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Role of the Mitogen-Activated Protein Kinase Hog1p in Morphogenesis and Virulence of *Candida albicans*

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The relevance of the mitogen-activated protein (MAP) kinase Hog1p in *Candida albicans* was addressed through the characterization of *C. albicans* strains without a functional *HOG1* gene. Analysis of the phenotype of *hog1* mutants under osmostressing conditions revealed that this mutant displays a set of morphological alterations as the result of a failure to complete the final stages of cytokinesis, with parallel defects in the budding pattern. Even under permissive conditions, *hog1* mutants displayed a different susceptibility to some compounds such as nikkomycin Z or Congo red, which interfere with cell wall functionality. In addition, the *hog1* mutant displayed a colony morphology different from that of the wild-type strain on some media which promote morphological transitions in *C. albicans*. We show that *C. albicans hog1* mutants are derepressed in the serum-induced hyphal formation and, consistently with this behavior, that *HOG1* overexpression in *Saccharomyces cerevisiae* represses the pseudodimorphic transition. Most interestingly, deletion of *HOG1* resulted in a drastic increase in the mean survival time of systemically infected mice, supporting a role for this MAP kinase pathway in virulence of pathogenic fungi. This finding has potential implications in antifungal therapy.

Fungi, like all living organisms, must be able to respond to changes in environmental conditions and hence develop a response which enables their adaptation to the new physiological situation. Signal transduction pathways serve as a molecular mechanism to accomplish this cellular response. In *Saccharomyces cerevisiae*, a model eukaryotic cell system, some of these pathways involve members of the MAP kinase family (from mitogen-activated protein kinase), a set of enzymes performing essential functions in cell physiology first discovered in mammalian cells but later shown to be also present in lower eukaryotes (3, 12). Among these, the high-osmolarity glycerol (HOG) response pathway (6) allows adaptation to high-osmolarity conditions and seems to be especially important in terms of ecological adaptation. This latter route is triggered in response to high external osmolarity (i.e., low water activity) and results in the accumulation of glycerol as an intracellular compatible solute in *S. cerevisiae*. Several elements of this cascade have been identified in recent years (see reference 3 for a recent review). The initial triggering events in the cascade are initiated by at least two different pathways (43): the first one involves Sln1p (58), Ypd1 (65), Ssk1, and Ssk2p/Ssk22p kinases (44), while the second involves Sho1p, a putative membrane protein able to interact with, and activate, the Pbs2p MAP kinase kinase via Ste11p (43, 64) and possibly Ste20p/Ste50p (57). Both signals converge at the Pbs2p level, which in turn phosphorylates and activates Hog1p, which mediates the intracellular accumulation of osmolytes such as glycerol (4). This response also involves reorganizations of the cytoskeleton (11) and, presumably, cell wall modifications, as suggested by the involvement of *PBS2* in β-(1,6)-glucan assembly (27, 35). Therefore, the HOG pathway participates in a pleiotropic response that enables a correct and rapid adaptation to osmotic stress. Functionally homologue-related cascades have been found in other fungal systems such as the fission yeast *Schizosaccharomyces pombe*. Interestingly, in this organism, this route not only is restricted to osmoadaptation but also links cell cycle control and sexual development (70, 71).

Candida albicans is a pathogenic yeast of great clinical interest in view of the increasing incidence of the infection that it causes in immunocompromised individuals (55). In addition, its ability to switch between a yeast-like and a hyphal form of growth has long been suspected to play a role in virulence (13, 30, 56). *C. albicans* has therefore been chosen as a model of pathogenic dimorphic fungi, although its diploid nature and lack of a sexual cycle have hampered molecular genetic studies, which have relied mostly on *S. cerevisiae*, a nonpathogenic yeast, as a host organism (62, 68). Knowledge of signal transduction pathways in pathogenic fungi is essential not only to understand their mechanisms of adaptation to a complex and changing environment such as the human body (and, therefore, their virulence) but also as a way to identify potential novel targets in antifungal therapy. In *C. albicans*, some genes homologous to the mating or pseudohyphal pathway genes (32, 36, 46, 72, 73, 77) or *PKC1* pathway (53, 60) have been identified in recent years. We have previously described the isolation of the *C. albicans* gene homologue of the *S. cerevisiae HOG1* gene (designated $HOG1_{Ca}$, previously) and shown its involvement in osmoadaptation by increasing the internal glycerol content upon osmotic stress (67). In the present work, we characterize the phenotype of *C. albicans hog1* mutants, showing their defects in the last stages of cytokinesis and cell wall biogenesis and repositioning certain elements of the budding machinery after osmotic stress. In addition, we show that the HOG pathway represses the serum-induced yeast-to-hypha transition in *C. albicans* and describe its role as a major determinant of virulence. These results suggest an additional role for this MAP kinase pathway in pathogenic fungi.

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TABLE 1. Strains used in this study

Microorganism	Strain	Genotype	Source
E. coli	DH5αF′	K-12 Δ(*lacZYA-argF*)*U169 supE44 thi1 recA1 endA1 hsdR17 gyrA relA1* (ϕ80*lacZ*Δ*M15*) F′	23
S. cerevisiae	L5366	*MAT***a**/*MAT*α *ura3*Δ*52*/*ura3*Δ*52*	33
	YPH499	*MAT***a** *ura3 leu2 his3 trp1 lys2 ade2*	M. Gustin
	JBY10	*MAT***a** *ura3 leu2 his3 trp1 lys2 ade2 hog1-*Δ*1*::*TRP1*	M. Gustin
	W303	*MAT*α *ura3 leu2 his3 trp1 ade2 can1*	M. Gustin
	J134	*MAT*α *ura3 leu2 his3 trp1 ade2 can1 hog1*::*LEU2*	M. Gustin
C. albicans	SC5314	Wild type	20
	CAI4	*ura3*Δ::*imm*^{434}/*ura3*Δ::*imm*^{434}	18
	RM100	*ura3*Δ::*imm*^{434}/*ura3*Δ::*imm*^{434} *his1*Δ::*hisG*/*his1*Δ::*hisG-URA3-hisG*	54
	RM1000	*ura3*Δ::*imm*^{434}/*ura3*Δ::*imm*^{434} *his1*Δ::*hisG*/*his1*Δ::*hisG*	54
	CNC11	*ura3*Δ::*imm*^{434}/*ura3*Δ::*imm*^{434} *his1*Δ::*hisG*/*his1*Δ::*hisG HOG1*/*hog1*::*hisG-URA3-hisG*	67
	CNC12	*ura3*Δ::*imm*^{434}/*ura3*Δ::*imm*^{434} *his1*Δ::*hisG*/*his1*Δ::*hisG HOG1*/*hog1*::*hisG*	67
	CNC13	*ura3*Δ::*imm*^{434}/*ura3*Δ::*imm*^{434} *his1*Δ::*hisG*/*his1*Δ::*hisG hog1*::*hisG-URA3-hisG*/*hog1*::*hisG*	67
	CNC15	*ura3*Δ::*imm*^{434}/*ura3*Δ::*imm*^{434} *his1*Δ::*hisG*/*his1*Δ::*hisG hog1*::*hisG*/*hog1*::*hisG*	This work
	CNC15-10	*ura3*Δ::*imm*^{434}/*ura3*Δ::*imm*^{434} *his1*Δ::*hisG*/*his1*Δ::*hisG hog1*::*hisG*/*hog1*::*hisG LEU2*/*leu2*::*HOG1 URA3*	This work
	CNCH1	*ura3*Δ::*imm*^{434}/*ura3*Δ::*imm*^{434} *his1*Δ::*hisG*/*HIS1 hog1*::*hisG*/*hog1*::*hisG LEU2*/*leu2*::*HOG1 URA3*	This work

