaatccaag |
| HOG1YFPF | aatgaaactgagggttccgaacaaccagacctgcaagtggagcaaaacaacttggattccgccaacggagctggtggtggttctaaaggtgaagaattatt |
| HOG1YFPR | gttaatagtaatagtaatacatatttcacttttaaatttatttctataattgctagcttgtatttttgaagctctagaaggaccacctttgattg |
| HOG1BamF | gcgcggatcccatgtctgcagatggagaatttacaagaacc |
| HOG1BamR | gcgcggatccttaagctccgttggcggaatcc |
| HSP12-F | atgtctgacgccggaag |
| HSP12-R | agtactatcagcgccgg |
| RHR2-F | gacaaagactcaacaaccag |
| RHR2-R | ccttgaattcgtcagtttcc |
| ACT1-F | gatgaagcccaatccaaaag |
| ACT1-R | ggagttgaaagtggtttggt |

copies of the myc-epitope, the *HOG1* gene was amplified by PCR by using the oligonucleotide primers HOG1PstF and HOG1HMR and ligated into CIp-C-ZZ (Blackwell *et al.*, 2003), which had been digested with *Pst*I and *Nhe*I to remove the TEV-protein A sequence. The resulting CIp-C-HOG1HM plasmid was linearized by digestion with *Hpa*I to target chromosomal integration at the *HOG1* locus in *C. albicans* RM1000 to generate strain JC4. Chromosomal insertion of the C-terminal $His_6$-myc tag was confirmed by PCR and DNA sequencing. To chromosomally tag Hog1 with YFP, *HOG1*-specific sequences were added to the universal primer sequences described previously (Gerami-Nejad *et al.*, 2001) to generate the oligonucleotide primers HOG1YFPF and HOG1YFPR. These primers were used in combination with the plasmid templates pYFP-URA3 and pYFP-HIS1 to generate *HOG1-YFP* cassettes by PCR (Gerami-Nejad *et al.*, 2001). The *HOG1-YFP* cassettes were sequentially transformed into *C. albicans* RM1000, to create strain JC63, and correct integration at both *HOG1* loci was confirmed by PCR and DNA sequencing.

### Plasmid Construction

To create pREP41MH-HOG1, the *C. albicans HOG1* gene was amplified by PCR from genomic DNA by using the oligonucleotide primers HOG1BamF and HOG1BamR and ligated into the *Bam*HI site of pREP41MH (Craven *et al.*, 1998).

### Stress Sensitivity Tests

For stress sensitivity assays, strains to be tested were grown at 30°C to mid-exponential phase. Cells were diluted in YPD media, and $10^3$ cells were spotted in 5 μl onto YPD agar containing the specific compound at the indicated concentration. Plates were incubated at 30°C for 24 h, unless indicated otherwise.

For stress cross-protection analysis, exponential cultures of wild-type (WT), Δ*hog1* (JC10), and Δ*hog1*+*HOG1* (JC52) cells were pretreated for 1 h with the following stress conditions: untreated control, oxidative stress (0.4 mM $H_2O_2$), osmotic stress (0.3 M NaCl), or heat stress (temperature shift from 23 to 37°C). Cells were collected by centrifugation and washed once in prewarmed YPD before resuspension in an equal volume of YPD. Aliquots (10 ml) were then incubated with increasing concentrations of $H_2O_2$. Cells were taken after 1 h, diluted, and plated onto YPD plates to determine surviving cell numbers. Plates were incubated for 24 h at 30°C, and survival was expressed as a percentage of viable cells at time = 0.

### Hog1 Phosphorylation Assays

Strains containing chromosomally tagged Hog1-$His_6$-myc (JC4) were grown to mid-exponential phase and exposed to a range of stress conditions for the indicated times. Hog1-$His_6$-myc was partially purified from cell extracts using $Ni^{2+}$-NTA agarose (QIAGEN, Valencia, CA), and phosphorylated Hog1 was detected by Western blot with an anti-phospho-p38 antibody (New England Biolabs, Beverly, MA) as described previously (Millar *et al.*, 1995). Blots were stripped and total levels of Hog1 were then determined by probing with an anti-myc 9E10 antibody (Sigma-Aldrich, St. Louis, MO).

### Hog1 Phosphorylation of Sty1 Substrates

To assay Hog1 activity in *S. pombe*, lysates were prepared from wild-type cells (CHP429) or *sty1*$^-$ cells (JM1160), transformed with the empty vector pREP41MH, or with pREP41MH-HOG1, before and after treatment with 0.6 M KCl for 10 min. Lysates (300 μg) were incubated with 5 μg of recombinant GST-Atf1 (Wilkinson *et al.*, 1996) or $His_6$-$Srk1^{K153A}$ (Smith *et al.*, 2002) (pre-bound to glutathione [GSH]-agarose or $Ni^{2+}$-NTA agarose, respectively) for 1 h at 4°C. The beads were washed three times with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM imidazole, 0.1% NP-40, 50 mM NaF, 2 mM $Na_2VO_3$, 1 mM phenylmethylsulfonyl fluoride, 0.07 trypsin inhibitor units/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin) and once with kinase buffer (20 mM HEPES, pH 7.5, 20 mM $MgCl_2$, 2 mM diothiothreitol, 20 μM ATP). Beads were resuspended in 20 μl of kinase buffer with 5 μCi of [$\gamma^{32}$-P]ATP, and kinase reactions were allowed to proceed for 30 min at 30°C. Samples were analyzed by SDS-PAGE on 10% gels, transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA), and autoradiographed.

### Fluorescence Microscopy

Localization of chromosomally yellow fluorescent protein (YFP)-tagged Hog1 was determined by fluorescence microscopy. Samples of exponentially growing *C. albicans* cells ($OD_{595}$ = 0.5) expressing chromosomally tagged Hog1-YFP (JC63), untreated or treated with a range of stress conditions for the indicated times were collected and fixed in 3.7% paraformaldehyde. Cells were spread onto poly-L-lysine–coated slides and coverslips mounted onto slides by using Vectashield mounting medium containing 1.5 mg/ml 4′-6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). DAPI and YFP fluorescence were captured by exciting cells with 365- and 450- to 490-nm wavelengths, respectively, by using an Axiovert microscope (Carl Zeiss, Jena, Germany), with a 100× oil immersion objective, and Axiovision imaging system.

### Northern Analysis

*C. albicans* RNA preparation and Northern analysis were performed as described previously (Brown *et al.*, 2001). Gene-specific probes were amplified by PCR from genomic DNA by using the oligonucleotide primers listed in Table 2.

## RESULTS

### *C. albicans* Hog1 Is Activated in Response to Diverse Stimuli

Previous studies have shown that the *S. pombe* Sty1 SAPK responds to a diverse range of stress conditions (reviewed in Toone and Jones, 1998). In contrast, the Hog1 SAPK, in the evolutionarily distant yeast *S. cerevisiae*, responds mainly to osmotic stress (Brewster *et al.*, 1993) and to a lesser extent to heat stress (Winkler *et al.*, 2002). We were interested in whether the related SAPK in *C. albicans*, Hog1, responds to a range of stress conditions (like the fission yeast Sty1 SAPK), or is primarily an osmosensing kinase (like the *S. cerevisiae* Hog1 SAPK). Therefore, we determined the levels of active phosphorylated Hog1 in *C. albicans* after exposure to a range of different environmental stresses. To facilitate this analysis the strain JC4 was created in which one copy of Hog1 is C-terminally tagged with six histidine residues and two copies of the myc epitope (Hog1-$His_6$-myc). This fusion was functional, because *hog1/HOG1-His$_6$-myc* cells displayed no increased sensitivity to various stress treatments compared with heterozygous *hog1/HOG1* cells (our unpublished data). JC4 cells were treated with a range of stress conditions for the indicated times, and Hog1 phosphorylation was monitored by Western blotting with an antibody that recognizes only the active, phosphorylated form of SAPKs (Millar *et al.*, 1995). The Hog1 fusion protein is tagged with the myc epitope, and hence blots were reprobed with an anti-myc antibody to confirm even loading.

