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The Hog1 Mitogen-Activated Protein Kinase Is Essential in the Oxidative Stress Response and Chlamydospore Formation in *Candida albicans*

Rebeca Alonso-Monge, Federico Navarro-García, Elvira Román, Ana I. Negredo,† Blanca Eisman, César Nombela, and Jesús Pla*

Departamento de Microbiología II, Facultad de Farmacia, Universidad Complutense de Madrid, E-28040 Madrid, Spain

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Candida albicans **mutants with mutations in mitogen-activated protein (MAP) kinase *HOG1* displayed an increased sensitivity to agents producing reactive oxygen species, such as oxidants (menadione, hydrogen peroxide, or potassium superoxide), and UV light. Consistent with this finding, *C. albicans* Hog1 was activated not only in response to an increase in external osmolarity, as happens with its *Saccharomyces cerevisiae* homologue, but also in response to hydrogen peroxide. The Hog1-mediated response to oxidative stress was different from that of transcription factor Cap1, the homologue of *S. cerevisiae* Yap1, as shown by the different sensitivities to oxidants and the kinetics of cell death of *cap1*Δ, *hog1*, and *hog1 cap1*Δ mutants. Deletion of *CAP1* did not influence the level of Hog1 phosphorylation, and deletion of *HOG1* did not affect Cap1 nuclear localization. Moreover, we show that the *HOG1* gene plays a role in chlamydospore formation, another oxygen-related morphogenetic event, as demonstrated by the fact that *hog1* cells were unable to generate these thick-walled structures in several media through a mechanism different from that of the *EFG1* regulator. This is the first demonstration of the role of the Hog1-mediated MAP kinase pathway in resistance to oxidative stress in pathogenic fungi, and it allows us to propose a molecular model for the oxidative stress response in *C. albicans*.**

One of the situations that often challenges growing yeast cells is oxidative stress, which is characterized by an abnormally high oxidative potential. This characteristic may be determined by the normal respiratory metabolism (the mitochondrial respiratory chain) but may also be generated by environmentally elevated oxygen pressure or by exposure to ionizing radiation. Under these conditions, the concentration of reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot -}$), the hydroxyl radical ($OH^{\cdot}$), or hydrogen peroxide (H_2O_2), is augmented, damaging—either directly or indirectly—several components of the cell, such as DNA, proteins, and lipids (28, 68). These effects are toxic and eventually lead to cell death. It is therefore not surprising that yeast cells have developed antioxidant mechanisms to cope with these conditions (see references 42 and 43 for recent reviews). Among the protective mechanisms are the activities of detoxifying enzymes (e.g., superoxide dismutases and catalases) or other, nonenzymatic scavenging substances that prevent the action of ROS. While this response has been characterized in some detail for bacteria (9, 16, 40), knowledge of these mechanisms in lower eukaryotes is lacking. It is clear, however, that this cellular response is accomplished, at least partially, at the transcriptional level. In the model yeast *Saccharomyces cerevisiae*, Yap1 (27), a transcription factor with a well-defined role in resistance to several drugs (43, 59), has been shown to mediate the oxidative stress response, a conclusion inferred not only from the susceptibility of *yap1* mutants to several oxidants but also from the Yap1-dependent transcription of different genes with a protective role (24, 31, 59, 63). Other elements, such as those encoded by the *SKO1* (55), *YAP2* (4, 63), *MSN2*/*MSN4* (18, 33), *HAL1* (72), or *SKN7* (36, 39, 44) genes, play a role in this adaptive response. In *Schizosaccharomyces pombe*, two mechanisms seem to control the response to oxidative stress—the activation of the StyI/Spc1 mitogen-activated protein (MAP) kinase pathway and the function of the Yap1 homologue—although these phenomena seem to be interdependent. The former pathway in this microorganism, the homologue of the HOG pathway, is responsible for sensing both osmotic stress and oxidative stress; the MAP kinase StyI is activated by phosphorylation (13, 57) to phosphorylate the transcription factor Atf1 (54, 60, 69), which is responsible for the transcription of different oxidative response genes. Pap1, the homologue of Yap1, is necessary to respond to oxidative stress through the transcription of another set of oxidative response genes, but its activity depends on its nuclear localization, which is hampered in the absence of StyI (65).

Candida albicans is a pathogenic yeast (49) of great clinical interest due to the incidence (21) and severity of the infections that it causes, especially in immunocompromised individuals; it is therefore a preferred model of a fungal pathogen. It is evident that the mechanisms of defense against oxidative stress are especially relevant for many pathogenic fungi, such as *C. albicans*, since the neutrophil-macrophage system is crucial in the control and outcome of the infections that they cause (66) through oxidative killing mechanisms. A major limitation in defining the molecular determinants of resistance to oxidative stress in *C. albicans* is the lack of a powerful genetic system, only partially developed in the last few years (11, 51). There

* Corresponding author. Mailing address: Departamento de Microbiología II, Facultad de Farmacia, Universidad Complutense de Madrid, Plaza de Ramón y Cajal s/n, E-28040 Madrid, Spain. Phone: 34 91 3941617. Fax: 34 91 3941745. E-mail: jesuspla@farm.ucm.es.

† Present address: Centro Nacional de Microbiología, Instituto de Salud Carlos III, E-28220 Majadahonda, Madrid, Spain.

TABLE 1. Strains used in this work

Strain	Genotype	Reference or source
Escherichia coli DH5αF′	K-12 Δ(*lacZYA-argF*)*u169 supE44 thi-1 recA1 endA1 hsdR17 gyrA relA1* (ϕ80*lacZ*ΔM15) F′	26
Candida albicans		
SC5314	Wild type (*HOG1*/*HOG1*)	23
CAF2	*URA3*/*ura3*Δ::λ*imm*^{434}	19
CAI4	*ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434}	19
RM1	*ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434} *his1*Δ::*hisG-URA3-hisG*/*HISI*	This work
RM10	*ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434} *his1*Δ::*hisG*/*HISI*	This work
RM100	*ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434} *his1*Δ::*hisG*/*his1*Δ::*hisG-URA3-hisG*	This work
RM1000	*ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434} *his1*Δ::*hisG*/*his1*Δ::*hisG*	This work
CNC11	*ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434} *his1*Δ::*hisG*/*his1*Δ::*hisG HOG1*/*hog1*::*hisG-URA3-hisG*	58
CNC13	*ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434} *his1*Δ::*hisG*/*his1*Δ::*hisG hog1*::*hisG-URA3-hisG*/*hog1*::*hisG*	58
CNC15	*ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434} *his1*Δ::*hisG*/*his1*Δ::*hisG hog1*::*hisG*/*hog1*::*hisG*	3
CNC16	*ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434} *his1*Δ::*hisG*/*his1*Δ::*hisG HOG1*:*URA3*/*hog1*::*hisG*	3
CCC1	*ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434} *his1*Δ::*hisG*/*his1*Δ::*hisG cap1*Δ::*URA3*/*cap1*Δ::*HIS1*	This work
CHC1	*ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434} *his1*Δ::*hisG*/*his1*Δ::*hisG hog1*::*hisG*/*hog1*::*hisG cap1*Δ::*URA3*/*cap1*Δ::*HIS1*	This work
RCG1	*ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434} *his1*Δ::*hisG*/*his1*Δ::*hisG CAP1*/*CAP1*-GFP-*URA3*	This work
HCG1	*ura3*Δ::λ*imm*^{434}/*ura3*Δ::λ*imm*^{434} *his1*Δ::*hisG*/*his1*Δ::*hisG hog1*::*hisG*/*hog1*::*hisG CAP1*/*CAP1*-GFP-*URA3*	This work

are some indications of the physiological response of *C. albicans* to oxidants in vitro (32), and recently, the roles of two *C. albicans* genes were analyzed in some detail. A *YAP1* homologue named *CAP1* was isolated and shown to play a role in multidrug and oxidative stress resistance (1, 2). Mutational analysis of this gene revealed a terminal cysteine residue essential for the regulated nuclear localization of the protein (71), similar to the carboxy-terminal cysteine-rich domain of Yap1 (15, 37). These authors also reported differences between Yap1 and Cap1, since *CAP1* did not completely complement the H_2O_2 sensitivity of *yap1* strains (71). The *CAT1* gene, which encodes a protein with catalase activity, was also shown to be involved in oxidant susceptibility, and its deletion generated cells that were less virulent in the mouse acute systemic infection model (70).

