The Hog1 MAP kinase controls respiratory metabolism in the fungal pathogen *Candida albicans*

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Signal transduction pathways mediated by mitogen-activated protein kinases (MAPKs) play crucial roles in eukaryotic cells. In the pathogenic fungus *Candida albicans* the HOG MAPK pathway regulates the response to external stresses (osmotic and oxidative among others) and is involved in morphogenesis and virulence. We show here that the lack of the Hog1 MAPK increases the sensitivity of this fungus to inhibitors of the respiratory chain. *hog1* mutants also show an enhanced basal respiratory rate compared to parental strains, and higher levels of intracellular reactive oxygen species despite an increased expression of detoxifying enzymes. We also demonstrate that although oxidative phosphorylation is essentially unaffected, *hog1* mutants have an altered mitochondrial membrane potential. Data indicate that *hog1*-defective mutants are more dependent on mitochondrial ATP synthesis, probably due to an increased cellular ATP demand. Our results therefore link a MAPK pathway with respiratory metabolism in pathogenic fungi.

INTRODUCTION

Candida albicans is an important human fungal pathogen. This microbe colonizes mucosa and skin in approximately 50 % of healthy individuals but can also cause opportunistic infections that range from superficial to systemic. Systemic infections are normally severe, difficult to treat and may eventually cause the death of the patient (Calderone & Gow, 2002; Odds, 1988). One of the main factors that influences virulence in *C. albicans* is its ability to adapt to different environmental conditions. *C. albicans* is able to change its morphology and cell wall composition in response to external signals. During infection, and depending on the body location, it will have to face challenges such as pH changes, nutrient fluctuations and oxidative and/or osmotic stress. Signal transduction pathways mediated by mitogen-activated protein kinases (MAPKs) play crucial roles in sensing and responding to changes in the environment (Alonso-Monge *et al.*, 2006; Hohmann, 2002; Qi & Elion, 2005). Two MAPKs become

phosphorylated upon oxidative stress conditions in *C. albicans*, Hog1 and Mkc1 (Alonso-Monge *et al.*, 2003; Navarro-García *et al.*, 2005). Both these MAPKs respond similarly to this challenge in terms of kinetics and levels of phosphorylation; nevertheless, the HOG pathway seems to mainly control this process since Mkc1 phosphorylation is partially dependent on the HOG pathway (Navarro-García *et al.*, 2005; Arana *et al.*, 2005). A non-functional HOG cascade leads to an increased sensitivity to osmotic and oxidative stress (caused by reactive oxygen species, ROS) and, as a consequence, to a decreased survival in the presence of phagocytes (neutrophils and macrophages) (Arana *et al.*, 2007). *hog1* mutants exhibit an increased catalase activity under basal conditions (Alonso-Monge *et al.*, 2003), as well as an enhanced expression of genes involved in the response to oxidative stress (Enjalbert *et al.*, 2006; Arana *et al.*, 2007). Although some transcription factors have been shown to participate in this response in *C. albicans* (Singh *et al.*, 2004; Alarco & Raymond, 1999; Zhang *et al.*, 2000) none of them has been shown to be a target of a MAPK pathway (Alonso-Monge *et al.*, 2003; Enjalbert *et al.*, 2006). Therefore, although the lack of a functional HOG cascade leads to alterations in the intracellular defences against oxidative stress, the reasons for this behaviour remain unknown.

ROS are not only generated external to the cell but intracellularly as a result of the oxidative metabolism in

Abbreviations: AOX, alternative oxidase; carboxy-H₂DCFDA, carboxy-2',7'-dichlorodihydrofluorescein; DNP, 2,4-dinitrophenol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HET, dihydro-ethidium; MAPK, mitogen-activated protein kinase; MCLA, 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*] pyrazin-3-one, hydrochloride; PQ, paraquat; Rh123, rhodamine 123; ROS, reactive oxygen species; SHAM, salicylhydroxamic acid.

