EUKARYOTIC CELL, Feb. 2006, p. 347–358 Vol. 5, No. 2
1535-9778/06/$08.00+0 doi:10.1128/EC.5.2.347–358.2006
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The Cek1 and Hog1 Mitogen-Activated Protein Kinases Play Complementary Roles in Cell Wall Biogenesis and Chlamydospore Formation in the Fungal Pathogen *Candida albicans*

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Received 23 May 2005/Accepted 20 November 2005

The Hog1 mitogen-activated protein (MAP) kinase mediates an adaptive response to both osmotic and oxidative stress in the fungal pathogen *Candida albicans*. This protein also participates in two distinct morphogenetic processes, namely the yeast-to-hypha transition (as a repressor) and chlamydospore formation (as an inducer). We show here that repression of filamentous growth occurs both under serum limitation and under other partially inducing conditions, such as low temperature, low pH, or nitrogen starvation. To understand the relationship of the HOG pathway to other MAP kinase cascades that also play a role in morphological transitions, we have constructed and characterized a set of double mutants in which we deleted both the *HOG1* gene and other signaling elements (the *CST20*, *CLA4*, and *HST7* kinases, the *CPH1* and *EFG1* transcription factors, and the *CPP1* protein phosphatase). We also show that Hog1 prevents the yeast-to-hypha switch independent of all the elements analyzed and that the inability of the *hog1* mutants to form chlamydospores is suppressed when additional elements of the *CEK1* pathway (*CST20* or *HST7*) are altered. Finally, we report that Hog1 represses the activation of the Cek1 MAP kinase under basal conditions and that Cek1 activation correlates with resistance to certain cell wall inhibitors (such as Congo red), demonstrating a role for this pathway in cell wall biogenesis.

Polymorphism, that is, the ability to acquire different morphologies, has long been considered a major virulence factor in the human fungal pathogen *Candida albicans*. This fungus is present on the skin and mucosal surfaces of many organisms, including humans, acquiring mainly a unicellular yeast-like form, while in infected tissues, different morphologies (yeast, mycelia, and even chlamydospores) have been observed (9, 13). These types of morphologies have distinct abilities to adhere, proliferate, invade, or escape phagocytic cells and, therefore, contribute by different degrees to the pathogenesis of the infection. The transfer from the yeast form to the filamentous form of growth is induced by certain chemicals (14, 18, 20, 48), a temperature close to 37°C (30), and a neutral pH (49), while chlamydospore formation is induced in vitro under special conditions, such as a low concentration of glucose, darkness, low temperature (24 to 28°C) and microaerophilia.

The molecular mechanisms involved in the regulation of polymorphism in *C. albicans* are very complex. Genetic analysis has shown the implication of several genes and regulatory cascades in this process (31, 37, 54, 56). These include, among others, the cyclic AMP (cAMP)-dependent protein kinase pathway and the mitogen-activated protein (MAP) kinase pathway. The cAMP pathway leads to an increase in intracellular cAMP (44) and controls the Efg1 transcription factor (16, 51, 52). *C. albicans efg1* mutants are defective in both filamentation and chlamydospore formation (50, 51) and have a reduced virulence in certain models of experimental infection (33). Other pathways involved in filamentation are mediated by MAP kinases and include the Cek1-mediated pathway and the HOG pathway. The Cek1 pathway involves the Cst20 PAK-like protein, the Hst7 MAP kinase kinase (26), the Cek1 MAP kinase (11, 55), and the Cph1 transcription factor (32). Mutants in these genes present defects in hyphal development to a different degree on certain media and have a reduced virulence in animal models. Other elements that have been partially characterized include the *CPP1* phosphatase (11) and the PAK-like kinase Cla4 (27, 34). The HOG (high-osmolarity glycerol response) MAP kinase pathway has also been involved in the morphological transition, as deletion of certain elements of the pathway results in enhanced hyphal growth on serum and altered colony morphologies on certain media (1, 4). In addition, *hog1* mutants are not able to form chlamydospores (2). In *Saccharomyces cerevisiae*, a similar situation occurs, and deletion of *HOG1* allows an efficient cross talk to the Kss1-mediated pathway and Fus3 mating pathway (40). In the present work, we demonstrate that the enhanced hyphal growth of *C. albicans hog1* mutants is independent of the *CEK1* pathway and the Efg1 transcription factor while, in close contrast, we show that the role of Hog1 in chlamydospore development is dependent on this pathway. We also propose that resistance of certain mutants of the HOG pathway to chitin-interfering compounds is linked to a hyperactivation of the Cek1 MAP kinase.

MATERIALS AND METHODS

Strains and growth conditions. Yeast strains are listed in Table 1. For clarity, and unless otherwise stated, a mutant in a *geneX* (*hog1*, *cst20*, etc.) will always indicate the homozygous *geneX/geneX* Ura$^+$ strain. Yeast strains were grown at 37°C (unless otherwise stated) in YPD medium (1% yeast extract, 2% glucose,

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Relevant genotype	Source or reference
RM100	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 his1*Δ::*hisG*/*his1*Δ::*hisG-URA3-hisG*	*his1*	2
RM1000	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 his1*Δ::*hisG*/*his1*Δ::*hisG*	*his1 ura3*	2
CNC13	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 his1*Δ::*hisG*/*his1*Δ::*hisG hog1*::*hisG-URA3-hisG*/*hog1*::*hisG*	*his1 hog1*	46
CNC15	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 his1*Δ::*hisG*/*his1*Δ::*hisG hog1*::*hisG*/*hog1*::*hisG*	*his1 ura3 hog1*	46
CLJ5	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 cla4*Δ::*hisG*/*cla4*Δ::*hisG*	*ura3 cla4*	28
CDH25	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 cst20*Δ::*hisG*/*cst20*Δ::*hisG*	*ura3 cst20*	26
CP29-1-7	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 cpp1*Δ::*hisG-URA3-hisG*/*cpp1*Δ::*hisG*	*cpp1*	10
CDH12	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 hst7*Δ::*hisG*/*hst7*Δ::*hisG*	*ura3 hst7*	26
JKC19	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 cph1*Δ::*hisG-URA3-hisG*/*cph1*Δ::*hisG*	*cph1*	25
CK43B-16	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 cek1*Δ::*hisG-URA3-hisG*/*cek1*Δ::*hisG*	*cek1*	11
HLC67	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 efg1*Δ::*hisG*/*efg1*Δ::*hisG*	*ura3 efg1*	33
HLC69	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 efg1*Δ::*hisG*/*efg1*Δ::*hisG cph1*Δ::*hisG*/*cph1*Δ::*hisG*	*ura3 efg1 cph1*	33
BEC13	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 cla4*Δ::*hisG*/*cla4*Δ::*hisGhog1*::*hisG-URA3-hisG*/*hog1*::*hisG*	*cla4 hog1*	This work
CP29-1-7-u	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 cpp1*Δ::*hisG*/*cpp1*Δ::*hisG*	*ura3 cpp1*	This work
JKC19-u	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 cph1*Δ::*hisG-URA3-hisG*/*cph1*Δ::*hisG*	*ura3 cph1*	This work
BEC15	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 cla4*Δ::*hisG*/*cla4*Δ::*hisGhog1*::*hisG*/*hog1*::*hisG*	*ura3 cla4 hog1*	This work
BEC23	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 cst20*Δ::*hisG*/*cst20*Δ::*hisG hog1*::*hisG-URA3-hisG*/*hog1*::*hisG*	*cst20 hog1*	This work
BEC25	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 cst20*Δ::*hisG*/*cst20*Δ::*hisG hog1*::*hisG*/*hog1*::*hisG*	*ura3 cst20 hog1*	This work
BEC33	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 cpp1*Δ::*hisG*/*cpp1*Δ::*hisGhog1*::*hisG-URA3-hisG*/*hog1*::*hisG*	*cpp1 hog1*	This work
BEC35	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 cpp1*Δ::*hisG*/*cpp1*Δ::*hisG hog1*::*hisG*/*hog1*::*hisG*	*ura3 cpp1 hog1*	This work
CHH13	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 hst7*Δ::*hisG*/*hst7*Δ::*hisG hog1*::*hisG-URA3-hisG*/*hog1*::*hisG*	*hst7 hog1*	This work
CCH13	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 cph1*Δ::*hisG*/*cph1*Δ::*hisG hog1*::*hisG-URA3-hisG*/*hog1*::*hisG*	*cph1 hog1*	This work
BEC53	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 cph1*Δ::*hisG*/*cph1*Δ::*hisG efg1*Δ::*hisG*/*efg1*Δ::*hisG hog1*::*hisG-URA3-hisG*/*hog1*::*hisG*	*cph1 efg1 hog1*	This work
BEC55	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 cph1*Δ::*hisG*/*cph1*Δ::*his efg1*Δ::*hisG*/*efg1*Δ::*hisG hog1*::*hisG*/*hog1*::*hisG*	*ura3 cph1 efg1 hog1*	This work
BEC43	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 efg1*Δ::*hisG*/*efg1*Δ::*hisG hog1*::*hisG-URA3-hisG*/*hog1*::*hisG*	*efg1 hog1*	This work
BEC45	*ura3*Δ::*imm434*/*ura3*Δ::*imm434 efg1*Δ::*hisG*/*efg1*Δ::*hisG hog1*::*hisG*/*hog1*::*hisG*	*ura3 efg1 hog1*	This work