Hog1 was originally described as regulating osmotic stress responses in *C. albicans* (San-Jose *et al.*, 1996). However, during the course of this study, it was reported that *C. albicans* Hog1 also responds to oxidative stress conditions (Alonso-Monge *et al.*, 2003). Here, we have confirmed and extended this analysis by showing that significant levels of Hog1 phosphorylation are observed after exposure to a wide range of conditions (Figure 1A). Moreover, the kinetics and magnitude of Hog1 activation depended upon the particular stress condition imposed on the cell. For example, exposure to osmotic stresses such as KCl and sorbitol, the superoxide generator menadione, the heavy metal $Cd^{2+}$, or the purine analogue caffeine resulted in rapid but transient activation of Hog1. In contrast, treatment with $H_2O_2$, the heavy metal $As^+$, or the drug staurosporine resulted in a rapid but prolonged activation of Hog1. Intriguingly, we also found that Hog1 exhibits a rapid and sustained activation upon exposure to the recently identified quorum-sensing molecule in *C. albicans*, the isoprenoid farnesol (Hornby *et al.*, 2001; Ramage *et al.*, 2002). Significantly, Δ*hog1* cells displayed increased sensitivity compared with wild-type cells when challenged with many of the same stresses, and this sensitivity was reversed upon introduction of the wild-type *HOG1* gene into the Δ*hog1* strain (Figure 1B). This emphasizes the importance of Hog1 signaling in the cellular responses to these stresses. However, we did note that Δ*hog1* cells and wild-type cells grew equally well in the presence of the quorum sensing molecule farnesol (our unpublished data).

Previous work has suggested that the Hog1 SAPK pathway also is involved in the regulation of yeast-hypha morphogenesis in *C. albicans*, because serum-induced hyphal

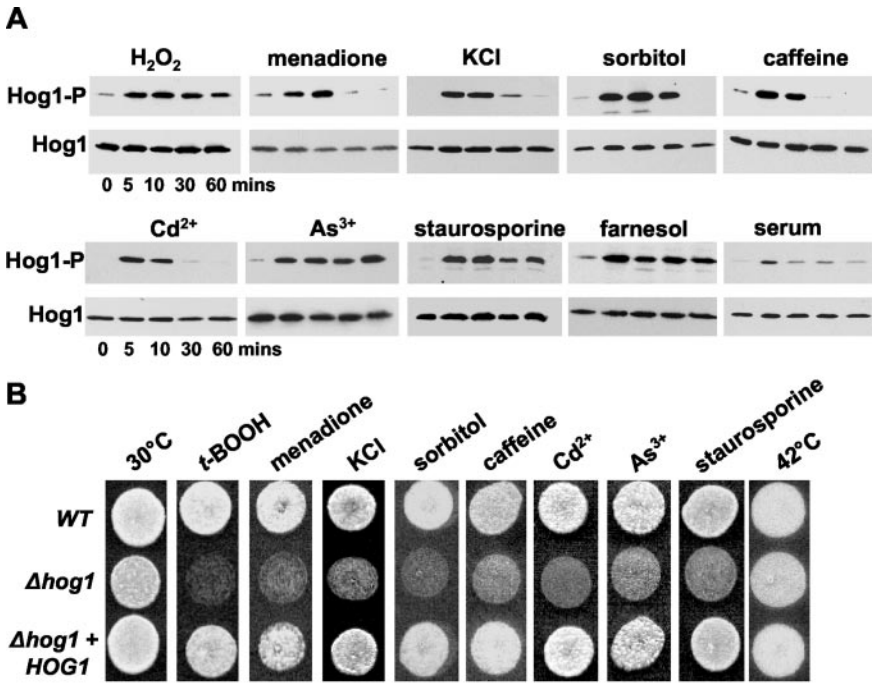


**Figure 1.** *C. albicans* Hog1 is phosphorylated in response to diverse stresses and Δ*hog1* cells are sensitive to these stresses. (A) Western blot analysis of $Ni^{2+}$-NTA agarose-purified Hog1-$His_6$-myc isolated from *C. albicans* cells after treatment with the following compounds: 5 mM $H_2O_2$, 0.3 mM menadione, 0.6 M KCl, 1.2 M sorbitol, 10 mM caffeine, 0.5 mM $CdSO_4$, 2 mM $Na_2As_3$, 2.0 $\mu$M staurosporine, 0.1 mM farnesol, or 20% serum. Western blots were probed with an anti-phospho p38 antibody, which only recognizes the phosphorylated, active form of *C. albicans* Hog1 (Hog1-P). Total levels of Hog1 protein was determined by stripping and reprobing the blot with an anti-myc antibody that recognizes both phosphorylated and unphosphorylated forms of Hog1 (Hog1). (B) Approximately $10^3$ cells from exponentially growing wild-type (WT), Δ*hog1* (JC50), or Δ*hog1*+*HOG1* (JC52) strains were spotted onto YPD plates containing the compounds listed above, with the exception that *t*-BOOH (1 mM) was used instead of $H_2O_2$. Plates were incubated at 30°C (or 42°C) for 24 h.

development is derepressed in *hog1* cells (Alonso-Monge *et al*., 1999). Hence, we also examined the response of Hog1 to serum. As illustrated in Figure 1A, we observed a slight increase in Hog1-phosphorylation after serum treatment, which is consistent with a role for Hog1 in regulating morphogenesis.

Clearly, the *C. albicans* Hog1 SAPK responds to a diverse range of stimuli (Figure 1). Based on studies in *S. pombe* (Chen *et al*., 2003), we reasoned that conditions that activate *C. albicans* Hog1 might result in the induction of a common set of genes that are regulated by this SAPK. However, previous experiments in *C. albicans* suggested that a common set of genes were not induced in response to osmotic stress, oxidative stress, and temperature upshift (Enjalbert *et al*., 2003). In this transcript-profiling study, the authors chose experimental conditions that had previously been shown to stimulate the ESR/CER in *S. cerevisiae* (0.3 M NaCl, 0.4 mM $H_2O_2$, and temperature shift from 23 to 37°C; Gasch *et al*., 2000; Causton *et al*., 2001). However, they did not examine activation of *C. albicans* Hog1 in response to these treatments. Therefore, we tested whether the Hog1 SAPK is activated by the osmotic stress, oxidative stress, and temperature upshift conditions used by Enjalbert *et al.* (2003). Significantly, only one condition strongly activated the Hog1 SAPK (treatment with 0.3 M NaCl; Figure 2). Indeed, the temperature shift appeared to cause a *decrease* in the basal level of phosphorylated Hog1 (Figure 2; see below). These findings probably account for the absence of a common transcriptional response to the three stress conditions used in the transcript-profiling study (Enjalbert *et al*., 2003).