aerobic cells. Mitochondria are the major source of cellular aerobic energy. ATP formation in respiring organisms is coupled to electron flow from reduced substrates through the respiratory chain to a terminal electron acceptor. In animals, the conventional respiratory chain comprises four enzyme complexes: NADH-ubiquinone oxidoreductase (complex I), succinate dehydrogenase (complex II), ubiquinol-cytochrome *c* oxidoreductase (complex III), and the terminal oxidase cytochrome *c* oxidase (complex IV) (Fig. 1). ROS production is coupled to the functioning of complex IV. Plants and fungi have more complex and flexible electron-transfer pathways, including alternative rotenone-insensitive NAD(P)H dehydrogenases (Kerscher, 2000) and alternative terminal oxidases (AOX). In animals, only the intramitochondrial NADH can be oxidized at the respiratory chain, while plants and fungi possess other NAD(P)H dehydrogenases that allow direct oxidation of the cytoplasmic NAD(P)H (Kerscher, 2000). Although the presence of an alternative NAD(P)H dehydrogenase in *C. albicans* has not been demonstrated, there is evidence that suggests its existence (Helmerhorst *et al.*, 2002, 2005). *C. albicans*, similarly to *Candida parapsilopsis*, contains a third respiratory pathway named PAR (parallel respiratory chain) (Ruy *et al.*, 2006). In addition, in *C. albicans* two genes coding for alternative oxidases have been identified, AOX1a and AOX1b (Huh & Kang, 1999, 2001). The AOX enzyme(s) constitute the cyanide-resistant respiratory pathway; these oxidases accept electrons from the ubiquinone pool and reduce oxygen to water, an activity that is not energy-conserving since it is not coupled to proton pumping.

In this work we demonstrate that *C. albicans* *hog1* mutants display a higher respiratory rate compared to wild-type

although the oxidative phosphorylation efficiency is maintained, suggesting that there is an increased cellular demand for mitochondrial ATP synthesis. We also show that disruption of the *HOG1* gene leads to an enhanced concentration of intracellular ROS and mitochondrial activity which, in turn, could lead to an enhanced ATP production. We therefore link a MAPK signalling pathway with cellular metabolism in this important human fungal pathogen.

METHODS

Strains and growth conditions. Yeast strains are listed in Table 1. For clarity and unless otherwise stated, *geneX* will always indicate the homozygous *geneX/geneX* Ura⁺ strain, except for *aox1a*, *aox1b* and *aox1a aox1b*, which represent the Ura⁻ strains. 'Reint' indicates the CNC15-10 strain, a *hog1* Ura⁺ strain derived from CNC15 (*hog1* Ura⁻), where the *HOG1* gene was reintroduced forcing recombination at the *LEU2* gene (Alonso-Monge *et al.*, 1999). The strains used as wild-type strains (in this context, not affected in signalling elements) were CAF2-1 and RM100. No differences were observed between them, and RM100 was used throughout the work since it is the parental strain of the *hog1* mutant that has been analysed in more depth.

Yeast strains were grown at 30 °C (unless otherwise stated) in YPD medium [1 % (w/v) yeast extract, 2 % (w/v) peptone and 2 % (w/v) glucose as a fermentable carbon source], YPG medium [1 % (w/v) yeast extract, 2 % (w/v) peptone and 2 % (v/v) glycerol, a non-fermentable carbon source], SD minimal medium [2 % (w/v) glucose, 0.67 % (w/v) yeast nitrogen base without amino acids] or SG [2 % (v/v) glycerol, 0.67 % (w/v) yeast nitrogen base without amino acids] with the appropriate auxotrophic requirements. Growth in liquid medium was estimated by OD₆₀₀ measurements.

Fig. 1. Schematic representation of the *C. albicans* mitochondrial respiratory chain. The electron-transport chain generates protons (H⁺) which are effluxed from the mitochondrial matrix while electrons are transported inside. Compounds that inhibit the respiratory electron flow are boxed and the steps where they exert their effects are indicated. AA, antimycin A; SHAM, salicylhydroxamic acid; Co Q, coenzyme Q. PAR indicates the secondary parallel chain. The alternative oxidase (AOX) branches off the conventional respiratory chain at the coenzyme Q level.