MATERIALS AND METHODS

Strains and growth conditions. The yeast and bacterial strains used in this study are listed in Table 1. For clarity and unless otherwise stated, the designation *hog1* will always indicate the homozygous *hog1*/*hog1* Ura$^+$ strain (strain CNC13). Although the genotypes of the strains were confirmed by Southern blot analyses, a control strain integrating the *HOG1* gene at the *LEU2* locus was constructed by homologous recombination using the restriction endonuclease *Kpn*I, thus obtaining strain CNC15-10. Strain CNCH1 was obtained by integrating a p34H derivative (constructed by inserting a 1.59-kbp *Ssp*I-*Ssp*I fragment from YEP-HISX [63] into the *Hin*dII site of p34H [76]) containing the *HIS1* marker at the *HIS1* locus in the genome of CNC15-10 by using *Nru*I as the restriction enzyme. Yeast strains were grown at 37°C (unless otherwise stated) in YED medium (1% yeast extract, 2% glucose) or SD minimal medium (2% glucose, 0.67% yeast nitrogen base without amino acids) with the appropriate auxotrophic requirements (50 μg/ml). The ability of cells to undergo the yeast-to-hypha transition was tested by using Lee's medium at pH 6.7 (38), SD medium plus 10% (vol/vol) fetal bovine serum, fetal bovine serum, Spider medium (1% mannitol, 1% nutrient broth, 0.2% K_2HPO_4, 1.35% agar) (39), SLAD medium (21), or YED medium plus fetal bovine serum at 1, 5, 10, and 20% as well as whole serum. To check the behavior of *C. albicans* strains with respect to dimorphic transition, cells were inoculated at 10^5 cells/ml in prewarmed liquid medium. Growth in liquid medium was estimated as the absorbance at 600 nm (A_{600}) or dry weight; in this case, 10 ml of the culture was filtered with a 0.45-μm-pore-size filter (Millipore) and dried at 42°C until a stable weight had been attained. Time lapse photography was performed, with images taken at defined intervals with cells deposited onto a thermostabilized chamber at 37°C containing solid yeast extract-peptone-dextrose (YEPD) medium supplemented with 0.75 M NaCl.

Molecular biology procedures and plasmid constructions. Standard molecular biology procedures were used (2). *C. albicans* was transformed as described previously (31). The plasmid YEp352 (a *URA3* 2μm-derived vector), pHOG1c24.2 (the *C. albicans HOG1* gene in YEp352), and pJB30 (the *S. cerevisiae HOG1* gene in a 2μm-derived vector) have been described previously (25, 67). The pRM-HOG1 plasmid, an episomic plasmid carrying the *C. albicans HOG1* gene and the 5′ regulatory regions, was obtained by inserting a *Hin*dIII fragment from pHOG1c24.2 into the *Sma*I site of pRM1 (63).

Confocal microscopy, flow cytometry, and fluorescent staining methods. Cells grown in YED medium plus 1 M NaCl were washed twice with 0.2 M NaCl and stained with primuline (Sigma, St. Louis, Mo.) at 50 ng/ml (final concentration) for 30 min at 37°C or calcofluor white (Bayer) at 4 ng/ml (final concentration) for 5 min at room temperature. Cells were briefly sonicated before the fluorescence was quantified with a Bio-Rad Bryte HS flow cytometer (for calcofluor white) or a Becton Dickinson (San José, Calif.) FACScan flow cytometer (for primuline). The same procedure was used for visualization of chitin under a Bio-Rad MRC 1000 confocal microscope. For analyses of DNA content, exponentially growing cells were transferred to YED medium supplemented or not with 1 M NaCl at 37°C. Aliquots were removed at defined intervals, collected by low-speed centrifugation, washed with phosphate-buffered saline (PBS), and resuspended in cold 70% ethanol for 1 min. They were then washed twice with PBS, resuspended in 500 μl of PBS containing 1 mg of RNase/ml, and incubated for 30 min at 37°C. Cells were briefly sonicated, washed twice with PBS, and stained with propidium iodide (Sigma) at a final concentration of 0.005% in PBS, and the DNA content was analyzed with a flow cytometer. Cell viability was assessed by staining the cells with propidium iodide at 0.005% in PBS.

Electron microscopy. Cells growing in YED medium at 37°C were transferred to YED medium supplemented with 1 M NaCl, and samples were taken at different times. Cells for scanning electron microscopy were prepared as described previously (75, 78) and visualized with a JEOL JSM-6400 microscope. Transmission electron microscopy samples were obtained as described previously (49) and embedded in Epon 812. Eighty-nanometer-thick sections were observed through a Zeiss 902 microscope from the Centro de Microscopía Electrónica Luis Bru (Universidad Complutense de Madrid [UCM]).

Antifungal assays. MICs were determined by the microdilution method in 96-well plates as described elsewhere (51, 52) by using SD medium without uridine. Drop tests were used to check susceptibility to Congo red and calcofluor white. They were performed by spotting 10^5, 10^4, 10^3, and 10^2 cells (in a 10-μl volume) onto YEPD solid medium plus Congo red or calcofluor white at 100, 120, and 150 μg/ml and incubated for 24 h at 24, 30, and 37°C for *S. cerevisiae* or at 30, 37, and 42°C for *C. albicans*.

Chitinase activity assays. Chitinase assays were carried out as described before (34). Hydrolysis of the substrate was determined after 1 h of incubation at 30°C. Units of activity are defined as nanomoles of 4-methylumbelliferone (the fluorescent product of hydrolysis of the substrate by chitinase) released per hour.

Virulence assays. Virulence assays were performed essentially as described previously (17).

RESULTS

Structural alterations in *C. albicans hog1* mutants. To characterize the influence of high solute concentrations on the structure and morphology of *C. albicans* and the role of the HOG pathway in *C. albicans* under these conditions, we performed a detailed characterization of the alterations of wild-type and *hog1* mutants. For this purpose, exponentially growing cells in YED medium were diluted and transferred to hyperosmolar conditions (1 M NaCl) and both the optical density (OD) and dry weight were measured at regular intervals. This shift to higher osmolarity caused both wild-type and *hog1* cultures to arrest growth (approximately 3 h for the wild type and 5 h for the mutants). Both wild-type and *hog1* strains resumed growth, with subsequent doubling times of 2 h for the wild type and 5 h for the *hog1* mutants (Fig. 1A). Mutant cells achieved a lower final OD (5.5 versus 10.1) or mass (4.5 versus 8.0 mg/ml) after 48 h of growth and entry into stationary phase. These results indicate that the high osmolarity-induced growth arrest is transient and that despite being osmosensitive and failing to accumulate glycerol intracellularly (67), *hog1* mutants are still able to grow in mass.