### *The C. albicans-specific Activation Profile of the Hog1 SAPK*

Our results indicate that the *C. albicans* SAPK pathway has a unique activation profile that differs from both the *S. pombe* Sty1 and *S. cerevisiae* Hog1 SAPKs. For example, both the *S. pombe* and *S. cerevisiae* SAPKs are activated in response to temperature upshift (Shieh *et al*., 1998; Shiozaki *et al*., 1998; Nguyen and Shiozaki, 1999; Winkler *et al*., 2002). However, the *C. albicans* Hog1 SAPK is not activated upon temperature upshift. In stark contrast, a significant and reproducible decrease in the basal level of Hog1 activation was observed upon shifting cells from 23°C to either 37 or 42°C (Figures 2 and 3A). Also, *C. albicans* Δ *hog1* cells did not display impaired growth compared with wild-type cells in response to temperature upshift (Figure 1B). This is in stark contrast with *S. pombe* cells lacking *sty1*$^+$, which struggle to grow at 36°C (Figure 4A). Furthermore, although the Sty1 SAPK is activated in response to low concentrations of $H_2O_2$ (Figure 3B; Quinn *et al*., 2002), *C. albicans* Hog1 activation is only seen at much higher levels of $H_2O_2$ (>2 mM) (Figure 3B). This unique activation profile of the *C. albicans* Hog1 SAPK pathway may have evolved to protect this human pathogen against host defenses and promote its survival in the human host (see *Discussion*).

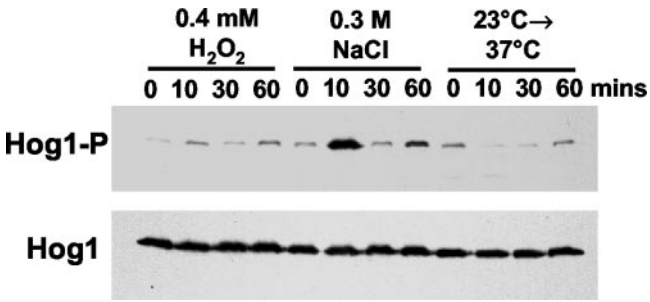


**Figure 2.** Activation profile of the Hog1 SAPK in response to the transcript-profiling conditions. Western blot analysis of $Ni^{2+}$-NTA agarose purified Hog1-$His_6$-myc from wild-type *C. albicans* cells after treatment with the same conditions used in the transcript-profiling study (Enjalbert *et al*., 2003), namely; 0.3 M NaCl, 0.4 mM $H_2O_2$, or temperature shift from 23 to 37°C, for the times indicated. Phosphorylated Hog1 (Hog1-P) and total Hog1 (Hog1) levels were detected as described in Figure 1.

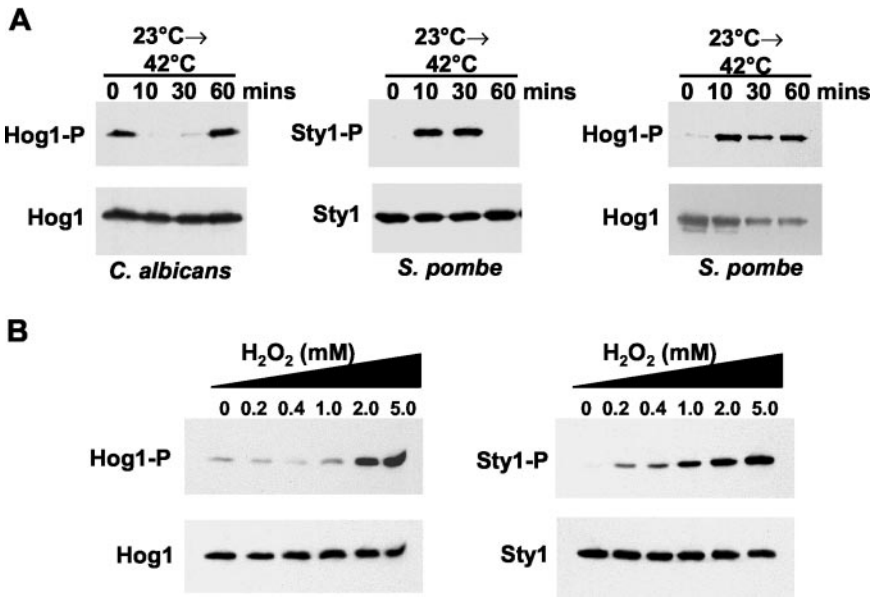


**Figure 3.** *C. albicans* Hog1 and the *S. pombe* Sty1 SAPKs respond differently to oxidative stress and temperature upshift. (A) Phosphorylation of *S. pombe* Sty1 and *C. albicans* Hog1 in response to temperature shift from 23 to 42°C. *C. albicans HOG1* also was expressed heterologously in *sty1*$^-$ cells and phosphorylation of Hog1 was analyzed under the same conditions. (B) Phosphorylation of *C. albicans* Hog1 and *S. pombe* Sty1 was compared after treatment with a range of $H_2O_2$ concentrations. Phosphorylated Hog1 (Hog1-P) and total Hog1 (Hog1) levels were detected as described in Figure 1. Phosphorylated Sty1 (Sty1-P) and total Sty1 (Sty1) levels were detected as described previously (Millar *et al*., 1995).

Heat shock-induced activation of the Sty1 SAPK is regulated via inhibition of Pyp1, the major tyrosine phosphatase that dephosphorylates and inactivates Sty1 (Nguyen and Shiozaki, 1999). The contrasting response of the *C. albicans* Hog1 SAPK to temperature upshift suggested that Hog1, or regulators of this SAPK pathway, respond in a different manner to this treatment. To explore this further, we examined the phosphorylation of the *C. albicans* Hog1 SAPK in *S. pombe sty1*$^-$ cells in response to temperature upshift (Figure 3A). In direct contrast to the effects of heat observed in *C. albicans*, Hog1 was rapidly phosphorylated when the host fission yeast cells were exposed to a temperature upshift from 23 to 42°C. These data strongly suggest that the contrasting responses of these fungi to temperature are mediated by divergence in the regulators that relay temperature upshift signals to the SAPK.

### *C. albicans HOG1 Complements Phenotypes Associated with Deletion of the S. pombe sty1*$^+$ *Gene*

The *C. albicans* and *S. pombe* SAPKs are both activated in response to several stresses. Therefore, we investigated whether expression of *C. albicans HOG1* in *S. pombe* could complement phenotypes associated with deletion of the *sty1*$^+$ gene. In addition to key roles in stress signaling (reviewed in Toone and Jones, 1998), Sty1 also regulates cell cycle progression in fission yeast. For example, *sty1*$^-$ cells are considerably elongated due to a delay in G2 (Millar *et al*., 1995; Shiozaki and Russell, 1995). *C. albicans HOG1* was expressed in *sty1*$^-$ cells under the control of a medium strength *nmt41*$^+$ thiamine-repressible promoter (Basi *et al*., 1993). In the absence of thiamine, ectopic expression of *HOG1* rescued both the sensitivity of *sty1*$^-$ cells to osmotic, oxidative and heat stresses, and the *sty1*$^-$-associated cell cycle defect (Figure 4, A and B).