Table 1. Strains used in this study

Strain	Genotype	Relevant genotype	Source
CAF2-1	<i>URA3/ura3Δ::λimm434</i>		Fonzi & Irwin (1993)
RM100	<i>ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG-URA3-hisG</i>	<i>his1</i>	Alonso-Monge <i>et al.</i> (2003)
RM1000	<i>ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG</i>	<i>his1 ura3</i>	Alonso-Monge <i>et al.</i> (2003)
CNC13	<i>ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG</i> <i>hog1::hisG-URA3-hisG/hog1::hisG</i>	<i>his1 hog1</i>	San José <i>et al.</i> (1996)
CNC15-10	<i>ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG</i> <i>hog1::hisG/hog1::hisG LEU2/leu2::HOG1URA3</i>	<i>his1</i>	Alonso-Monge <i>et al.</i> (1999)
CCC1	<i>ura3Δ::λimm434/ura3Δ::λimm434 his1Δ::hisG/his1Δ::hisG</i> <i>cap1::URA3/cap1::HIS1</i>	<i>cap1</i>	Alonso-Monge <i>et al.</i> (2003)
CHC1	<i>ura3Δ::λimm434/ura3Δ::λimm434 his1Δ::hisG/his1Δ::hisG</i> <i>hog1::hisG/hog1::hisG cap1::URA3/cap1::HIS1</i>	<i>hog1 cap1</i>	Alonso-Monge <i>et al.</i> (2003)
CM16-13	<i>ura3Δ::imm434/ura3Δ::imm434 mkc1Δ::hisG-URA3-hisG/mkc1Δ::hisG</i>	<i>mkc1</i>	Navarro-García <i>et al.</i> (1995)
WH304	<i>ura3Δ::imm434/ura3Δ::imm434 aox1aΔ::hisG/aox1aΔ::hisG</i>	<i>ura3 aox1a</i>	Huh & Kang (1999)
WH314	<i>ura3Δ::imm434/ura3Δ::imm434 aox1bΔ::hisG/aox1bΔ::hisG</i>	<i>ura3 aox1b</i>	Huh & Kang (2001)
WH324	<i>ura3Δ::imm434/ura3Δ::imm434 aox1aΔ::hisG/aox1aΔ::hisG</i> <i>aox1bΔ::hisG/aox1bΔ::hisG</i>	<i>ura3 aox1a aox1b</i>	Huh & Kang (2001)

Drug susceptibility assays. Susceptibility to inhibitors of mitochondrial function was determined using exponentially growing or stationary-phase cells in YPD medium at 30 or 37 °C. Equal numbers of cells were taken from these cultures and serial dilutions were spotted onto YPD or YPG plates supplemented with the compound to be tested. Plates were incubated at 30 or 37 °C overnight.

Protein extracts and immunoblot analysis. Overnight cultures were refreshed and yeast strains were grown to an OD₆₀₀ of 1 at 37 °C in YPD medium. Then H₂O₂ was added to the medium at a final concentration of 10 mM and samples were taken 10 min after the challenge. Cell extracts were obtained as previously indicated (Martín *et al.*, 1993, 2000). Protein concentration was assessed as A₂₈₀ and equal amounts of proteins were loaded onto gels. Ponceau Red staining of the membranes was performed prior to blocking and detection. Blots were probed with phospho-p42/44 MAPK (Thr202/Tyr204) (Ab-p42-44P, Cell Signaling Technology), ScHog1 polyclonal antibody (Ab-ScHog1, Santa Cruz Biotechnology) and Ab-p38-P (Thr180/Tyr182) 28B10 monoclonal antibody (Anti-p38-P, Cell Signaling Technology) and developed according to the manufacturer's conditions using the Hybond ECL kit (Amersham-Pharmacia Biotech).

Measurement of oxygen consumption. Oxygen consumption was measured using an oxygen electrode (Hansatech Instruments). Cells were grown in SG minimal liquid medium at 30 °C to an OD₆₀₀ of 1, recovered by low-speed centrifugation (5000 g, 3 min), washed twice with cold water and suspended in 1 mM potassium phosphate buffer (pH 7.0) to an OD₆₀₀ of 1.7. All the experiments were performed at 30 °C. Five-microlitre samples of cells were introduced into the electron chamber. Respiration due to the cytochrome oxidase pathway was inhibited with antimycin A or KCN while respiration due to AOX was inhibited with 5 mM salicylhydroxamic acid (SHAM). The maximal respiratory capacity was determined from the stimulation of respiration induced by the uncoupling reagents 2,4-dinitrophenol (DNP) or carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). Mitochondrial ATP synthesis was inhibited with 25 µg oligomycin ml⁻¹. To induce the AOX, cells were grown for 1 h at 30 °C in the presence of 5 µg antimycin A ml⁻¹. Respiratory rates are expressed as nmol O₂ min⁻¹ per 10⁶ cells.

Chemiluminescence measurements. Exponential- or stationary-phase cultures grown in YPD medium at 37 °C were collected (5000 g, 3 min), washed twice with PBS and suspended to 0.5 × 10⁷ cells in 500 µl PBS containing 10 mM glucose or 50 mM glycerol and maintained at 37 °C. Then 5 µl 1 mM 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazol[1,2-*α*]pyrazin-3-one (MCLA) was added to the mixture and a measurement was taken to quantify the chemiluminescence generated endogenously. After 2–5 min, 5 µl of a 0.5 M solution of paraquat (PQ) was added and measurements were taken until the signal became stable. Luminescence was measured in an OPTOCOMP I luminometer for 30 s in integration mode. Results are expressed as the signal emitted per cell.