In this work, we demonstrate that the Hog1 MAP kinase is essential for *C. albicans* resistance to oxidative stress through a mechanism different from those found in other yeast models, defining an additional role for this MAP kinase besides the previously described functions in osmoadaptation (58) and morphogenesis (3).

MATERIALS AND METHODS

Strains and growth conditions. Yeast strains are listed in Table 1. For clarity, and unless otherwise stated, *hog1* will always indicate the homozygous *hog1*/*hog1* Ura$^+$ strain (strain CNC13). Construction of *his1*Δ, *ura3*Δ *his1*Δ, *cap1*Δ, and *hog1 cap1*Δ strains is indicated below. Unless otherwise stated, RM100 was used as the wild-type strain. *his1*Δ strains behaved in the same manner as strain CAF2 or SC5314.

Yeast strains were grown in YPD medium (1% yeast extract, 2% peptone, 2% glucose) or SD minimal medium (2% glucose, 0.67% yeast nitrogen base without amino acids) with the appropriate auxotrophic requirements. Histidine and uridine were routinely added to liquid and solid media for phenotypic assays. The growth temperature was 37°C unless otherwise indicated. Usually, overnight cultures were refreshed to an optical density at 600 nm (OD_{600}) of 0.05, and experiments were performed when cultures reached an OD_{600} of 1 when exponential-phase cells were required. When stationary-phase cells were required, cells from a 3-day-old culture were routinely used. The viability of stationary-phase cultures was checked by staining with propidium iodide (14).

Deletion of the *HIS1* gene. A *Kpn*I fragment from YEpHISX carrying the *HIS1* gene (52) was subcloned in the *Kpn*I site of pUC19 to generate pUCHISA. A 0.7-kbp *Eco*RV fragment of pUCHISA (carrying the *HIS1* gene) was substituted with a blunt-ended *Bam*HI *hisG-URA3-hisG* cassette (obtained from pCUB6-K [58]) to generate plasmid pHP4. pHP4 was linearized by using *Sac*I and *Bam*HI to obtain a 7-kbp fragment for *HIS1* deletion in strain CAI4. Homologous recombination was achieved by using 2,170- and 730-bp flanking regions (5′ and 3′ fragments, respectively) to obtain strain RM1. Strains RM10, RM100, and RM1000 (Table 1) were obtained by using a previously described procedure (19). Correct disruptions were confirmed by PCR with primers oYBR7 (5′-AACAG TGTCGCCAGAATGTGCCG-3′) and oURA3 (5′-AGATCCAGATATTGAA GGTAAAAG-3′) for Ura$^+$ strains or oYBR7 and oHG1 (5′-GTTTTCCGCCA TCGCAATCAGGC-3′) for Ura$^-$ strains.

Deletion of the *CAP1* gene. The *CAP1* gene was isolated in a functional screening for the identification of azole-resistant clones in *S. cerevisiae* (to be described elsewhere). For deletion of this gene, we used a strategy in which the 5′ and 3′ regions of the gene to be deleted were amplified by PCR with oligonucleotides oCAP1 (5′-AGAGCGTCGACGGGCCCATGCAGATATTAAAA GAAAT-3′) and oCAP2 (5′-AGGCAGTCGACAAGCTTCAACTCATTTTTC AACACGTCTAC-3′) for the 5′ region and oligonucleotides oCAP3 (5′-GCG GACTAGTGATTTTGTCAAGAATTCATTACCT-3′) and oCAP4 (5′-ATAC CTGCGGCCGCTTAATGTTTTATACTTCGCTC-3′) for the 3′ region. These pieces of DNA were first independently subcloned in the pGEMT vector, generating plasmids pGEMT-5CAP1 and pGEMT-3CAP1. Then, the 5′ region was excised from pGEMT-5CAP1 by using the restriction sites *Sal*I and *Apa*I, and the 3′ region was excised from pGEMT-3CAP1 by using the restriction sites *Spe*I and *Not*I. The fragments were then included in a four-fragment ligation in plasmid pCAPUCf2 flanking the *URA3* marker. Similarly, the fragments were placed in plasmid pCAPHKn1 flanking the *HIS1* marker (to be described elsewhere). To delete the *CAP1* gene, strain RM1000 (*ura3 his1*) was cotransformed with plasmids pCAPUCf2 and pCAPHKn1 (digested with *Xba*I and *Not*I) by electroporation (34). Histidine and uracil prototrophs were isolated after 4 to 5 days of growth at 30°C and then replica plated on plates containing 4 mM hydrogen peroxide to detect knockouts. The correct integration of the construction in the sensitive clones was confirmed by PCR analysis with appropriate oligonucleotides from regions outside of the integration cassette. *hog1 cap1*Δ strains were obtained by deleting *CAP1* in strain CNC15 (3). Deletion of the *CAP1* gene was assessed by PCR with primers oCAP1 and oURA3 (described above) or oHIS1 (5′-GCTGTAACTTATTGAGTGGTGCCG-3′).

***CAP1*-GFP integration.** *CAP1*-green fluorescent protein (GFP)-expressing strains RCG1 and HCG1 were obtained through the integration of plasmid pDS569 (W. S. Moye-Rowley and D. Sanglard, unpublished data) in the *CAP1* locus by using *Sph*I to cut the *CAP1*-GFP construct. Strains showing correct integration were detected by PCR with oligonucleotides oCAP1 (see above) and $oGFP_{LO}$ (5′-CCAGTAGTACAAATAAATTTTAAGGTC-3′). Expression of the *CAP1*-GFP fusion was ascertained by Western blotting (see conditions below) with an anti-GFP monoclonal antibody (JL-8; Clontech, Palo Alto, Calif.).

Oxidative stress assays. Hydrogen peroxide, menadione sodium bisulfite (MD), and potassium superoxide (KO_2) were obtained from Sigma. Dilutions were performed by using sterile double-distilled H_2O. Susceptibility to hydrogen peroxide was measured by using exponential- or stationary-phase growing cells in YPD medium at 37°C. A total of 10^7 cells were transferred to an Eppendorf tube,

and hydrogen peroxide was added to a final concentration of 100 mM. The tube was incubated at 37°C, and 10-μl samples were collected at different times and spotted onto YPD plates. The plates were incubated for 24 h at 37°C and photographed. Susceptibility to MD and other oxidants at various concentrations was quantified in a similar way. Cell death was estimated by CFU counting or by propidium iodide staining and flow cytometry (14).

Protein extracts and immunoblot analysis. Yeast strains were grown to an optical density of 1 at 37°C in YPD medium. NaCl or hydrogen peroxide was added to the medium at a final concentration of 1.2 M or 10 mM, respectively. Samples were taken 10 min after the challenge or at different times. Cell extracts were obtained by using glass beads in a Fast Prep cell breaker as indicated previously (41). Equal amounts of proteins were loaded onto gels, as assessed by 280-nm measurements of the samples and Ponceau red staining of the membranes prior to blocking and detection. Blots were probed with phospho-p38 MAP kinase (Thr180-Tyr182) monoclonal antibody 28B10 (Cell Signaling Technology, Inc.) and a polyclonal antibody to *S. cerevisiae* Hog1 (Santa Cruz Biotechnology) and developed with a Hybond ECL kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The anti-*S. cerevisiae* Hog1 signal was used both as an internal control in the experiments concerning the activation of *C. albicans* Hog1 and as a loading control. Image densitometry was performed by Bio-Rad Multi-Analyst 1.1 software analysis of the bands scanned with a Bio-Rad GS-690 imaging densitometer.