Cell viability was determined by both flow cytometry quan-

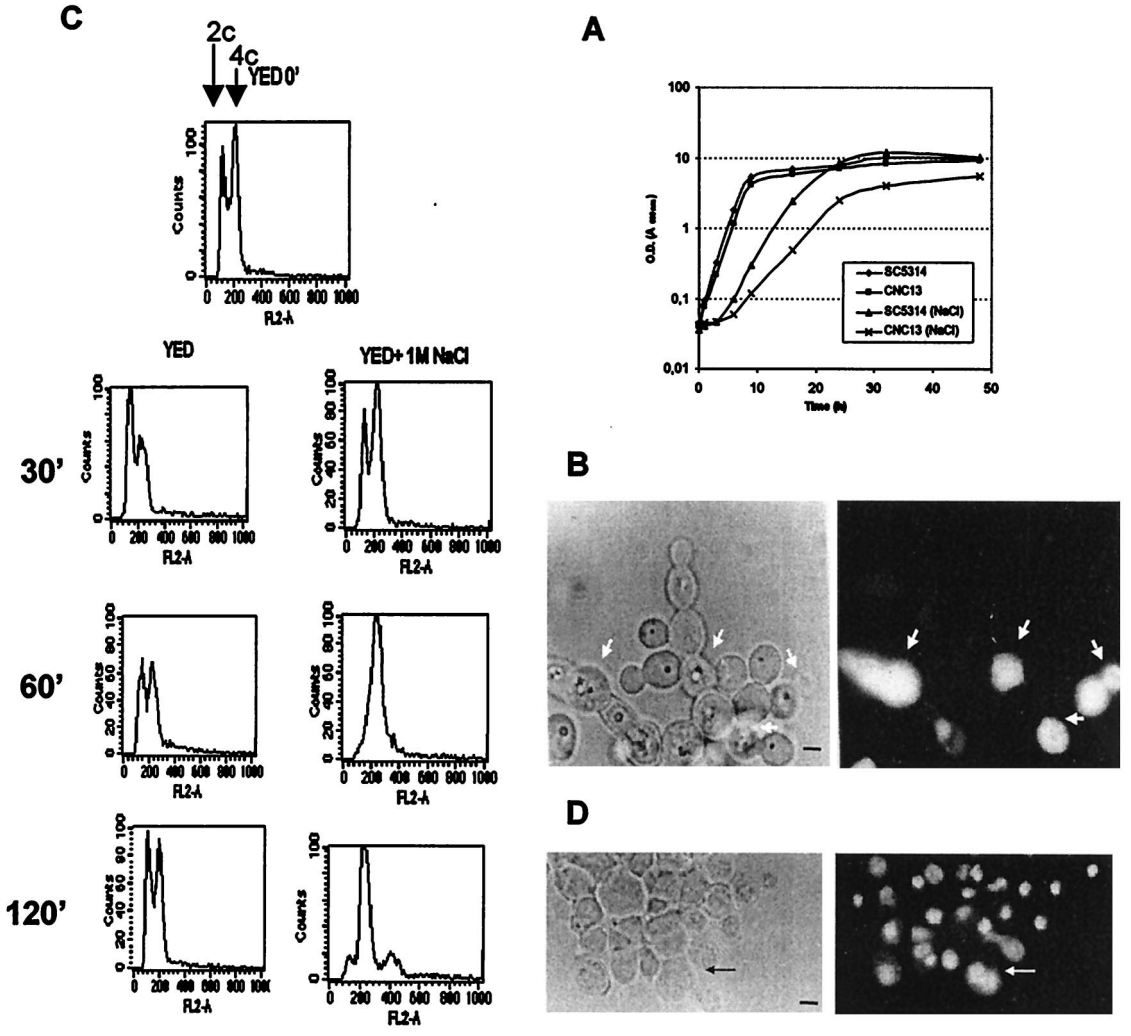

FIG. 1. Terminal phenotype of *hog1* mutants. (A) Effect of osmostress (1.0 M NaCl) on the growth of wild-type cells (SC5314) or the *hog1* mutant (CNC13) in liquid YED medium. The OD (estimated as the A_{600}) is plotted versus time. The *y*-axis scale is logarithmic. (B) Propidium iodide-stained cells of the *hog1* mutant after 24 h of growth under restrictive conditions (right panel). Arrows indicate dead cells in the clusters observed under phase-contrast microscopy (left panel). (C) Flow cytometric analysis of the DNA content of mutant cultures grown in parallel on YED medium or YED medium plus 1 M NaCl. The peaks observed for control cells (labeled YED 0′) represent 2n (left) and 4n (right) DNA content, while the numbers indicate the time in minutes after the transfer to the restrictive conditions. (D) Microscopic analysis of *hog1* mutant cells under restrictive conditions (left panel; phase-contrast image), showing nuclei (right panel; fluorescence image). The arrow indicates a detail of a nucleus in the process of segregation to the new bud. A representative cluster of cells is shown. Bars, 1 μm.

tification of dead cells stained with propidium iodide (a fluorochrome able to enter dead cells due to the loss of selective permeability) (16) and direct standard plating (CFU counting) on YED medium (data not shown). Visual microscopic examination was also used to monitor the behavior of the culture. No obvious differences were observed in the morphology of the wild-type or *hog1* cells at 2 or 4 h after the change to the 1 M NaCl medium. However, after 24 h, individual mutant cells appeared rounder and smaller than wild-type cells (as determined by flow cytometry). At the same time, several mutant cells remained attached after budding due to an apparent defect in cell separation (Fig. 1B, left panel), a phenotype not observed in the wild-type strain (data not shown). Consistent with the previous data on growth, cell viability in the *hog1* culture was high (more than 75% after 24 h under hyperosmotic conditions). Dead cells revealed by propidium iodide uptake had no specific localization within the clusters of cells (Fig. 1B, right panel). The DNA content was also quantified. Under nonrestrictive conditions (YED medium alone), *hog1* cells displayed a DNA pattern characteristic of an asynchronous culture (Fig. 1C, left panels), while on YED medium plus 1 M NaCl, the peak containing a 2n DNA content disappeared in 60 min, and after this period, only 4n cells were detected (Fig. 1C, right panels). After this period, the DNA content increased as the result of normal DNA replication but failure of the cells to segregate, therefore appearing as a single cytometric count. Cells under the microscope appeared to have a single nucleus (Fig. 1D). The observed increase in DNA content therefore seems to be the result of impaired cell division and not sensitivity to the high osmolarity of normal DNA replication. This effect was not observed under permissive conditions or in osmostressed wild-type cells (data not shown). Interestingly, some of the *hog1* cells also displayed altered budding patterns in which the polarity of bud emergence was lost, some cells budding away from the distal poles (Fig. 2A). Scanning transmission microscopy showed that daughter cells