Flow cytometry assays. Cells from cultures grown at 30 °C in SG medium were collected, washed twice with PBS and treated with 20 µM dihydroethidium (HET), 50 µM rhodamine 123 (Rh123) or 5 µM JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide); an untreated sample was used as control. Samples were maintained for 30 min at 30 °C in the dark and then cooled on ice, washed twice with PBS and propidium iodide was added (except HET-marked cells) to stain dead cells prior to analysis using a Becton Dickinson FACScan flow cytometer. In parallel, 10 µM carboxy-2',7'-dichlorodihydrofluorescein (carboxy-H₂DCFDA) was added to the medium and incubated for 30 min at 30 °C in the dark. Cells were collected, washed twice with PBS, propidium iodide was added and fluorescence was measured with a Becton Dickinson FACScan flow cytometer. The data shown correspond to the fluorescence histograms (relative number of yeast cell versus relative fluorescence intensity expressed in arbitrary units on a logarithmic scale). The emission of fluorescence was quantified only in live cells and standard-sized cells.

Confocal microscopy. Cells grown overnight at 30 °C in SD or SG medium were collected, washed with PBS and stained with JC-1 (5 µM for 30 min). Cells were then observed using a Leica confocal microscope. The maximal fluorescence excitation/emission was 514/529 nm for the monomer form and 585/590 nm for the J-aggregate form (Pina-Vaz *et al.*, 2001).

RESULTS

hog1 mutants are more sensitive to inhibitors of mitochondrial function

In order to establish a connection between adaptation to oxidative stress and aerobic metabolism, we analysed the behaviour of different pathways involved in sensing and responding to oxidative stress in the presence of inhibitors of oxidative phosphorylation. We tested *hog1* and *mkc1* mutants (defective in Hog1 and Mkc1 MAPKs, which are activated upon oxidative stress) as well as *cap1* mutants (defective in the Cap1 transcription factor). As shown in Fig. 2, the lack of the *HOG1* gene caused an increased sensitivity to inhibitors of mitochondrial oxidative phosphorylation: *hog1* and *cap1 hog1* mutants were significantly more sensitive to Na_3N and KCN, inhibitors of complex III and IV, as well as to oligomycin, an inhibitor of the ATP

synthase (Fig. 2a, b). Similar results were observed for antimycin A (data not shown). This effect was evident when cells were grown with either a fermentable (glucose) or a non-fermentable (glycerol) carbon source. In contrast, no significant differences were observed in the growth of *mkc1* and *cap1* mutants compared to the wild-type strain. Analogous results were obtained when the plates were incubated at 30 or 37 °C or when stationary-phase cells (instead of exponential-phase cells) were used (data not shown).

We also tested the role that the cyanide-insensitive alternative pathway had on these strains by the specific AOX inhibitor SHAM. SHAM had no effect on the growth of any of the strains (Fig. 2b). When both pathways, conventional and AOX, were inhibited, all the strains hardly grew on glycerol (Fig. 2b). However, disruption of the *HOG1* gene rendered mutant cells significantly more

Fig. 2. Susceptibility of wild-type and mutant *C. albicans* strains to inhibitors of mitochondrial oxidative phosphorylation. Cells growing exponentially in YPD liquid medium at 37 °C were suspended in YPD or YPG and serial 10-fold dilutions were made. Cells were spotted onto YPD or YPG plates supplemented with the inhibitors as indicated and incubated at 37 °C for 24 h. (a) Effect of 0.025 % Na_3N and 3 μg oligomycin ml^{-1} . (b) Effect of 10 mM KCN and 2 mM SHAM. (c) Effect of 4 mg chloramphenicol ml^{-1} .

sensitive to the combination of these inhibitors. Once again, the results were similar at 30 °C or 37 °C or when cells were in the exponential or stationary phase of growth (data not shown). Comparable results were observed when assays were performed in liquid cultures: the *hog1* mutant always displayed an elevated sensitivity to inhibitors of oxidative phosphorylation (data not shown).