Fluorescence microscopy. Yeast strains were grown at 30°C in SD minimal medium to an optical density of 0.8. Samples were centrifuged and washed twice with phosphate-buffered saline. Cells were resuspended in phosphate-buffered saline and maintained at 30°C for 15 min. For H_2O_2-treated cells, H_2O_2 was added to a final concentration of 0.4 mM. Cell fixation and nuclear staining (with propidium iodide) were performed as described previously (3). Fluorescence microscopy was carried out with a Nikon Eclipse TE2000-U microscope at a magnification of ×100. Images were captured by using a Hamamatsu ORCA-ER CCD camera with AquaCosmos 1.3 software. All images were processed in the same manner and mounted by using Adobe Photoshop 4.0.

Catalase assays. Strains were grown to early exponential phase (OD_{600}, 0.4), and H_2O_2 was added to a final concentration of 0.4 mM. Samples were taken at 0, 15, 30, and 60 min after H_2O_2 addition. Total protein extracts of different strains were obtained by glass bead breakage in the presence of protein inhibitors and used for measurement of catalase activity as indicated previously (8). The amounts of proteins in the extracts were quantified by measuring the absorbance at 280 nm.

Chlamydospore formation. Chlamydospore formation was assayed essentially as indicated previously (62). The borders of more than 50 colonies were examined for each strain tested.

RESULTS

***HOG1* mediates resistance to oxidative stress.** In order to understand the lack of virulence of *C. albicans hog1* mutants in a systemic model of infection (3), we examined other phenotypic traits of this mutant. Invasion of epithelial cells was not impaired in *hog1* strains (F. Navarro-García, F. García del Portillo, C. Nombela, and J. Pla, unpublished observations), suggesting that other defects should be responsible for the loss of virulence. Thus, we tested the sensitivity to oxidative stress of *hog1* mutants, since ROS production is one of the mechanisms used by cells of the immune system for killing microbial pathogens (66). We subjected exponential- or stationary-phase growing cells to externally added hydrogen peroxide (100 mM H_2O_2) to test the viability of the cultures at different times. In both cases, the *hog1* mutant strain displayed enhanced susceptibility to this compound compared to the wild-type strain. As shown in Fig. 1A, exponentially growing cells were found to be more susceptible to H_2O_2, the *hog1* cells being unable to grow after a treatment of 0.5 to 1 min; in contrast, longer times (more than 2 min; data not shown) were required to reduce the viability of the wild-type cells to the same level. In stationary phase, the times required for the same reductions in viability were 8 min for the mutant and 30 min for the wild type (Fig. 1A). It has been shown that oxidant sensitivity diminishes when yeast cells enter stationary phase as a consequence of metabolic changes that enhance the endogenous production of ROS, thus generating an adaptive response to oxidants (42). Our results indicate that the entry of *C. albicans* cells into stationary phase, similar to what is seen for *S. cerevisiae*, increases cell resistance to oxidants. Although this is also true for *hog1* mutant cells, they are still more sensitive than wild-type cells to H_2O_2 (Fig. 1A). The higher sensitivity of *hog1* mutants was also observed when cells were spotted onto YPD plates containing 1 mM H_2O_2 (data not shown). We also found that pretreatment of the cells at 42°C for 30 min did not affect sensitivity to hydrogen peroxide (no to hypertonic medium) in assays on solid medium (data not shown), indicating that the types of responses generated to heat and oxidants are, to a certain extent, separate phenomena in this organism.

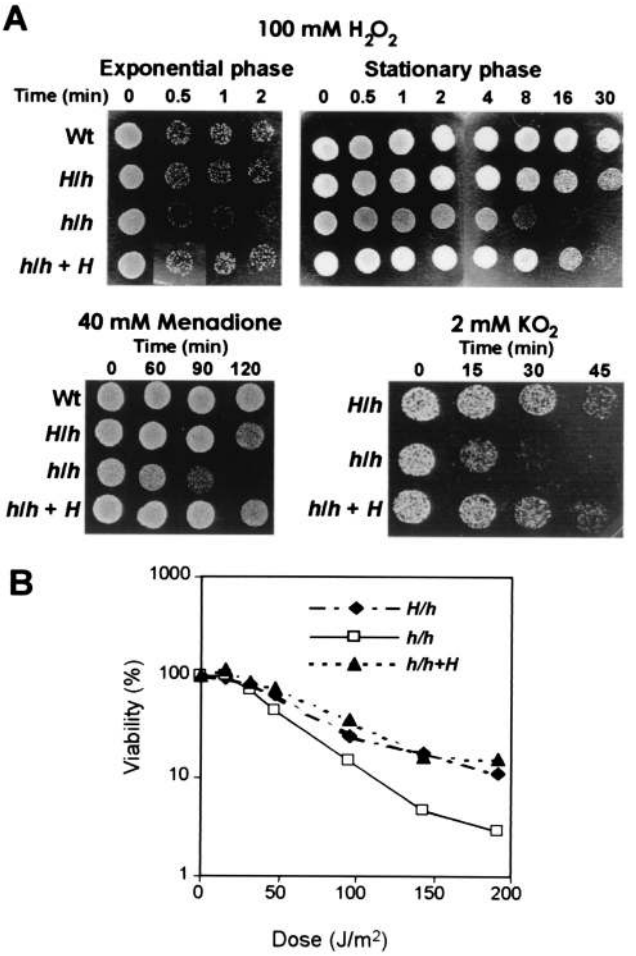

FIG. 1. *C. albicans hog1* mutants are sensitive to oxidative stress. (A) *C. albicans* cells were exposed to 100 mM hydrogen peroxide, 40 mM MD, and 2 mM KO_2 in liquid YPD medium. Samples were taken at different times, diluted, and spotted onto YPD plates (10^5 cells in 10 μl). (B) A total of 100 to 200 *C. albicans* cells were plated on YPD plates and irradiated at 254 nm at different times by using a UV source. Plates were incubated at 37°C, and the percent survival was calculated by CFU counting after 24 h. The results are the means of three experiments. The standard error was always less than 10%. Strains used were as follows: Wt, *HOG1*/*HOG1*; *H*/*h*, *HOG1*/*hog1*; *h*/*h*, *hog1*/*hog1*; and *h*/*h* + *H*, *HOG1* reintegrated in a *hog1*/*hog1* strain (Table 1).