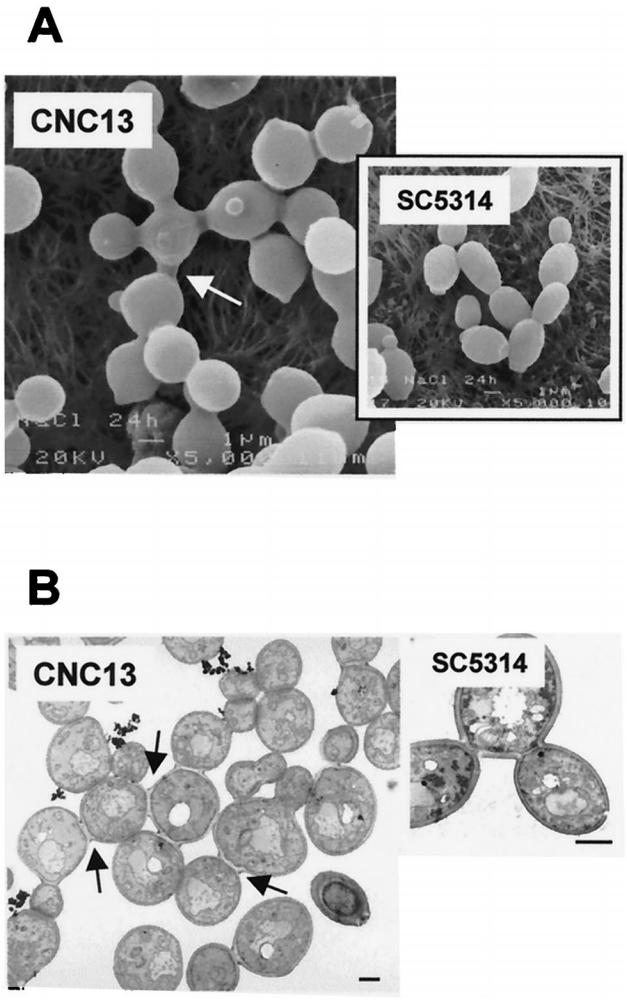

FIG. 2. Scanning and transmission electron microscopy of *hog1* mutants. (A) Scanning electron microscopy of *hog1* mutant (CNC13) (left panel) or wild-type (SC5314) (right panel) cells after 24 h of growth under restrictive conditions. The arrow indicates a characteristic cell with a symmetric type of division. (B) Transmission electron microscopy of similar samples. Arrows indicate almost completely separated but still connected cells with well-formed septa. Bars, 1 μm.

were apparently completely formed but remained attached to the mother cell, unable to complete cytokinesis (Fig. 2A). Transmission electron microscopy (Fig. 2B) revealed that the septum between the mother and daughter cells was physically completed and that the outer cell wall still connected both cells. Sonication for different periods of time or digestion with glusulase [a β-(1,3)-glucanase enriched lytic preparation] did not result in segregation of these clusters, but interestingly, treatment with a commercial preparation of chitinase did (data not shown).

The influence of osmostressing conditions on polarity was investigated in more detail. For this purpose, growing cells were plated onto YEPD solid medium supplemented with 0.75 M NaCl and photographs were taken at different intervals. While the wild-type strain was able to grow normally after a short time of adaptation to the new physiological situation (data not shown), *hog1* mutant cells stopped growing for a prolonged time. In this mutant, a small percentage of the population displayed a defect which consisted in small newly formed buds ceasing growth while mother cells emitted a new bud that completed its growth. By contrast, during the same period, the first bud did not resume growth at all (Fig. 3). These observations are consistent with the idea that certain components of the bud positioning or emergence machinery (but not DNA replication) are dependent on a functional HOG pathway (7).

***C. albicans hog1* mutants are resistant to certain cell wall inhibitors.** The susceptibility of *C. albicans hog1* mutants to antifungals with different structures and mechanisms of action was determined. No differences were found between wild-type and *hog1* cell susceptibilities to the following antifungals under nonosmostressing conditions: cilofungine (an inhibitor of β-glucan biosynthesis; MIC, 1 μg/ml), trichodermine (an inhibitor of protein synthesis; MIC, 1 μg/ml), fluconazole and miconazole (inhibitors of ergosterol biosynthesis; MICs, 2.5 and 1 μg/ml, respectively), canavanine (a toxic amino acid analog; MIC, 6.25 μg/ml), 5-fluorocytosine (an inhibitor of nucleic acid synthesis; MIC, 0.0625 μg/ml), or amphotericin B (inhibitor of membrane functionality; MIC, 2.9 μg/ml). However, a drastic difference was observed in the susceptibilities of *hog1* and wild-type cells to nikkomycin Z, an inhibitor of chitin biosynthesis. When assayed at 30°C, both wild-type and mutant cells displayed high levels of resistance to nikkomycin Z (MIC, >800 μg/ml). However, when susceptibility was assayed at 37 or 42°C, the wild type became sensitive (nikkomycin Z MICs, 3.12 μg/ml at 37°C and 0.78 μg/ml at 42°C) but the *hog1* strain remained resistant to nikkomycin Z (MIC, >800 μg/ml). The MICs of nikkomycin Z for the heterozygous *HOG1*/*hog1* mutant and the wild-type strain, SC5314, were the same. It should be noted that these effects were observed under normal conditions, i.e., nonosmostressed cells.

In addition, *C. albicans hog1* cells were consistently more resistant than the corresponding heterozygous or wild-type strains to Congo red, a dye which also interacts with the fungal cell wall (Fig. 4A) at all the temperatures tested, and very slightly to calcofluor white (data not shown). These differences were not found in *S. cerevisiae hog1* mutants in two different backgrounds. Unexpectedly, overexpression of the *C. albicans HOG1* gene (or the homologous *S. cerevisiae HOG1* gene) in *S. cerevisiae* by using an episomal vector also resulted in increased resistance to these compounds, especially to calcofluor white (Fig. 4B).

To further explore the relationship between the cell wall and the *HOG1* gene, the chitin levels in both wild-type and mutant strains under restrictive (1.0 M NaCl) and nonrestrictive conditions were quantified by flow cytometry with both calcofluor white and primuline, but no significant variation in the chitin content was observed. In addition, confocal microscopy analyses showed the predicted chitin accumulation on scars and mother-bud necks (data not shown). In view of the presence of a well-formed septum between mother and bud but incomplete cytokinesis in mutant cells, we measured chitinase activity in osmostressed cells. As shown in Fig. 4C, a significant reduction in the enzymatic activity of chitinase was observed in total cell extracts. These results suggest that the cell separation defects observed in this mutant could be the result of defective chitinase activity. Chitinase activity has been shown, in fact, to be required for cell separation in *S. cerevisiae* (34), although no dependence on the HOG pathway has been described. Collectively, our results suggest a link between cell wall metabolism and the HOG pathway in *C. albicans*.

A role for *HOG1* in morphological transitions. An important biological question to be addressed in *C. albicans* is its ability to undergo the dimorphic transition, a cell differentiation program that allows yeast cells to generate hyphal forms. Dimorphism, which has long been suspected to play a role in *C. albicans* pathogenesis (see reference 56 and references there-

