Studies on the regulation of the two-component histidine kinase gene \textit{CHK1} in \textit{Candida albicans} using the heterologous \textit{lacZ} reporter gene

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The two-component histidine kinase Chk1p of \textit{Candida albicans} has been implicated in the regulation of cell wall biosynthesis. Deletion of \textit{CHK1} results in avirulence that in part may be due to the increased sensitivity of mutant strains to polymorphonuclear leukocytes. The mutant also does not adhere to human oesophageal tissue \textit{in vitro}, probably as a consequence of its altered cell wall. In the current study, a \textit{CHK1} promoter-lacZ reporter (\textit{CHK1prlacZ}) construct was expressed in wild-type \textit{C. albicans} strain CAI4 and in two-component signal transduction mutants to determine the effect of environmental stress conditions on the regulation of \textit{CHK1} and the co-regulatory activities among these proteins. It is shown that \textit{lacZ} expression varied according to the type of growth conditions and incubation time; expression was also influenced by the strain background. \textit{lacZ} expression in CAI4 was greater at 37 °C and at a pH of 3.5 and in the presence of 4 mM \textit{H}_2\textit{O}_2, 0.1 mM menadione, 10 % serum or 1.5 M NaCl compared to cells grown at 30 or 42 °C. The increases in expression were time-dependent and not observed until cells were incubated for 120 min in these conditions (\textit{P}<0.05). As a correlate of the increase in transcription of \textit{CHK1}-\textit{lacZ} in the presence of \textit{H}_2\textit{O}_2, the \textit{chk1} mutant was more sensitive than wild-type and revertant cells to \textit{H}_2\textit{O}_2 \textit{in vitro}. In addition to strain CAI4, we also measured \textit{CHK1prlacZ} reporter activity of mutants deleted in genes encoding other two-component proteins such as the response regulator gene \textit{SSK1}, the histidine kinases, \textit{SLN1} and \textit{NIK1}, and the \textit{HOG1} MAP kinase. Of these proteins, Ssk1p and Sln1p are presumed to mediate phosphotransfer to the HOG1 [hyperosmotic glyceral] MAP kinase pathway during oxidative and perhaps osmotic stress in \textit{C. albicans}. Compared to strain CAI4, \textit{lacZ} reporter activity increased significantly in the \textit{ssk1} mutant under all growth conditions after a 10 and 120 min incubation (\textit{P}<0.0001). \textit{lacZ} expression in the \textit{ssk1} mutant was less at 42 °C compared to all other growth conditions (\textit{P}<0.05). Furthermore, \textit{lacZ} reporter activity also increased in the \textit{hog1} mutant of \textit{C. albicans}. These data suggest that \textit{SSK1} and \textit{HOG1} indirectly or directly negatively regulate \textit{CHK1} under most growth conditions tested. In the \textit{sin1} mutant, downregulation of \textit{CHK1} was observed in all growth conditions compared to strain CAI4 (\textit{P}<0.05), while regulation of \textit{lacZ} in the \textit{nik1} mutant was similar to strain CAI4 except when cells were incubated in the presence of 4 mM \textit{H}_2\textit{O}_2 for 120 min (\textit{P}<0.05). Western blot analysis was used to determine the role of Chk1p in phosphorylation of Hog1p under oxidative or osmotic stress. It was found that Hog1p was phosphorylated in the \textit{chk1} mutant similar to wild-type CAF2-1 cells, although the temporal events of phosphorylation differed slightly in mutant cells. These results show that transcription of \textit{CHK1}, as measured by the \textit{lacZ} reporter assay, is statistically increased when cells are exposed to several types of stress or when incubated in 10 % serum in a mutant-specific background and at a specific time point. Of importance, our data also suggest that \textit{lacZ} expression is indirectly or directly regulated by the \textit{HOG1} MAP kinase pathway, although a determination of its position in this pathway or in a cross-talking pathway awaits additional studies.
INTRODUCTION

Invasive candidiasis appears most often in the immunocompromised patient. The disease carries with it a high attributable mortality, due to at least two factors (Bodey et al., 1966; Wenzel, 1995). First, the infection often goes undetected and second, either therapy is started too late or drug failure occurs. While Candida albicans predominantly causes disease in debilitated patients, nevertheless, the organism expresses several virulence factors needed for its invasiveness and ability to colonize tissues. The expression of some of these virulence factors has been observed both in vitro and in vivo (Calderone & Fonzi, 2001; Navarro-Garcia et al., 2001). Signal transduction pathways that regulate virulence factor expression, such as morphogenesis, stress adaptation and cell wall biosynthesis, have been elucidated, but the upstream signalling events, such as the perception of environmental signals by receptors, are rather poorly characterized (Lengler et al., 2000).