Since the mitochondrial protein synthesis machinery shares functional similarities with prokaryotic machinery, high chloramphenicol concentrations affect mitochondrial protein synthesis (Yunis, 1989; Turton *et al.*, 2002). We tested susceptibility to chloramphenicol and, as shown in Fig. 2(c), lack of the *HOG1* gene rendered mutant cells more sensitive to chloramphenicol while lack of *MKC1* or *CAP1* genes did not alter the susceptibility pattern. This effect was more evident when cells were grown on the non-fermentable carbon source glycerol. Taken together, these results indicate that *hog1* mutants are clearly more sensitive to inhibitors of oxidative phosphorylation.

hog1 mutants display enhanced O₂ consumption

Analysis of the rate of O₂ consumption of exponentially growing cells revealed that the *hog1* mutant exhibited higher respiration rates than the wild-type strain (2.5-fold) (Fig. 3a). This effect was specific to the *HOG1* gene since introduction of a wild-type copy (strain CNC15-10) reversed this effect, with respiration rates similar to wild-type observed. All strains tested exhibited increased O₂ consumption in exponential growth compared to stationary-phase cultures (data not shown). The increased respiratory rate of the *hog1* mutant was not due to a lower coupling of the oxidative phosphorylation since the addition of uncoupling agents (96 µM DNP or 50 µM FCCP) caused the same stimulation of respiration, 2.3-fold in the wild-type strain and 2.4-fold in the *hog1* mutant (Fig. 3b).

In order to analyse the contribution of the AOX to mitochondrial oxygen consumption, the AOX inhibitor SHAM was added to cells at maximum respiration rate. The addition of 5 mM SHAM caused only 16 % inhibition

Fig. 3. Respiratory profiles of wild-type and the *hog1* mutant. (a) Basal rate of respiration for the wild-type strain, the *hog1* mutant and the reintegrant (reint) strain, in which the *HOG1* gene was reintroduced into the the *hog1* mutant. (b) Maximum respiratory capacity of the wild-type and *hog1* mutant determined from the rate of respiration in the presence of an uncoupler (50 µM FCCP). Data are presented as the fold stimulation compared with the basal respiratory rate. (c) Contribution of the AOX to the respiration of the wild-type and the *hog1* mutant strain. The inhibitors SHAM (5 mM) and antimycin A (AA; 5 µg ml⁻¹) were added sequentially to cells respiring in the presence of the uncoupler FCCP. Data are expressed as the percentage of the uncoupled rate of respiration. All these data were observed for cells growing in SG at 30 °C. (d) Induction of the AOX. Cultures grown in SG at 30 °C were incubated with antimycin A (5 µg ml⁻¹) for 1 h and then treated to measure O₂ consumption in the oxygen electrode. The graph shows both the basal respiration rate and the rates after the addition of 5 µg antimycin A ml⁻¹ or 5 mM SHAM. The respiration is expressed as a percentage of the basal rate of respiration. (e) Respiration rates expressed as nmol O₂ min⁻¹ per 10⁶ cells of the wild-type and the *hog1* mutant pre-incubated for 1 h in the presence of antimycin A. In all cases, data represent the mean ± SEM of three or four determinations.

of respiration while the subsequent addition of $5 \mu\text{g ml}^{-1}$ antimycin A or 5 mM KCN (Fig. 3c and data not shown) completely abolished respiration. This behaviour is comparable to results previously reported (Helmerhorst *et al.*, 2002), indicating that under our growth conditions the contribution of the AOX pathway in oxygen consumption is low. In the *hog1* mutant the pattern was not significantly altered (17.5 % inhibition with SHAM), and therefore the relative contribution of the AOX to the observed high respiratory activity remained unchanged (Fig. 3c).

The AOX pathway can be induced with inhibitors of the electron-transfer pathway. Cells from wild-type and *hog1* mutant were treated with $5 \mu\text{g ml}^{-1}$ antimycin A for 1 h and then O_2 consumption was analysed. Both strains showed a similar profile, and after addition of the drug most of the respiration rate became dependent on the AOX, since O_2 consumption was slightly inhibited by antimycin A or KCN but severely impaired by the addition of SHAM (80 % in the wild-type and 82 % in the *hog1* mutant: Fig. 3d). This result suggested that both strains were able to induce the AOX. Interestingly, the respiration rate of the *hog1* mutant was still twofold higher compared to the wild-type strain (Fig. 3e). Taking these results together might indicate that the increased electron transfer observed in the *hog1* mutant takes place upstream of coenzyme Q.

