The Pbs2 MAP kinase kinase is essential for the oxidative-stress response in the fungal pathogen Candida albicans

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The human fungal pathogen Candida albicans responds to stress by phosphorylation of the Hog1 MAP kinase. PBS2 was cloned and shown to encode the MAP kinase kinase that is involved in this activation, as determined by immunoblot analyses using antibodies that recognize the active form of the target Hog1 protein. Characterization of pbs2 mutants revealed that they were sensitive to both osmotic and oxidative stress and that they, interestingly, displayed differential behaviour from that of hog1 mutants, losing viability when exposed to an oxidative challenge more rapidly than the hog1 strain. Hog1 and Pbs2 were also shown to be involved in the mechanism of adaptation to oxidative stress, as evidenced by the enhanced susceptibility to oxidants of pbs2 and hog1 mutants, compared with the wild-type strain, when cells were previously exposed to a low, sub-lethal concentration of hydrogen peroxide and by the PBS2-dependent diminished activation of Hog1 MAP kinase in the adaptive process. Studies with a chimaeric Hog1–green fluorescent protein fusion revealed that this protein was localized throughout the cell (being excluded from the vacuole), but concentrated in the nucleus in response to NaCl stress, a process that was dependent on the Pbs2 protein. Both Hog1 and Pbs2 also play a role in controlling the phosphorylation state of the other MAP kinases Mkc1 and Cek1, involved respectively in cell-wall integrity and invasive growth. Furthermore, it is demonstrated that PBS2 plays a role in cell-wall biogenesis in this fungal pathogen, as its deletion renders cells with an altered susceptibility to certain cell wall-interfering compounds.

INTRODUCTION

Eukaryotic cells respond to environmental changes through signal-transduction pathways. MAPK (mitogen-activated protein kinase) routes consist of a three-kinase module: the MAP kinase kinase kinase, the MAP kinase kinase and the MAP kinase, which are activated by sequential phosphorylation in response to different extracellular signals. These pathways are conserved through evolution (Kültz & Burg, 1998) and their functionality is well-documented in some model organisms, such as Saccharomyces cerevisiae and Schizosaccharomyces pombe (Banuett, 1998; Gustin et al., 1998). In budding yeast, six MAPK pathways have been described that perform essential functions in fungal physiology, such as mating, pseudohyphal/invasive growth, cell-wall integrity, STE vegetative growth (SVG), spore-wall assembly and adaptation to high osmolarity (HOG) (Gustin et al., 1998; Posas et al., 1998). This last route is activated in response to osmotic and – as described recently – oxidative stress (Haghnazari & Heyer, 2004; Singh, 2000) and mutant cells in some elements of the pathway are sensitive to both kinds of stresses. Osmosensitivity is partially explained by the inability to increase intracellular glycerol under restrictive conditions in order to counteract the extracellular turgor (Albertyn et al., 1994). Under hyperosmotic conditions, hog1 and pbs2 mutant strains display different alterations, such as a defective bud repositioning (Brewster & Gustin, 1994), shmoo projections (O’Rourke & Herskowitz, 1998) and induction of pseudohyphal growth. Some phenotypes are, however, evident under non-restrictive conditions (Jiang et al., 1995; K Apteyn et al., 2001; Lai et al., 1997). S. cerevisiae is also able to sense the opposite situation – hypo-osmotic conditions – through the cell-integrity pathway mediated by the Slt2 MAP kinase, which is activated in response to high temperatures, low osmolarity and cell-wall degrading compounds (Davenport et al., 1995; Kamada et al., 1995; Martin et al., 2000). Recently, activation of the Slt2 MAP kinase by oxidizing agents (hydrogen peroxide and diamide) has been reported (Vilella et al., 2005).

In fission yeast, a functional-homologue route to the HOG pathway is mediated by the Sty1 MAP kinase, which responds to several stresses, such as heat and cold shock and osmotic and oxidative stress (Samejima et al., 1997; Shiozaki

Abbreviations: MAPK, mitogen-activated protein kinase; NES, nuclear export signal; NLS, nuclear localization sequence; SVG, STE vegetative growth.
& Russell, 1996; Soto et al., 2002). sty1− mutants are sterile and exhibit a G2 cell-cycle delay, indicating additional roles for Sty1 in meiosis and cell-cycle progression (Millar, 1999; Shiozaki & Russell, 1995, 1996). The range of environmental insults that activate Sty1 is similar to the stimuli that activate the mammalian SAPKs: JNKs and p38/CSBP (Galcheva-Gargova et al., 1994; Kyriakis & Avruch, 1996), which control a wide variety of physiological and pathological conditions.

_Candida albicans_ is a pathogenic yeast of great clinical interest (Fox, 1993; Odds, 1988). Four MAP kinases have so far been identified in this organism: Mck1, the homologue to the Slt2/Mpk1 MAP kinase from _S. cerevisiae_ (Navarro-García et al., 1995), Cek1, homologue to Kss1 (Csank et al., 1998), Cek2, homologue to Fus3 (Chen et al., 2002) and Hog1, homologue to the Hog1 MAP kinase. Hog1 has been implicated in different functions in _C. albicans_, such as glycerol accumulation, morphological transitions, cell-wall biogenesis and virulence (Alonso-Monge et al., 1999; San José et al., 1996). It has also been shown how this MAP kinase is phosphorylated when exposed to NaCl and hydrogen peroxide (Alonso-Monge et al., 2003). Recent studies have suggested that it also exerts a regulatory role on other MAP kinases, Mck1 and Cek1 (F. Navarro-García, B. Eisman, S. Fiuza, C. Nombela & J. Pla, unpublished data). However, the overall organization of the HOG pathway in _C. albicans_ is not clear. Calera and co-workers reported the isolation of Ypd1 (Calera et al., 2000a) and the Ssk1 response regulator (Calera et al., 2000b), a member of a two-component signal-transduction pathway that was recently shown to play a role in the transmission of oxidative stress (Chauhan et al., 2003). The role of other putative components is not clear (Chauhan et al., 2003; Yamada-Oka be et al., 1999), despite their role in cell-wall construction (Kruppa et al., 2003, 2004; Yamada-Oka be et al., 1999).