Two-component signal transduction has been studied in both non-pathogenic and pathogenic fungi (Santos & Shiozaki, 2001). Of interest, the absence of these proteins in mammalian cells offers some degree of specificity in the development of antifungal drugs (Barrett & Hoch, 1998; Koretke et al., 2000). Two-component signal transduction proteins of C. albicans include three histidine kinases (Snl1p, Nik1p and Chk1p), two response regulator proteins (Ssk1p and Skn7p) and a phosphohistidine intermediate protein (Ypd1p) (Calera & Calderone, 1999b; Santos & Shiozaki, 2001). Snl1p, Ypd1p and Ssk1p are probably orthologues of the HOG1 MAP kinase pathway proteins [heterosmotic glycerol] that in Saccharomyces cerevisiae are used for the adaptation of cells to osmotic stress (Hohmann, 2002). In C. albicans these proteins have additional functions which include adaptation to oxidant stress, morphogenesis, virulence, adherence and cell wall biosynthesis (Alex et al., 1998; Alonso-Monge et al., 1999, 2003; Bernhardt et al., 2001; Calera et al., 1998, 1999, 2000a, b; Calera & Calderone, 1999a; Chauhan et al., 2003; Kruppa et al., 2003, 2004b; Li et al., 2002; Nagahashi et al., 1998; Selitrennikoff et al., 2001; Singh et al., 2004; Srikantha et al., 1998; Torosantucci et al., 2002; Yamada-Okabe et al., 1999). All of the two-component genes except SKN7 have been implicated in the virulence of the organism in a haematogenously disseminated murine model. Deletions of nik1 or snl1 attenuate virulence, while deletion of chk1 abolishes virulence (Calera et al., 1999; Yamada-Okabe et al., 1999). Ssk1p, while not essential for adaptation to osmotic stress in C. albicans as it is in Saccharomyces cerevisiae, regulates adaptation to oxidant stress and the expression of cell wall proteins such as Als1p, Flo1p and Mnn4p (Chauhan et al., 2003). Downregulation of Als1p (Kapteyn et al., 2000) in the ssk1 mutant is offered as a partial explanation for the decreased adherence of the mutant to human oesophageal tissue in vitro (Li et al., 2002). Of the histidine kinases of C. albicans, Chk1p and Nik1p are not found in Sac. cerevisiae (Santos & Shiozaki, 2001). Nik1p of C. albicans, a homologue of Neurospora crassa nik1, is partially required for phenotypic switching and morphogenesis (Srikantha et al., 1998; Santos & Shiozaki, 2001). In addition to C. albicans, a nik1 orthologue has been reported in Aspergillus fumigatus (Pott et al., 2000).

Chk1p is homologous to the Mak2p and Mak3p of Schizosaccharomyces pombe. In that organism, these proteins are used for adaptation to oxidant stress (Buck et al., 2001). C. albicans strains deleted of CHK1 have an altered cell wall phenotype characterized by a truncation of acid-stable cell wall mannan side chains, as well as a reduction in the amount of 1,3-β-glucan and an increase in the amount of 1,6-β-glucan (Kruppa et al., 2003). The primary lesion of the chk1 null mutant has not been defined and is currently being investigated. That the mutant displays several changes in cell wall composition is often typical of fungal cell wall mutants. It would seem, therefore, that Chk1p is part of a signal pathway that regulates cell wall biosynthesis. The changes in cell wall composition may explain the reduced ability of the chk1 null mutant to adhere to human oesophageal tissue in vitro (Li et al., 2002).

While a presumed pathway similar to the HOG MAP kinase of Sac. cerevisiae is postulated in C. albicans that includes Snl1p, Ypd1p and Ssk1p, the relationship of Chk1p to this pathway as well as with Nik1p is unknown. To determine the relationship of Chk1p to the HOG1 MAP kinase and to the other histidine kinases, a CHK1 promoter-lacZ reporter construct was used to transform wild-type and the null mutants in ssk1, sln1 and nik1 and measure expression of lacZ under stress conditions and in serum. Furthermore, the relationship of Chk1p to Hog1p was investigated by determining the phosphorylation of Hog1p using Western blotting in wild-type and the chk1 mutant as well as lacZ expression in the hog1 mutant of C. albicans.