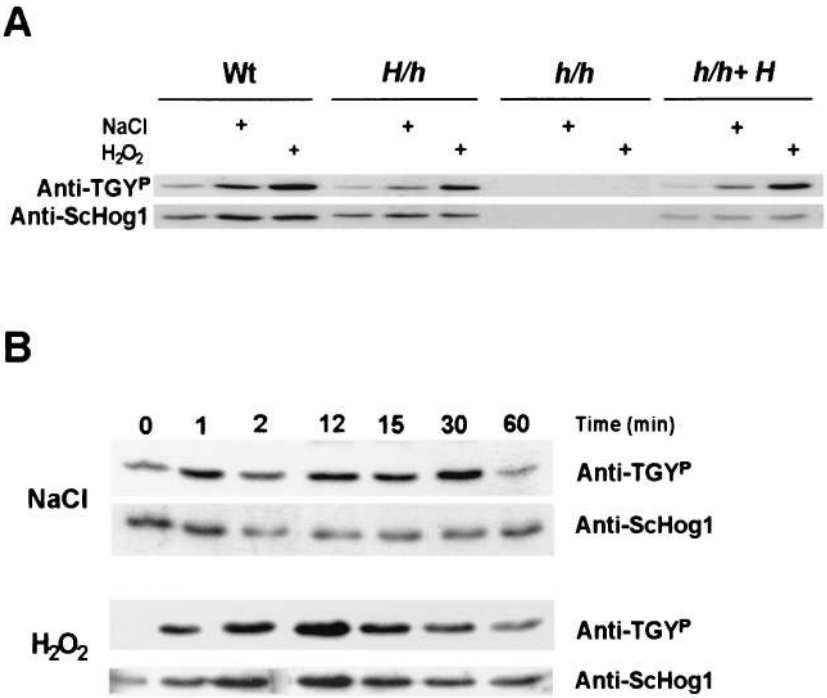

FIG. 2. Hog1 is phosphorylated in the presence of NaCl and H_2O_2 with similar kinetics. Western blots show the activation of Hog1 after H_2O_2 (10 mM) or NaCl (1.2 M) treatment for 10 min (A) or in a kinetic study at different times (B). Intermediate times were analyzed in the kinetic study, but only representative time points are shown. The same blots were assayed with a monoclonal antibody raised against phospho-p38 (Anti-TGY^P) and a polyclonal antibody raised against *S. cerevisiae* Hog1 (Anti-ScHog1) (see Materials and Methods). See the legend to Fig. 1 for strain designations.

Oxidants can be generated inside the cell by different mechanisms based either on the cellular metabolism or the effect of external agents, such as UV light. These can generate free radicals (O_2^-) that are detoxified by the sequential action of superoxide dismutases ($O_2^=$ generators) and catalases (O_2 generators) (42). We therefore determined the effects of other agents different from peroxides on *hog1* strains. To address the capacity of *hog1* cells to detoxify superoxide anion ($O_2^{\cdot -}$), we tested sensitivity to MD (40 mM), a superoxide anion generator that affects *S. cerevisiae* but in a manner different from that of hydrogen peroxide (30). *hog1* cells were found to be more sensitive than wild-type cells to this agent at a 40 mM concentration; control experiments showed that sodium bisulfite, either in liquid or on solid media, had no effect (data not shown). As shown in Fig. 1B, mutant cells were unable to grow after 120 min of treatment (growth defects were already evident at 90 min), while wild-type cells and the single-knockout strains grew normally under these conditions. Consistently, the effect on viability of another substance producing superoxide anion was qualitatively similar, as evidenced when cells were exposed to 2 mM KO_2. While heterozygous strains could grow after 30 min of treatment, the *hog1* mutant died (Fig. 1). As already described for other genes in *C. albicans* (35, 47, 58), the presence of one copy of the wild-type *HOG1* gene, either in the heterozygous strain or in the strain with reintegrated *HOG1*, conferred an intermediate phenotype. The *hog1* mutants also showed increased susceptibility to UV irradiation, as indicated by the faster loss of viability for these mutants than for the heterozygous mutants (which showed sensitivity similar to that of wild-type strains; data not shown) (Fig. 1). All of these findings demonstrate that Hog1 function is important for survival in the presenc of oxidative stress.

The HOG pathway in *C. albicans* is activated in response to oxidative stress. The experiments described above indicated that Hog1 plays a role in the protective response against oxidants in *C. albicans*, suggesting that such a function for the HOG pathway would also exist in *C. albicans*. A likely—but not exclusive—explanation for the protective role of Hog1 is that oxidative stress itself could activate the pathway mediated by this MAP kinase. This is in fact the situation for the nonpathogenic fungus *S. pombe* (12, 57). We therefore analyzed the activation of this pathway by using a monoclonal antibody raised against phospho-p38, a mammalian homologue of the Hog1 MAP kinase (22, 25). This antibody recognizes the TGY motif characteristic of stress-activated MAP kinases activated by phosphorylation in threonine and tyrosine (6), thus providing a demonstration of the activation of the pathway. Protein extracts were obtained from *C. albicans* wild-type and *hog1* cells exposed or not exposed to osmotic stress and oxidative stress and analyzed by Western blotting with the aforementioned antibody. We detected a band of about 43 kDa in response to osmotic stress (1.2 M NaCl, 10 min). No bands of the same size could be detected in extracts of *hog1* mutants or in extracts of nontreated strains. Reintroduction of a functional copy of *HOG1* into the genome of a *hog1* mutant restored the detection of the same band in extracts of osmotically treated cells (Fig. 2A). After stripping of the anti-phospho-p38 antibody, we immunoblotted the same membrane with a com-

mercial *S. cerevisiae* Hog1 polyclonal antibody, which recognized a band of the same size (≈43 kDa) in extracts of *HOG1* strains but not *hog1* strains (Fig. 2A). This was the only band that did not appear in *hog1* strain protein extracts under basal or stress conditions when tested with the *S. cerevisiae* Hog1 polyclonal antibody. The detection of the band of phosphorylated Hog1 with the phospho-p38 monoclonal antibody under conditions of osmotic stress is consistent with the originally described role of the HOG pathway in *S. cerevisiae* and *C. albicans* (5, 58). The similarity of *C. albicans* Hog1 to the *S. cerevisiae* homologue (≈80%) is the reason for the cross-reactivity with the Hog1 polyclonal antibody.

For oxidatively stressed cells (10 mM H_2O_2, 10 min), the anti-phospho-p38 antibody detected a 43-kDa band that cross-reacted with the *S. cerevisiae* Hog1 polyclonal antibody as well (Fig. 2A). Moreover, the phosphorylation signal increased significantly upon exposure of the cells to oxidative stress compared to osmotic stress (Fig. 2A). Image densitometry revealed that the intensities of the signals in sodium chloride stress and in oxidative stress were two times and three times higher, respectively, than that under basal conditions.

To explore the characteristics of the responses to both stimuli, we performed kinetic studies of the activation of the pathway by monitoring Hog1 phosphorylation. The results showed that the onset of activation was similar and fast in both cases; phosphorylation was detected after 1 min of treatment (Fig. 2B). In the presence of both stimuli, the signal extinction was also similar, although H_2O_2-induced phosphorylation was still high after 1 h of H_2O_2 treatment (Fig. 2B). We also found that Hog1 is phosphorylated over a broad range of H_2O_2 concentrations (from 0.4 to 100 mM; unpublished data). All of these results indicate the specificity of Hog1 phosphorylation induced by either osmotic or oxidative stress.

Deletion of *CAP1* in a *hog1* background leads to additive phenotypes in response to oxidative stress. Yap1, a bZIP transcription factor, has been shown to be involved in resistance to oxidative stress in *S. cerevisiae* (59, 63) through the transcription of different genes in an oxidant-specific fashion (10), a phenomenon that is correlated with its nuclear location (37). The homologue of Yap1 in *C. albicans*, Cap1, has been cloned (1) and characterized. This protein also localizes in the nucleus in response to oxidative stress (71). Deletion of *CAP1* renders the cells hypersensitive to different compounds—in particular, hydrogen peroxide—(2). In view of the facts that *CAP1* is the best-characterized *C. albicans* gene involved in oxidative stress and that transcription factors are common targets of MAP kinase pathways, we addressed the possibility of the existence of an epistatic relationship of *CAP1* with the Hog1 pathway. To this end, we deleted the *CAP1* gene from both a wild-type background (RM1000) and a *hog1* background. Strain RM1000, used for *CAP1* deletion, was derived from CAI4 and carried a deletion of both alleles of the *HIS1* gene constructed by *URA3* gene Blaster methodology (19) (see Materials and Methods). Disruption of *HIS1* did not affect growth rate (data not shown) or virulence (3). Deletion of *CAP1* was accomplished by using the two-marker strategy (48), and the correct (homologous) integration of the genetic construction was checked by PCR with appropriate internal primers (see Materials and Methods). Strains with a deletion of the *CAP1* gene did not show a significant reduction in growth either in normal minimal medium or in rich liquid medium at 37°C; the doubling times of these strains were similar to those of their parental strains (data not shown). Restoration of *CAP1* in either *cap1*Δ or *hog1 cap1*Δ strains led to a recovery of the *CAP1*-deficient phenotype, that is, sensitivity to both cadmium stress and oxidative stress (data not shown).