Because *HOG1* can complement phenotypes associated with loss of *sty1*$^+$, it was likely that Hog1 can phosphorylate many, if not all, key Sty1 substrates. Hence, we tested whether the *C. albicans* SAPK phosphorylates two previously identified substrates of Sty1; Atf1 and Srk1. Atf1 is a transcription factor (Shiozaki and Russell, 1996; Wilkinson *et al*., 1996), and Srk1 is a serine/threonine protein kinase (Smith *et al*., 2002). Extracts were prepared from wild-type *S. pombe* cells (CHP429), *sty1*$^-$ cells (JM1160), and *sty1*$^-$ cells expressing *HOG1*, both before and after treatment with 0.6 M KCl for 10 min. Extracts were incubated with either recombinant GST-Atf1 or $His_6$-tagged Srk1, and the phosphorylation of the fusion proteins was assayed (Figure 4C). In agreement with previous studies, no stress-induced phosphorylation of Atf1 and Srk1 was observed using *sty1*$^-$ extracts. However, significant Atf1 and Srk1 phosphorylation was seen with extracts prepared from wild-type cells or from *sty1*$^-$ cells expressing *HOG1* (Figure 4C, lanes 2 and 6). Hence, *C. albicans* Hog1 can phosphorylate targets of *S. pombe* Sty1. Therefore, *C. albicans* Hog1 is a functional homologue of *S. pombe* Sty1.

### *Cellular Localization of Hog1*

Previous studies of the *S. pombe* Sty1 SAPK (Gaits *et al*., 1998) and the *S. cerevisiae* Hog1 SAPK (Ferrigno *et al*., 1998; Reiser *et al*., 1999) revealed that these kinases are regulated at the level of cellular localization. In particular, both were found to translocate to the nucleus after activation. For example, the Sty1 SAPK accumulates in the nucleus after exposure to oxidative and osmotic stresses (Gaits *et al*., 1998; Smith *et al*., 2002), whereas the *S. cerevisiae* Hog1 SAPK accumulates in the nucleus after osmotic stress (Ferrigno *et al*., 1998; Reiser *et al*., 1999). Therefore, the cellular localization of the *C. albicans* Hog1 SAPK was examined in response to a range of stress conditions. To facilitate these studies, both copies of the *HOG1* gene were chromosomally tagged with *YFP*, and the cellular localization of Hog1-YFP was examined by fluorescence microscopy. *HOG1-YFP/HOG1-YFP* cells displayed no increased sensitivity to various stress treatments compared with *HOG1/HOG1* cells, illustrating that the Hog1–YFP fusion protein is functional in *C. albicans* (our unpublished data).

As illustrated in Figure 5, Hog1 did not accumulate in the nuclei of cells treated with stresses that do not activate Hog1, such as low levels of $H_2O_2$ (0.4 mM) or temperature shift from 23 to 42°C. In contrast, Hog1 rapidly accumulated in the nucleus after exposure to the stresses that do activate this SAPK. For example, exposure to high doses of $H_2O_2$ (5 mM), osmotic stress (0.6 M KCl), or heavy metal stress (0.5 mM $CdSO_4$) resulted in rapid nuclear accumulation of Hog1.

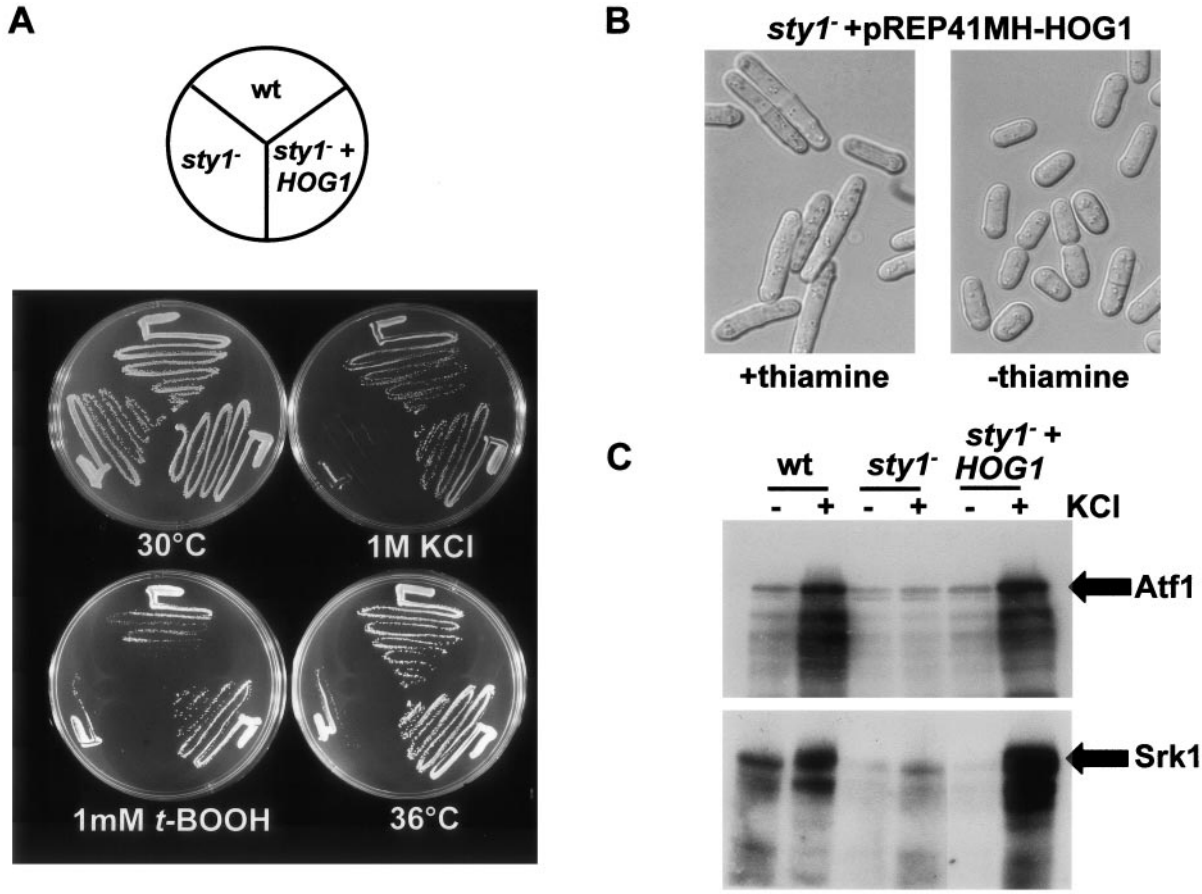


**Figure 4.** *C. albicans* Hog1 is a functional homologue of *S. pombe* Sty1. *sty1*$^-$ cells were transformed with pREP41MH-HOG1, in which the expression of *C. albicans HOG1* is under the control of the fission yeast thiamine-repressible *nmt41* promoter. (A) Expression of *HOG1* in *sty1*$^-$ cells complements *sty1*$^-$-associated stress phenotypes. The growth of wild-type cells, *sty1*$^-$ cells, and *sty1*$^-$ cells transformed with pREP41MH-HOG1 was compared upon exposure to osmotic stress (0.6 M KCl), oxidative stress (1 mM *t*-BOOH), temperature upshift (36°C), or no stress (30°C). (B) Expression of *HOG1* in *sty1*$^-$ cells rescues the *sty1*$^-$-associated cell cycle defect. *sty1*$^-$ cells, transformed with pREP41MH-HOG1, were grown in the presence or absence of thiamine and differential interference contrast images captured. (C) Active Hog1 phosphorylates Sty1 substrates. Extracts prepared from wild-type (*wt*), *sty1*$^-$, and *sty1*$^-$ cells transformed with pREP41MH-HOG1, that had been harvested before (−) or after (+) treatment with 0.6 M KCl for 10 min, were incubated with recombinant GST-Atf1 prebound to GSH-agarose or $His_6$-$Srk1^{K153A}$ prebound to $Ni^{2+}$-NTA agarose beads for 1 h at 4°C. A kinase assay was performed and phosphorylation of GST-Atf1 and $His_6$-$Srk1^{K153A}$ was assessed by SDS-PAGE and autoradiography.