METHODS

Strains and growth conditions. Candida albicans CAI4 and all other strains listed in Table 1 have been described previously and were used in transformations with the pChk1lacZ or pACT1prlacZ (pAU36) plasmids. Ura3− clones from each mutant that were used for transformation were obtained by selection on 5′-fluoroorotic acid as described by Fonzi & Irwin (1993). Strain CAI4 and all mutant strains were grown at 30°C in SD medium supplemented with uridine (25 µg ml−1). Ura3− transformants were maintained and grown on SD medium without uridine. SD medium contains 2% glucose, 0-67% yeast nitrogen base without amino acids and 0-12% of additional supplements. CAP2-1 was used in phosphorylation and oxidant sensitivity studies. It is Ura3− (Table 1).

Plasmid construction. Parent plasmid pAU36 has been described by Uhl & Johnson (2001). It contains the lacZ gene from Streptococcus thermophilus, which was codon-optimized for expression in C. albicans. The lacZ gene was placed under control of the ACT1 promoter of C. albicans and used to transform C. albicans CAI4 and strains SSK22 (ssk1Δ), NIK22 (nik1Δ), SLN22 (sln1Δ) and CNC15 (hog1Δ) (Table 1), all of which are Ura3−. The plasmid pChk1lacZ was constructed by amplifying a 1.4 kb fragment upstream of the start site of CHK1 from CAI4 genomic DNA that was then used to replace a KpnI–SmaI fragment in pAU36 (Fig. 1a, top). The primers used to amplify the 1.4 kb CHK1 promoter were

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Table 1. Strains of *C. albicans* used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
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<tbody>
<tr>
<td>CAI4</td>
<td>Δura3::imm434/Δura3::imm434</td>
<td>Fonzi &amp; Irwin (1993)</td>
</tr>
<tr>
<td>CAF2-1</td>
<td>Δura3::imm434/URA3</td>
<td>Fonzi &amp; Irwin (1993)</td>
</tr>
<tr>
<td>SSK22</td>
<td>Δura3::imm434/Δura3::imm434 Δssk1::hisG/Δssk1::hisG</td>
<td>Calera <em>et al.</em> (2000a)</td>
</tr>
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<td>SLN22</td>
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<td>This study</td>
</tr>
<tr>
<td>NIK22</td>
<td>Δura3::imm434/Δura3::imm434 Δnik1::hisG/Δnik1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>CNC15</td>
<td>Δura3::imm434/Δura3::imm434 Δhog1::hisG/Δhog1::hisG</td>
<td>Alonso-Monge (2003)</td>
</tr>
<tr>
<td>CHK21</td>
<td>Δura3::imm434/Δura3::imm434 Δchk1::hisG/Δchk1::hisG</td>
<td>Calera &amp; Calderone (1999a)</td>
</tr>
<tr>
<td>CHK23</td>
<td>Δura3::imm434/Δura3::imm434 Δchk1::hisG/CHK1::URA3::hisG</td>
<td>Calera &amp; Calderone (1999a)</td>
</tr>
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Fig. 1. (a) Top. Construction of the pChk1lacZ plasmid. The stippled (non-integrated) and dark (integrated) rectangles indicate the *CHK1* promoter. The *CHK1*, *URA3* and *lacZ* ORFs are indicated as clear rectangles. Middle. Restriction map of the pChk1-lacZ cassette and primers (P1–P4) used for PCR. The 550 bp probe used for Southern hybridizations is shown as a dotted arrow above the cassette. Bottom. The wild-type *CHK1* allele is indicated with diagonal lines, indicating portions of the ORF. (b) Southern hybridizations of *CHK1-lacZ* transformants in CAI4 and the mutants described in Table 1. Genomic DNA from these strains was digested with *Bcl* I and hybridized with a 550 bp PCR fragment (indicated by a dotted arrow in Fig. 1a, middle). The lower band of approximately 1.84 kb is the wild-type allele of *CHK1*. Hybridizing fragments of approximately 4-9 and 5-6 kb are seen in each transformant.
a 617 bp PCR fragment (data not shown).