Confirming previous observations (2), *cap1*Δ mutants were found to be hypersensitive to hydrogen peroxide and a variety of other oxidants in solid medium. In a standard diffusion method, *cap1*Δ and *hog1* mutants behaved similarly; double mutants were more sensitive (data not shown). However, the differences between both types of mutants were evident in experiments in which a rapid adaptive response was required. Incubation of exponentially growing cells (optical density, 0.5) with 50 mM H_2O_2 resulted in a rapid death of *hog1* cells, which were unable to grow after 4 min of incubation, while cells of heterozygous strains could grow longer (Fig. 3A). The *cap1*Δ mutant behaved in a manner similar to that of *hog1* mutants; however, the *hog1 cap1*Δ double mutant died soon after treatment, indicating an increased sensitivity to H_2O_2, higher than that of *hog1* or *cap1*Δ single mutants (Fig. 3A). A higher temperature aggravated this phenotype; the growth defect was more evident at 42 and 37°C than at 30°C, and the effect was additive for *hog1 cap1*Δ with respect to *hog1* (data not shown).

In order to define precisely the response to oxidative stress, we performed a detailed kinetic analysis of cell death when cells were exposed to hydrogen peroxide in liquid cultures. Viability was monitored by propidium iodide staining. As shown in Fig. 3B, the addition of 100 mM H_2O_2 resulted in a clear reduction after 4 h in the viability of *hog1* mutants (34% viability) compared to *cap1*Δ mutants (50%) and wild-type and heterozygous strains (70 and 62%, respectively). The viability of the *hog1 cap1*Δ double mutant was lower (20%) than that of *hog1* or *cap1*Δ mutants (Fig. 3B). These results indicate that there are clear differences in the adaptation to oxidative stress between both types of mutants; *cap1*Δ mutants are able to survive the challenge, probably through the production of an alternative response that *hog1* or *hog1 cap1*Δ mutants cannot elicit. Since *hog1 cap1*Δ mutants showed exacerbated sensitivity to H_2O_2, the Cap1- and Hog1-mediated mechanisms seem to be at least partially independent, and each contributes to the susceptibility observed.

Hog1 and Cap1 mediate different oxidative stress response mechanisms. The differences in the type and intensity of stress responses between *cap1*Δ and *hog1* mutants also suggest differences in phenotypes for other types of stress.

We addressed the sensitivities of single and double mutants to specific types of stress previously described as harmful for each of the traits under consideration, namely, high osmolarity for *hog1* and heavy metals for *cap1*Δ. As shown in Fig. 4A, only *hog1* and *hog1 cap1*Δ mutants were hypersensitive to osmotic stress (1 M NaCl), whereas *cap1*Δ mutants behaved like the wild-type strain. In contrast, only *cap1*Δ and *hog1 cap1*Δ mutants did not grow in the presence of 150 μM cadmium (Fig. 4A). It follows that the responses to osmotic stress and this heavy metal do not overlap in *C. albicans*.

We also studied the responses of these strains to two types of oxidative agents: hydrogen peroxide and MD. The responses to peroxide were similar in the wild type and *cap1*Δ mutants, which were less sensitive than *hog1* and *hog1 cap1*Δ mutants to

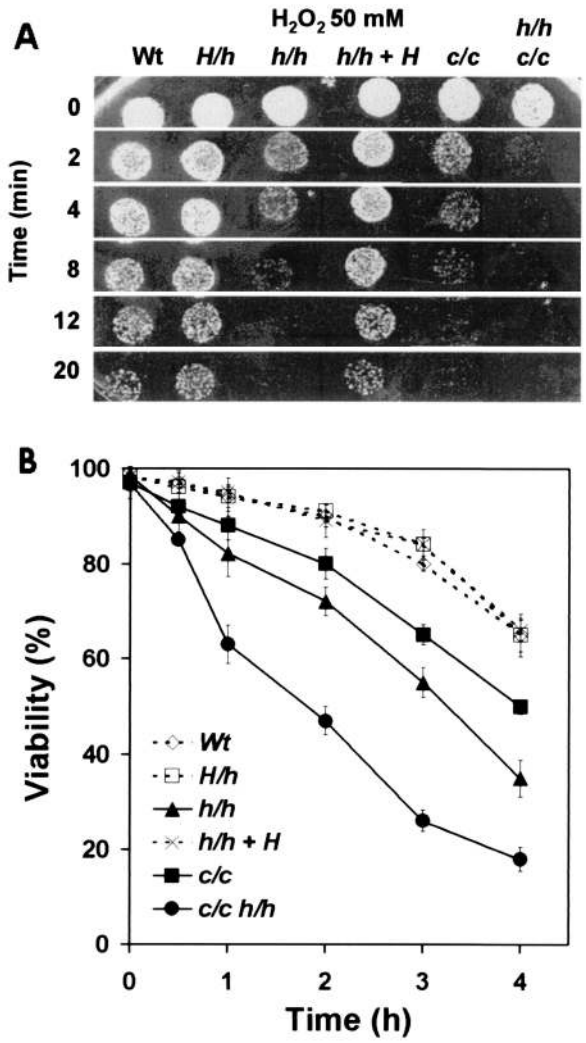

FIG. 3. *hog1 cap1*Δ knockout strains are more sensitive to oxidative stress than *hog1* or *cap1*Δ knockout strains. (A) *C. albicans* exponentially growing cells were exposed to 50 mM hydrogen peroxide in liquid YPD medium as indicated in Materials and Methods and spotted onto YPD plates. The plates were incubated at 37°C for 24 h. (B) The viability of different strains in YPD medium containing 100 mM hydrogen peroxide was determined by fluorescence-activated cell sorting analysis with propidium iodide as a cell death marker. Error bars indicate standard errors. *c*/*c*, *cap1*Δ/*cap1*Δ strain; *h*/*h c*/*c*, *hog1*/*hog1 cap1*Δ/*cap1*Δ strain; see the legend to Fig. 1 for other strain designations.

5 mM H_2O_2 (Fig. 4B). On the other hand, the responses to MD were different; *cap1*Δ mutants were slightly sensitive but more resistant than *hog1* mutants (Fig. 4B). Interestingly, the *hog1 cap1*Δ double mutant could not grow at all in the presence of the same concentration of MD, showing that its ability to overcome superoxide stress is markedly impaired (Fig. 4B). These results represent a clear indication that Cap1 and Hog1 operate through different mechanisms to overcome oxidative stress, since the double mutants were much more sensitive than the single-knockout mutants but maintained the defects of the individual mutants.

To search for potential targets of the Hog1 or Cap1 activity (as has been done for *S. cerevisiae* or *S. pombe*), we measured catalase activity, which is one of the enzymatic mechanisms that cells use to eliminate peroxides and which has also been indicated to be an important element in resistance to neutrophils in *C. albicans* (70). Catalase activity was induced similarly in *cap1*Δ and wild-type cells exposed to 0.4 mM H_2O_2 (Fig. 4C). Interestingly, the *hog1* and *hog1 cap1*Δ strains displayed higher levels of catalase activity, in some cases twice the levels found in wild-type and *cap1*Δ strains (Fig. 4C). Basal catalase activity was also higher in the *hog1* and *hog1 cap1*Δ strains (2.8 and 4.4 μmol of H_2O_2 degraded per min per mg of protein, respectively, versus 2.1 and 1.4 for wild-type and *cap1*Δ strains). It follows that catalase activity depends on neither Cap1 nor Hog1 function but is increased in the absence of Hog1.