In this work, we have identified the gene homologue to _PBS2_ in _C. albicans_ and shown this protein to play a role in cell-wall construction. We also present evidence that Pbs2 mediates phosphorylation of Hog1 in response to both osmotic and oxidative stress, a phenomenon that correlates with translocation of the Hog1 MAP kinase. These results indicate that the HOG pathway is involved in the development of an adaptive response in this fungal pathogen.

**METHODS**

**Strains and growth conditions.** Yeast strains are listed in Table 1. For clarity and unless otherwise stated, hog1 always indicates the homozygous hog1/hog1 Ura+ strain (strain CNC13), pbs2 the homozygous pbs2/pbs2 Ura+ strain (strain BDR3) and hog1 pbs2 the double mutant hog1/hog1 pbs2/pbs2 Ura+ (strain BDR7). pbs2+/PBS2 refers to the pbs2/pbs2 strain Ura− (BDR8), where the PBS2-GFP gene was reintegrated, whilst pbs2+ PBS2AN refers to the pbs2/pbs2 Ura− strain, where the PBS2-GFP gene lacking the prlone-rich domain was reintegrated. Unless otherwise stated, _C. albicans_ RM100 was used as a wild-type strain in control experiments.

Yeast strains were grown in YPD medium (1% yeast extract, 2% peptone, 2% glucose) or SD minimal medium (2% glucose, 0-67% yeast nitrogen base without amino acids) with the appropriate auxotrophic requirements. The ability of cells to invade was tested by using Spider medium (1% mannitol, 1% nutrient broth, 0-2% KH2PO4, 1-35% agar) (Liu et al., 1994) or SLAD medium (Kirsch & Whitney, 1991). Uridine was added routinely to liquid and solid media used for phenotypic assays. Growth temperature was 37°C unless indicated. Usually, overnight cultures were refreshed to an OD600 of 0-1 and experiments were performed when cultures reached an OD600 of 1 when exponential-phase cells were required. In the case of stationary-phase cells, cells from a 24 h culture were routinely used.

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

Filamentation assays were performed by using overnight cultures in liquid YPD medium at 30 °C that were refreshed to 10^-6 cells ml^-1 in liquid YPD medium supplemented with 5% fetal bovine serum and incubated at 24, 30 and 37°C. **Deletion of the PBS2 gene.** A _C. albicans_ genomic library (Navarro-García et al., 1995) was used in a screening designed to complement the osmosensitivity of a _S. cerevisiae_ strain ssk2 ssk22 in a ura3 background. Clone 66 was identified to bear the 10-43 kb plasmid in which the PBS2 gene was identified. A 4-46 kb Snail–Clal fragment carrying the _S. cerevisiae_ URA3 marker gene was eliminated, generating the c66-YEP-URA+ plasmid. A 891 bp Acl–Spel fragment from the PBS2 ORF was replaced with a 3-35 kb Acl–Spel fragment from pD1 carrying the CaURA3 marker flanked by the chloramphenicol acetyltransferase (cat) gene from _Escherichia coli_ (B. Eisman, personal communication). A 5-4 kb KpnI–XbaI fragment carrying the deletion construction was used to force homologous recombination and deletion of the PBS2 gene in the RM1000 and CNC15 strains. Strains BDR1–8 were obtained following the procedure described previously for genetic transformation (Köhler et al., 1997). Correct disruptions were confirmed by PCR using primers o-PSBRV (5′-CTCGGTTGACCATCTTGATC-3′) and o-URA3CR (5′-GGTTGACGAATCAGGCTACAGC-3′) for _Ura+ strains_ or o-PSBRV and o-CF (5′-GATGTTGGCTGACCTG-3′) for _Ura− strains_, as well as by Southern blot. In this case, genomic DNA was digested with _PvuII_ and the probe was obtained by PCR using the primers o-NPBXS XO (5′-CTCGAGTCGCTAGATATTATTTAATATGTTG-3′) and o-NPBSCLA (5′-ATATCGATATCCACCTACTTTAATTTAG-3′).

**HOG1-GFP and PBS2-GFP integration.** The _HOG1-GFP_ chimaera was constructed by PCR amplification of the ORF using the primers o-5′ HHOGI (5′-CCAAAGCTTTAGCTCGAGTGGAGAATTTACA-3′) and o-HOGI fusion2 (5′-GGAAGCTTCCACCAAGCTTACGTGAGGCTGAA-3′). After incorporation of this fragment into the pGEM-T vector (Promega), it was excised as a 1138 bp fragment that was subsequently introduced in the large _HindIII_ fragment of the pGFP-URA3 plasmid (Gerami-Nejad et al., 2001). This construction was integrated at the _HOGI_ locus after digestion with _SalI_ to render the construction linear. Correct integration was detected by PCR with primers o-ChI (5′-TCCGCA-AAGCTTAAAAATGTCTGAGTGGAG-3′) and o-GFPlo (5′-CGTAGATAGACATTTTAATATACATTT-3′). _ACTIp-HOGI-GFP_ was constructed by amplifying the _HOGI-GFP_ fusion using the primers o-5′-ACHTHO (5′-CCTCTTAACTATGCTGAGTGGAG-3′) and o-3-TADH (5′-CCTCTTAACTATGCTGAGTGGAG-3′) and cloning it into the _XbaI_ site of the plasmid _PIR4_ (L. Rios, personal communication) derived from pRM1 (Pla et al., 1995), where the _ACT1_ promoter was inserted. The construction was
Table 1. Yeast strains used in this study