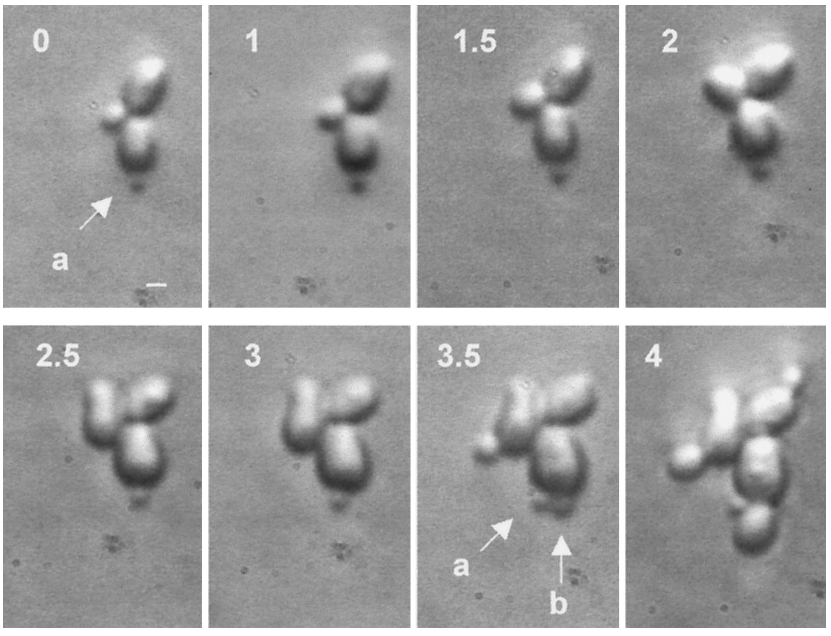

FIG. 3. Defects in bud site selection after osmotic shock. Time lapse photography of *hog1* mutant cells under solid YPD medium supplemented with 0.75 M NaCl. Numbers indicate the time (in hours) after the transfer to restrictive conditions. Arrows labeled "a" indicate small buds, while the arrow labeled "b" indicates a newly formed bud. Bars, 1 μm.

in), can be induced by environmental factors such as the pH or the temperature or can be induced in response to serum, proline, or *N*-acetylglucosamine. To analyze the role of *HOG1* in these transitions, we observed the colony morphology of *hog1* cells under different growth conditions. First, on normal YED plates, *hog1* cells did not show the limited agar invasion displayed by SC5314 wild-type cells (Fig. 5). A similar difference was also found when both strains were plated in Spider medium, which has been described as inducing hyphal formation (39). Mutant colonies, although able to invade the agar, appeared smooth with small grains, while wild-type colonies showed clear invasive borders. However, the mutant strain observed under an optical microscope appeared as large filaments, similar to the wild type. Most interestingly, mutant cells grown on a nitrogen limiting medium such as SLAD medium (which has been shown to induce pseudohyphal formation in *S. cerevisiae* [21]) penetrated the agar medium and were hence more invasive than wild-type cells (Fig. 5), frequently appearing under the microscope as short filaments or pseudohyphae. None of these effects were observed in the heterozygous strain (strain CNC11) or the strain in which the wild-type *HOG1* gene was reintroduced (data not shown).

To explore further the filamentation in *hog1* cells, we analyzed their behavior in the true dimorphic transition. When assayed on liquid media that induce hyphal formation, such as Lee's medium at pH 6.7 or serum, no significant differences were found between the mutant (CNC13), the heterozygous (CNC11 and CNC15-10), and the wild-type (SC5314) strains. However, we performed experiments in which the cells were exposed to limiting serum concentrations. On 100% serum, both mutant and wild-type cells generated long filaments and no differences could be observed with respect to the timing of appearance of the germinative tubes. In contrast, on YED medium containing 1, 5, and 10% serum, *hog1* mutant cells (strain CNC13) displayed a clear filamentous phenotype, with several cells appearing as long true polynucleated filaments with several septa, the frequency of this occurrence in wild-type cells (data not shown) or the heterozygous strain (CNC11) was much lower (Fig. 6A). Consistent with this, on solid medium containing 10% bovine fetal serum, *hog1* cells (strain CNC13) generated wrinkled colonies (Fig. 5) with frequent invaginations towards the inner regions of the colony that were absent in the wild-type cells. This presumably regulatory (repressive) role of *HOG1* in morphological transitions was also evidenced by the suppression of pseudohyphal growth in *S. cerevisiae*. Overexpression of the *C. albicans HOG1* gene from a multicopy plasmid partially suppressed the pseudodimorphic transition (invasion) of the diploid *S. cerevisiae* strain L5366 (Fig. 6B) on nitrogen-deprived medium (SLAD medium). These results clearly support the regulatory role of the HOG pathway in morphogenetic programs in *C. albicans*.

Virulence of *hog1* cells in a mouse model. To analyze the role of the HOG pathway in virulence, we checked the behavior of *hog1* cells by use of a mouse model of fungal infection. Both BALB/c and DBA/2 mice were challenged with different doses of the parental (SC5314) and a *hog1* mutant (CNC13) strain by inoculation into the lateral vein of the tail. These two mouse strains have been shown to differ in their susceptibility to fungal infections, BALB/c mice being more resistant than DBA/2 mice (24). Standard death curves were obtained after the infection, and representative death curves are shown in Fig. 7. In BALB/c mice, a challenge with a dose of 10^6 cells resulted in a rapid mortality for the wild-type strain (mean survival time [MST] of 3 days). In contrast, mice challenged with *hog1* cells showed a drastic decrease in mortality, being able to survive up to 60 days (Fig. 7A). These differences were also observed for mice given a larger inoculum (10^7 blastospores) (Fig. 7B), with MSTs of 1 day for the wild-type strain and 30 days for the mutant (see also Table 2). A lower dose (10^5 blastospores) did not lead to any differences in the mortality of mice challenged with either yeast strain.