AOX1 deletion mutants do not mimic the *hog1* mutant response to oxidative stress

The *hog1* mutant displays a high sensitivity to osmotic and oxidative stress (Alonso-Monge *et al.*, 2003; San José *et al.*, 1996). Thus, when exponential-phase cultures were spotted

onto plates supplemented with sorbitol or H_2O_2 there was a marked inhibition of growth (Fig. 4a). In the wild-type strain, the Hog1 and Mkc1 MAPKs became activated upon addition of 10 mM H_2O_2 for 10 min while Cek1 was dephosphorylated (Fig. 4b). The *hog1* mutant could hardly activate Mkc1 and showed higher levels of phosphorylated Cek1 as previously described (Román *et al.*, 2005; Arana *et al.*, 2005). Since the induction of the AOX pathway is a protective mechanism against oxidative stresses (Wagner & Moore, 1997), we attempted to characterize the relation between the two mechanisms by the analysis of mutants lacking components of the AOX pathway: *aox1a*, *aox1b* and *aox1a aox1b*. None of these mutants was sensitive to osmotic or oxidative agents as occurs with *hog1* mutants (Fig. 4a), although a slight sensitivity was observed for the *aox1b* mutant growing on plates supplemented with H_2O_2 . All *aox* mutants displayed the same pattern of MAPK phosphorylation as the wild-type strain after a challenge with H_2O_2 (Fig. 4b). These results indicate that AOX activity could play a minor role in oxidative stress resistance and suggests its independence of the control of the MAPK system.

The *hog1* mutant displays enhanced mitochondrial activity and intracellular ROS production

Mitochondrial activity was quantified by flow cytometry using three different fluorescent dyes, rhodamine 123 (Rh123), dihydroethidium (HET) and carboxy- H_2DCFDA , which have been previously used to quantify mitochondrial transmembrane potential (Rh123) or intracellular ROS (HET and carboxy- H_2DCFDA) in *C. albicans* and other *Candida*

Fig. 4. Response of *C. albicans* *aox* mutant strains to stress. (a) Effect of 1 M sorbitol and 6 mM H_2O_2 on the growth in YDP. (b) Effect of oxidative stress on the MAPK phosphorylation pattern of the strains indicated. Cells grown in YPD were exposed (+) or not (–) to 10 mM H_2O_2 for 10 min and then samples were processed. Phosphorylated MAPKs are marked with an asterisk (*).

species (Veerman *et al.*, 2004; Helmerhorst *et al.*, 2001; Brun *et al.*, 2003). Rh123 selectively accumulates in mitochondria depending on the membrane potential. HET is rapidly oxidized by ROS into its fluorescent derivative, while carboxy-H₂DCFDA indirectly measures O₂^{•−} generated in mitochondria that has dismutated to H₂O₂ through the action of superoxide dismutase (Chance *et al.*, 1979; Turrens *et al.*, 1985). Overnight cultures grown in SG liquid medium were treated as described in Methods and stained with the mentioned fluorescent dyes. Flow cytometry analysis showed that the emission of fluorescence was significantly higher in the *hog1* mutant compared to the parental or reintegrated strain (Fig. 5a) for the three fluorochromes tested. The increase in the fluorescence signal was remarkably high when Rh123 was tested (1386 mean intensity compared to 321 for the wild-type strain).

Additionally, ROS production was quantified in AOX-induced cultures. For this purpose, cells grown overnight in SG at 30 °C were incubated with 5 µg antimycin A ml^{−1} for 1 h and then processed for cytometry assay. No significant differences were observed between cells treated or not with antimycin A; nevertheless, differences among strains were still observed (data not shown). This reinforces

the idea that the increased electron transfer observed in the *hog1* mutant takes place upstream of coenzyme Q.

We also tested the level of superoxide anion generated by wild-type and *hog1* mutant strains in response to the superoxide generator paraquat (PQ) using a chemiluminescence method (Aoki *et al.*, 2002). The *hog1* mutant showed a remarkably enhanced ability to generate superoxide compared to the parental strain (Fig. 5b). This effect was more evident in stationary-phase cells, where the chemiluminescence level in the *hog1* strain was sixfold higher than in the wild-type, whereas in exponential-phase cells the increase was only twofold. Similar results, although less pronounced, were obtained when glycerol was used as carbon source (data not shown). The generation of superoxide by PQ was completely dependent on the conventional respiratory pathway since its production was practically suppressed by the addition of KCN (data not shown).