<table>
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<tr>
<th>Organism/strain</th>
<th>Genotype</th>
<th>Source</th>
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</thead>
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</tr>
<tr>
<td>MY007</td>
<td>MATa ura3 leu2 his3 ssk2::LEU2 ssk22::LEU2 sho1::HIS3</td>
<td>Posas &amp; Saito (1997)</td>
</tr>
<tr>
<td>FP50</td>
<td>MATa ura3 leu2 his3 ssk2::LEU2 ssk22::LEU2 ste11::HIS3</td>
<td>Posas &amp; Saito (1997)</td>
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<tr>
<td><strong>C. albicans</strong></td>
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<td></td>
</tr>
<tr>
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<td>Ura3Δ::imm434/ura3Δ::imm434</td>
<td>Negredo et al. (1997)</td>
</tr>
<tr>
<td>RM1000</td>
<td>his1Δ::hisG/his1Δ::hisG-Ura3-hisG</td>
<td>Negredo et al. (1997)</td>
</tr>
<tr>
<td>CNC13</td>
<td>RM1000</td>
<td>San José et al. (1996)</td>
</tr>
<tr>
<td>CNC15</td>
<td>hog1::hisG-Ura3-hisG/hog1::hisG</td>
<td>Alonso-Monge et al. (1999)</td>
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<td>This work</td>
</tr>
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<td>This work</td>
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integrated at the LEU2 locus after digestion with KpnI to render the plasmid linear.

The PBS2-GFP fusion was constructed by amplifying the PBS2 ORF by PCR using the primers o-PBS2N-upper (5'-CGGCGCGCTTAACTA-3') and o-PBS2N-lower (5'-CGGCGCGAGATTATTTAAGAGCCTCT-3'); the fragment was inserted into the Nol site of pACT1-GFP (E. Román, personal communication), a plasmid derived from pRM1, where the ACT1 promoter and the GFP gene (Cormack et al., 1997) were first inserted. The construction was then integrated at the LEU2 locus of the C. albicans genome after digestion with KpnI. Similarly, the PBS2-GFPAN fusion was obtained by amplifying an N-terminally truncated version of the PBS2 ORF by using the primers o-PBS2-NSH3 (5'-CGGCGCGAGATTATTTAAGAGCCTCT-3') and o-PBS2N-lower. The PCR product was also inserted at the Nol site of pACT1-GFP and integrated into the LEU2 locus after digestion with KpnI.

Nikkomycin Z assay. MICs were determined by the microdilution method in 96-well plates as described previously (Navarro-García et al., 1998; NCCLS, 1992). Cells (10^5 well) were inoculated into SD minimal medium without uridine.

β-1,3-Glucanase-sensitivity assay. To measure the inhibition of growth caused by Zymolyase, cell cultures from an exponentially growing culture were inoculated to an OD_{600} of 0.025 in YPD medium supplemented with different amounts of Zymolyase 100T (ICN Biomedicals). Zymolyase was suspended in Tris/HCl (pH 7.5), 5% glucose.

Oxidative-stress assays. Hydrogen peroxide and menadione sodium bisulphite (MD) were obtained from Sigma. Dilutions were performed by using sterile, double-distilled H2O. Susceptibility to hydrogen peroxide was measured by using exponential- or stationary-phase growing cells in YPD medium at 37°C. Cells (10^7) were then transferred to an Eppendorf tube and hydrogen peroxide was added.
to a final concentration of 50 mM. Tubes were incubated at 37 °C and 5 μl samples were collected at different times and spotted onto YPD plates, which were then incubated for 24 h at 37 °C and photographed. Susceptibility to MD and other oxidants was quantified in a similar way by using different concentrations (indicated in the figure legends).

Protein extracts and immunoblot analysis. Yeast strains were grown to an OD$_{595}$ of 1 at 37 °C in YPD medium (or SD medium in the case of the HOG1–GFP detection). NaCl or hydrogen peroxide was added to the medium at the final concentration indicated. Samples were taken 10 min after the challenge or at different time points, as indicated in the figures. Cell extracts were obtained as described previously (Martin et al., 2000). Equal amounts of proteins were loaded onto gels, as assessed by $A_{260}$ measurement of the samples and Pierce Red Staining of the membranes prior to blocking and detection. Blots were probed with a phospho-p38 MAP kinase (Thr180/Tyr182) 28B10 mAb (Cell Signaling Technology) (Ab-p38P in the figures), an ScHog1 polyclonal antibody (Santa Cruz Biotechnology) (Ab-Hog1 in the figures) and phospho-p44/42 MAP kinase (Thr202/Tyr204) (Cell Signaling Technology) (Ab-p44/42P) and Ab-GFP (JL-8) (Clontech) mAbs, and developed according to the manufacturer’s conditions by using a Hybond ECL kit (Amersham Biosciences).

RESULTS

Isolation of the C. albicans PBS2 gene

In order to isolate upstream elements of the HOG pathway in C. albicans, screening was performed by using a C. albicans genomic library in the plasmid YEp352 and S. cerevisiae as host (E. Román, C. Nombela & J. Pla, unpublished data). Several osmosensitive clones were identified when assayed in plates supplemented with 0-9 M NaCl to an ssk2 ssk22 sho1 ura3 mutant of S. cerevisiae. Plasmids were isolated from these clones and shown to be responsible for the osmosensitive phenotype by using a standard recombination test, using the same strain as host. The sequence of the flanking regions of clone 66 revealed homology to contig 6–2513, in which the putative MAP kinase homologue of the S. cerevisiae PBS2 was found. This gene was shown to be responsible for the osmosensitive phenotype by using standard restriction-mapping and deletion experiments; we therefore called this gene PBS2 (CaPBS2 will be used on some occasions where it could be confused with the S. cerevisiae homologue). The plasmid isolated in the screening also complemented the osmosensitive phenotype of an ssk2 ssk22 sho1 mutant of S. cerevisiae, indicating that overexpression of C. albicans PBS2 (CaPBS2) bypasses the requirement of phosphorylation by the putative upstream MAPK kinase kinases of the pathway in S. cerevisiae. The mentioned plasmid also rescued the osmosensitivity of an S. cerevisiae pbs2 mutant, but not a hog1 strain (data not shown), indicating that ScPBS2 and CaPBS2 are, indeed, functional homologues.