Moreover, the kinetics of nuclear accumulation of Hog1 depended on the stress-condition imposed on the cell and also correlated with the kinetics of phosphorylation (compare Figure 1A with Figure 5). For example, the rapid but transient phosphorylation of Hog1 in response to heavy metal and osmotic stress was mirrored by a rapid but transient nuclear accumulation of Hog1, which peaked at 10 min after exposure to stress (Figure 5). However, after exposure to high doses of $H_2O_2$, significant amounts of Hog1 remained in the nucleus after 30 min (Figure 5), which paralleled the sustained phosphorylation of Hog1 under this condition (Figure 1A).

### *Identification of Hog1-regulated Genes*

Our data show that the *C. albicans* Hog1 SAPK is phosphorylated and accumulates in the nucleus in response to a range of stress conditions. We predict that this will lead to the regulation of common gene targets of this SAPK pathway. However, to date, genes regulated by the Hog1 SAPK in *C. albicans* have not been identified. To identify candidate Hog1 gene targets, we looked for *C. albicans* genes that were induced in response to the osmotic stress conditions used in the transcript-profiling experiments (Enjalbert *et al*., 2003) and that seem to be homologues of known Hog1 targets in *S. cerevisiae* (Posas *et al*., 2000; Rep *et al*., 2000). Two genes were selected for analysis, *HSP12* (*IPF16067*), which encodes a putative heat shock protein; and *RHR2*, which encodes a putative glycerol phosphate phosphatase (http://genolist.pasteur.fr/CandidaDB). Northern analyses were performed to examine the expression of these genes in wild-type and Δ*hog1* cells after exposure to low or high doses of oxidative stress (0.4 and 5 mM $H_2O_2$), osmotic/saline stress (0.3 M NaCl), heavy metal stress (0.5 mM $CdSO_4$), or temperature upshift (from 23 to 37°C).

The *RHR2* mRNA was induced in response to osmotic stress, which is in agreement with the previously published microarray data (Enjalbert *et al*., 2003). We found that high levels of oxidative stress or heavy metal stress also stimulated induction of *RHR2*. Moreover, *RHR2* induction is dependent on the Hog1 SAPK because no detectable expression of *RHR2* mRNA was observed in Δ*hog1* cells treated with the same stresses (Figure 6A). This lack of induction of *RHR2* in Δ*hog1* cells is reversed

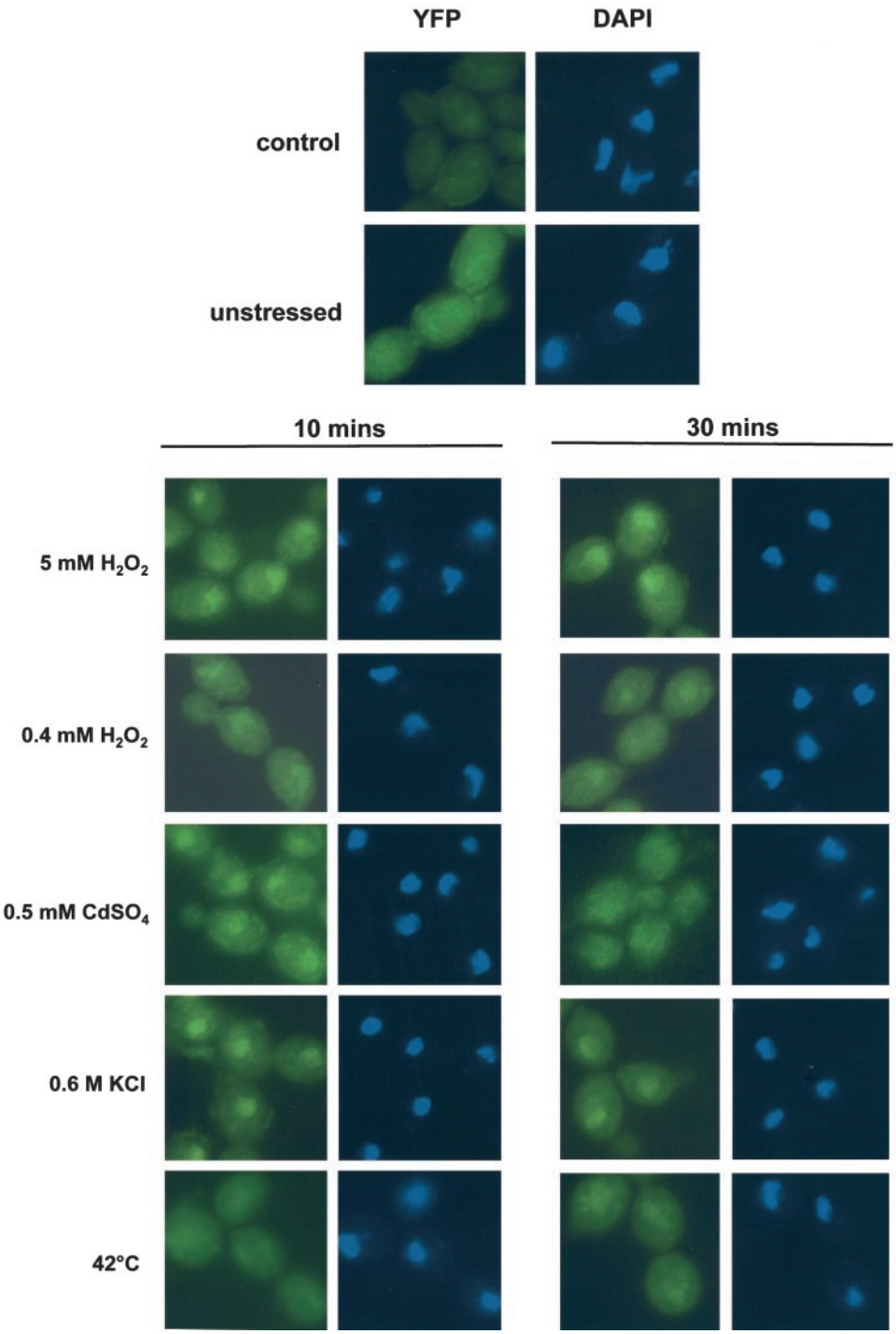


**Figure 5.** *C. albicans* Hog1-YFP accumulates in the nucleus in response to specific stresses. The localization of YFP-tagged Hog1 was determined by fluorescence microscopy in cells treated with the indicated stresses at 10-and 30-min time points (YFP). The position of the nuclei in these cells was visualized with DAPI. An autofluorescence control also is shown (control).

upon integration of the wild-type *HOG1* gene in the *hog1* mutant (Figure 6B). Furthermore, the kinetics of *RHR2* induction in wild-type cells paralleled the kinetics of Hog1 activation and nuclear localization (compare Figure 1A with Figures 5 and 6). These data indicate that Hog1 positively regulates *RHR2* expression in response to osmotic, oxidative, and heavy metal stresses. We also found that *RHR2* is slightly induced in response to temperature upshift, but this induction was not dependent on the Hog1 SAPK. This suggests that a separate, Hog1-independent signaling pathway regulates *RHR2* expression in response to temperature upshift.