In the preceding experiments, we showed that the defective phenotype of *cap1*Δ strains is not as dramatic as the *hog1* mutant phenotype under conditions of oxidative stress. Regarding the possible influence of Cap1 in Hog1 activation, we must take into account the increase in oxidative radicals in the

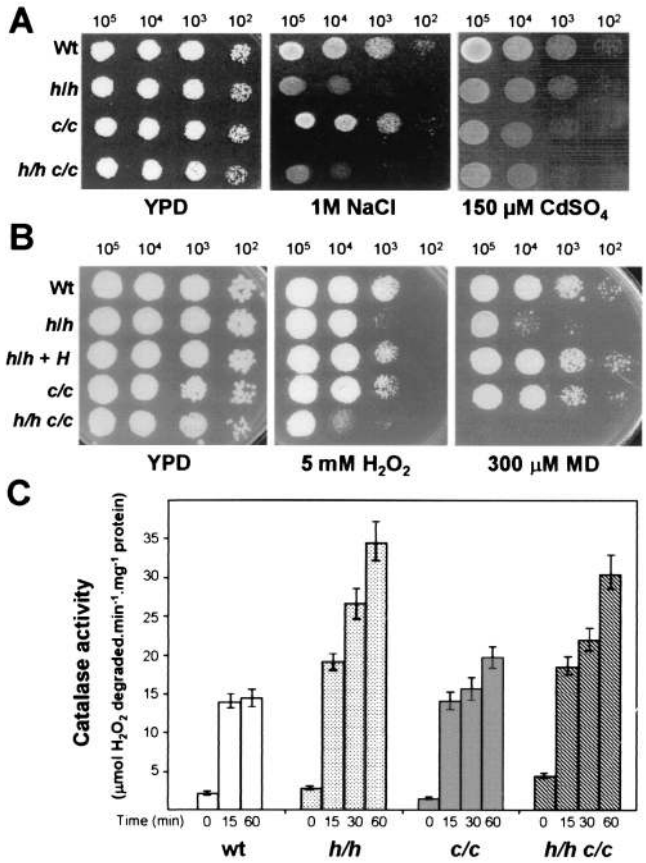

FIG. 4. *hog1* and *cap1*Δ knockout strains behave differently, the *hog1 cap1*Δ double mutant having some mixed phenotypes. (A) Responses to high osmolarity and heavy metal stress of wild-type and mutant strains. Serial 10-fold dilutions of exponentially growing cells of the strains indicated were spotted onto YPD plates containing 1 M NaCl or 150 μM $CdSO_4$. (B) Responses to different types of oxidative stress of wild-type and mutant strains. Serial 10-fold dilutions of exponentially growing cells of the strains indicated were spotted onto YPD plates containing 5 mM H_2O_2 or 300 μM MD. (C) Catalase activities in *hog1*, *cap1*Δ, and *hog1 cap1*Δ mutants. The results are the means and standard deviations of three experiments.

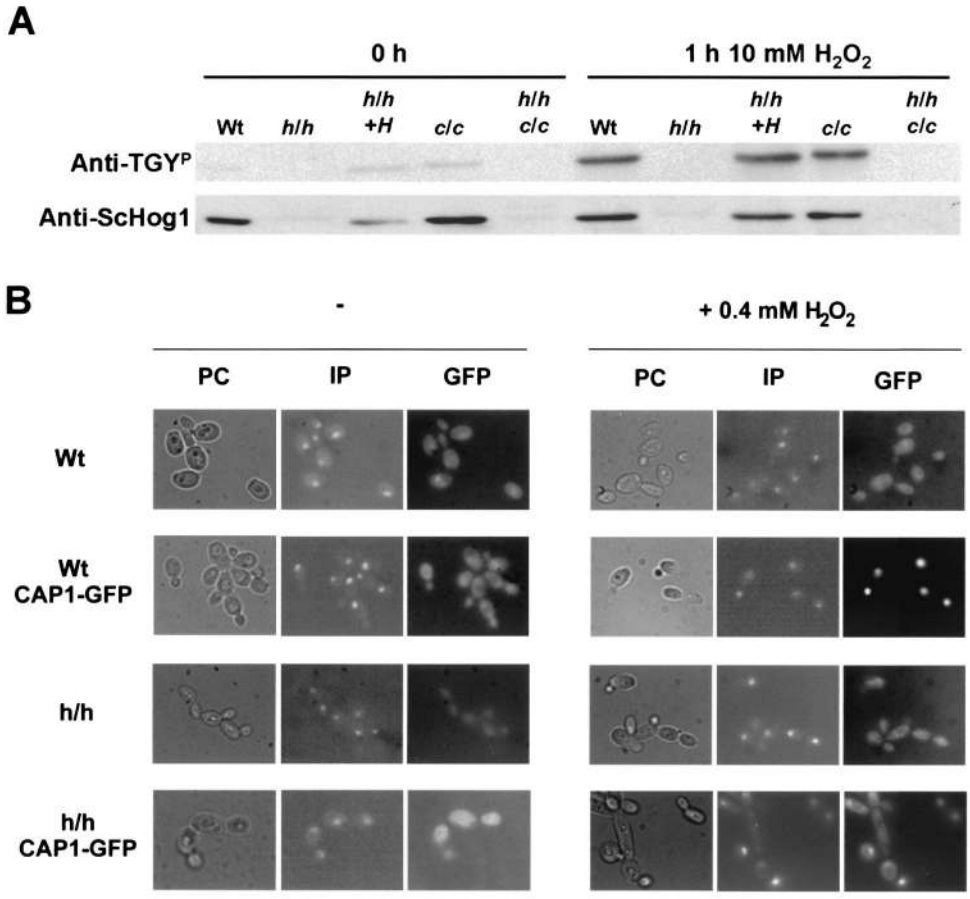

FIG. 5. Upon oxidative stress, Cap1 did not affect the phosphorylation of Hog1, and Hog1 did not affect the nuclear localization of Cap1. (A) Hog1 is phosphorylated in the presence of hydrogen peroxide independently of Cap1. A Western blot shows the Cap1-independent activation of Hog1 after 1 h in the presence of 10 mM H_2O_2. The same blot was assayed with a monoclonal antibody raised against phospho-p38 (Anti-TGY^P) and a polyclonal antibody raised against *S. cerevisiae* Hog1 (Anti-ScHog1) (see Materials and Methods). (B) Cap1 localization is not affected in a *hog1* mutant upon oxidative stress. Cap1 localizes to the nucleus in the presence of oxidative stress (0.4 mM H_2O_2, 15 min) irrespective of the genetic background. Cells were observed by phase-contrast microscopy (PC), and nuclei were observed by using propidium iodide (IP) to ascertain the localization of the Cap1-GFP fusion (GFP). More than 300 cells were observed, and representative images of different strains were chosen.

medium, which cannot be counteracted by the contribution of Cap1, so that it is feasible that it could enhance the response of Hog1 by a compensatory mechanism. To address this question, we measured the phosphorylation of Hog1 in different strains (Fig. 5A). This activation is very fast (1 min) (Fig. 2B), so that one could expect an increased level of phosphorylation in *cap1*Δ mutants only under basal conditions or if the stimulus were present. Neither of those possibilities was confirmed; in both cases, the levels of Hog1 phosphorylation seemed to be equal (Fig. 5A). Thus, the absence of Cap1 function does not seem to influence the Hog1 pathway in terms of activation.