C. albicans PBS2 has been annotated in the Institute Pasteur Database (Fungal Galail Group) as IPF3329. This gene is 1638 bp long and encodes a 545 aa protein that shares high identity (63 %) and similarity (77 %) within the tyrosine kinase domain with the S. cerevisiae orthologue. Interestingly, differences appear in the ends, as the CaPBS2 C-terminal domain is 37 aa longer than the S. cerevisiae homologue. Although the N-terminal domain of ScPBS2 is longer than that of the C. albicans protein, both kinases conserve the proline-rich domain through which Pbs2 binds the transmembrane protein Sho1 in S. cerevisiae (Maeda et al., 1995; Posas & Saito, 1997). In this organism, the N-terminal domain also contains the docking site for Ssk2 and Ssk22 and the nuclear export signal (NES), whilst the nuclear localization sequence (NLS) is located in the C-terminal domain of the protein (Tatebayashi et al., 2003). Similar domains can be identified in the protein sequence of the C. albicans Pbs2, suggesting a functional conservation between both proteins (the putative NES domain can be located between aa 8 and 10, whilst the potential NLS domain is located between aa 76 and 83).

The Pbs2 proline-rich domain is dispensable to transmit the signal to the Hog1 MAP kinase

In S. cerevisiae, the N-terminal proline-rich domain is responsible for the interaction with the Sho1 adaptor protein and transmission of the signal (Maeda et al., 1995) towards the MAP kinase cascade. In order to check the functionality of this region in CaPBS2, we constructed a mutant allele of PBS2 in which this domain was deleted (aa 1–51). As described above, this fragment of CaPBS2 also includes the putative NES domain; deletion of the NES domain in S. cerevisiae confines the protein permanently to the nucleus (Tatebayashi et al., 2003). When wild-type and mutant alleles were introduced in a pbs2 strain under the control of the ACT1 promoter, they complemented all phenotypes attributed to pbs2 and restored signalling in response to hydrogen peroxide and sodium chloride, as with the wild-type allele (Figs 1, 4 and to be described through the present work). From these observations, we conclude that the proline-rich domain is dispensable to transmit the signal towards the downstream MAP kinases.

Deletion of PBS2 confers an osmosensitive phenotype in C. albicans

To explore the function of the MAPK kinase Pbs2 in C. albicans, the PBS2 gene was deleted in both a wild-type
strain and a hog1 mutant strain to define the epistatic relationship of these genes, generating the BDR3 and BDR7 strains (Table 1).

As expected, the absence of the PBS2 gene generated cells that were defective in growth under hyperosmotic conditions. As shown in Fig. 1, no differences were found in osmosensitivity between the hog1, pbs2 and hog1 pbs2 mutant strains. This phenotype was not restricted to sodium chloride, but was also evident with sorbitol, an osmostressing agent of a non-ionic nature. Such defects were also observed when cells were grown in liquid YPD medium supplemented with 1 M sorbitol. After 24 h growth in this medium, pbs2 mutants reached an OD$_{600}$ of 4.83 (9 in YPD medium), in contrast to the values observed for wild-type strains (6.89 and 9.5, respectively). These values were 1.21 for wild-type and 0.43 for the pbs2 mutant when 2 M sorbitol was used and 1.77 and 0.94, respectively, when 1.5 M NaCl was used. Under these restrictive conditions, cells displayed an altered morphology, remaining attached to their mother cell after budding, a phenomenon that resulted in clumped cells. This phenotype closely resembles the one previously observed for hog1 mutants (Alonso-Monge et al., 1999), indicating a defect in cell separation (Fig. 1b). The pbs2, hog1 and pbs2 hog1 mutants displayed a similar degree of osmosensitivity when assayed by using growth on both solid (Fig. 1a) and liquid (data not shown) media, suggesting that Hog1 is the only target of Pbs2 in response to osmotic stress. These defects were reversed in pbs2 and pbs2 hog1 strains in which functional Pbs2–GFP – or even Pbs2ΔN–GFP – protein was reintroduced (Fig. 1a). pbs2 cells were also unable to accumulate glycerol in response to osmotic stress when exponentially growing cells were challenged with 1 M sodium chloride (data not shown), indicating that this pathway mediates glycerol accumulation and suggesting that glycerol is a major compatible solute in this organism.

In order to define the role of PBS2 within the MAP kinase network, we performed Western blot assays to measure the phosphorylation state of the Hog1 MAP kinase by using antibodies against the TGY motif that is characteristic of stress kinases. No phosphorylated Hog1 could be detected in pbs2 mutants under either basal or activating conditions (1 M NaCl) (Fig. 2d), indicating that deletion of this kinase impairs signalling of osmotic stress towards the Hog1 MAP kinase.
Collectively, these results indicate that Pbs2 is an upstream element of the HOG pathway in C. albicans and an essential mediator of osmotic-stress tolerance in this organism.

**Hog1 is translocated to the nucleus upon saline shock**

In order to investigate the intracellular localization of the MAP kinase Hog1 under different environmental conditions, we fused the ORF of HOG1 to the N terminus of the green fluorescent protein (GFP). When it was expressed under its own promoter, the HOG1-GFP fusion was able to rescue the osmosensitivity of the hog1 mutant cells. Western blot assays demonstrated that the protein fusion was expressed correctly, as it generated a polypeptide of the expected molecular mass (69.7 kDa) that was wholly functional (as it was able to complement hog1 mutant phenotypes similarly to a wild-type Hog1 protein) (data not shown).