Like *RHR2*, *HSP12* was induced in response to osmotic stress (again in agreement with Enjalbert *et al*., 2003), and in response to high levels of oxidative stress, or heavy metal stress in wild-type cells. We noted that *HSP12* also was slightly induced in response to temperature upshift. However, in contrast to *RHR2*, Hog1 seems to repress *HSP12* because high basal levels of *HSP12* expression were observed consistently in Δ*hog1* cells, even under nonstressed

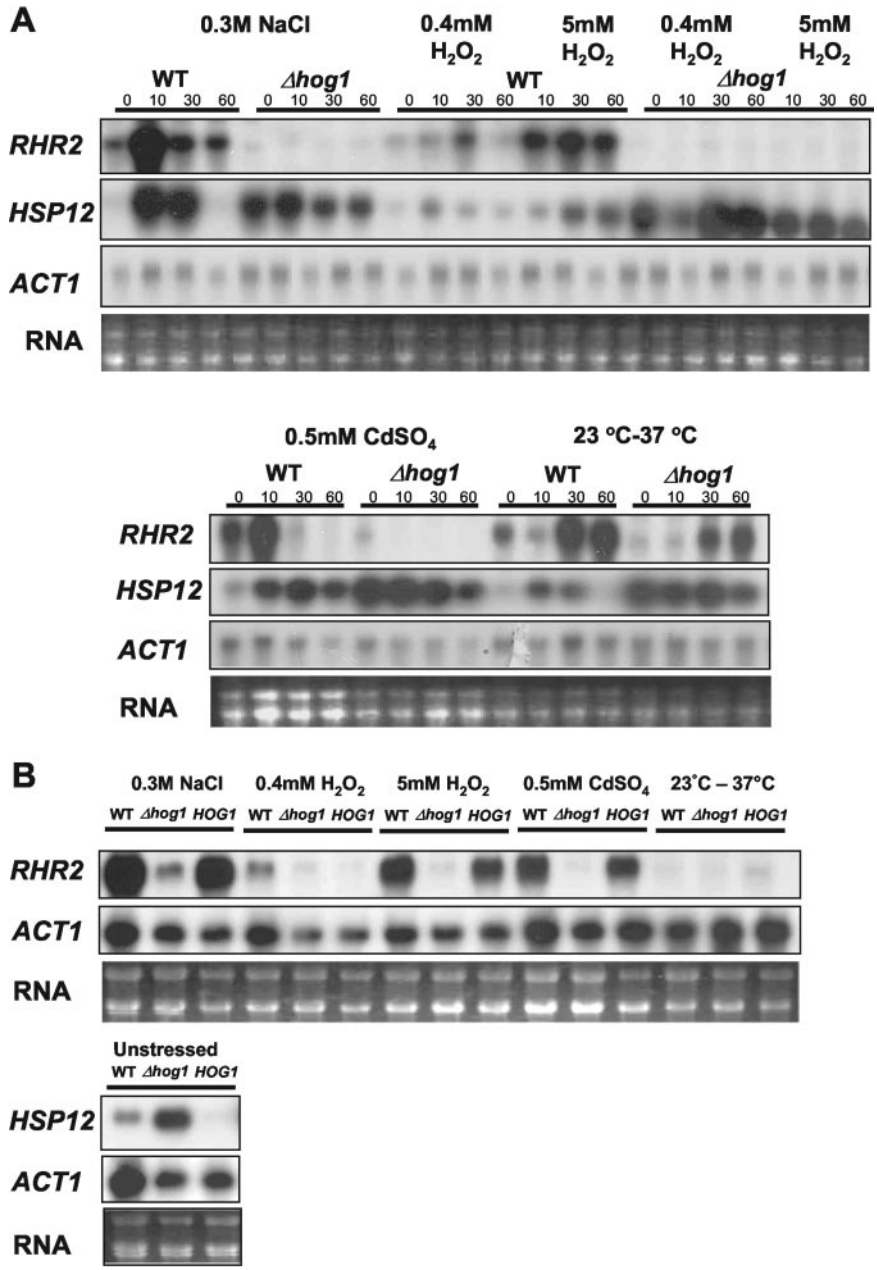


**Figure 6.** Expression of Hog1-regulated genes in *C. albicans*. (A) Northern blot analysis of RNA isolated from mid-log cultures of wild-type (WT) and Δ*hog1* (JC10) cells treated with the indicated stresses for 0, 10, 30, and 60 min by using probes specific for *RHR2*, *HSP12*, and *ACT1* (loading control). rRNA was stained with ethidium bromide (RNA). (B) Northern blot analysis comparing *RHR2* and *HSP12* expression in Δ*hog1*+*HOG1* (JC52), wild-type (WT), and Δ*hog1* (JC50) cells. Cells were treated with the indicated stresses for 10 min.

conditions (Figure 6A). The high basal level of *HSP12* expression in the Δ*hog1* strain was reversed upon integration of the wild-type *HOG1* gene in the *hog1* mutant (Figure 6B). It is not yet clear whether the role of Hog1 in the regulation of *HSP12* expression is direct. However, as *HSP12* is only strongly induced in response to stress conditions that activate Hog1, it is possible that stress-induced phosphorylation of Hog1 functions to alleviate Hog1-mediated repression.

Collectively, this Northern analysis indicates that Hog1 plays different roles in the regulation of specific stress-activated genes in *C. albicans*.

### *Stress Cross-Protection in C. albicans Mediated by the Hog1 SAPK*

The existence of a general stress response has been proposed to explain the phenomenon of cross-protection, wherein exposure to a non-lethal dose of one stress can protect against a potentially lethal dose of a seemingly unrelated stress. Importantly, cross-protection has been observed in *S. cerevisiae* (Lewis *et al*., 1995) and *S. pombe* (Chen *et al*., 2003; M. Toone, personal communication), both of which have been shown to mount general stress responses. Previous work in *C. albicans* demonstrated that pretreatment of cells with a mild stress, such as oxidative or temperature upshift, stimulates adaptation that protects against subsequent exposure to higher levels of the *same* stress (Jamieson *et al*., 1996; Arguelles, 1997). However, Enjalbert *et al*. (2003) could detect no stress cross-protection in *C. albicans* under the conditions used in their transcript-profiling study, reinforcing their suggestion that this fungus might not mount a general stress response. However, we have shown that only one of the stress conditions used in their transcript-profiling study

activates Hog1 (0.3 M NaCl; Figure 2). We predicted that stress cross-protection would be seen in *C. albicans*, but only upon exposure to stress conditions that activate the Hog1 SAPK. To test this prediction, cells were pretreated with the range of stress conditions used in the transcript-profiling study (Enjalbert *et al*., 2003) and then subjected to acute doses of $H_2O_2$. As demonstrated previously (Jamieson *et al*., 1996), cell survival to acute doses of $H_2O_2$ was notably improved by pretreatment of cells with a low dose of $H_2O_2$ (0.4 mM) (Figure 7A). Significantly, however, we also found that pretreatment of cells with an osmotic stress which activates Hog1 (0.3 M NaCl) provided substantial protection against exposure to 100 mM $H_2O_2$. However, pretreatment of cells with a temperature upshift (which does not activate Hog1) conferred no cross-protection against oxidative stress (Figure 7A).