The mitochondrial membrane potential is altered in the *hog1* mutant

In order to examine mitochondrial activity in more depth, we used another sensor of mitochondrial potential. JC-1 is

Fig. 5. Quantification of intracellular ROS and mitochondrial activity. (a) Cells grown overnight in SG at 30 °C were stained with fluorescent dyes as described in Methods. Fluorescence emission was quantified by flow cytometry and is represented in absolute units versus count numbers. The figure shows a representative experiment. Bold lines, *hog1* mutant; normal lines, *hog1* reintegrand; dotted lines, wild-type. (b) Luminescence signal emitted by wild-type and *hog1* mutant strains after the sequential addition of MCLA and PQ, for stationary-phase and exponentially growing cultures. Data represent the mean ± SEM of four determinations.

a cationic dye that exhibits potential-dependent accumulation in mitochondria; mitochondrial polarization is thus indicated as an increase in the red (J-aggregated)/green (monomer) fluorescence intensity ratio. This fluorescent dye has been used in a variety of cell types, including *C. albicans*, to quantify the mitochondrial membrane potential (Pina-Vaz *et al.*, 2001). Cells grown in SD or SG were stained with JC-1 prior to fluorescence detection by flow cytometry. Cells grown in SD mainly showed green fluorescence, indicating a predominant fermentative metabolism in the presence of glucose, as the mitochondria are depolarized (Table 2). The red/green ratio increased when cells were grown with glycerol as carbon source (Table 2), indicating aggregation of the dye due to the polarization of the mitochondrial membrane. The lack of Hog1 caused an increase in the fluorescence emission in both channels, red and green (Fig. 6a). Nevertheless when the red/green ratio was calculated the *hog1* mutant displayed a lower ratio compared to the wild-type and the reintegrated strain (Table 2). This unexpected result was confirmed by confocal microscopy. The *hog1* mutant cells showed an enhanced fluorescence emission at both wavelengths (529 and 590 nm), green and red, compared with the wild-type strain grown and treated in the same way (Fig. 6b). These results suggest an impaired mitochondrial potential when Hog1 is absent.

DISCUSSION

The HOG pathway has been shown to mediate the response of the fungal pathogen *C. albicans* to different types of stress such as osmotic, oxidative, metal or a decrease in the growth temperature (Alonso-Monge *et al.*, 2003; Smith *et al.*, 2004). Previous investigations suggested that the *hog1* mutant displayed increased levels of antioxidant defences as a consequence of an altered gene expression and/or an enhanced enzymic activity (Alonso-Monge *et al.*, 2003; Arana *et al.*, 2007; Smith *et al.*, 2004). Thus, the lack of Hog1 caused an elevated expression of genes such as *CTA1*, *TRR1* or *TRX1* involved in detoxifying ROS. Although these genes were overexpressed in the absence of stress in the *hog1* mutant, their expression levels

were further increased when cells were challenged with the appropriate stimuli (Arana *et al.*, 2007; Enjalbert *et al.*, 2006).

The present work provides evidence that *C. albicans* mutants lacking the MAPK Hog1 show an altered mitochondrial metabolism whose two main features are an increased rate of respiration and a remarkably high rate of mitochondrial ROS production. The increased respiratory activity is not due to a decreased efficiency of the oxidative phosphorylation since neither the respiratory control nor the relative contribution of the AOX is significantly altered. This increased respiration does not have a role in preventing ROS generation, since intracellular ROS also increase in the *hog1* mutant. In fact, *hog1* mutants show a higher dependence on mitochondrial ATP production as determined from the increased sensitivity to drugs that impair oxidative phosphorylation. These effects are less pronounced when the cells grow on fermentable carbon sources, thus indicating that glycolytically generated ATP can, to some extent, sustain the increased energy demand.

The defect of the *hog1* mutant that leads to an increase in ROS levels is not due to an inability to induce the AOXs. *C. albicans* possesses two genes encoding AOXs: *AOX1a*, whose expression is constitutive, and *AOX1b*, whose expression depends on the growth phase and which is induced by cyanide, antimycin A, H_2O_2 , menadione or PQ (Huh & Kang, 2001). Treatment with antimycin A induced the cyanide-resistant respiratory pathway since the O_2 consumption was slightly inhibited with antimycin A but drastically impaired with SHAM. The enhanced ROS production by the *hog1* mutant persists when cells respire through the AOX pathway; these data indicate that the *hog1* mutant is impaired upstream of coenzyme Q. On the other hand, *AOX1a* and *AOX1b* mutants did not display the characteristic *hog1* mutant phenotypes (sensitivity to oxidative and osmotic stress) and the pattern of MAPK phosphorylation was unaltered. However, these results do not eliminate the possibility that the expression of the *AOX1a* or *AOX1b* genes depends on the HOG pathway, as suggested by Huh and co-workers (Huh *et al.*, 1994; Huh & Kang, 2001). Genome-wide transcriptional studies by Enjalbert *et al.* (2006) showed that *AOX1* and other genes whose products are localized in the mitochondria or are related to their activity were downregulated by Hog1. The absence of this MAPK caused a slight overexpression of genes such as *DLD3* (D-lactate ferricytochrome *c*), *ALD5* (aldehyde dehydrogenase oxidoreductase), *MRPL39* (mitochondrial 60S ribosomal protein) and *CAT2* (carnitine O-acetyltransferase). This study was performed by growing cells on a fermentable carbon source (glucose), and presumably the expression of the mentioned genes must be increased for cells growing in non-fermentable carbon sources. It must be pointed out, however, that other ROS-detoxifying systems are activated in *hog1* mutants (Arana *et al.*, 2007; Enjalbert *et al.*, 2006), such as the thioredoxin reductase *TRR1* gene, and consistent with this, we detected