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**Fig. 2.** (a) HOG1-GFP fusion reverts the defective phenotypes of a hog1 mutant both in a plasmid (p) or integrated in the genome (i). Serial dilutions of cells were spotted onto YPD plates supplemented with the stressing compounds indicated and incubated for 24 h. (b) A hog1 strain where the ACT1p-HOG1-GFP fusion was integrated in the genome was grown in SD medium and exposed to 1 M NaCl or 4 mM hydrogen peroxide for 5 min. Cells were fixed, stained with DAPI and visualized under a fluorescence microscope. (c) Similar experiments were performed with the hog1 pbs2 HOG1-GFP strain. (d) Immunodetection of Hog1–GFP under the conditions described above (b, c).
Nevertheless, the GFP signal was not intense enough to visualize the protein intracellularly. However, expression of the HOG1-GFP fusion under the ACT1 promoter, a constitutively strongly expressed promoter, revealed that the chimaera was localized throughout the cytoplasm and nucleus (being excluded from the vacuole) in non-stressed cells. When cells were exposed to an osmotic shock (1 M NaCl), the fluorescent signal concentrated in the nucleus (Fig. 2b). Translocation of Hog1 after osmotic challenge was rapid and could be observed in both fixed and viable cells (Figs 2 and 3). As shown in Fig. 3, both the phosphorylation and nuclear localization of Hog1 took place within the first minute after the shock and remained for approximately 15 min. These results suggest that these phenomena are temporally linked. The translocation of the Hog1–GFP chimaera did not take place in a hog1 mutant in response to osmotic stress, and neither activation (Fig. 2d) nor nuclear accumulation (Fig. 2c) was observed. These data indicate that PBS2 is required for both events to occur.

_C. albicans_ Hog1 is also activated in response to oxidative stress (Alonso-Monge et al., 2003). To determine whether the nuclear translocation of Hog1–GFP is dependent on the nature of the stimulus, we checked the intracellular distribution of the Hog1 GFP-tagged protein upon oxidative challenge. Although the HOG1-GFP fusion complemented the sensitivity to oxidative agents that is characteristic of hog1 mutants (Fig. 2a) and phosphorylation was detected by immunoblot (Fig. 2d), no translocation to the nucleus was observed when testing different doses of hydrogen peroxide (0.07–5 mM) and different times (a few seconds to 10 min) of detection.

### _hog1_ and _pbs2_ mutant strains displayed different behaviour under oxidative stress

One of the most important challenges that _C. albicans_ must face during progression of an infection is oxidative stress. Previous work from our group has shown that the Hog1 MAP kinase plays a crucial role in the response and adaptation to oxidative stress (Alonso-Monge et al., 2003). Not only is Hog1 phosphorylated when cells are exposed to hydrogen peroxide, but this MAP kinase is also needed to survive under oxidative-stress conditions. Is this role dependent exclusively on Hog1? To answer this question, we performed different experiments.

First, cells were spotted onto YPD plates supplemented with hydrogen peroxide or MD, the latter being a generator of superoxide ions. As can be observed in Fig. 4(a), hog1, pbs2 and hog1 pbs2 cells were sensitive to these compounds. The mutant strains were hardly able to grow on 300 μM MD or 6 mM hydrogen peroxide plates.

A slight but reproducible increase in sensitivity was observed in the pbs2 mutant, compared with hog1 and hog1 pbs2 mutant strains. To further characterize this different phenotype, we performed experiments in which the kinetics of viability were measured among the different strains. Exponential- and stationary-phase cultures were exposed to a lethal concentration of oxidant agents in liquid medium and samples were taken at different times and spotted onto YPD plates. As shown in Fig. 4(b and c), the pbs2 mutant lost viability faster than the wild-type and, again, faster than the hog1 mutant. Remarkably, the double mutant hog1 pbs2 displayed an intermediate phenotype. Western blot assays showed that Hog1 phosphorylation is also Pbs2-dependent in response to hydrogen peroxide (Figs 4d and 5b–c), as well as in response to osmotic stress (NaCl). These results suggest that, although Pbs2 and Hog1 function within the same pathway, both proteins have additional and separate roles in mediating oxidative stress.

In _C. albicans_, as in other organisms (Hoefen & Berk, 2002; O’Rourke & Herskowitz, 1998), there is a certain degree of crosstalk between pathways. In fact, Hog1 plays a regulatory role in the activation of the Mkc1 and Cek1 MAP kinases (F. Navarro-García, B. Eisman, S. Fiuza, C. Nombela & J. Pla, unpublished data). Mkc1 is involved in the response to oxidative stress, in addition to its role in cell-wall integrity (F. Navarro-García, B. Eisman, S. Fiuza, C. Nombela & J. Pla, unpublished data). The phosphorylation of Mkc1 is detectable in a hog1 strain only in a narrow interval of hydrogen peroxide concentrations. In contrast, Cek1 is activated under standard growth conditions in a hog1 mutant strain. Western blot analyses confirm that Pbs2 also plays this regulatory role. The signal of phosphorylated Mkc1 in response to hydrogen peroxide is reduced significantly in pbs2 and pbs2 hog1 mutants. In addition, Cek1 is activated constitutively in the mentioned mutants, similarly to previous descriptions for a hog1 strain (Figs 4d and 5b–c).