2% peptone) and 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 using Lee's medium at different pHs (4.3 to 5.8 and 6.7) (30), SD adjusted to the pHs indicated, fetal bovine serum, or YPD medium plus fetal bovine serum at 5%. To check the dimorphic transition, cells were inoculated in prewarmed liquid medium at 10^5 cells per ml. Growth in liquid medium was estimated as the absorbance at 600 nm (A_{600}). Uridine and histidine were routinely added to liquid and solid media used for phenotypic assays to minimize the differences between strains. Usually, overnight cultures were inoculated into fresh medium to an optical density of 0.1 (measured at 600 nm), and experiments were performed when cultures reached an optical density of 1 (600 nm) when exponential-phase cells were required. A 24-h culture was routinely used in the case of stationary-phase cells.

Sensitivity to different compounds (oxidative agent, NaCl, sorbitol, Congo red, or calcofluor white) was tested on solid YPD medium. Serially diluted (1/10) cell suspensions were spotted to examine the growth of the different strains. Plates were incubated overnight at 37°C unless otherwise indicated.

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

Construction of strains. All strains generated in the present study were obtained by disrupting the *HOG1* gene in various single-mutation strains of *C. albicans*. *HOG1* gene disruption was performed as previously reported (46) following the Fonzi and Irwin strategy (17) and using the transformation method developed by Köhler et al. (24). Gene deletion was verified by Southern blotting. Genomic DNA was digested with EcoRI and HpaI, and the probe was obtained by PCR using the primers o-HOG1 ext (GAGTAGTAGTTTTGGATAAAT GTA) and HE2r2 (GATTTGCTTCCTGTACTCAACGTT).

The appropriate strains were transformed with the plasmids pRC2312 (7) (as control vector), pRC2312P-H (51) (to overexpress the *EFG1* gene), or *ACT1p-HOG1-GFP* (4) (to overexpress the *HOG1* gene).

Protein extracts and immunoblot analysis. Overnight cultures were refreshed to an optical density of 0.1 (measured at 600 nm), and samples were collected when cultures reached an optical density of 1 (600 nm). Alternatively, cultures in stationary growth phase were refreshed in YPD or YPD plus Congo red, and then samples were taken from the stationary-phase culture and after 1 and 2 h of growth in these conditions. Cell extracts were obtained as previously indicated (36). Equal amounts of proteins were loaded onto each lane, as assessed by 280-nm measurement of the samples and Ponceau red staining of the membranes prior to blocking and detection. Blots were probed with phospho-p42/44 MAP kinase (Thr202/Tyr204) (Cell Signaling Technology, Inc.), ScHog1 polyclonal antibody (Santa Cruz Biotechnology), and Ab-CaCek1 (developed in our lab) and developed according to the manufacturer's conditions using the Hybond ECL kit (Amersham Pharmacia Biotech).

β-1,3-Glucanase sensitivity assay. To measure the inhibition of growth caused by Zymolyase, cells from an exponentially growing culture were inoculated to an optical density at 600 nm (OD_{600}) of 0.025 in YPD medium supplemented with different amounts of Zymolyase 100T (ICN Biomedicals, Inc.). The assay was performed in a 96-well plate in duplicate rows and incubated overnight at 37°C. Zymolyase was suspended in Tris-HCl (pH 7.5)/glucose 5%. Growth is depicted as the percentage of growth in YPD supplemented with Zymolyase compared with growth in YPD alone. Graphs represent the means of the results from at least three independent experiments.

RESULTS

***hog1* mutants are derepressed in the yeast-to-hypha transition.** We have previously shown that *hog1* mutant cells are derepressed in hyphal formation when cells are exposed to limiting concentrations of serum (1). This result indicated that the threshold level to activate filamentation in *hog1* mutant cells was lower than in the wild type. In the present work, we investigated whether this effect was exclusive to serum or could also be mimicked by other conditions known to promote morphological transitions in *C. albicans*, such as pH and temperature. When cells were grown in minimal medium at 37°C, both the wild type and *hog1* mutants were able to induce hyphal growth at pH 6.7; when the pH was lowered to 4.5, only the *hog1* mutant was able to form filaments (Fig. 1A). A similar behavior was observed when cells were grown in liquid Lee's

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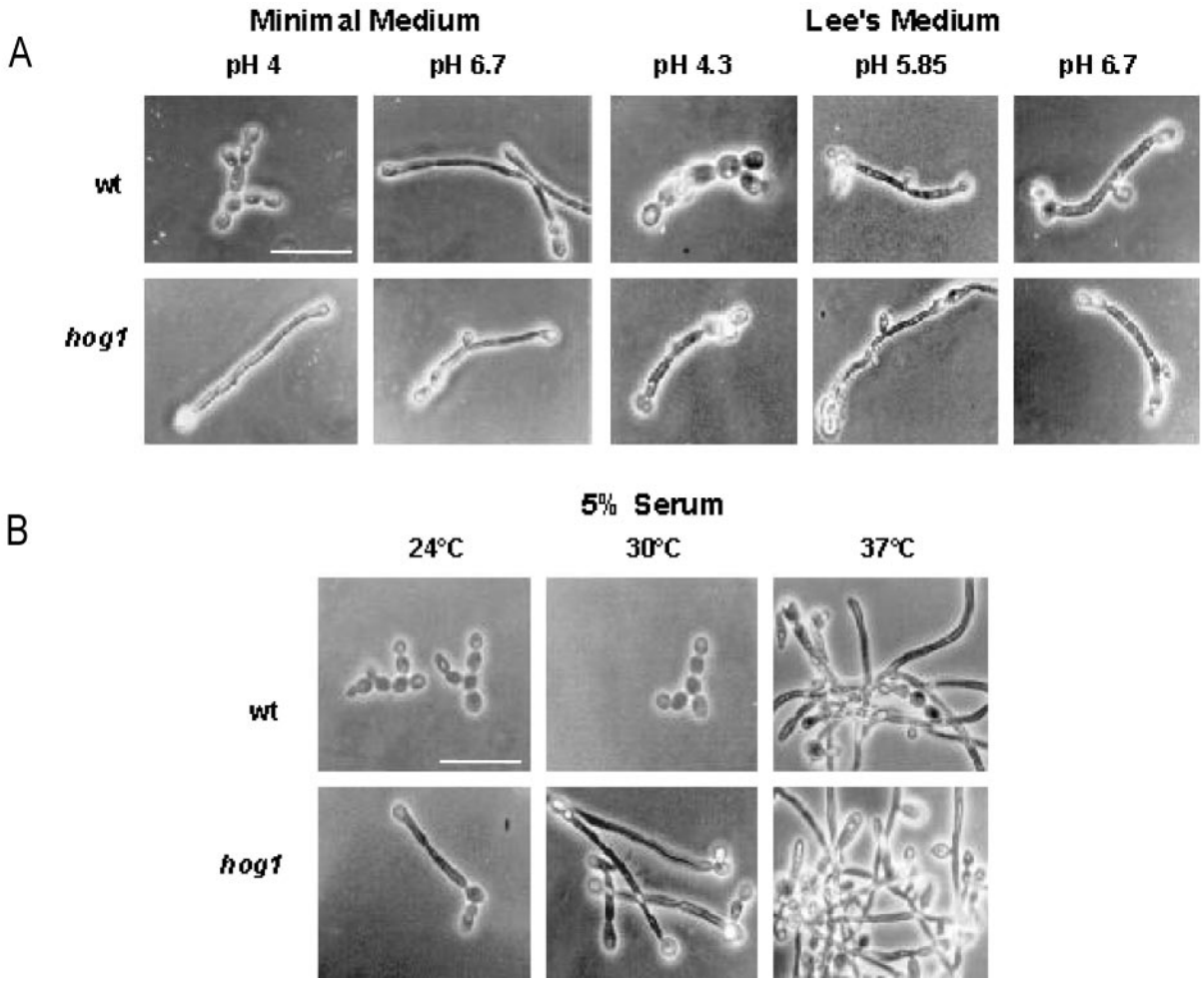