Table 2. Measurement of polarization/depolarization of the mitochondrial membrane

Means of the green (FL-1) and red (FL-2) fluorescence emission are tabulated. The FL-2/FL-1 ratio represents the ratio of polarized/depolarized mitochondria.

Strain	SG			SD		
	FL-1	FL-2	FL2/FL1	FL-1	FL-2	FL2/FL1
wt	44.19	147.79	3.34	31.05	3.82	0.11
<i>hog1</i>	131.8	355.16	2.69	37.5	3.76	0.1
<i>hog1</i> ^{reint}	45.73	169.11	3.70	36.71	3.77	0.1

Fig. 6. Bivariate JC-1 analysis of mitochondrial membrane potential. (a) Dot plots of *C. albicans* strains grown in SG at 30 °C and stained with JC-1. Green fluorescence (FL1) versus red fluorescence (FL2) is represented. (b) Visualization by confocal microscopy of wt or *hog1* mutant cells stained with JC-1. Cells were grown overnight in SG medium at 30 °C, washed with PBS and stained with 5 μ M JC-1 for 30 min.

enhanced *TRR1* gene expression under our growth conditions (SD or SG at 30 °C) using a *TRR1*-pLUC reporter fusion (Arana *et al.*, 2007) (data not shown).

Although Hog1 plays an important role in the response to oxidative stress, other elements may be crucial to orchestrate the appropriate response; two relevant candidates are the MAPK Mkc1 and the transcription factor Cap1. Cap1 controls the expression of many genes also regulated by Hog1, although the HOG cascade and Cap1 seem to act independently (Enjalbert *et al.*, 2003, 2006; Alonso-Monge *et al.*, 2003). Mkc1 becomes phosphorylated in response to different oxidative and nitrosative agents (Navarro-Garcia *et al.*, 2005), thus indicating that this MAPK plays an important role in the adaptive response to this type of stress. Its phosphorylation in response to H₂O₂ depends partially on the HOG pathway (Arana *et al.*, 2005; Navarro-Garcia *et al.*, 2005). Nevertheless *mkc1* mutants do not show an enhanced sensitivity to H₂O₂, menadione or inhibitors of the mitochondrial respiratory chain, suggesting that the role of Mkc1 is not as direct as the one played by Hog1 in the response to oxidants.

The results presented here also indicate that the *hog1* mutant might demand elevated ATP production. It must be emphasized that this occurs under basal (unstressed) conditions, and therefore reflects an underlying deficiency of *hog1* mutants which is independent of the triggering of the pathway by MAPK phosphorylation. In line with these observations, the analysis of the global transcriptional response has revealed alterations even in the absence of stress. Thus, the transcription factor *TYE7*, whose orthologue in *Saccharomyces cerevisiae* promotes expression of

the glycolytic genes, and the glycolytic genes *PGK1* and *FBA1* are downregulated in the *hog1* mutant and there is also a decrease in the expression of genes involved in glycerol synthesis (*RHR2* and *GAP1*). In contrast, *GLC2*, which induces glycogen synthesis, and *UGP1*, involved in a wide variety of metabolic pathways including biosynthesis of glycogen and trehalose or the formation of cell wall β -glucans and glucomannoproteins, are also upregulated (Enjalbert *et al.*, 2006). Reinforcing these data, the *hog1* mutant displays enhanced levels of intracellular trehalose in the absence of external stress (P. Gonzalez-Parraga & J. C. Arguelles, personal communication). An elevated ATP demand could be necessary to promote the hyphal growth and cell wall remodelling characteristic of *hog1* mutants.

In conclusion, we have shown that the HOG pathway is involved in regulating ROS production and ATP demand by mitochondria. This not only provides an explanation for the observed avirulence of *hog1* mutants, which are more sensitive to immune cells that act by oxidative-killing mechanisms (Arana *et al.*, 2007), but, most importantly, links a signal transduction MAPK pathway with respiratory metabolism in an important fungal pathogen.

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