### The HOG pathway mediates adaptation to stress

When cells are exposed to sub-lethal stress, they mount an adaptive response that enables toleration to subsequent, more severe stresses. In order to determine the role of Pbs2 in this process, exponentially growing cells were exposed to a high concentration of hydrogen peroxide (50 mM) after a previous pre-treatment with a sub-lethal concentration of hydrogen peroxide (5 mM). Samples were taken at different times and spotted onto YPD plates at 37°C. Only in the wild-type strain was an increase in tolerance observed when cells were pre-treated (Fig. 5a). Mutants in the MAP kinase and MAPK kinase (hog1, pbs2 and hog1 pbs2) lost viability even faster than samples that were not previously exposed to oxidative stress. As shown, hog1, pbs2 and hog1 pbs2 mutants died within seconds after addition of 50 mM hydrogen peroxide, a result that can be explained because of the failure of these strains to respond to the previous hydrogen peroxide treatment. Similar results were obtained when the assay was performed at 30°C (data not shown). This observation suggests that hog1, pbs2 and hog1 pbs2 mutants are not only intrinsically more sensitive to oxidative agents, but also defective in the...
Fig. 3. Kinetics of Hog1 activation/translocation under saline stress. (a) hog1 HOG1-GFPi (hAHGI) strain grown in SD medium was spotted onto a slide where NaCl was added at approximately 1 M. Hog1–GFP translocation was visualized under a fluorescence microscope and pictures were taken at the indicated times (min). (b) Immunodetection of Hog1–GFP. hAHGI strain was grown in SD medium and was challenged with 1 M NaCl; samples were taken at the times indicated. The membrane was subsequently incubated with the antibodies specified. A hog1 strain grown in SD medium was added as a control.
Fig. 4. HOG-pathway mutants are more sensitive to oxidative stress. (a) Serial dilutions of indicated C. albicans strains were spotted onto YPD plates supplemented with MD or hydrogen peroxide to the specified concentrations and incubated overnight at 37 °C. (b, c) Exponentially growing cells were exposed to (b) 50 mM hydrogen peroxide and (c) 10 mM MD in liquid YPD medium and kept at 37 °C. Samples were taken at different times and spotted onto YPD plates (10^5 cells in 10 μl) and incubated for 16 h at 37 °C. (d) Indicated strains were exposed (+) or not (−) to 5 mM hydrogen peroxide for 5 min and processed for immunoblot assay. The same membrane was subsequently incubated with the different antibodies.
Fig. 5. Adaptation to oxidative challenge. (a) Exponentially growing cells were exposed or not (+/−) to an oxidative stress (5 mM hydrogen peroxide) for 30 min at 37 °C and then challenged with 50 mM hydrogen peroxide. Samples were taken at different times and spotted onto YPD plates. (b) A similar procedure was followed for the immunoblot assay: cells were exposed to 5 mM hydrogen peroxide and samples were taken at different times. A second hydrogen peroxide challenge was added to 50 mM (b) and 5 mM (c). Phosphorylation of the different MAP kinases was detected by using the antibodies described.
development of an adaptive response. One interpretation of this result is that oxidative challenge causes a high concentration of ROS in the mutant cells, which are unable to restore the basal/physiological levels of these reactive species because of the absence of Pbs2 and/or Hog1.

We also monitored the phosphorylation of MAP kinases by Western blot during this adaptation process. Cells were challenged with a low dose of hydrogen peroxide (5 mM) and afterwards were treated with a 50 mM hydrogen peroxide shock. As observed in Fig. 5(b), phosphorylation of Mkc1 is detected in response to the first oxidative shock, whereas a subsequent exposure fails to activate this MAP kinase. The Hog1 protein became phosphorylated after the first and second shocks, although with different kinetics, as this was delayed and less prolonged (only detectable within 5 min) after the second addition of hydrogen peroxide. Untreated cells activated Mkc1 and Hog1 at 50 mM hydrogen peroxide (data not shown).

The adaptive signal is relatively dose-independent: when using a lower concentration (5 mM hydrogen peroxide) as the second challenge, we observed similar results (Fig. 5c). Mkc1 became activated after the first oxidative shock, but not the second, whereas Hog1 was phosphorylated after both oxidative challenges. No activation of Hog1 was detected in pbs2 cells and, obviously, not in hog1 or hog1 pbs2 mutants. Phosphorylation of Mkc1 was significantly lower in these mutant strains than in the wild-type strain, as described above. These results imply that the cells adapted to oxidative stress in a relatively dose-independent manner.

**Pbs2 and Hog1 play different roles in cell-wall biogenesis**

The HOG pathway has been suggested to be involved in construction of the cell wall in both *S. cerevisiae* and *C. albicans* (Alonso-Monge *et al.*, 1999; Jiang *et al.*, 1995). In *S. cerevisiae*, pbs2 and hog1 mutants have been reported to be resistant to calcofluor white (Garcia-Rodriguez *et al.*, 2000) and sensitive to cell wall-degrading enzymes (Zymolyase and Quantazyme) (Alonso-Monge *et al.*, 2001; Kaptyn *et al.*, 2001). In *C. albicans*, disruption of the Hog1 MAP kinase results in cells that are resistant to certain cell-wall biogenesis inhibitors (Alonso-Monge *et al.*, 1999).

To determine whether this phenotype is exclusive to the Hog1 MAP kinase, we checked the growth of *pbs2* cells in the presence of Congo red and calcofluor white, two compounds that interfere with cell-wall assembly. We checked different concentrations of Congo red (150 and 200 μg ml⁻¹) and calcofluor white (12, 24 and 50 μg ml⁻¹). Perhaps unexpectedly, only those mutants where the *HOG1* gene was absent showed resistance to Congo red (Fig. 6a), whilst the *pbs2* mutant displayed an intermediate resistance, observed only at the lowest Congo red concentration tested (150 μg ml⁻¹). Reintroduction of the *PBS2* gene into the *pbs2* mutant strain restored the wild-type phenotype. In the case of calcofluor white, the mutant strains behaved similarly, although differences were more subtle but still reproducible (Fig. 5b). The arrangement of strains according to decreasing sensitivity to calcofluor white was: wild-type, *pbs2*, *hog1 pbs2*, *hog1*. Transformation of *pbs2* mutant with the *PBS2-GFP* construct restored the wild-type phenotype (Fig. 5). These data indicate a significant difference between *hog1* and *pbs2* mutants in *C. albicans*, in contrast to the situation in *S. cerevisiae*. It is noteworthy that no phosphorylation of Hog1 was detected in response to Congo red after 1 h incubation in YPD medium supplemented with 15 μg Congo red ml⁻¹ (data not shown).

Mutants in the HOG pathway were resistant to Nikkomycin Z, an inhibitor of chitin biosynthesis. Wild-type strain, as well as the *pbs2* strain where *PBS2-GFP* or *PBS2AN-GFP* was reintegrated, displayed sensitivity to this antifungal agent with a MIC of 6·25 μg ml⁻¹ at 37 °C. In contrast, *hog1*, *pbs2* and *hog1 pbs2* strains displayed MICs of greater than 400 μg ml⁻¹.