DBA/2 mice were similarly infected. In this case, as ex-

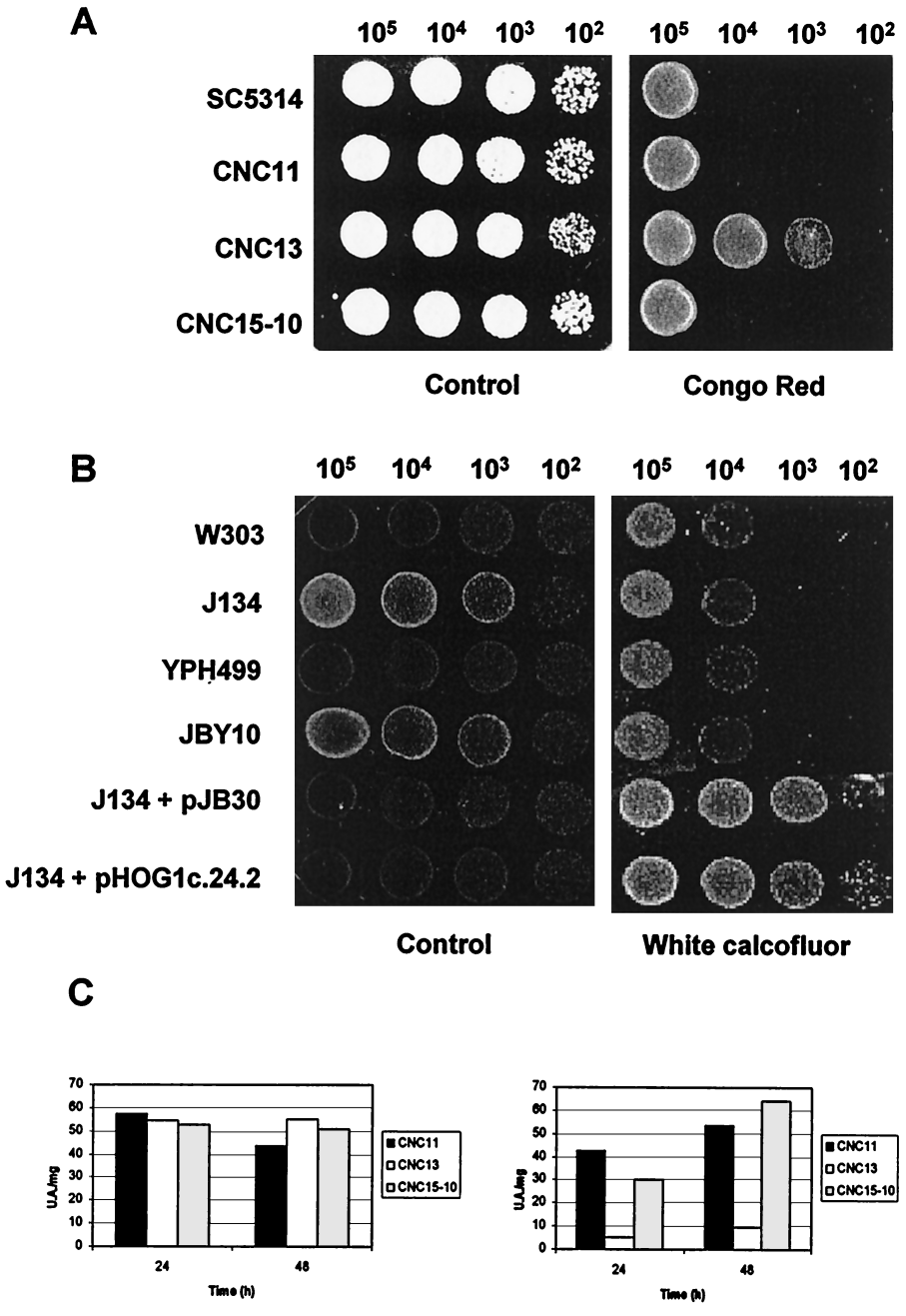

FIG. 4. Antifungal susceptibility and cell wall architecture. (A) Different amounts of cells (indicated at the top of the rows) from the indicated *C. albicans* strains were spotted onto YEPD medium (as a control) or YEPD medium supplemented with Congo red at 150 μg/ml and incubated at 37°C. (B) Experiments similar to those described for panel A were done with the *S. cerevisiae* strains indicated; cells were spotted onto YEPD medium supplemented with a 150-μg/ml final concentration of calcofluor white and incubated at 30°C for 24 h. (C) Chitinase activity in cell extracts after 24 and 48 h of growth under nonrestrictive (1 M NaCl) (left panel) and restrictive (right panel) conditions. Units of activity (UA) (see Material and Methods) per milligram of dried extract are given in the y axis. Data are the mean value of two independent experiments.

pected, differences at the 10^7 cell dose were not observed due to the increased susceptibility of DBA/2 mice to *C. albicans* infections, both strains producing a high mortality. However, differences in MST were observed for the mutant and wild type at a challenge dose of 10^6 cells (Table 2), and the use of this mouse strain allowed us to detect differences between both *C. albicans* strains when a small inoculum dose (10^5) was used (Table 2). In all these experiments, *ura3* auxotrophic strains were avoided due to the effect that this nutritional requirement (especially in certain genetic backgrounds) has on *C. albicans* virulence (29, 69). However, since the *hog1* knockout strains were obtained in a *his1* background (RM1000), it was confirmed that *HIS1* did not play a role in the observed reduction in virulence, as shown for the control strains RM100 and CNCH1 (Fig. 7A). In addition, the *hog1* heterozygous strain CNC15-10 (a strain obtained through the reintroduction of the *HOG1* gene in the genome of strain CNC15 [see Materials and Methods] to serve as an internal control of the knockout de-

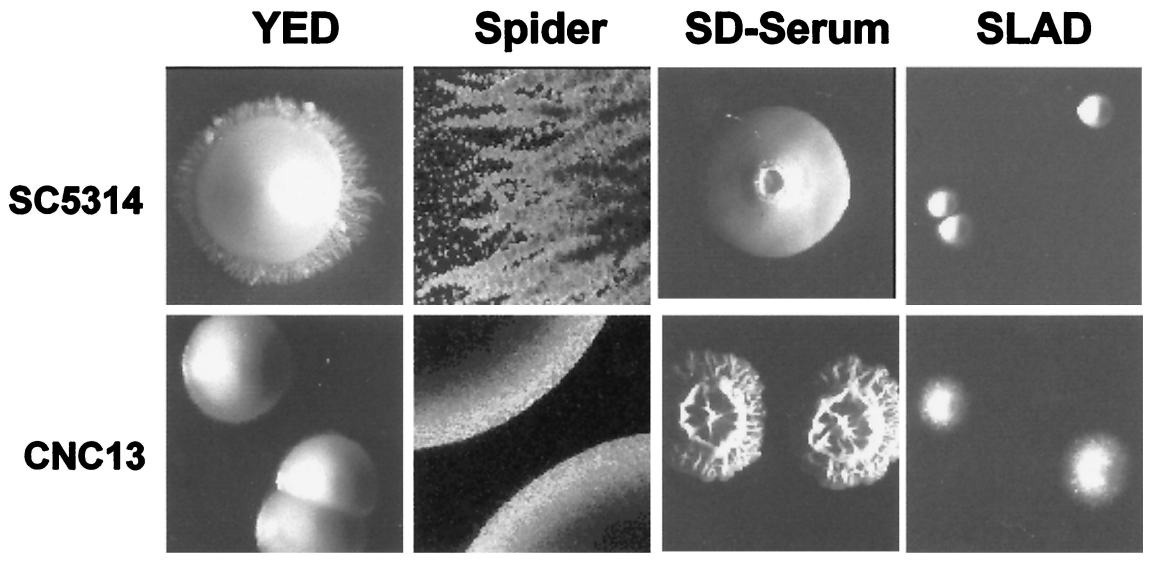

FIG. 5. Colony morphology of *hog1* mutants. Colony morphology of wild-type (SC5314) and mutant *hog1* (CNC13) cells on different solid media. Approximately 50 CFU were spread onto either YED medium, Spider medium, minimal SD medium plus 10% bovine fetal serum, or SLAD medium on petri dishes and incubated for 7 days at 37°C before photographs were taken. The colony borders are shown for cells on Spider medium.

letion scheme used) displayed virulence similar to that of wild-type (SC5314) cells (Fig. 7A), indicating that a single copy of the *HOG1* gene is enough to restore full virulence in this animal model. The fungal burden was quantified in the kidney and brain, representative organs of *C. albicans* infections (59). Organs were recovered at different times postinfection, and viable cells were quantified. As shown in Table 3, strain CNC13 colonizes tissues less efficiently than SC5314 does and it is cleared from the brains of BALB/c mice in a few days (i.e., 7 days, even with the high dose of 10^6 cells). Collectively, these data indicate that a functional HOG pathway is essential for the maintenance of full virulence in *C. albicans*.