**Transformation.** Transformation methods followed those of Calera & Calderone (1999a). AvrI-linearized pChk1pr::lacZ (5 μg) was used to transform CA4 and each of the mutant strains (skl, nkl, shl, hog1) using lithium acetate. Also, 5 μg of the Bgl fragment obtained from linearized pUA36 that includes the ACT1 promoter sequence in-frame with lacZ was introduced into CA4 and the mutant strains to compare lacZ expression under the ACT1 and CHK1 promoters. Competent cells were obtained by growing each strain to early exponential phase in 50 ml SD medium containing uracil (25 μg ml⁻¹). Cells were collected, washed twice with water and then resuspended in 0.1 M lithium acetate with shaking at 30 °C for 1 h. The volume of the suspension was reduced to 0.5 ml. AvrI-linearized pChk1pr::lacZ (5 μg) or Bgl-linearized pAct1pr::lacZ (5 μg) and 100 μg denatured salmon sperm DNA were added to 100 ml competent CA4 or mutant strains and incubated at 30 °C for 30 min. A total of 0.6 ml 40% PEG was added and the cell suspensions were incubated at 30 °C for 60 min and then heat-shocked at 42 °C for 5 min. Transformants were centrifuged and plated on SD medium lacking uridine and cultured at 30 °C for 2 days.

**Southern hybridization and PCR.** Verification of the correct integration of the CHK1pr::lacZ and Act1pr::lacZ cassettes in all transformants of CA4 and mutants was accomplished by Southern hybridization using standard methods (Calera & Calderone, 1999a). For the CHK1-lacZ transformants, genomic DNA from all strains was digested with BglI and probed with the 550 bp fragment that is located at approximately 175 bp upstream of the KpnI restriction site (Fig. 1a). For each transformant, two hybridizing bands of 4.9 and 5.6 kb were observed in Southern hybridizations (Fig. 1b). The ACT1-lacZ transformants were restriction-digested with HindIII and probed with an 800 bp sequence derived from the ACT1 promoter (~1210 to ~410 bp, not shown). In addition to Southern analyses, two PCRs using primers P1–P4, indicated in Fig. 1(a), bottom, were also used to confirm the correct integration of each cassette mentioned above. First, a 1.79 kb fragment was amplified with primer CHK5 (5'-GACACTCTCTAATAACTCAC-3') and Act5 (3'-CTCGGCCGATCTTACTTAC-3') at ~200 bp downstream of the lacZ start codon (Fig. 1a, bottom, primer indicated as primer pair P1 and P2). The second PCR was performed using primers P3 (from pBSII KS+) and P4 (CHKP3', 5'-CTCGGCCGATCTTACTTAC-3') at about 350 bp downstream of the KpnI site (Fig. 1a, bottom, primer pair P3 and P4). This PCR fragment was 387 bp in size. Using the same strategy, ACT1-promoter-lacZ transformants were confirmed by two other PCRs. Primer ACTPr3' (5'-GAGAGATTTGAATGATCAG-3'), located at ~198 bp upstream of 5' end of the ACT1 promoter-integrating site, and the Stlacz3' primer amplified a product of approximately 1.58 kb (data not shown). A second PCR utilizing the primers ACTPr3' (5'-TACAGACAACTCCTAAACAC-3'), ~590 bp downstream of the integrating site, and T3 resulted in a 617 bp PCR fragment (data not shown).

**β-Galactosidase assays.** Three transformants of CA4 and each mutant strain were chosen for determinations of β-galactosidase activity. Quantitative determinations of β-galactosidase activity were performed by measuring the hydrolysis of the substrate ONPG from broth cultures as described by Uhl & Johnson (2001) using mid-exponential-phase cells obtained in the following manner. Fresh YNB medium (5 ml) with or without uridine was inoculated with 100 μl of an overnight culture of CA4 or each transformant. Cultures were incubated at 30 °C with vigorous shaking for 3 h to achieve an OD₆₀₀ of approximately 0.3. Cultures were then supplemented with 4 mM H₂O₂, 0.1 mM mendadione (ViK₃) or 1.5 M NaCl. With other cultures, cells were collected by centrifugation and resuspended in 10% serum or M199 medium at pH 3.5. All cultures were then incubated at 30 °C. Control cultures consisted of cells without supplements and maintained at 30 °C in YNB. For other assays, cultures were shifted to 37 or 42 °C and expression was compared to cells grown at 30 °C. Cells incubated as described above under each condition were harvested at 10, 30 60 or 120 min for lacZ assays. The cells were collected and resuspended in 5 ml Z buffer (pH 7.0, 0.01 M sodium phosphate, supplemented with KCl, MgSO₄ and β-mercaptoethanol) (Uhl & Johnson, 2001). Then, triplicate samples of cells (0.8 ml per strain and for each growth condition) were permeabilized with 25 μl chloroform and 25 μl 0.1% SDS. Cells were equilibrated at 37 °C for 5 min, 0.2 ml (4 mg ml⁻¹) of the ONPG substrate was added and the cells were mixed and incubated at 37 °C for 20 min. The reactions were stopped by the addition of 0.5 ml 1 M Na₂CO₃, the cells were then centrifuged in a Sorvall Biofuge Pico for 5 min at 3000 r.p.m. and A₅₇₀ was determined for each reaction. The units of β-galactosidase activity were determined by the following equation:

\[ U = \frac{1000 \times A_{\text{420}}}{\text{v} \times \text{OD}_{\text{600}}} \]

where \( t \) is time of reaction and \( v \) is volume of culture used in assay.

Visual screens for C. albicans transformants were carried out by patching colonies onto X-Gal-modified medium (XMM) plates with 5 μl (1×10⁶) mid-exponential-phase cells for each sample (Uhl & Johnson, 2001). XMM contained 1.7 g yeast nitrogen base (without amino acids), 20 g glucose, 5 g ammonium sulfate and 20 g agar in 930 ml H₂O. After autoclaving, 70 ml 1 M potassium phosphate (pH 7.0) and 2 ml X-Gal (20 mg ml⁻¹) solution were added.

**Phosphorylation of Hog1p.** The assay follows our protocol as described previously (Chauhan et al., 2003). The parental strain CAF2-1 and the chk1 null (CHK21) were grown in YPD medium supplemented with either 10 mM H₂O₂ or 1.5 M NaCl as described previously (Chauhan et al., 2003). At designated times following incubation (t=0 min), cells were collected, proteins extracted and equal amounts separated by SDS-PAGE. The electrophoresed proteins were then transferred to nylon membranes and first probed with a ScHog1p polyclonal antibody (anti-ScHog1; Santa Cruz Biotechnology). Subsequently, the blots were stripped and reacted with a phospho-p38 MAP kinase (Thr180/Tyr182) 28B10 mAb (anti-TGYP; Cell Signalling Technology Inc.). Blots were then developed as recommended by the manufacturer (Amersham Pharmacia Biotech).

**In vitro sensitivity assays.** To determine the sensitivity of the mutants to oxidant or osmotic stress, we used in vitro drop plate assays containing 2–10 mM H₂O₂, 1–5 mM NaCl or 0–1 mM mendadione in YPD agar (Chauhan et al., 2003). To these media, inocula of 50–5 × 10⁷ yeast cells of CAF2-1, CHK21 (chk1/chk1), CHK23 (chk1/CHK1) and, for comparison, two other histidine kinases, the sh1 and nkl mutants, were spotted onto the agar media. Plates were then incubated at 30 °C for 48 h at which time growth was assessed at each cell concentration.

**Statistical analysis.** To determine the significance of lacZ expression in different growth conditions or in strains, we used a non-parametric analysis-of-variance technique with multiple comparison tests (SAS 8.2, SAS Institute, Cary, NC, USA). All outcomes were considered statistically significant at P<0.05.
RESULTS

Construction of \textit{C. albicans} CHK1-lacZ reporter gene

We used the \textit{Str. thermophilus} lacZ gene in reporter assays with the CHK1 promoter to determine the environmental signals that regulate transcription of \textit{CHK1} in \textit{C. albicans} wild-type cells (strain CAI4) as well as in strains that were deleted of other histidine kinase genes (\textit{shn1}, \textit{nik1}), the response regulator gene (\textit{ssk1}) or the \textit{hog1} MAP kinase mutant. These mutants were chosen for study to identify co-regulatory activities of Chk1p and other two-component proteins as well as to determine if transcription of \textit{CHK1} was influenced by different environmental conditions. As an internal control, we used \textit{ACT1-promoter-lacZ} that was also transformed into strain CAI4 and each mutant. Southern hybridization was used to ensure that the correct integration occurred with all transformants, but without the integration of tandem cassettes. BglII-digested DNA of untransformed as well as transformed CAI4 and mutants revealed a 1·84 kb fragment and in each mutant two hybridizing bands of 4·9 and 5·6 kb were observed (Fig. 1b) using the probe indicated in Methods. The \textit{ACT1-promoter-lacZ} transformants were digested with HindIII and probed as described in Methods. Two hybridizing fragments of 5·73 and 5·88 kb as well as a 3·5 kb fragment, the latter representing the wild-type allele, were observed. We also used PCR to verify the transformants in the background of each mutant strain and wild-type cells (CA14). Primer set P1 and P2 amplified a 1·79 kb fragment, while a 350 bp fragment was amplified by primer set P3 and P4 (data not shown).