We also checked the susceptibility of the different strains to the β-1,3-glucanase-enriched preparation Zymolyase (which also contains additional β-1,6-glucanase and protease activities). Cells were grown in YPD medium supplemented with different amounts of Zymolyase and incubated overnight. In this case, strains defective in *PBS2* and *HOG1* genes were significantly more sensitive to Zymolyase than wild-type and control strains in which the corresponding genes were reintroduced. *hog1*, *pbs2* and *hog1 pbs2* mutant strains lyed at 50 U Zymolyase ml⁻¹, whereas control strains were able to grow at concentrations higher than 200 U ml⁻¹.

**Pbs2 represses hyphal formation in *C. albicans***

Morphological studies revealed that *pbs2* mutants display an enhanced ability to develop hyphae under different conditions. Serum was added at limiting concentrations (1, 5, 10 and 20 %) to YPD liquid medium and cultures were incubated at different temperatures (24, 30 and 37 °C). Deletion of *PBS2* increased hyphal growth under all conditions tested; *pbs2* and *hog1 pbs2* cells exhibited an enhanced ability to form filaments, even greater than that of the *hog1* mutant. This phenotype is depicted in Fig. 7, in which the microscopic appearance of the cultures in YPD medium supplemented with 5 % fetal bovine serum is shown. HOG-pathway mutants filament at any temperature (24, 30 or 37 °C). The frequency of branched hyphae was similar between all strains and calcofluor staining of these filaments showed no differences compared with wild-type (data not shown), indicating that correct localization of chitin deposition in these structures also takes place in HOG-pathway mutants. They are, at least by using these criteria, similar to wild-type filaments.

A different colony morphology was observed on several media (Fig. 8). Mutants in the Hog1 MAP kinase display smooth or less invasive colony borders on Spider plates (Alonso-Monge *et al.*, 1999), a medium that induces
invasive/filamentous growth in the wild-type strain (Liu et al., 1994). On SD medium, the wrinkly pattern of the colony surface is different in wild-type, hog1 and pbs2 strains and the double mutant hog1 pbs2 displayed the same morphology as the pbs2 mutant strain. On nitrogen minimal medium (SLADH), a medium that induces invasive growth in S. cerevisiae (Gimeno et al., 1992), all HOG-pathway mutants showed a distinct colony morphology, being more invasive than the wild-type strain. The morphology of the colonies revert to wild-type when either the PBS2 gene or the N-terminally truncated PBS2ΔN version was reintroduced into the pbs2 mutant strain.

**DISCUSSION**

In this work, we have identified the PBS2 gene of C. albicans as the MAPK kinase that mediates signalling to the Hog1 MAP kinase and have characterized its function in this fungal pathogen. We present evidence that Pbs2 plays an important role in the response to oxidative and osmotic stress. Deletion of the PBS2 gene in C. albicans leads to the expected osmosensitive phenotype that has already been described for the hog1 mutant (San José et al., 1996), as also occurs with homologous routes in other organisms (Brewster et al., 1993; Millar et al., 1995; Shiozaki & Russell, 1995). The hierarchical relationship between Pbs2 and Hog1 regarding osmotic stress seems to be linear, as pbs2 and hog1 mutants displayed a similar osmosensitivity, independent of the osmolyte and the state of the medium (liquid or solid). Reinforcing this idea, the double mutant hog1 pbs2 did not aggravate the osmosensitive phenotype of single mutants. Moreover, Pbs2 phosphorylates Hog1, as suggested by the fact that Hog1 became unphosphorylated in a pbs2 mutant independently of the stimuli tested, either osmotic or oxidative. The putative proline-rich domain is not required for transmission of the signal to the MAP kinase or to complement the phenotype of a pbs2 mutant. A homologue domain in the Pbs2 MAPK kinase from S. cerevisiae has been reported to mediate the interaction with the SH3 domain in the cytoplasmic region of the transmembrane protein Sho1 (Maeda et al., 1995). The role of the proline-rich domain could be masked by the role of the Ssk1 branch in mediating adaptation to osmotic and oxidative stress (Chauhan et al., 2003).

We previously demonstrated the involvement of Hog1 in the morphological transition, a physiologically relevant aspect in C. albicans (Alonso-Monge et al., 1999). pbs2 cells, as well as hog1 mutant cells, display a hyperfilamentous behaviour in limiting concentrations of serum. This result, together with the altered colony morphology exhibited by the mutants, reinforces the repressor role of the HOG pathway in the morphological transition (Alonso-Monge et al., 1999).

In C. albicans, the Hog1 MAPK has been implicated in the response to oxidative stress (Alonso-Monge et al., 2003).
**Fig. 7.** PBS2 and HOG1 disruption enhance hyphal growth in *C. albicans*. Cultures on YPD liquid medium grown at 30 °C were refreshed in YPD plus 5% fetal bovine serum (FBS) and incubated at 24, 30 and 37 °C. Samples were taken after 1 h incubation.

**Fig. 8.** Colony morphology on different media. Approximately 20 c.f.u. was spread onto either SD, SLADH or Spider petri-dish plates and incubated at 37 °C for 7 days before photographs were taken.
and recent experiments demonstrate that hydrogen peroxide signalling to Hog1 is dependent on the Ssk1 branch (Chauhan et al., 2003). The fact that Hog1 became phosphorylated when exposed to hydrogen peroxide in a Pbs2-dependent manner demonstrates the implication of this route in the response to oxidative stress. However, the differential sensitivity to oxidative agents displayed by the pbs2 and hog1 mutants suggests that Pbs2 may play an additional role that is independent of Hog1. This role could be mediated by Mkc1, as the level of phospho-Mkc1 in response to hydrogen peroxide is reduced significantly in pbs2, hog1 and pbs2 hog1 mutant strains. Another possible mechanism could be through Hog1. Hog1 could repress gene transcription in its non-phosphorylated state and, consequently, lack of the protein could cause a phenotype different from the inactive (not phosphorylated) Hog1, similar to that observed for other MAP kinases (Madhani et al., 1997). Genes not repressed by Hog1 could then be expressed and, consequently, protect cells from a certain degree of oxidative stress. The oxidative-stress responses appear to be regulated, at least in part, at the transcription level, with Yap1 and Skn7 being two important oxidative-stress regulators in S. cerevisiae (Costa et al., 2002). The Yap1 homologue in C. albicans, Cap1, does not appear to be Hog1-dependent (Alonso-Monge et al., 2003), whilst the epistatic relationship between Hog1 and Skn7 has not been analysed in this pathogen. CaHog1 may control transcription through other transcription factors, such as Msn2 and Msn4 or Sko1.