FIG. 1. Morphological transition of *hog1* mutant under different conditions. *C. albicans* strains were inoculated to 10^5 cells/ml into Lee's medium or minimal medium at different pHs and incubated at 37°C (A) or liquid YPD supplemented with 5% serum and incubated at 24, 30, or 37°C (B). Microphotographs were taken after 3 h of incubation. Bars, 10 μm. wt, wild type.

medium (Fig. 1A). A pH below 5 prevented filamentation of the wild-type strain. In contrast, the *hog1* mutant was able to undergo the morphological transition at any pH. Finally, the enhanced hyphal formation of the *hog1* mutant was also evident using temperature as an inducer of filamentation. As shown in Fig. 1B, when cells were grown in 5% serum at low temperature (24 or 30°C), only the *hog1* mutant displayed a filamentous phenotype, while the wild-type cells were able to display only hyphae-like structures at 37°C (Fig. 1B). We conclude from these observations that the absence of the Hog1 MAP kinase leads to an enhanced hyphal formation evidenced under several conditions (low serum concentration, low pH, and low temperature), and therefore, Hog1 does play a constitutive/basal role in repressing the morphological transition.

The repression of filamentation mediated by Hog1 is not dependent on the Cek1 MAP kinase. In *S. cerevisiae*, Hog1 prevents cross talk between the HOG and the pheromone response/invasive growth pathways (19, 40). We explore the existence of a similar mechanism in *C. albicans* by analyzing (i) the phosphorylation state of the MAP kinases under different conditions and (ii) the ability to undergo the yeast-to-hypha transition in response to physiological stimuli. For the first purpose, antibodies that recognize the TEY motif of growth MAP kinases (Cek1 and Mkc1) (4) were used, and whole-cell extracts obtained from cells obtained under different conditions were analyzed. Immunodetection studies showed a constitutive basal activation of Cek1 when exponentially growing cells of the *hog1* mutant (but not wild type) were used (4, 38). The levels of phospho-Cek1 were 2 to 4 times higher in *hog1* cells than in wild-type strain cells (as determined by autoradiography), suggesting that the enhanced hyphal growth of *hog1* mutants may be the result of a constitutive activation of the *CEK1*-mediated pathway. We tested this assumption genetically through the construction of double *hog1* mutants with other signaling elements. For this purpose, a *HOG1-hisG-URA3-hisG* disruption construction was used to perform the disruption of the *HOG1* gene in *cla4*, *cst20*, *hst7*, *cpp1*, *cph1*, *efg*, and *cph1 efg1* mutants. We checked the basal state of Cek1 phosphorylation in the mutant strains generated. Activation of Cek1 completely disappeared in *hst7*; furthermore, this signal was also absent in *hst7 hog1* mutants (Fig. 2A), indicating that the Hst7 MAP kinase kinase is required to phosphorylate the

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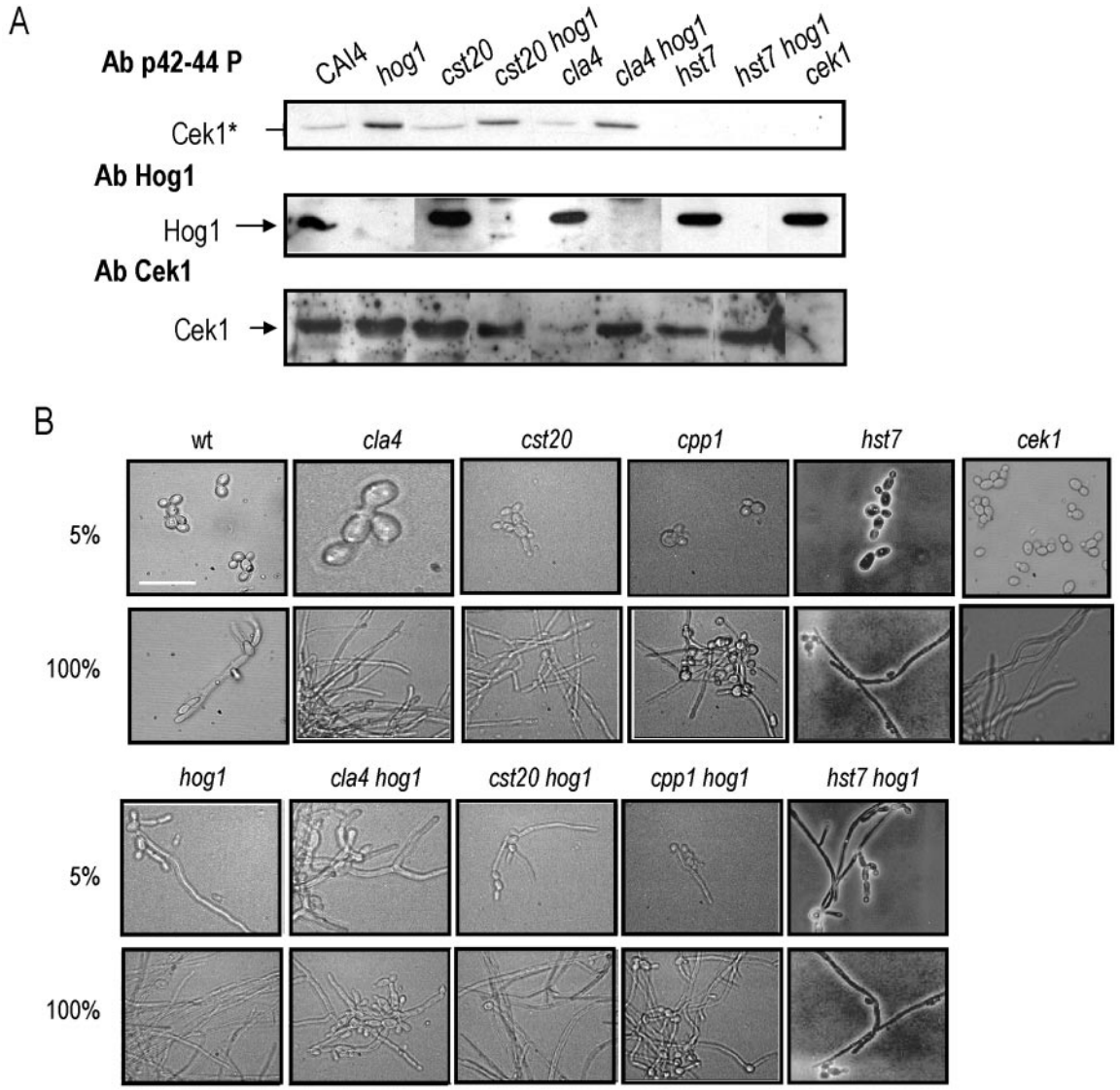

FIG. 2. MAP kinase activation and cell morphologies. (A) Ten milliliters of cell cultures growing exponentially (1 OD) was taken and processed for immunoblot assay. The same membrane was incubated subsequently with the antibodies (Ab) indicated. Ab p42-44 P, phospho-p42/44 MAP kinase; Ab Hog1, ScHog1 polyclonal antibody; Ab Cek1, Ab-CaCek1; Cek1*, Cek1 phosphorylated. (B) Cell morphology of different mutants under subinducing conditions. Cells were inoculated at 10^6 cells/ml in YPD plus 5% serum or serum and incubated at 30°C for 5 h before being photographed. Bars, 10 μm. wt, wild type.

Cek1 MAP kinase (MAPK). In contrast, deletion of *CST20*, *CPH1*, and *CPP1* had no evident effect on Cek1 phosphorylation. Single mutants (*cla4*, *cst20*, *cph1*, and *cpp1*) displayed a phosphorylation of Cek1 similar to that of the wild type (Fig. 2A), and the deletion of the *HOG1* gene in these backgrounds also showed an increased phospho-Cek1 similar to the *hog1* single mutant. These immunodetection assays also revealed a significant and reproducible reduction in the amount of Cek1 protein in *cla4* extracts; remarkably, the Cek1 protein level is restored in the *cla4 hog1* double mutant. The increased activation of Cek1 is not exclusive to *hog1* mutants, as it was recently reported in other mutants of the HOG pathway, such as the *ssk1* mutant (45) and the *pbs2* mutant (4). We conclude from these observations that the HOG pathway represses the activation of the *CEK1*-mediated pathway.