\(\beta\)-Galactosidase activity in CAI4 and mutant strains

\(\beta\)-Galactosidase (CHK1-lacZ) activity in all strains was measured from cells grown in broth media (Fig. 2a, b). In addition, essentially similar results were obtained with the agar plate X-Gal agar assays (data not shown). The level of \(\beta\)-galactosidase in each strain indicated in Fig. 2 is expressed as absorption units for cultures that reached a similar optical density in each of the growth conditions described in Methods. For all determinations represented in Fig. 2(a–d), three clones of each transformant (mutants or CA14) were assayed in triplicate and mean values for each of the three transformants are indicated. Experiments were repeated twice with similar results. We also measured the expression of \textit{ACT1-promoter-lacZ} in mutant strains and CAI4 under all growth conditions to determine the changes in gene expression relative to \textit{CHK1-promoter-lacZ} (data not shown). Thus, all data shown in Fig. 2(a–d) are normalized to the expression of \textit{ACT1-promoter-lacZ}. Expression of \textit{ACT1-promoter-lacZ} was similar for all strains under all growth conditions (\(P=0.76\)), although \textit{ACT1-promoter-lacZ} expression was higher in all strains when cells were incubated in 10% serum or 4 mM \(\text{H}_2\text{O}_2\) (\(P<0.05\)) (data not shown). We measured the temporal expression of \textit{lacZ} under all growth conditions and in each mutant at 0, 10, 30, 60 and 120 min and found that for all strains, \textit{lacZ} expression was highest after 10 min incubation. For comparisons among strains and under each environmental condition, we have included expression data in Fig. 2(a–d) from both 10 and 120 min. In Fig. 2(a), \textit{lacZ} expression of CAI4 is compared to the \textit{shn1} and \textit{nik1} mutants. At 10 min, the expression of \textit{lacZ} in the \textit{shn1} mutant is lower than that of CAI4 under all growth conditions (\(P<0.05\)). However, expression of \textit{lacZ} was not changed under any growth condition in CAI4 or either mutant. After 120 min under stress, expression of \textit{lacZ} varied according to the strain and environmental growth conditions (Fig. 2b). We observed that the expression of \textit{lacZ} in CAI4 increased when cells were grown in 0·1 M menadione, 10% serum, 4 mM \(\text{H}_2\text{O}_2\), 1·5 M NaCl, pH 3.5, and at 37°C (\(P<0.05\)). In the \textit{shn1} mutant, expression of \textit{lacZ} was lower than in CAI4, again under all growth conditions (Fig. 2b) (\(P<0.05\)). This observation indicates that Ssn1p positively affects \textit{lacZ} expression. On the other hand, \textit{lacZ} expression in the \textit{nik1} mutant was similar to CAI4, except when cells were grown in the presence of 4 mM \(\text{H}_2\text{O}_2\) or 1·5 M NaCl for 120 min (Fig. 2b) (\(P<0.05\)).

When compared to strain CAI4, we found that \textit{lacZ} expression increased in the \textit{ssk1} and \textit{hog1} mutants under all growth conditions, including temperature, oxidants (peroxide and menadione), M-199 (pH 3.5), 10% serum and hyperosmotic stress (1·5 M NaCl) at 10 min (Fig. 2c) and 120 min (Fig. 2d) (\(P<0.0001\)), but was significantly less when cells were incubated at 37 and 42°C than under all other growth conditions (\(P<0.05\)). The data in Fig. 2(c) and (d) indicate that SSK1 and HOGL negatively regulate expression of \textit{CHK1}.