StyI controls the localization of Pap1 to the nucleus in *S. pombe* (65). We tested the possibility that Hog1 could influence the nuclear localization of Cap1. For that purpose, we integrated a *CAP1*-GFP construct into the *CAP1* locus in either a wild-type or a *hog1* background to generate strain RCG1 or HCG1, respectively. The correct integration was confirmed by PCR (data not shown), and the expression of the full-length fusion was detected by using anti-GFP antibodies (data not shown; see Materials and Methods for details). Cap1 localized to the nucleus in the presence of H_2O_2 in both wild-type and *hog1* backgrounds (Fig. 5B), showing that in the absence of Hog1, Cap1 is still able to enter the nucleus. All of these data confirm that Hog1 and Cap1 act in parallel—and independently—in sensing and responding to oxidative stress damage in *C. albicans*.

***hog1* mutants are defective in chlamydospore formation, an oxygen-dependent morphogenetic program.** Alonso-Monge et al. previously showed that *C. albicans* Hog1 functions as a repressor of morphological transitions in both *S. cerevisiae* and *C. albicans* (3), a result in close agreement with the situation for *S. cerevisiae* Hog1 (50). In view of the involvement of this MAP kinase in both morphogenesis and oxidative stress responses, a specific phenotype for chlamydospore formation was anticipated for Hog1-deficient strains. Chlamydospores are thick-walled structures that develop under certain experimental conditions (low temperature, defined nutritional media and, probably related to oxidative stress, the absence of light and the presence of a microaerophilic environment). Wild-type cells (SC5314) were able to form chlamydospores after 4 to 5 days of growth at 24°C on cornmeal agar (Fig. 6). These structures appeared at the tips of growing hyphae (either long [Fig.

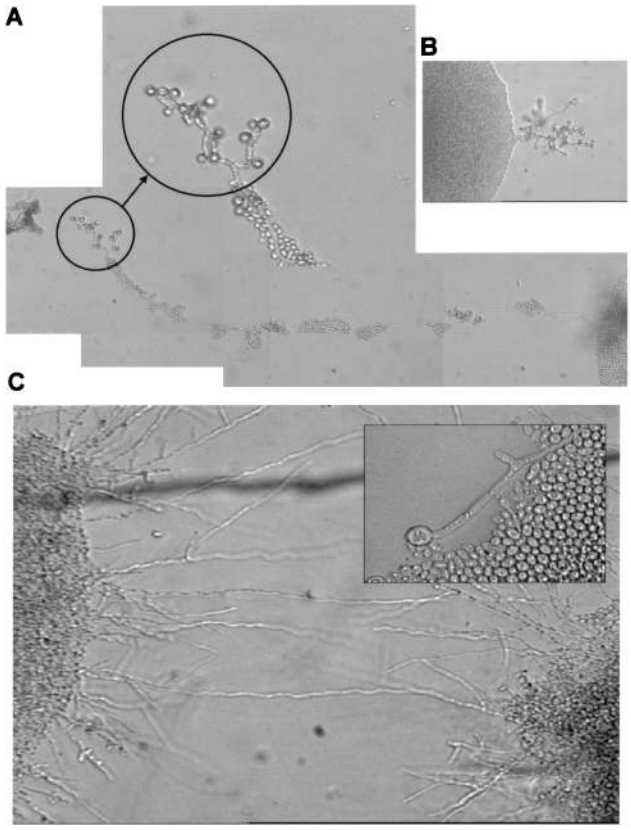

FIG. 6. *hog1* mutants show defects in chlamydospore formation. Images show the wild-type strain (A and B) and *hog1* mutants (C) on corn meal agar. No formation of chlamydospores can be observed at the end of the *hog1* mutant hyphae, in contrast to those of the wild-type strain.

6A] or short [Fig. 6B]) under microaerophilic conditions. We observed the formation of abundant chlamydospores, which were round and had thick walls, in wild-type cells. In contrast, in *hog1* mutants, chlamydospore formation was completely abolished. The colonies displayed the hyperfilamentous phenotype characteristic of *hog1* mutants and, very infrequently, some structures at some hyphal tips slightly resembled chlamydospores and might have represented immature chlamydospores (Fig. 6C, inset). *cap1*Δ mutants were able to form chlamydospores, but *hog1 cap1*Δ cells were not. Since the only gene that has been described to date to play a role in this process is *EFG1* (62), which encodes a transcription factor also shown to be involved in morphogenesis (64), it is feasible that the activation of *EFG1* is *HOG1* dependent. The overexpression of *EFG1* under the control of the regulated *PCK1* promoter resulted in enhanced filamentous growth, as expected, but did not suppress the defect in chlamydospore formation (data not shown). These results support a role for *HOG1* in chlamydospore formation in *C. albicans* through a mechanism independent from *EFG1*.

DISCUSSION

Novel mechanism for oxidative stress resistance in a pathogenic fungus. In this work, we have shown for the first time that the Hog1 MAP kinase plays a role in the responses to oxidants of the pathogenic fungus *C. albicans*. This finding is particularly relevant for pathogenesis, since adaptation to the human body requires the pathogen to cope with host natural defenses, such as macrophages or neutrophils, whose capacity for oxidative killing of the invader is essential to controlling the outcome of fungal infections (66, 70). Other responses of pathogenic fungi to natural oxidative stress are the blocking of the macrophage oxidative burst in *Histoplasma capsulatum* (29); the production of polyols, such as mannitol, to scavenge reactive oxygen intermediates in *Cryptococcus neoformans* (7); and the activation of a TEY-MAP kinase-mediated pathway in *Pneumocystis carinii* (20). Our results show a novel way (activation of a "stress-activated" MAP kinase route [38]) in which a pathogenic fungus can escape from oxidative stress. An analysis of the killing of *Candida hog1* mutants by macrophages and/or neutrophils could explain the reduced virulence of *hog1* mutants (3) as being due to the reduced ability of these mutants to respond to oxidative stress, as shown by the higher sensitivity of *hog1* mutants to peroxide- or superoxide-producing compounds and UV light.

Cells of the immune system produce oxidants on a continuous basis, thus maintaining constant concentrations (up to 0.5 mM $ONOO^-$ per min [67] or 0.5 mM H_2O_2 [56]) in the phagolysosomes of macrophages. Our experiments in which a small amount of H_2O_2 was maintained could mimic this phenomenon in vivo. In other experiments, we used larger amounts of oxidants (10 to 100 mM) to study the acute response of the cells to a single challenge of oxidants, which is probably exhausted over the time course of the experiments. In both situations, *hog1* strains showed increased sensitivity to oxidants, indicating that Hog1 function is needed for both acute and adaptive responses to oxidants. Pretreatment of *hog1* cells with a sublethal amount of oxidant (10 mM hydrogen peroxide for 1 h) resulted in enhanced survival when the cells were challenged with higher doses of oxidants (100 mM). The viability of *hog1* cells increased from 52% to 83% when the cells were pretreated with 10 mM hydrogen peroxide for 1 h. On the contrary, the viability of *hog1 cap1*Δ cells was not increased by the same treatment (data not shown). Adaptation to oxidative stress does not simply imply a Hog1-dependent transcriptional adaptive mechanism but probably implies underlying altered levels of antioxidant defenses. However, our evidence clearly shows the specificity of the Hog1-mediated response to oxidative stress, because Hog1 was activated by phosphorylation in the presence of H_2O_2 (Fig. 2). We also showed that an increase in external osmolarity is signaled by Hog1 phosphorylation. It should be noted that this is the first time that stimuli previously presumed to activate a MAP kinase route in *C. albicans* have been demonstrated biochemically. Most of the data concerning the involvement of MAP kinase routes in different functions in this microorganism have been deduced from epistasis experiments and extrapolation of data obtained for model yeasts, such as *S. cerevisiae* (46).