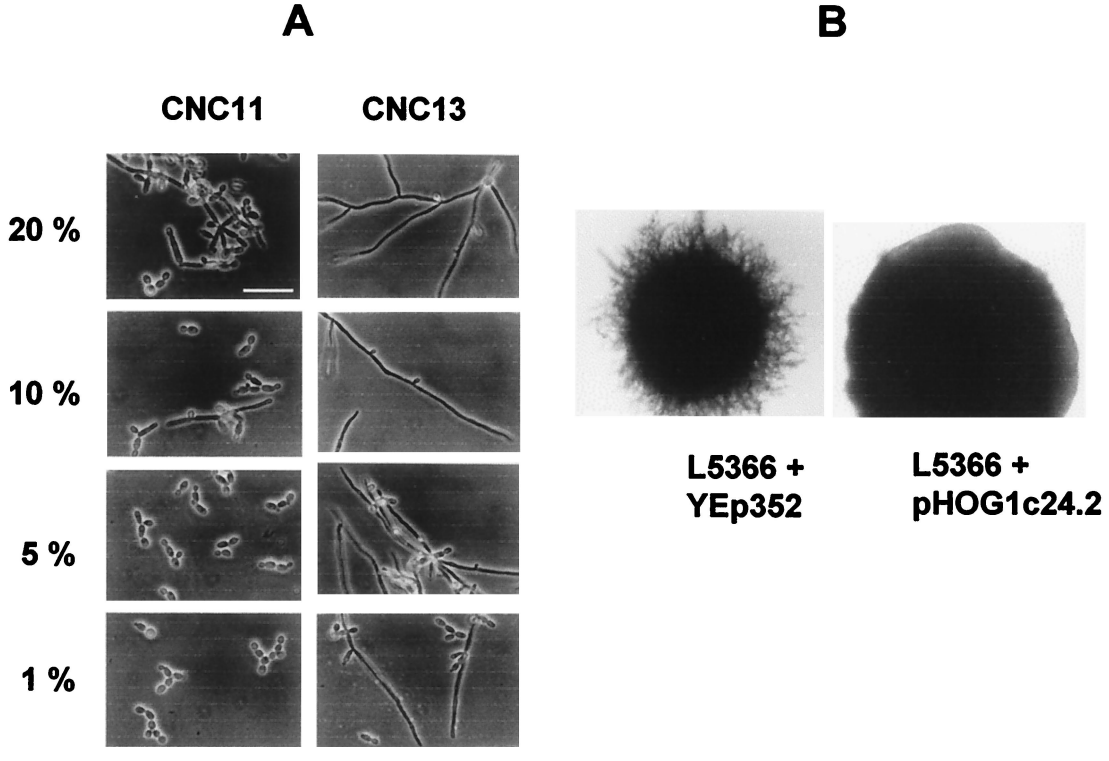

FIG. 6. Effect of Hog1p on the serum-induced dimorphic transition. (A) Cells from the indicated strains were inoculated in YED medium plus bovine fetal serum at different concentrations (20, 10, 5, and 1%), and phase-contrast microphotographs were taken after 6 h of incubation at 37°C. (B) Border colony morphologies of *S. cerevisiae* L5366 transformed with vector YEp352 (left picture) or the multicopy plasmid pHOG1c24.2 (bearing the *C. albicans HOG1* gene) (right picture) are shown. Cells were plated onto SLAD medium, and pictures were taken after growth for 6 days at 30°C. Bars, 10 μm.

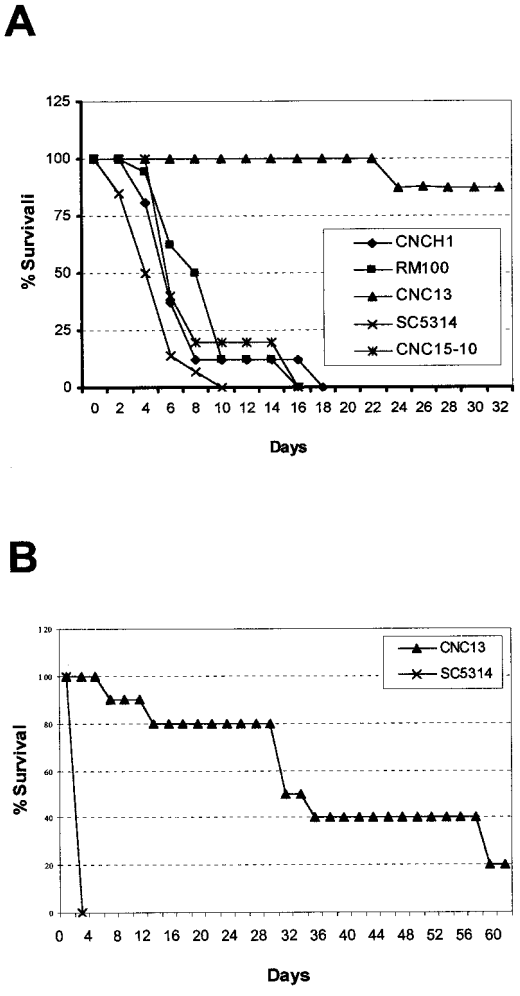

FIG. 7. Virulence assays. Standard survival curves of BALB/c mice infected systemically with 10^6 (A) or 10^7 (B) cells of the *C. albicans* strains indicated in the figure. Since strains CNC15-10, RM100, and CNCH1 at a dose of 10^7 gave curves similar to the one shown for SC5314 in panel B, these results are therefore not shown for clarity.

DISCUSSION

In this work, we have addressed the role of the HOG pathway through the characterization of the phenotype of *C. albicans* mutants defective in the central MAP kinase gene of this pathway, the *HOG1* gene, under both restrictive and permissive conditions. DNA replication was insensitive (at least for the period analyzed) to high osmolarity, and the increased DNA content detected by flow cytometry was the result of a block in cytokinesis, similar to that in *S. cerevisiae* mutants (7). We also found that the defects in cell separation are localized to the last stages of cytokinesis but are, apparently, not due to abnormal septum formation (Fig. 3A and B); instead, we show that chitinase activity is low and may be limiting under these conditions. This is supported not only by the enzymatic analysis of osmostressed cells but also by the effect that externally added chitinase (and not other cell wall lytic enzyme preparations such as zymolyase or glusulase) exerts on cell separation. Also, certain components of the bud polarity machinery appear to be nonfunctional in the mutant cells under restrictive conditions, a phenomenon similar to that of *S. cerevisiae*, where transfer of the cells to high-osmolarity conditions often results in the selection of a new polarization region for bud emergence to occur (7).

An interesting conclusion from our studies is the suggestion of a link between cell wall metabolism and the HOG pathway. This is inferred from the resistance to compounds which interact with the cell wall of the mutant strain in ways different from those of the wild type. A possible explanation for this result is that *hog1* mutants are altered in their permeability by certain compounds. In fact, nikkomycin Z is a nucleoside-peptide antibiotic inhibitor of chitin synthase (8, 19) which is imported into the cell through a peptide transport system (48, 61, 79). Alterations in membrane permeability cannot, in principle, be excluded, although these effects are also obtained with dyes such as calcofluor white or Congo red, which show affinity for external cell wall polymers. The HOG pathway could, therefore, play an as-yet-undefined role in cell wall metabolism. Such a relationship has, in fact, been postulated to occur in *S. cerevisiae*, since *PBS2* (the *HOG1* MAP kinase kinase gene) may regulate β-(1,6)-glucan assembly (27, 35). No defects in chitin synthase activity (35) were observed in this study in *pbs2*Δ mutants, in agreement with our results on quantification of the chitin content in *hog1* mutants.