To determine if the enhanced hyphal formation of the *hog1* mutant correlated with Cek1 phosphorylation, we performed specific filamentation assays. The ability of these strains to form filaments was tested using a subinducing serum concentration (5%) and incubation at 30°C. These conditions were chosen because they allowed us to clearly discern between the behavior of *hog1* and wild-type strains. Assays in liquid media revealed that all strains tested grew as yeast cells when grown in YPD medium, but under 100% serum, they all formed filaments (Fig. 2B). This result contrasts with previous published data showing that *cla4* mutants were unable to form

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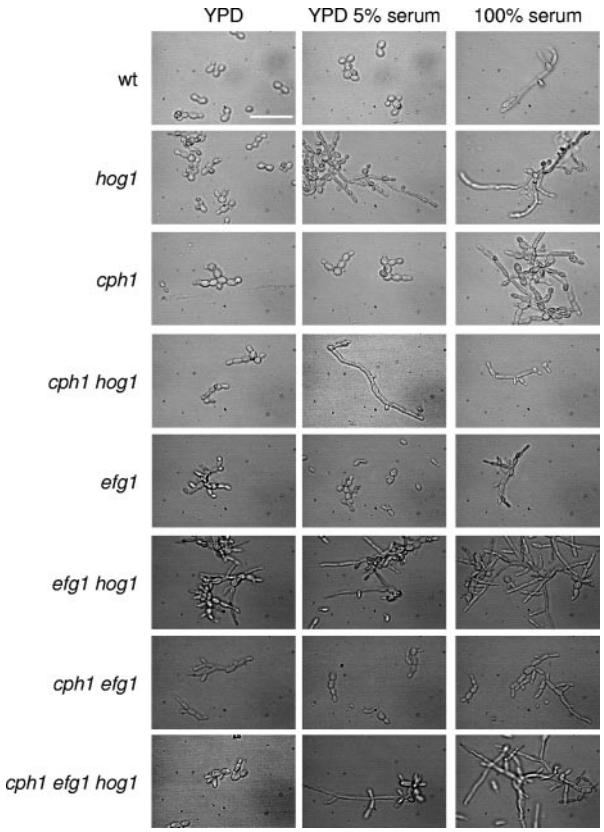

FIG. 3. Filamentation of *hog1*, *cph1*, and *efg1* mutants. Cells were inoculated at 10^6 cells/ml in YPD, YPD plus 5% serum, or 100% serum and incubated at 30°C. Photomicrographs were taken after 5 h of incubation. Bars, 10 μm. wt, wild type.

filaments (28); in our laboratory, *cla4* cells were able to form filaments when grown in 100% serum. However, under limiting serum concentrations, all of the mutant strains lacking the *HOG1* gene were able to form true filaments (Fig. 2B), including those where the phosphorylation of Cek1 was not detected, such as the *hst7 hog1* mutant. These data indicate that the hyperfilamentous phenotype is not due to activation of *CEK1*-mediated pathway in *C. albicans*.

The role of the Cph1 and Efg1 transcription factors, implicated in the morphological transition, was also analyzed in relation to the *HOG1* gene. The double *cph1 efg1* mutant was unable to form filaments under any laboratory conditions (although hyphal forms have been isolated in vivo from the throat of gnotobiotic piglets) (43); nevertheless, the disruption of the *HOG1* gene in this background resulted in the characteristic derepressed phenotype of *hog1* mutants (Fig. 3). The *cph1 hog1* and *efg1 hog1* double mutants also displayed an enhanced ability to form true filaments. These data suggest that Hog1 is a dominant repressor of filamentation, probably acting through other transcription factors.

Blockage of the CEK1-mediated pathway suppresses the defect in chlamydospore formation of *hog1* mutants. Given that the *CEK1*-mediated pathway has been implicated in the dimorphic transition and that there is cross talk with the *HOG1* pathway, we aimed to determine its role in chlamydospore formation. When single mutants *cla4*, *cst20*, *hst7*, *cek1*, *cph1*, and *cpp1* were analyzed, they were all found to form a similar abundance of these structures to a similar degree of maturity in comparison to wild-type cells. The behavior of *cpp1* mutants has also been recently reported (47). Interestingly, the analysis of double mutants implicated the Cek1 pathway in chlamydospore formation, since the double *hog1 cst20*, *hog1 hst7*, and *hog1 cpp1* mutants were able to form such structures. In contrast, deletion of the *HOG1* gene in a *cla4* mutant generated a *hog1* phenotype, that is, the inability to form chlamydospores (Fig. 4). This result indicates that the mechanism inhibiting the formation of chlamydospores in *hog1* cells is *CST20*, *HST7*, and *CPP1* dependent.

The epistatic relationship between Hog1 and Efg1 was also analyzed using this approach. Both *efg1* and *hog1* mutants have been shown to block this process. The double *efg1 hog1* (as well as a *cph1 efg1 hog1* mutant) was unable to form chlamydospores. Overexpression of the *EFG1* gene under the control of *PCK1* promoter in the double *efg1 hog1* (as well as in a *hog1* mutant) did not suppress the *hog1* phenotype (Fig. 5). Furthermore, overexpression of the *HOG1* gene under the control of the strong constitutive *ACT1* promoter did not restore this capacity in the double mutant (*efg1 hog1*) (not shown). Both results suggest that chlamydospore formation could be controlled by two independent pathways, one mediated by Efg1 and the other by Hog1.

The role of Hog1 in mediating resistance to osmotic and oxidative stresses is independent of Cek1. The HOG pathway is required for the adaptation of cells to oxidative and osmotic stresses (1) in *C. albicans* (46). The role of *CLA4* and other elements of the putative *CEK1*-mediated pathway in response to osmotic and oxidative stress has not been reported previously. None of the *cek1*, *hst7*, *cst20*, *cla4*, *cph1*, *efg1*, or *cph1 efg1* mutants displayed sensitivity to osmotic stress (Fig. 6) or to oxidants (data not shown) compared to wild-type cells. In addition, the single *cla4*, *cst20*, and *hst7* mutations did not impair the signaling to other MAPKs (Hog1 and Mkc1) in response to NaCl or H_2O_2 (data not shown). Furthermore, combining these mutations in a *hog1* background did not aggravate the susceptibility of the *hog1* mutant to both osmotic (NaCl and sorbitol) or oxidative (H_2O_2 and menadione) stress. Those results suggest that the role of the HOG pathway in the response to stress is at least partially independent of Cla4, Cst20, Hst7, Cpp1, Cph1, and Efg1.

Congo red resistance is dependent on Cek1 activation. The Cek1 MAP kinase is involved in the biogenesis of the cell wall, since mutants defective in this MAP kinase, and other elements that mediate its activation, show sensitivity to certain cell wall assembly inhibitors such as Congo red and calcofluor white (45). As *hog1* mutants also present cell wall alterations (1) and constitutively activate the Cek1 MAP kinase (4, 45), we reasoned that both phenomena could be linked. This hypothesis was genetically tested by performing assays of sensitivity to Congo red and calcofluor white on solid media. As shown in Fig. 7, the *cst20*, *cla4*, *hst7*, *cek1*, *cph1*, and *efg1* mutant strains showed impaired growth in the presence of these compounds, while a *cpp1* mutant displayed a phenotype close to that of the wild-type strain. Deletion of *HOG1* in these strains resulted in two different phenotypes (Fig. 7). An *hst7 hog1* mutant showed an *hst7* phenotype; therefore, the lack of the *HOG1* gene did not improve the growth in the presence of cell wall-disturbing