Relationship of Hog1 to other proteins involved in the oxidative stress response. Cap1 and Cat1 are the only proteins that had been associated with the oxidative stress response in *C. albicans* (1, 2, 70, 71). Our results from epistasis analyses also demonstrated that Hog1 and Cap1 play separate roles in oxidative stress defense and operate through different mechanisms. There are several arguments in favor of these conclu-

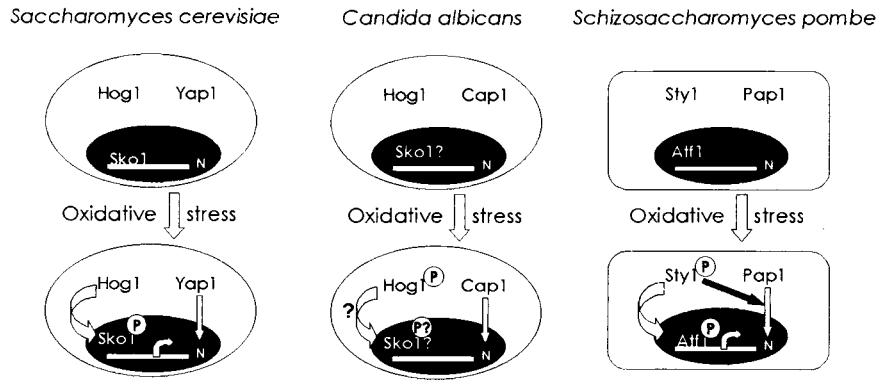

FIG. 7. *C. albicans* seems to respond to oxidative stress in a manner different from those of *S. cerevisiae* and *S. pombe*. The scheme shows the different ways in which *C. albicans*, *S. cerevisiae*, and *S. pombe* respond to oxidative stress. N, nucleus. The white bar inside the nucleus represents DNA. See Discussion for a detailed explanation.

sions. First, the patterns of susceptibility to the same oxidant agent (either hydrogen peroxide or MD) are different in *cap1*Δ and *hog1* mutants, and the phenotype of the double mutant is aggravated (Fig. 3 and 4). Second, Hog1 and Cap1 respond—and maintain—separate responses to different stimuli, indicating that they do indeed participate in different physiological processes, such as heavy metal resistance, the osmotic response (Fig. 4A), or chlamydospore formation (Fig. 6), a morphological process related to oxygen availability and in which only the *EFG1* regulator has been shown to play a role (62). *hog1* and *hog1 cap1*Δ mutants are defective in chlamydospore formation or growth in high-osmolarity media, defects that are not observed in *cap1*Δ mutants. Third, in *cap1*Δ mutants, oxidative stress-induced Hog1 activation is not substantially altered in response to the stimulus (Fig. 5A). Fourth, Cap1 nuclear localization is not affected in *hog1* mutants (Fig. 5B). All of these data suggest distinct and complementary roles of Hog1 and Cap1 in resistance to oxidative stress, as recently described for the homologous proteins in *S. pombe* (54).

With regard to catalase, we found that the activity of this enzyme was increased in both *hog1* and *cap1*Δ mutants (Fig. 4C), although both mutants were still sensitive to H_2O_2. These results suggest that this activity is independent of both proteins and that there are alternative ways to sense and respond to oxidative stress that could induce the activity of catalase independently of Hog1 or Cap1. Such a mechanism would operate as an emergency mechanism for cell rescue in the absence of the capacity (which the HOG pathway seems to provide) to sense and respond to oxidative stress in *C. albicans*. The higher basal levels and the greater increase in the levels of catalase activity in *hog1* and *hog1 cap1*Δ mutants (Fig. 4C) could suggest this explanation. Although catalase activity seems to be important for resistance to oxidative stress (70), the results shown in Fig. 4C indicate that in *C. albicans*, mechanisms other than catalase activity are important for overcoming oxidative stress challenge, since *hog1* and *cap1*Δ strains were very sensitive, despite their ability to increase their catalase activity.

A molecular model for the oxidative stress response in *C. albicans*. Several data indicate that the response of *C. albicans* to oxidative stress has several specificities compared to those of the classical species used as yeast model systems. For example, *C. albicans hog1* mutants display intermediate sensitivity to UV light (Fig. 1). Whereas *S. cerevisiae hog1* mutants are not sensitive to UV irradiation (61), *S. pombe sty1*$^-$ mutants are very sensitive, StyI being phosphorylated in wild-type strains in the presence of UV light (12). Moreover, *cap1*Δ mutants can increase their catalase activity in the presence of H_2O_2 (Fig. 4C), while *S. pombe pap1*$^-$ mutants cannot (although the expression of *ctt1*$^+$ increases) (45). In addition, *pap1*$^-$ mutants show intermediate sensitivity to acute oxidative stress similar to that of *cap1*Δ mutants (Fig. 3B); both types of mutants are less sensitive than *sty1*$^-$ or *hog1* strains (Fig. 3B) (54). Thus, the mechanism of the oxidative stress response in *C. albicans* is different from those in *S. cerevisiae* or *S. pombe*.

Models of the oxidative stress responses in *S. cerevisiae* and *S. pombe* are depicted in Fig. 7. In *S. cerevisiae*, Hog1 is not phosphorylated upon oxidative stress (data not shown) (61), although *hog1* mutants are sensitive to H_2O_2 (55). A bZIP transcription factor, Sko1, has been shown to be a repressor of the transcription of some oxidative stress response genes. Interestingly, they are also downstream targets of Yap1 (17), and their expression—even in *sko1*Δ mutants—is dependent on Yap1. The phosphorylation of Sko1 by Hog1 disturbs the Ssn6-Tup1-Sko1 repressor complex (53), probably allowing the transcription of these genes (55). *S. cerevisiae* Sko1 is the homologue of *S. pombe* Atf1 (Fig. 7). In *S. pombe*, StyI plays two roles: it activates through phosphorylation the transcription factor Atf1 (54, 60, 69), and it influences the nuclear localization of Pap1 during oxidative stress (Fig. 7) (65). In addition, *sty1*$^-$ mutants show high sensitivity to H_2O_2, which is higher than that of the *atf1*$^-$ or *pap1*$^-$ single mutants but very similar to that of the *atf1*$^-$ *pap1*$^-$ mutants, indicating that StyI controls both mechanisms of the oxidative stress response in *S. pombe* (54).

The phenotypes of *C. albicans hog1* mutants share some characteristics with counterparts in the two model systems, so that a mixed scheme can be envisioned for *C. albicans* on the basis of important distinctive features: Hog1 is phosphorylated in the presence of oxidative stress, and no relationship that mediates different responses to oxidative stress seems to exist between Hog1 and Cap1 (Fig. 7). Moreover, the nuclear localization of Cap1 upon oxidative stress is not affected in *hog1* mutants, and neither is the basal phosphorylation status of Hog1 in *cap1*Δ mutants, thus reinforcing our hypothesis of

Cap1 and Hog1 sensing and responding to oxidative stress in independent ways. The existence in the genome of *C. albicans* of an Sko1 homologue whose structure is more similar to that of Atf1 than to that of *S. cerevisiae* Sko1 could suggest a mechanism of transmission of the signal to the nucleus more like that of *S. pombe*, but regulated by *C. albicans* Hog1. *C. albicans* Sko1 has a region which is similar to the Hog1 phosphorylation region in *S. cerevisiae* Sko1 (53). We are currently examining this possibility through the cloning and disruption of *SKO1* in different backgrounds.

Given the fact that these three species live in different environments, it is not strange that they have developed different strategies to respond to oxidative stress, using the same elements but in distinct ways. In any case, our data indicate that this pathway is essential in a pathogenic fungus for resistance to oxidative stress, a result with potential implications in the field of antifungal therapy.

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R. Alonso-Monge and F. Navarro-García contributed equally to this work.

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