Phosphorylation of Hog1p in parental cells and the \textit{chk1} mutant

The \textit{lacZ} reporter assays indicated that Ssk1p, Hog1p and Sln1p affect expression of \textit{CHK1-promoter-lacZ}. To further define this interaction, we determined the phosphorylation of Hog1p in both CAF2-1 and the \textit{chk1} mutant (strain CHK21), since Hog1p is a MAP kinase that is downstream of Sln1p and Ssk1p in \textit{Sac. cerevisiae} and, presumably, \textit{C. albicans}. Both strains were stressed with either 10 mM \(\text{H}_2\text{O}_2\) or 1·5 M NaCl and phosphorylation of Hog1p was measured. We knew from previous studies that Ssk1p is required for phosphorylation of Hog1p in cells under oxidant stress (Chauhan \textit{et al}., 2003). In Fig. 3 (upper and lower panels), Hog1p is phosphorylated in CAF2-1 within 2 min after the shift to either stress condition. The phosphorylation signal then decreases by 60 min. In strain CHK21 the temporal phosphorylation of Hog1p is somewhat different. Under oxidative stress, phosphorylation of Hog1p persists, even at 60 min, while under osmotic stress, phosphorylation of Hog1p is delayed and a weak signal is first seen at 10 min which then persists for at least 60 min. Thus, under both types of stress, Chk1p is not required for phosphorylation of Hog1p, although minimal temporal changes occur in the \textit{chk1} mutant compared to wild-type cells.

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Fig. 2. β-Galactosidase activity in strains transformed with the CHK1p-lacZ cassette. CAI4 (white bars) is compared to the histidine kinase mutants sln1 (black bars) and nik1 (grey bars) after 10 (a) or 120 min (b) incubation under each of the conditions indicated. (c, d) lacZ expression in CAI4 (white bars) compared to the ssk1 (grey bars) and the hog1 (black bars) mutants after 10 (c) or 120 min (d) incubation. All strains were grown under the conditions indicated below each bar graph.

Fig. 3. Western blot analysis. Yeast cells of strains CAF2-1 and CHK21 (chk1/chk1) grown in YPD broth were collected, washed and shifted to YPD broth (0 time) containing 10 mM H2O2 (upper panels) or 1.5 M NaCl (lower panels) to induce oxidative or osmotic stress, respectively. At the times indicated above each lane (min), protein samples were obtained and Western blots were performed with an anti-Sac. cerevisiae Hog1p antibody (lower band, loading control). The blots were then stripped and reacted with an anti-TGY antibody (upper band) that recognizes the phosphorylated p38 protein of mammalian cells (Hog1p is a homologue of this protein).
Sensitivity of the chk1 mutant to oxidants and osmotic stress

Drop plate assays were performed to determine the sensitivities of CAF2-1, the chk1, sln1 and nik1 mutants, and strain CHK23 (reconstituted with a single copy of CHK1) to H2O2 (2–10 mM), NaCl (1–1.5 M) and 0·1 mM menadione. Concentrations above and below 4–5 mM H2O2 were either too toxic or had no effect on the strains. Thus, we evaluated the sensitivities of each strain using different inocula concentrations (5 x 10^4–50 cells) at 5 mM H2O2 (Fig. 4). Of all the strains, the chk1 mutant (CHK21) exhibited the greatest sensitivity to H2O2. The sln1 mutant was similar in its sensitivity to CAF2-1 while the nik1 mutant was only slightly more resistant to peroxide than CHK21. The growth of all strains in 1·5 M NaCl or 0·1 mM menadione, germination in 10% serum and growth at 30, 37 or 42 °C, and in M-199, pH 3·5, was similar to strain CAF2-1 (data not shown).

DISCUSSION

Reporter gene assays provide an approach to understanding the relationships among proteins. In C. albicans, several heterologous reporter genes have been used, including Kluyveromyces lactis LAC4 and Str. thermophilus lacZ (both reporters express β-galactosidase), Renilla reniformis luciferase, several versions of the green fluorescent protein and the Flp/FRT in vivo expression system (Leuker et al., 1992; Srikantha, 1996; Uhl & Johnson, 2001). For a discussion of the advantages and disadvantages of each reporter system in C. albicans, readers are directed to the review by Berman & Suddbery (2002). In this report, we used the lacZ gene of Str. thermophilus as a reporter to identify regulatory interactions of Chk1p with other two-component proteins.