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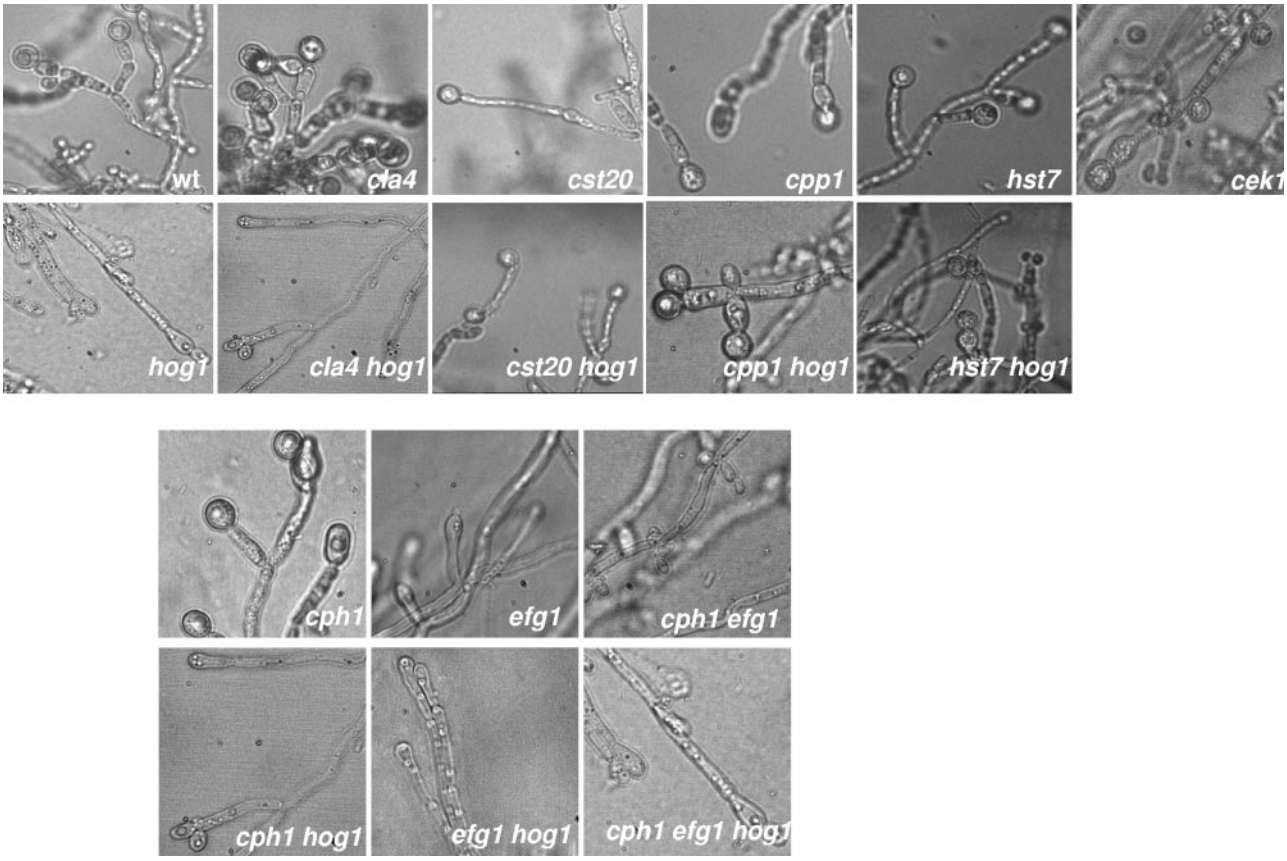

FIG. 4. Chlamydospore formation. CFU (25 to 50) were spread onto cornmeal agar and incubated in darkness at 24°C for 72 h. wt, wild type.

agents, which clearly correlated with the absence of Cek1 activation. However, in *cst20*, *cla4*, and *cph1* mutants, the absence of the *HOG1* gene enhanced growth in the presence of Congo red and calcofluor white, consistent with the fact that these mutants displayed Cek1 phosphorylation levels similar to those of the *hog1* mutant (Fig. 2A).

The role of the Cph1 and Efg1 transcription factors was also analyzed. As mentioned above, the sensitivity of the *cph1* mu-

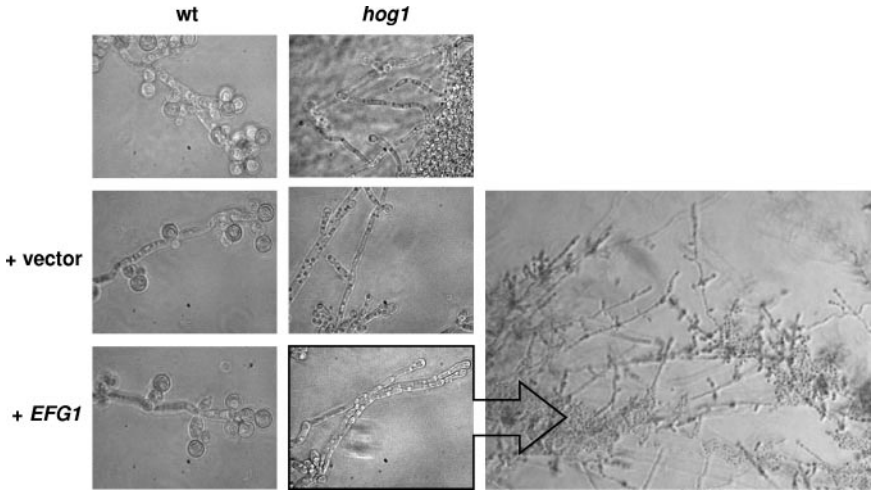

FIG. 5. Influence of *EFG1* overexpression on chlamydospore formation. Wild-type and *hog1* mutant strains were transformed either with (+) the plasmid pRC2312P-H (which consists of the *EFG1* gene under the control of *PCK1* promoter) or the vector. The transformants obtained were spread onto cornmeal agar and incubated in darkness for 72 h before photographs were taken. The arrow points to an offset wider field of the *hog1* mutant carrying the *EFG1*-overexpressing plasmid. wt, wild type.

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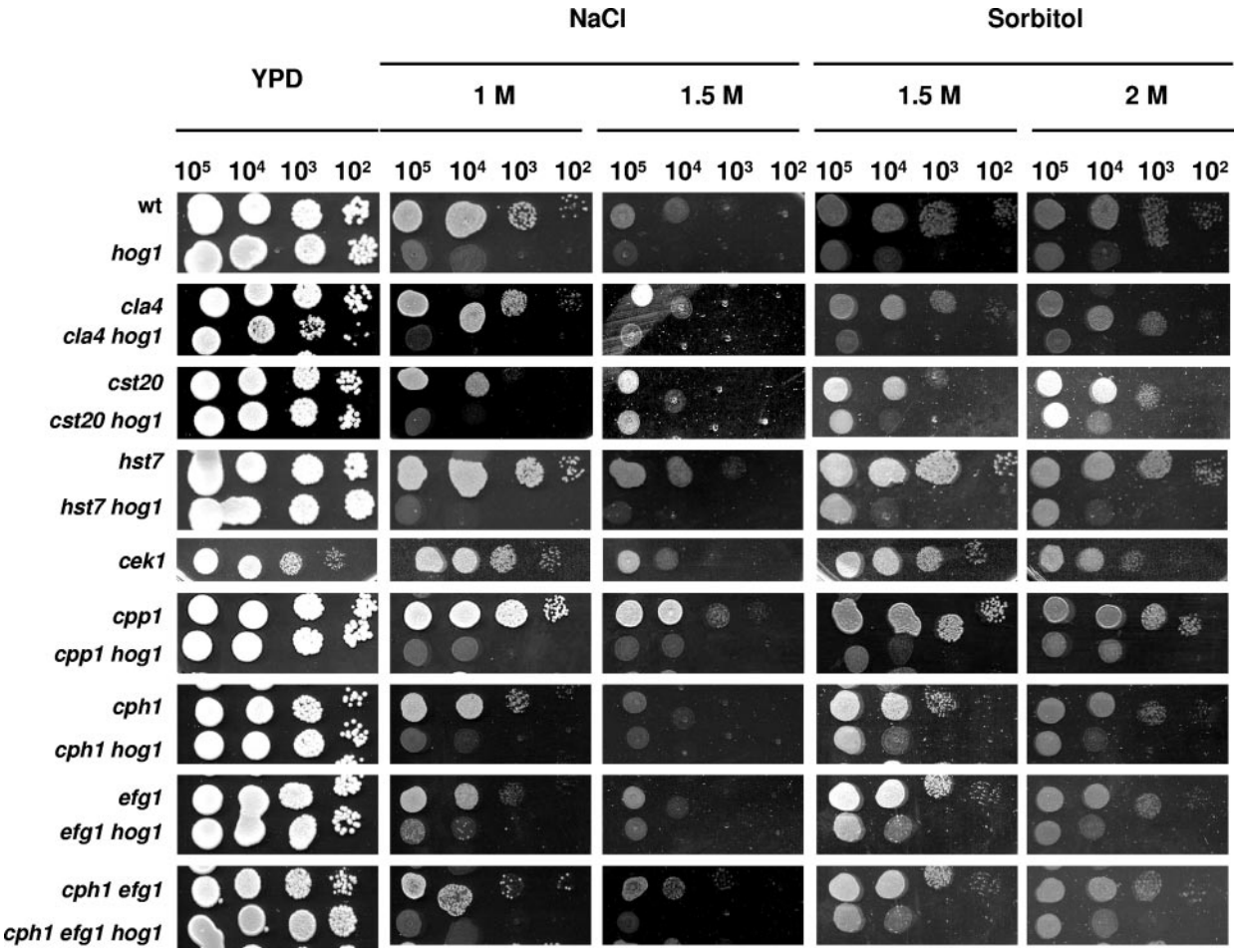