To investigate the role of Hog1 in stress-cross protection, cell survival experiments were repeated in Δ*hog1* cells. The protection afforded by pretreatment of cells with a low dose of $H_2O_2$ (0.4 mM) against acute doses of the *same* agent was retained in Δ*hog1* cells (Figure 7B). Hence, adaptation to an oxidative stress is not dependent upon Hog1. This correlates with our observation that Hog1 is not activated in response to low doses of $H_2O_2$. However, the protection against an acute dose of $H_2O_2$, conferred by an osmotic stress of 0.3 M NaCl, was lost in Δ*hog1* cells (Figure 7B). Importantly, integration of the wild-type *HOG1* gene into the *hog1* mutant restored wild-type levels of stress cross-protection (Figure 7C). Therefore, stress cross-protection does exist in *C. albicans*, and this phenomenon is mediated by the Hog1 SAPK.

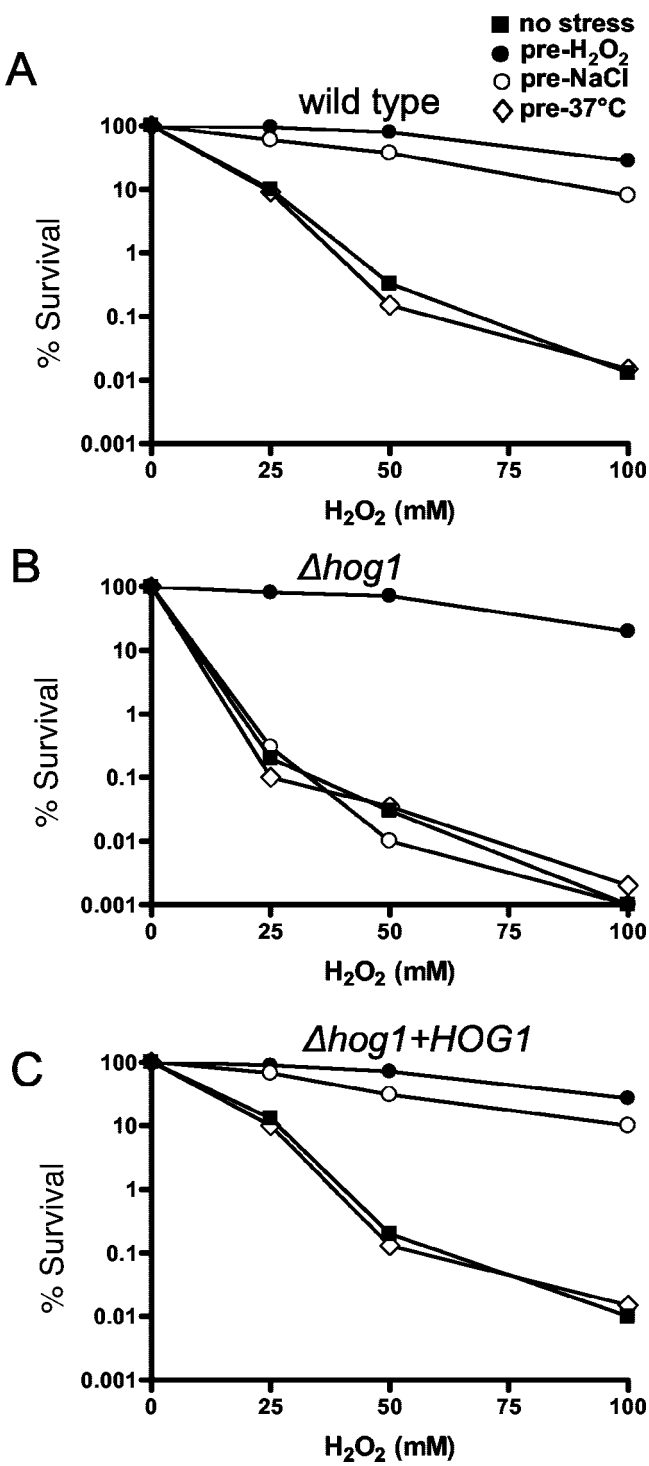


**Figure 7.** Stress cross-protection in *C. albicans*. Wild-type (WT), Δ*hog1* (JC10), or Δ*hog1*+*HOG1* cells (JC52) were either unstressed or pretreated for 1 h with osmotic stress (0.3 M NaCl), low-dose oxidative stress (0.4 mM $H_2O_2$), or temperature upshift (23–37°C), and cell survival was calculated after a subsequent 1-h exposure to increasing doses of $H_2O_2$. The experiments were each repeated at least three times, and the data from one representative experiment are shown.

## DISCUSSION

In this article, we demonstrate that the human pathogen *C. albicans* mounts a core environmental stress response. We show that the *C. albicans* Hog1 SAPK is activated and translocates to the nucleus in response to a diverse range of stress conditions (Figure 5). These observations confirm and considerably extend a recent study that found that *C. albicans* Hog1 is phosphorylated in response to oxidative as well as osmotic stress stimuli (Alonso-Monge *et al*., 2003). We also have shown that the *C. albicans* Hog1 SAPK is a functional homologue of the *S. pombe* Sty1 SAPK, which is responsible for activating the core environmental response in this yeast. In addition, we have identified examples of Hog1-regulated genes that are stereotypically induced in response to the same diverse stress conditions that activate Hog1 (Figure 6). Furthermore, we find that stress cross-protection, a classical hallmark of a general stress response, does occur in *C. albicans* with stresses that activate the Hog1 SAPK (Figure 7). Hence, collectively our data indicate that *C. albicans* mounts a core stress response and that the Hog1 SAPK is pivotal to this response.

A previous transcript-profiling study suggested that *C. albicans* "lacks the strong general stress response exhibited by *S. cerevisiae*" (Enjalbert *et al*., 2003). These authors observed that no genes were commonly induced by oxidative stress (0.4 mM $H_2O_2$), osmotic stress (0.3 M NaCl), and temperature upshift (23–37°C) treatments, although these conditions had previously been found to stimulate strong core stress responses in *S. cerevisiae*. However, we have established that only one of the three conditions (0.3 M NaCl) used in their study activates the Hog1 SAPK in *C. albicans* (Figure 2), which presumably explains why they did not observe a general stress response under these conditions. We have shown that other conditions do stimulate the activation of the Hog1 SAPK (Figure 1), and under such conditions *C. albicans* displays phenotypes associated with a general stress response (Figures 6 and 7).

The identification of target genes for *C. albicans* Hog1 has uncovered both activator and repressor functions for this SAPK. We show that both *RHR2* (encoding a putative glycerol phosphate phosphatase) and *HSP12* (encoding a putative heat shock protein) are Hog1 targets (Figure 6). However, Hog1 represses *HSP12* under nonstress conditions, whereas *RHR2* is induced in a Hog1-dependent manner in response to conditions that activate this SAPK. We note that the repression of *HSP12* by Hog1 in *C. albicans* is in striking contrast to the situation in *S. cerevisiae*, where Hog1 is required to activate *HSP12* (Varela *et al*., 1995). The fact that *C. albicans* Hog1 has both activator and repressor functions suggests that this kinase has multiple cellular targets. We predict that Hog1-regulated genes such as *RHR2* and *HSP12* form the basis of the core stress response in *C. albicans*, and genomic-based approaches are currently underway in our laboratories to identify further Hog1 target genes.