An alternative possibility is that either Pbs2 or Hog1 could phosphorylate another kinase involved in response to oxidative damage. In Schizosaccharomyces pombe, Sty1, the homologue of Hog1, interacts with and phosphorylates the protein kinase Cmk2 under oxidative insult (Sánchez-Piris et al., 2002). Although the role of Cmk2 in oxidative stress is not clear, it has been suggested that Sty1 may regulate translation through Cmk2 to display oxidative stress-induced responses in a similar way to that in S. cerevisiae. SchHog1 activates the Rck2 kinase (Bilsland-Marchesan et al., 2000; Teige et al., 2001), which, in turn, inhibits protein biosynthesis. Although the role of Rck2 was initially defined in the context of osmostress, current studies show that the major role of Rck2 may be to deal with oxidative stress (Bilsland et al., 2004). In S. cerevisiae, the signalling cascade is Pbs2–Hog1–Rck2 (Bilsland-Marchesan et al., 2000; Jiang et al., 2004), although Rck2 phosphorylation is not absolutely HOG1-dependent. In C. albicans, there are two homologue proteins to Rck2 and two to Cmk2 that could be controlled by Hog1 or Pbs2, generating a specific response to the stimulus. Both NaCl and hydrogen peroxide cause growth arrest in C. albicans (R. Alonso-Monge, unpublished data), possibly through some of these Ser/Thr protein kinases. Although both signals converge at the Pbs2 kinase and therefore to Hog1, the mechanisms implicated in the responses generated are not the same and additional elements must be involved to discriminate between stimuli. Moreover, the localization pattern observed for Hog1 depends on the insult. Clearly, CaHog1 is translocated to the nucleus when cells are exposed to saline stress, whereas no nuclear accumulation was observed under oxidative shock. This result disagrees with that observed by Smith et al. (2004). This group showed CaHog1 translocation upon different stress conditions, including hydrogen peroxide, CdSO4 and KCl. Different explanations for the discrepancy between our results and those reported previously could be the genetic approach used or simply the sensitivity of the system. In any case, the translocation of Hog1 upon oxidative stress has been reported recently in S. cerevisiae as being less pronounced and slower than that upon osmotic stress (Bilsland et al., 2004; Smith et al., 2004).

Our work suggests that the HOG pathway is also important for developing tolerance to oxidative stress. Hydrogen peroxide induces a specific protein oxidation in yeast cells; oxidative proteins accumulate in the cells, even at low adaptive levels. Treatment of C. albicans with low concentrations of either hydrogen peroxide or MD induces an adaptive response that protects cells from the lethal effects of a subsequent challenge with higher concentrations of these oxidants (Jamieson et al., 1996). This protection was not evident in cells lacking the HOG1 and/or PBS2 genes. Furthermore, although two MAPKs, Hog1 and Mkc1, are implicated in the oxidative-stress response in C. albicans, the activation pattern after a second oxidative challenge is different. Mkc1 is phosphorylated after the first oxidative insult alone, whilst Hog1 activation is observed after the first and second hydrogen peroxide challenges. This observation may suggest that the response generated by Mkc1 confers tolerance to the stress, although disruption of MKC1 does not lead to hypersensitivity to oxidative agents (F. Navarro-García, B. Eisman, S. Fiuza, C. Nombela & J. Pla, unpublished data).

Another interesting observation is the differential phenotype related to the cell wall that was detected in hog1 and pbs2 mutants. The link between the HOG pathway and cell-wall architecture has been reported in both S. cerevisiae (Alonso-Monge et al., 2001; García-Rodríguez et al., 2000; Jiang et al., 1995; Kapteyn et al., 2001) and C. albicans (Alonso-Monge et al., 1999). We describe growth differences between hog1 and pbs2 in the presence of Congo red and calcofluor white. No other significant differences between hog1 and pbs2 mutants were observed when susceptibility to Nikkomycin Z or Zymolyase was assessed. Nevertheless, it is remarkable to note the resistance to the chitinase inhibitor Nikkomycin Z that was displayed by mutants in the HOG pathway compared with the wild-type strain and, in contrast, the higher sensitivity to the β-1,3-glucanase Zymolyase. The lack of HOG1 and/or PBS2 genes may cause alterations in the cell-wall composition or architecture and these changes in the distribution and proportion of the polymers that form the cell wall may lead to this phenotype. For example, the S. cerevisiae phr1 mutant exhibits a higher proportion of chitin and...
sensitivity to Nikkomycin Z and calcofluor white (Popolo & Vai, 1998), whereas mutants in chitin synthases chs2 and chs8 have less chitin synthase activity and are therefore hypersensitive to calcofluor white (Munro et al., 2003). In S. cerevisiae, mutants defective in the HOG pathway are resistant to calcofluor white, although no differences were detected in chitin synthesis during vegetative growth or during calcofluor treatment (Garcia-Rodriguez et al., 2000). Additionally, overexpression of the PBS2 gene leads to higher β-1,3-glucan synthase activity and resistance to β-1,3-glucanases (Lai et al., 1997). In C. albicans, therefore, the HOG pathway may contribute to maintenance of the cell-wall architecture under non-inducing conditions, probably through interaction with other MAP kinase routes.

In summary, our results reveal that Pbs2 and Hog1 play different roles in cell-wall construction and in the oxidative-stress response. Further work will be aimed at characterizing their roles during pathogenesis.

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Characterization of the Pbs2 MAPK kinase in C. albicans