FIG. 6. Susceptibility to osmotic stress. Tenfold serial dilutions from exponentially growing cultures were spotted on YPD plates supplemented or not (control) with the osmotic agents indicated and incubated at 37°C for 24 h. wt, wild type.

tant to cell wall-interfering agents is reversed to resistance when *HOG1* gene is lacking (Fig. 7). This effect does not occur in the case of *efg1*, since both *efg1* and *efg1 hog1* mutants display an increased sensitivity to Congo red and calcofluor white, suggesting a possible epistatic relationship between Hog1 and Efg1. Remarkably, the double *cph1 efg1* mutant was resistant to these compounds, arguing for the implication of both transcription factors in the architecture of the cell wall. This result suggests a different mechanism for both proteins in the biogenesis of the cell wall. Deletion of the *HOG1* gene in a *cph1 efg1* background did not significantly alter the resistant phenotype of the double *cph1 efg1* mutant.

Recently, Cek1 activation has been shown to correlate with cellular growth and/or the transition from stationary to exponential phase (45). Congo red inhibits the growth of *C. albicans* in a dose-dependent manner in liquid cultures. We therefore tried to correlate both phenomena (Cek1 activation and growth in optical density) using a compound that had a different effect on wild-type and *hog1* mutants. Cells were allowed to enter stationary phase and were then diluted in media containing different amounts of Congo red. Samples were taken at 1 and 2 h and processed for Western blot analyses. As shown in Fig. 8, levels of activated Cek1 were found to be inversely dependent on Congo red concentration, consistent with the inhibition of growth caused by this compound. In addition, Cek1 phosphorylation was always higher in the *hog1* strain versus the wild-type strain (independent of time of sample withdrawal), and finally, it appeared earlier in this mutant at the same concentration (see, for example, lanes at 1 h). As shown in the growth curves, *hog1* mutant cells suffered a less pronounced growth delay in the presence of Congo red than the wild-type strain (Fig. 8B).

Previous studies have revealed that mutants in the HOG pathway (both in *C. albicans* and *S. cerevisiae*) are sensitive to Zymolyase, a β-1,3-glucanase-enriched enzyme preparation (3, 4, 23). To characterize in more detail the relationship between the cell wall composition/architecture and the Cek1- and Hog1-MAPK pathways, we performed the following assay. Cells were grown overnight in YPD medium supplemented with different amounts of Zymolyase, and cell growth was quantified by the final OD reached. *cst20*, *hst7*, *cek1*, and *cph1* mutants were found to be more sensitive to Zymolyase than the wild type. The deletion of the *HOG1* gene in *hst7* and *cph1* mutants slightly aggravated the Zymolyase-sensitive phenotype (Fig. 9); however, the *cst20 hog1* double mutant displayed an increase in the resistance to glucanase. In agreement with the

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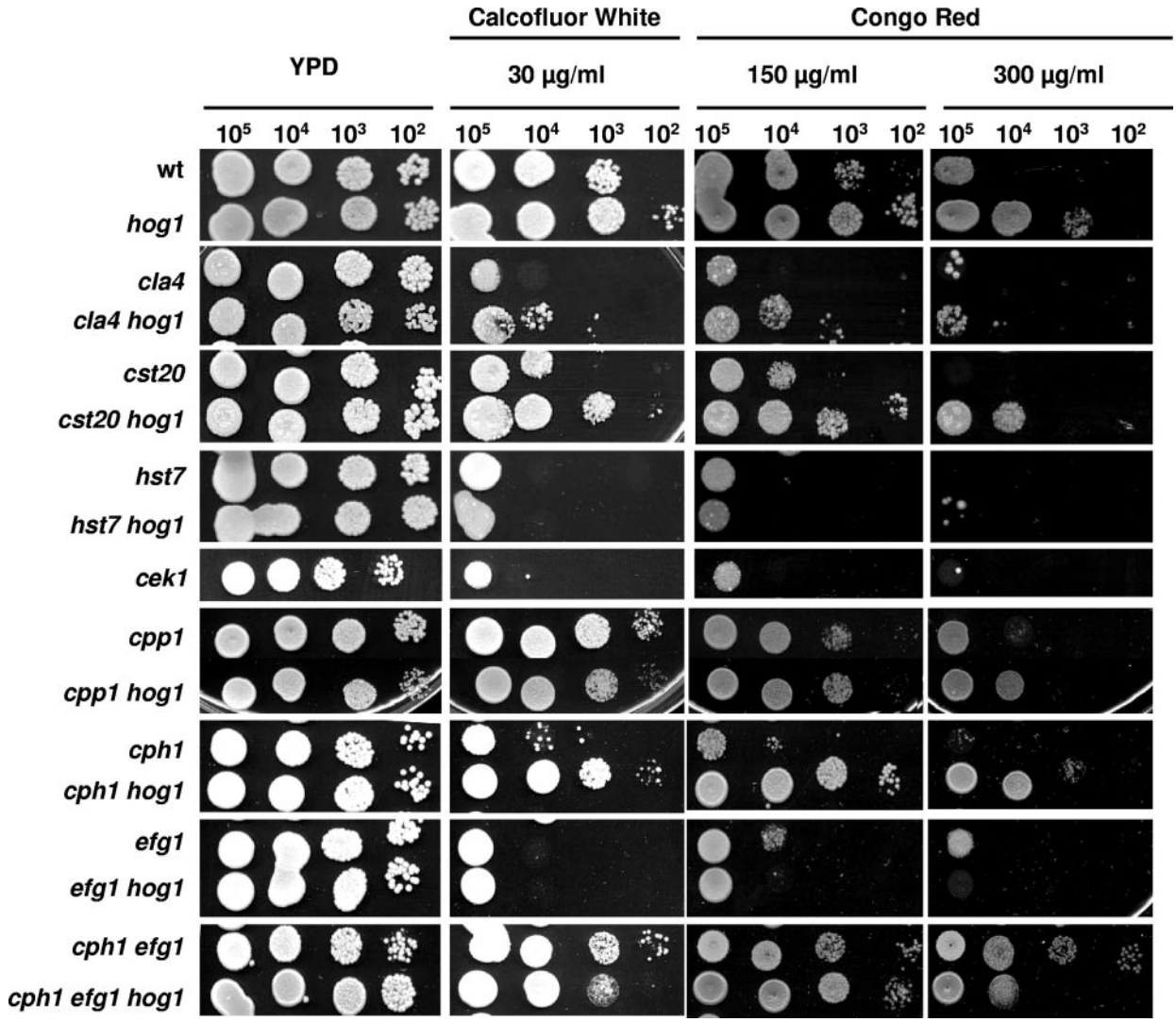

FIG. 7. Growth in the presence of cell wall-disturbing compounds. Serial dilutions of cells were spotted on plates supplemented with calcofluor white or Congo red, and plates were incubated at 37°C for 24 h before photographs were taken. wt, wild type.

phenotype observed on Congo red and calcofluor white plates, a *cpp1* mutant was not sensitive to β-1,3-glucanase. *efg1* and *cph1 efg1* mutants showed similar sensitivities to Zymolyase but a lower sensitivity than *cph1* mutants; deletion of *HOG1* aggravated these phenotypes to a sensitivity similar to that of the *hog1* mutant. This observation suggests that Hog1 plays a role in glucan assembly/regulation independent of Efg1 and Cph1.

Deletion of *CLA4* rendered cells drastically sensitive to cell wall-interfering compounds, and further deletion of the *HOG1* gene slightly improved growth in the presence of these compounds (still far beyond the levels attained in the *hog1* mutant), suggesting that Cla4 and Hog1 contribute independently to cell wall biogenesis (Fig. 7). This idea was reinforced when the susceptibility to glucanase was tested. A *cla4* mutant was as resistant as the wild-type strain, while the double *cla4 hog1* mutant displayed the sensitive phenotype characteristic of *hog1* mutants (Fig. 9).