Our work indicates that the core stress response in *C. albicans* has evolved, in a niche-specific manner, to promote survival of this pathogen in the mammalian host. This conclusion is supported by two main findings. First, the conditions that stimulate the core stress response in *C. albicans* are different to those that induce similar responses in *S. cerevisiae* and *S. pombe*. Second, the molecular circuitry that senses and transduces stress signals has evolved to respond to those conditions that are important for the growth and survival of *C. albicans*.

*C. albicans*, *S. cerevisiae*, and *S. pombe* occupy contrasting niches, and hence one would expect the conditions which these yeast perceive as a stress to have diverged in a niche-specific manner. This is highlighted by our analysis of the response of the *C. albicans* Hog1 SAPK pathway to oxidative stress and heat treatments. For example, Hog1 activation was only observed at relatively high levels of $H_2O_2$ (>2 mM; Figure 3). This may be related to the fact that *C. albicans* is relatively resistant to oxidative stress compared with *S. cerevisiae* (Jamieson *et al*., 1996) and *S. pombe* (J.Q., unpublished observation). Low doses of $H_2O_2$ (≤0.5 mM) stimulate core stress responses in both *S. cerevisiae* and *S. pombe* (Gasch *et al*., 2000; Causton *et al*., 2001; Chen *et al*., 2003), but not in *C. albicans* (Enjalbert *et al*., 2003). We predict that the relative resistance of *C. albicans* to oxidative stress, and corresponding activation of the Hog1 SAPK only in response to high doses of $H_2O_2$, reflects the ability of this fungal pathogen to survive the relatively high levels of reactive oxygen species that are generated by the host immune system, for example, during phagocytic attack.

Regarding the effects of temperature upshift upon *C. albicans*, it is relevant that this pathogen has evolved to thrive in warm-blooded mammalian hosts, where temperature homeostasis is maintained, for example, at 37°C in humans. Consequently, the SAPK pathway in *C. albicans* is not activated in response to a temperature upshift; in fact, we see a consistent *reduction* in the basal levels of Hog1 phosphorylation after temperature increase (Figures 2 and 4A). Presumably this adaptation prevents the continuous stimulation of the Hog1 SAPK in response to temperatures of 37°C, which might desensitize the pathway to other significant stresses in the mammalian host. In contrast to the environmental niche occupied by *C. albicans*, the growth environments shared by *S. cerevisiae* and *S. pombe* are subject to significant temperature fluctuations. Not surprisingly, therefore, temperature upshift stimulates strong core stress responses in *S. pombe* and *S. cerevisiae* (Gasch *et al*., 2000; Causton *et al*., 2001; Chen *et al*., 2003), and the SAPK pathways in both of these organisms respond to temperature upshift (Shieh *et al*., 1998; Shiozaki *et al*., 1998; Nguyen and Shiozaki, 1999; Winkler *et al*., 2002). Therefore, it seems that the *C. albicans* Hog1 SAPK pathway has evolved not to respond to temperature upshift as a result of the selective pressures in warm-blooded mammalian hosts. To investigate this further, we examined the response of the *C. albicans* Hog1 SAPK to temperature upshift when it is expressed in *S. pombe*. Similar to the Sty1 SAPK, *C. albicans* Hog1 becomes rapidly phosphorylated upon temperature upshift in *S. pombe*. This result suggests that there has been a divergence in the circuitry that relays temperature upshift signals to the SAPK module in *C. albicans* and *S. pombe*. However, we cannot exclude the possibility that the *C. albicans* SAPK interacts differently with the regulators that relay temperature upshift signals in *C. albicans* and *S. pombe*.

In addition to stress responses, the *C. albicans* Hog1 SAPK pathway also has been implicated in the regulation of serum-induced dimorphism, because *hog1* mutants display derepressed serum-induced hyphal formation (Alonso-Monge *et al*., 1999). Consistent with this we see an increase in the levels of active, phosphorylated Hog1 after exposure to serum (Figure 1). However, it should be noted that serum-induced levels of phosphorylated Hog1 are relatively low compared with other stress conditions (Figure 1), and a direct role for Hog1 in the regulation of morphogenic gene targets has not been established. Importantly, the deletion of *HOG1* in *S. cerevisiae* results in inappropriate activation of the mating and filamentation mitogen-activated protein kinase pathways (O'Rourke and Herskowitz, 1998; Davenport *et al*., 1999). Hence, derepression of morphogenesis seen in *C. albicans hog1* mutants may arise from a similar indirect phenomenon. We did, however, detect a significant response of the *C. albicans* Hog1 SAPK pathway to the quorum-sensing molecule farnesol. Quorum sensing is predicted to form the basis of cell density-dependent morphogenesis seen in *C. albicans*. The mechanism(s) underlying farnesol-mediated quorum sensing is not known (Hornby *et al*., 2001; Ramage *et al*., 2002), but our data suggest that the Hog1 SAPK pathway might play a key role in cell-to-cell signaling in *C. albicans*. Further experimentation is needed to investigate this intriguing possibility.

Together, our results suggest that a niche-specific Hog1-mediated core stress response has evolved in *C. albicans* to protect this human pathogen against host defenses and to promote its survival in the host. The evolution of this niche-specific response is reflected in the specific environmental conditions that trigger the Hog1-regulated core stress response in *C. albicans* and by differences in the molecular circuitry that drive this response (Figure 8). Many conserved regulatory modules have evolved to execute different cellular roles in *C. albicans*. Examples include Gcn4 (Tripathi *et al*., 2002), Rfg1 (Kadosh and Johnson, 2001) and Tup1 (Braun and Johnson, 2000; Murad *et al*., 2001). Because the Hog1 SAPK is required for virulence in *C. albicans* (Alonso-Monge *et al*., 1999), this pathway may represent a further example of a regulatory module that has been redirected toward virulence in this pathogenic fungus.

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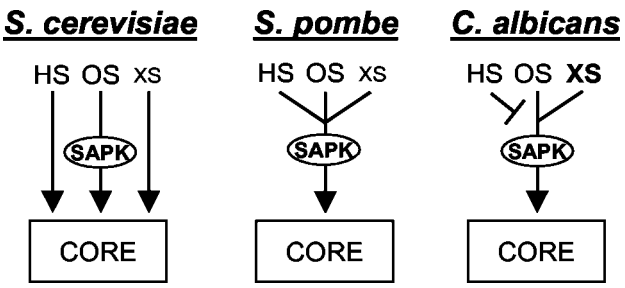


**Figure 8.** Regulation of core stress responses in *S. cerevisiae*, *S. pombe*, and *C. albicans*. The SAPK pathways in *S. pombe* and *C. albicans* play a central role in regulating core stress responses. However, these pathways have adapted to respond in a niche-specific manner, reflecting the different growth environments of these two yeasts. In contrast, core stress responses in *S. cerevisiae* and *S. pombe* are stimulated by similar conditions, presumably as these organisms inhabit very similar environments. In *S. cerevisiae*, however, core stress responses are regulated by stress-specific pathways. HS, heat stress; OS, osmotic stress; **XS**, high doses of oxidative stress; xs, low doses of oxidative stress.

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