Chk1p is not found in Sac. cerevisiae, and while orthologues have been identified in Schizosaccharomyces pombe, Mak2p and Mak3p have not been assigned to a signal pathway (Buck et al., 2001). Based upon our current data, we postulate that the regulation of Chk1p by proteins of the HOG1 MAP seems likely, but the alignment of Chk1p within or downstream of this pathway remains uncertain. Sln1p and Ssk1p are both components of the HOG1 pathway, yet their effects on Chk1p transcription are opposite but reminiscent of their interactions in the Sac. cerevisiae HOG1 osmosensing pathway (Hohlmann, 2002). In that organism, in unstressed cells, the downstream Ssk1p is inactive (unable to bind to the MAPKKK of the HOG1 pathway), because Ssk1p is phosphorylated by the phosphohistidine intermediate protein, Ypd1p, via the histidine kinase, membrane receptor protein Sln1p. In osmotically stressed cells, Ssk1p is not phosphorylated and is able to activate the HOG1 MAP kinase pathway, which in turn results in an osmoadaptation. If SLN1 is deleted, then Ssk1p is constitutively active, since it is unphosphorylated in both stressed and unstressed cells; this leads to inviability in Sac. cerevisiae but not in C. albicans. Thus, in the C. albicans sln1 mutant, Ssk1p is presumably unphosphorylated and, hence, active, resulting in the downregulation of CHK1 transcription. Likewise, if SSK1 is deleted, then transcription of CHK1 increases. Support for the CHK1-lacZ expression profile in the sln1 mutant has been demonstrated in previous studies by microarray analysis, since CHK1 transcription increases in the sln1 mutant (Chauhan et al., 2003). If this model of CHK1 regulation is correct, then Hog1p (downstream of Ssk1p) should likewise negatively regulate CHK1. In fact, the hog1 deletion mutant behaves similarly to the sln1 mutant: CHK1 transcription is increased. The effects of the SLN1 or SSK1 deletions on CHK1 in C. albicans are observed in both stressed and unstressed cells, but the level of expression of CHK1p-lacZ is changed under some stress conditions at a specific time point.

We attempted to correlate the activity of Chk1p and Hog1p by Western blot analysis of phosphorylated and unphosphorylated Hog1p. Those data indicate that Chk1p is not required for Hog1p phosphorylation, although the deletion of CHK1 caused a minimal temporal change in phosphorylation of Hog1p. These data suggest that Chk1p is transcriptionally regulated but perhaps downstream of the HOG1 signal pathway.

The relationship of Nik1p to CHK1p-lacZ transcription is less apparent, since changes in the latter only occurred in a narrow range of growth conditions in the nik1 mutant. From previous microarray data with the sln1 mutant, CHK1 transcription is increased, while NIK1 is unchanged compared to CAF2-1 (Chauhan et al., 2003). On the other hand, in the nik1, sln1 and chk1 mutants, the transcription profile of six mannosyl transferases increased similarly in each, and Western blotting profiles of acid-stable, but not...
Chk1p may be a receptor for quorum sensing caused by recently, Kruppa than parental cells in vitro chk1 mutant does not exhibit the same cell wall phenotype as ssk1 like the farnesol pathway, independent of HOG1, since the wall biosynthesis (Kruppa et al., 2003, 2004b). These changes have been confirmed by both biochemical and immunological determinations. Associated with the changes in cell wall structure is the reduced adherence of the mutant to human oesophageal tissues (Li et al., 2002; Bernhardt et al., 2001), increased sensitivity to human polymorphonucleocytes (Torosantucci et al., 2002) and avirulence (Calera et al., 1999). We now also show that the chk1 mutant is more sensitive to peroxide than parental cells in vitro using drop plate assays. More recently, Kruppa et al. (2004a) have demonstrated that Chk1p may be a receptor for quorum sensing caused by the autoinducer farnesol. The chk1 mutant is refractory to farnesol in comparison to wild-type cells and the sln1, ssk1 and niki two-component mutants whose germination is inhibited by farnesol. This observation indicates a signalling pathway for Chk1p that does not include HOG1 two-component proteins. The Chk1p functions and relationship to the Hog1 pathway are summarized in Fig. 5. It appears that Chk1p participates indirectly or directly in at least two signal pathways whose activation may depend upon the environmental signal, or Chk1p may be downstream of Hog1p and transcriptionally regulated via the HOG1 MAP kinase pathway.

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REFERENCES


Fig. 5. Chk1p functions and its relationship to the HOG1 signal pathway of C. albicans. Hog1 is activated by either oxidant or osmotic stress via the Sln1p histidine kinase sensor protein. Both Ssk1p and Hog1p downregulate CHK1 transcription, while Sln1p upregulates transcription, since in the sln1 mutant Ssk1p is probably constitutively active. On the other hand, the quorum sensing pathway which is induced by the isoprenoid farnesol is activated via Chk1p and does not require the HOG1 MAP kinase pathway (Kruppa et al., 2004b). Another major function of Chk1p is its regulatory activity in cell wall biosynthesis (Kruppa et al., 2003, 2004b). This activity is, like the farnesol pathway, independent of HOG1, since the ssk1 mutant does not exhibit the same cell wall phenotype as the chk1 mutant. Thus, Chk1p participates in at least three signal pathways.