DISCUSSION

The aim of the current work was to investigate the relationship between the HOG and the Cek1-mediated MAPK pathways. Both routes have been implicated in important cellular functions such as morphogenesis and cell wall construction. The data obtained is this work are summarized in the model shown in Fig. 10.

In *S. cerevisiae*, a genetic interaction between both routes has been described previously (15, 40). When cells are exposed to osmotic stress, in the absence of either the *HOG1* or *PBS2* gene, cells display an invasive growth on solid media, *shmoo* projection, and expression of mating type-specific genes, and these phenotypes are dependent on the transmission of the signal through Sho1 to Ste20 and Ste11 and Ste7-Kss1. We demonstrate that, in *C. albicans*, the mechanism of cross talk is different. In this organism, deletion of some of the predicted elements of the pathway (*CST20*, *HST7*, *CEK1*, and *CPH1*)

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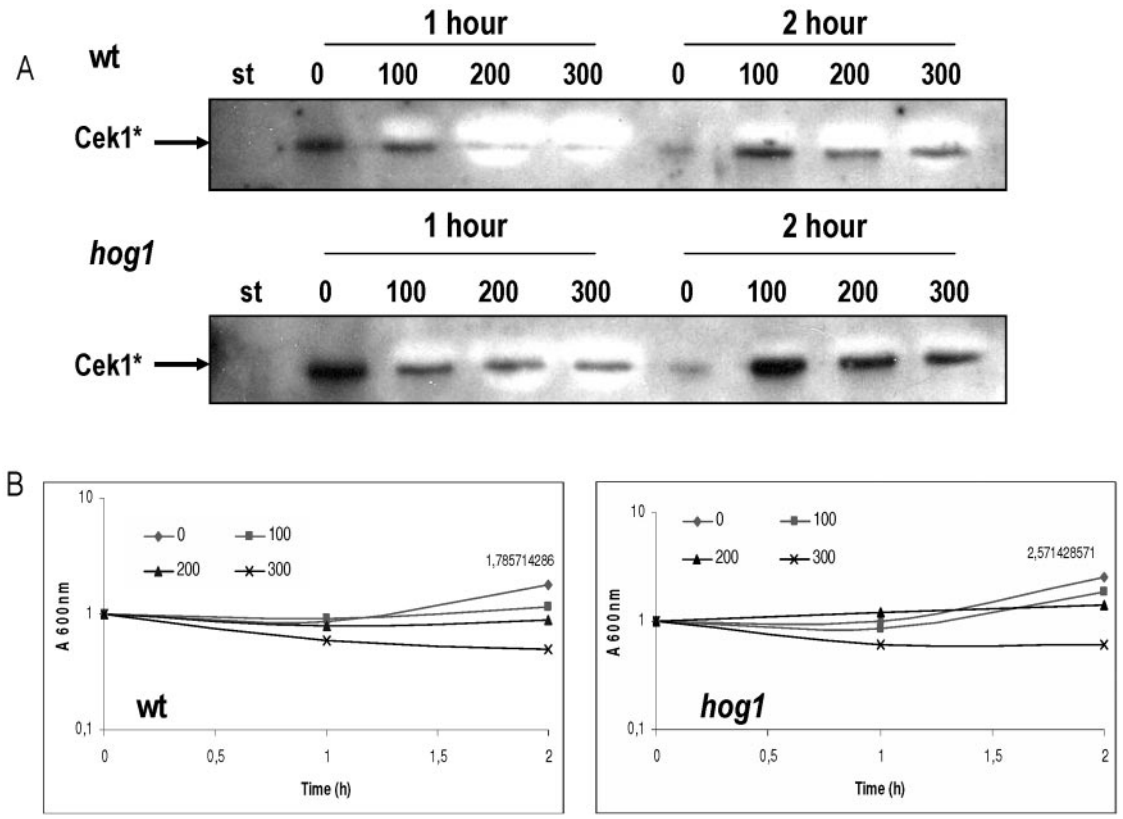

FIG. 8. Congo red effect on Cek1 phosphorylation and growth. Stationary-phase cells were diluted at 0.1 OD in YPD supplemented with Congo red at 0, 100, 200, and 300 μg/ml. Samples were collected after 1 and 2 h of growth at 37°C and processed for Cek1 phosphorylation by Western blotting analysis (A) or growth measurement (B). wt, wild type; Cek1*, Cek1 phosphorylated.

generate mutants that show defects on certain solid media that induce morphological transitions, although they retain the ability to form filaments on serum. In addition *hog1* and *pbs2* mutants display an enhanced ability to form filaments (1, 4) independent of the stimuli (either pH, temperature, or serum concentration) tested (Fig. 1). This occurs even in the absence of osmotic stress, suggesting that the activation of Hog1 does not have an effect on filamentation. However, genetic analysis of double mutants in the HOG and *CEK1*-mediated pathways show that the derepressed behavior of *hog1* cells is not mediated by the Cek1 pathway, since the *hog1* hyperfilamentous phenotype is dominant when the Cek1 pathway is impaired (Fig. 2 and 3). A similar situation is observed when the *HOG1* gene is deleted in concert with the *EFG1* and *CPH1* genes (Fig. 2 and 3). Deletion of *EFG1* and *CPH1* renders cells unable to form filaments under most laboratory conditions tested, although not in vivo (43). The triple deletion mutant *cph1 efg1 hog1* was able to form filaments under subinducing conditions, similar to *hog1*. These data indicate that the *HOG1* gene might carry out its repressing effect on additional elements, not Efg1 or Cph1. Potential candidates include *RBF1* (21) or *TUP1*, which have not been accommodated in any signaling pathway mediated by MAP kinases. Deletion of these genes led to enhanced (*RBF1*) (22) or even constitutive (*TUP1*) (5, 6) hyphal growth. The Tup1 protein is a strong candidate, as the Ssn6-Tup1 repressor has been involved in *S. cerevisiae* in the induction of certain *HOG1*-dependent genes (35); Hog1 could signal environmental changes to Tup1 in *C. albicans* and consequently relieve the repression of certain filamentation-responsive genes.

We have also shown that the HOG pathway is involved in the formation of chlamydospores, a process that occurs under defined environmental conditions, such as low temperature, oxygen concentration, and rich media. It can also occur, apparently, in vivo, as chlamydospore-like cells were isolated from the gastrointestinal tract of cyclophosphamide-treated mice (9). It has been suggested that chlamydospores are resistant forms, since they displayed a thickened cell wall which could protect against environmental challenges. Moreover, most of the *C. albicans* clinical isolates are able to induce the formation of chlamydospores, arguing for an important role of chlamydospores in *C. albicans* biology. Both the *EFG1* and *HOG1* genes are essential in the formation of chlamydospores (2, 50), involving a MAPK signal transduction pathway and the cAMP pathway in this process. We present data suggesting that both proteins, Hog1 and Efg1, act independently, since overexpression of the *EFG1* gene did not restore the ability to form chlamydospores in the *hog1* mutant, and similarly, overexpression of *HOG1* gene does not restore the formation of chlamydospores in the *efg1* mutant. The reasons for the inability of *hog1* mutants to form chlamydospores are not yet known (2). One possible explanation could be oxidative stress: chlamydospore formation is favored under microaerophilia and absence of light, a result that suggests that reactive oxygen species impair