Another aspect of biological relevance that we investigated is dimorphism, a long-suspected mechanism of virulence (see references 13 and 30). The repressive effect that this pathway exerts on pseudohyphal formation is evidenced by the hyperfilamentous phenotype of *C. albicans* strains on different media such as SLAD medium (21) (a similar phenotype has been observed for *S. cerevisiae hog1* mutants [cited in reference 42]) as well as by the suppression of the pseudodimorphic transition in *S. cerevisiae* when the *C. albicans HOG1* gene is overexpressed. Alterations in the colony morphology on different solid media may also support this observation, although the apparently contradictory results observed could be explained by the involvement of different signal transduction pathways in these processes. Alterations in colony morphology are also observed in response to serum, and the conditions used in this assay (1 to 20% serum versus 100% serum) may better reflect the complex environmental conditions that a pathogen finds inside the human body, where different locations may have different concentrations of an inducer(s). Our results indicate the repression that the HOG pathway exerts on the serum-induced dimorphic transition in *C. albicans*. Given our current knowledge of signal transduction pathways mediated through MAP kinases, it is tempting to speculate about the final targets of this cascade. In *C. albicans*, hyphal formation seems to be a complex process in which both positive and negative signals do

TABLE 2. Quantification of virulence in experimental infection assays

Challenge dose	*C. albicans* strain	BALB/c mice		DBA/2 mice	
		MST (days)	No. of dead mice/total no.	MST (days)	No. of dead mice/total no.
1×10^7	SC5314	1	15/15	1	8/8
	CNC13	30	8/10	1	13/13
1×10^6	SC5314	3	14/14	1	14/14
	CNC13	>60	1/8	4	11/11
1×10^5	SC5314	>60	3/16	4	17/17
	CNC13	>60	0/12	>60	1/12
5×10^4	SC5314	>60	3/12	7	12/12
	CNC13	>60	0/11	>60	1/17

TABLE 3. Virulence in a systemic mouse model of experimental infection in BALB/c and DBA/2 mice

Challenge dose	Strain	Time[a]	Fungal burden (log CFU)[b] in:			
			BALB/c mice		DBA/2 mice	
			Kidney	Brain	Kidney	Brain
1×10^7	SC5314	1	6.03 ± 0.84	4.47 ± 0.13	NT[c]	NT
	CNC13	1	4.39 ± 0.24	3.24 ± 0.2	5.27 ± 0.21	3.98 ± 0.13
1×10^6	SC5314	1	5.93 ± 0.18	4.04 ± 0.22	6.02 ± 0.92	3.81 ± 0.16
	CNC13	1	3.26 ± 0.24	1.89 ± 0.31*	4.43 ± 0.09	3.16 ± 0.13
		7	3.94 ± 1.13	*		
		60	5.11 ± 0.98**	*		
1×10^5	SC5314	1	3.61 ± 0.10	3.34 ± 0.36	5.17 ± 0.76	3.70 ± 0.19
		7	4.10 ± 0.84	*		
		60	4.32 ± 1.51*	*		
	CNC13	1	2.53 ± 0.21	1.78 ± 0.00**	3.76 ± 0.08	2.35 ± 0.18
		7	*	*	3.60 ± 0.98*	3.34 ± 0.00**
		60	4.98 ± 0.00***	*	4.35 ± 0.35**	*
5×10^4	SC5314	7			6.45 ± 0.56	4.53 ± 0.50
		60	4.87 ± 0.96*	*		
	CNC13	1	*	*	3.71 ± 0.73	3.66 ± 0.00**
		7	*	*	*	*
		60	*	*	3.92 ± 0.00**	*

[a] Day postchallenge (up to MST) when mice were killed by cervical dislocation for CFU quantification.
[b] Only log CFU values that are statistically significantly different at $P = 0.003$ (kidney) and $P < 0.0001$ (brain) are shown (analysis of variance test with *C. albicans* strain; covariates were time, mice, and dose). *, some or all mice cleared infection; **, more than 50% mice cleared infection.
[c] NT, not tested.

play a role (see reference 45 for an elegant model). For example, the HOG pathway could be involved in the repression of the pathway that leads to a pseudofilamentous or filamentous growth pathway in *C. albicans*, interacting with those elements of the mating-hyphal pathway presumably involved in dimorphic transition. In fact, in *S. cerevisiae*, elements of the mating pathway are used for pseudofilamentous growth (32, 36, 40), and it has been shown that the HOG pathway represses the activity of the mating pathway in *S. cerevisiae* (22). Furthermore, recent studies in *S. cerevisiae* (57) reveal that *HOG1* prevents cross talking between both the mating and HOG pathways. The repressive role of the HOG pathway in *C. albicans* hyphal formation could be its involvement in the activation of *RBF1*, a transcription factor whose deletion generates hyphal forms (26), or, alternatively, a putative *SSN6-TUP1* complex in *C. albicans*. In fact, it has been recently shown that the *S. cerevisiae* Ssn6-Tup1 repressor complex (28) plays a role in the repression of different osmolarity-inducible genes in *S. cerevisiae* (some of which are *HOG1* dependent) and that *ssn6* or *tup1* mutants partially suppress the characteristic osmotic sensitivity associated with *hog1* mutants (47). More interestingly, the *C. albicans TUP1* gene has been shown to play a role in hyphal formation in *C. albicans* since deletion of this gene results in a gene dosage-dependent filamentous growth (5). A possible explanation for our results would be the *HOG1*-dependent expression or activation of a DNA binding protein able to recruit the Ssn6-Tup1 complex for the repression of specific hyphal genes. In any case, these similarities must be analyzed carefully because of the pleiotropic role of transcription factors like *TUP1* in fungal cell physiology and the fact that phenotypes associated with *S. cerevisiae* and *C. albicans* mutants may clearly diverge, as occurs with *tup1* mutants (5).

It is noteworthy that other elements of this pathway have been found to play a role in hyphal development. For example, deletion of the *nik-1*$^+$ gene, a *Neurospora crassa* homologue of the *SLN1* gene (58), results, in addition to osmotic sensitivity, in restricted mycelial growth, the loss of conidiophore development, and in aberrant hyphal structures under restrictive conditions (1). Recently, two-component *C. albicans* kinase gene homologues of *SLN1* have been identified (10, 50, 74) and effects on the efficiency of the transition process have been observed (74).

Our results also demonstrate that the HOG pathway plays a major role in *C. albicans* virulence. The genes involved in virulence currently identified are functionally diverse, probably reflecting the character of a commensal opportunistic pathogen instead of a primary pathogen of *C. albicans*. Dimorphism provides a morphological switch that has been related to certain features undoubtedly related to pathogenicity, such as adhesion, escape from phagocytic cells, and invasion (9, 30, 56, 66). It is therefore not surprising that strains defective in hyphal formation (under certain conditions) should display a reduced virulence, as has been shown for some signal transduction protein kinases (15, 36, 37). However, although the role that hyphal formation must play in virulence is evident, our results clearly demonstrate that this trait is not enough for virulence, since a functional (and even enhanced) in vitro hyphal development does not necessarily correlate with virulence

in this animal model, as suggested recently (14). It should be emphasized that both the dose of cells used in the virulence experiments and the length of the period in which mice infected were followed up indicate the complete avirulence of the mutant strain, in comparison with the standard defined in other recent studies (14, 41), and suggest that the HOG pathway participates in other as-yet-unraveled cellular processes which are essential for virulence. Although it is difficult to define what this role is at this stage, it is tempting to speculate that Hog1p behaves like a general stress kinase, similar to the *S. pombe* homologue, and that this cellular response is essential for the successful establishment of an infection in the host. In conclusion, we show in this work that the pathway(s) controlling osmotic sensitivity in *C. albicans* also plays a role in differentiation programs and virulence in this pathogenic fungus, a result which identifies this route of primary importance in the search for novel antifungal targets.

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