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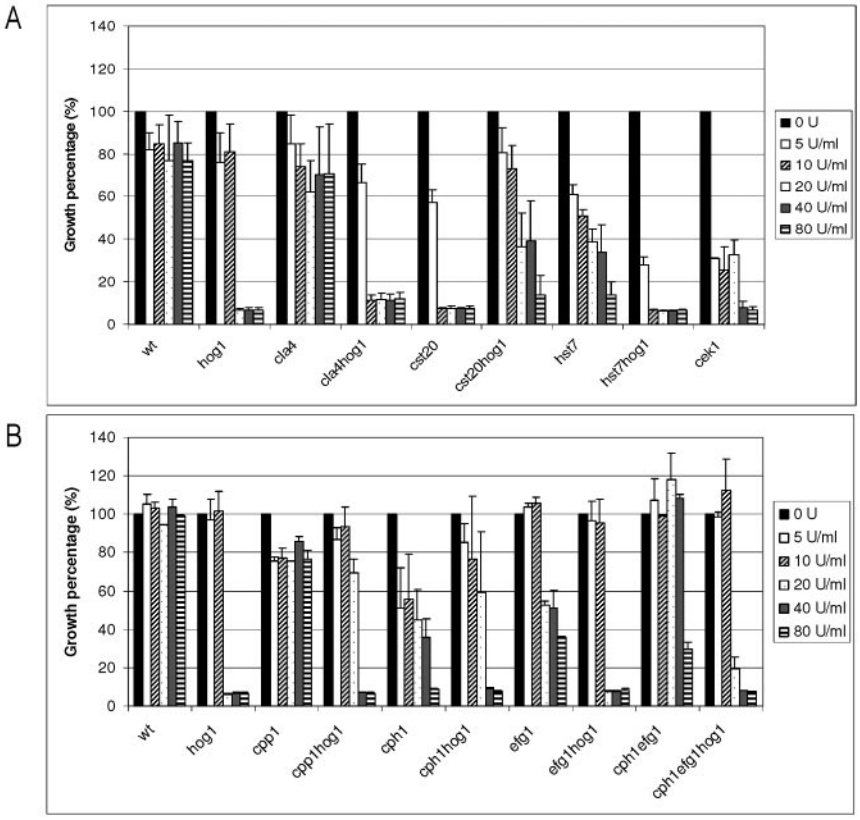

FIG. 9. Susceptibility to Zymolyase. The strains indicated, PAKs and MAP kinases (A) or phosphatases and transcription factors (B), were grown overnight at 37°C in the presence of different amounts of Zymolyase starting with an OD of 0.025. Growth is depicted as the percentage of growth in YPD supplemented with Zymolyase compared to growth in YPD alone. wt, wild type.

this process. The absence of Hog1-dependent defense mechanisms in *hog1* mutants could generate a higher concentration of reactive oxygen species and, therefore, the inability to form chlamydospores. An additional and alternative explanation could be a repressive role of the Cek1 pathway in chlamydospore formation, as this pathway is constitutively active in *hog1* mutants (Fig. 2) and *pbs2* mutants (4). This suggests that a coordinate balance between both pathways is necessary to generate such structures. In a recent study, a number of different genes have been reported to be required for chlamydospore formation, such as *SUV3*, *SCH9*, and *ISW2*, which are involved in mitochondrial function, glycogen accumulation, and chromatin remodeling, respectively (39). It is reasonable to assume that the expression of some of these genes may be dependent on *HOG1* and/or *CEK1*. It must be stated, however, that the effect of the Cek1 pathway seems to be independent of oxidative stress, since Cek1 pathway mutants do not show altered sensitivity to oxidants nor increase the sensitivity of *hog1* cells to these compounds (data not shown).

The results presented in this work also show that *hog1* mutants display increased resistance to certain cell wall inhibitory compounds, such as Congo red and calcofluor white, indicating its relationship with cell wall biogenesis. We propose that Cek1 activation is responsible for this effect, as evidenced by biochemical and genetic analyses. The failure to activate Cek1 (as occurs in *hog1 hst7* cells) would suppress the resistance phenotype in *hog1* mutants, while deletion of the *CPP1* phosphatase gene or the *CST20* PAK gene would have minor effects according to the activation pattern determined by Western blot analyses. However, the stimuli (either extra- or intracellular) involved in Cek1 activation remains unclear. In *S. cerevisiae*, Kss1 (a Cek1 homologue) participates in the SVG (*s*terile *v*egetative *g*rowth) pathway, which is involved in cell wall biogenesis (12, 29). Defects in protein glycosylation cause its constitutive and *SHO1*-dependent activation. Cek1 activation could be triggered in response to those physiological situations that require active cell wall remodeling, such as exit from the stationary phase and entrance to the exponential phase of growth, and this sensing mechanism is fully functional in *hog1* mutants (Fig. 2 and 8), despite its derepressed behavior on Cek1 activation. The stimuli that could lead to an activation of Cek1 are not yet clear, as recent data (38) indicate that Cek1 is activated in response to Zymolyase, a β-glucanase-enriched enzymatic preparation. Furthermore, Zymolyase, as well as Congo red, also activates the cell integrity Mkc1 MAP kinase, similar to what is observed in *S. cerevisiae* for the Slt2 protein (36).

Interestingly, *cst20* and the *cst20 hog1* mutants activate Cek1 similar to the wild-type and *hog1* mutant strains, respectively, indicating that Cst20 is not the only mediator of Cek1 activation. In *C. albicans*, the PAK Cla4 protein is a putative transduction element that has been reported to be involved in morphogenesis and virulence in this fungus (28, 41). Our results, as

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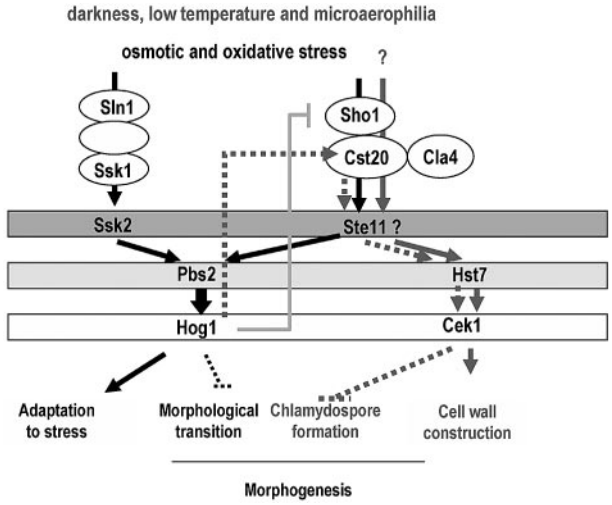

FIG. 10. Proposed model of interaction between the pathways mediated by Hog1 and Cek1 MAP kinases. Osmotic stress triggers Hog1 activation through both branches, enabling the cell to adapt to hyperosmotic conditions (black arrow). The Cek1 pathway is involved in the construction of the cell wall (gray arrow); the stimulus is not known and is depicted as a question mark. Regarding morphogenesis, the HOG pathway plays an inhibitory role over yeast-to-hypha transition; this role is independent or dominant over the *CEK1* pathway (discontinuous black bar) and the transcription factor, Efg1. Under specific conditions, such as low glucose concentration, darkness, low temperature (24°C to 28°C), and microaerophilia, Hog1 plays an inducing role in the formation of chlamydospores; this positive role may be played, presumably, through Cst20, Ste11, Hst7, and Cek1 (discontinuous thick gray arrow). Under standard growth conditions, Hog1 controls the activation of Cek1 (light gray bar).

revealed by the pattern of MAPK activation, chlamydospore formation, cell wall resistance phenotypes, and filament formation, suggest that Cla4 is not a member of the pathway mediated by Cek1 or that there is redundancy at this level. Other elements implicated in the transmission of the signal at the level of Cst20, such as Cdc42 (53) or Ste50, could play a role in this process (42).

Unfortunately, the construction of the double *hog1 cek1* mutant was not possible despite continued genetic attempts (data not shown), suggesting either synthetic lethality or that the mutant is strongly counterselected under the normal experimental conditions of isolation. Since a *hst7 hog1* mutant is viable and a BLAST analysis reveals no functional homologue to Hst7 in the *C. albicans* genome, one possible explanation for lethality could invoke a downstream mediator of Hst7. Cek2 is a candidate for such a role, since this MAP kinase has been shown to complement the mating deficiency defect of a *fus3 kss1* mutant in *S. cerevisiae* and a *C. albicans cek1 cek2* mutant is also mating deficient (8). Whether Cek2 is functionally redundant to Cek1 in nonmating functions (such as chlamydospore formation or filamentation) is, however, open to speculation, since it is also possible that other downstream mediators compensate for the absence of Cek1.

In conclusion, the data obtained in this work indicate that the Hog1 and the Cek1-mediated pathways play independent roles in processes such as filamentation and osmotic/oxidative stress resistance but play complementary roles in cell wall biogenesis and chlamydospore formation in *C. albicans*. Further work will be aimed toward the definition of the elements of the HOG pathway responsible for Cek1-mediated signaling.

ACKNOWLEDGMENTS

We thank Alistair J. P. Brown, G. R. Fink, and M. Whiteway for generously providing strains.

R.A.-M. is a recipient of the Ramón y Cajal Program. E.R. and D.A. hold fellowships associated with the projects 2RO1 AI043465-05 A2 (NIH) and BIOTEC0992-2003 (MEC), respectively. This work was supported by grant BIOTEC0992-2003.

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