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Studies on the regulation of the two-component histidine kinase gene *CHK1* in *Candida albicans* using the heterologous *lacZ* reporter gene

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The two-component histidine kinase Chk1p of *Candida albicans* has been implicated in the regulation of cell wall biosynthesis. Deletion of *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 *in vitro*, probably as a consequence of its altered cell wall. In the current study, a *CHK1* promoter-*lacZ* reporter (*CHK1prlacZ*) construct was expressed in wild-type *C. albicans* strain CAI4 and in two-component signal transduction mutants to determine the effect of environmental stress conditions on the regulation of *CHK1* and the co-regulatory activities among these proteins. It is shown that *lacZ* expression varied according to the type of growth conditions and incubation time; expression was also influenced by the strain background. *lacZ* expression in CAI4 was greater at 37 °C and at a pH of 3·5 and in the presence of 4 mM H_2O_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 ($P<0·05$). As a correlate of the increase in transcription of *CHK1-lacZ* in the presence of H_2O_2, the *chk1* mutant was more sensitive than wild-type and revertant cells to H_2O_2 *in vitro*. In addition to strain CAI4, we also measured *CHK1p-lacZ* reporter activity of mutants deleted in genes encoding other two-component proteins such as the response regulator gene *SSK1*, the histidine kinases, *SLN1* and *NIK1*, and the *HOG1* MAP kinase. Of these proteins, Ssk1p and Sln1p are presumed to mediate phosphotransfer to the HOG1 [hyperosmotic glycerol] MAP kinase pathway during oxidative and perhaps osmotic stress in *C. albicans*. Compared to strain CAI4, *lacZ* reporter activity increased significantly in the *ssk1* mutant under all growth conditions after a 10 and 120 min incubation ($P<0·0001$). *lacZ* expression in the *ssk1* mutant was less at 42 °C compared to all other growth conditions ($P<0·05$). Furthermore, *lacZ* reporter activity also increased in the *hog1* mutant of *C. albicans*. These data suggest that *SSK1* and *HOG1* indirectly or directly negatively regulate *CHK1* under most growth conditions tested. In the *sln1* mutant, downregulation of *CHK1* was observed in all growth conditions compared to strain CAI4 ($P<0·05$), while regulation of *lacZ* in the *nik1* mutant was similar to strain CAI4 except when cells were incubated in the presence of 4 mM H_2O_2 for 120 min ($P<0·05$). 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 *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 *CHK1*, as measured by the *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 *lacZ* expression is indirectly or directly regulated by the HOG1 MAP kinase pathway, although a determination of its position in this pathway or in a cross-talking pathway awaits additional studies.

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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 (Sln1p, Nik1p and Chk1p), two response regulator proteins (Ssk1p and Skn7p) and a phosphohistidine intermediate protein (Ypd1p) (Calera & Calderone, 1999b; Santos & Shiozaki, 2001). Sln1p, Ypd1p and Ssk1p are probably orthologues of the HOG1 MAP kinase pathway proteins [hyperosmotic 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 *sln1* 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 Sln1p, 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. CAF2-1 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 (*ssk1*Δ), NIK22 (*nik1*Δ), SLN22 (*sln1*Δ) and CNC15 (*hog1*Δ) (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 *Kpn*I–*Sma*I fragment in pAU36 (Fig. 1a, top). The primers used to amplify the 1·4 kb *CHK1* promoter were

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

Strain	Genotype	Reference
CAI4	Δ*ura3*::*imm434*/Δ*ura3*::*imm434*	Fonzi & Irwin (1993)
CAF2-1	Δ*ura3*::*imm434*/*URA3*	Fonzi & Irwin (1993)
SSK22	Δ*ura3*::*imm434*/Δ*ura3*::*imm434* Δ*ssk1*::*hisG*/Δ*ssk1*::*hisG*	Calera *et al.* (2000a)
SLN22	Δ*ura3*::*imm434*/Δ*ura3*::*imm434* Δ*sln1*::*hisG*/Δ*sln1*:*hisG*	This study
NIK22	Δ*ura3*::*imm434*/Δ*ura3*::*imm434* Δ*nik1*::*hisG*/Δ*nik1*::*hisG*	This study
CNC15	Δ*ura3*::*imm434*/Δ*ura3*::*imm434* Δ*hog1*::*hisG*/Δ*hog1*::*hisG*	Alonso-Monge (2003)
CHK21	Δ*ura3*::*imm434*/Δ*ura3*::*imm434* Δ*chk1*::*hisG*/Δ*chk1*::*hisG*	Calera & Calderone (1999a)
CHK23	Δ*ura3*::*imm434*/Δ*ura3*::*imm434* Δ*chk1*::*hisG*/*CHK1*::*URA3-hisG*	Calera & Calderone (1999a)

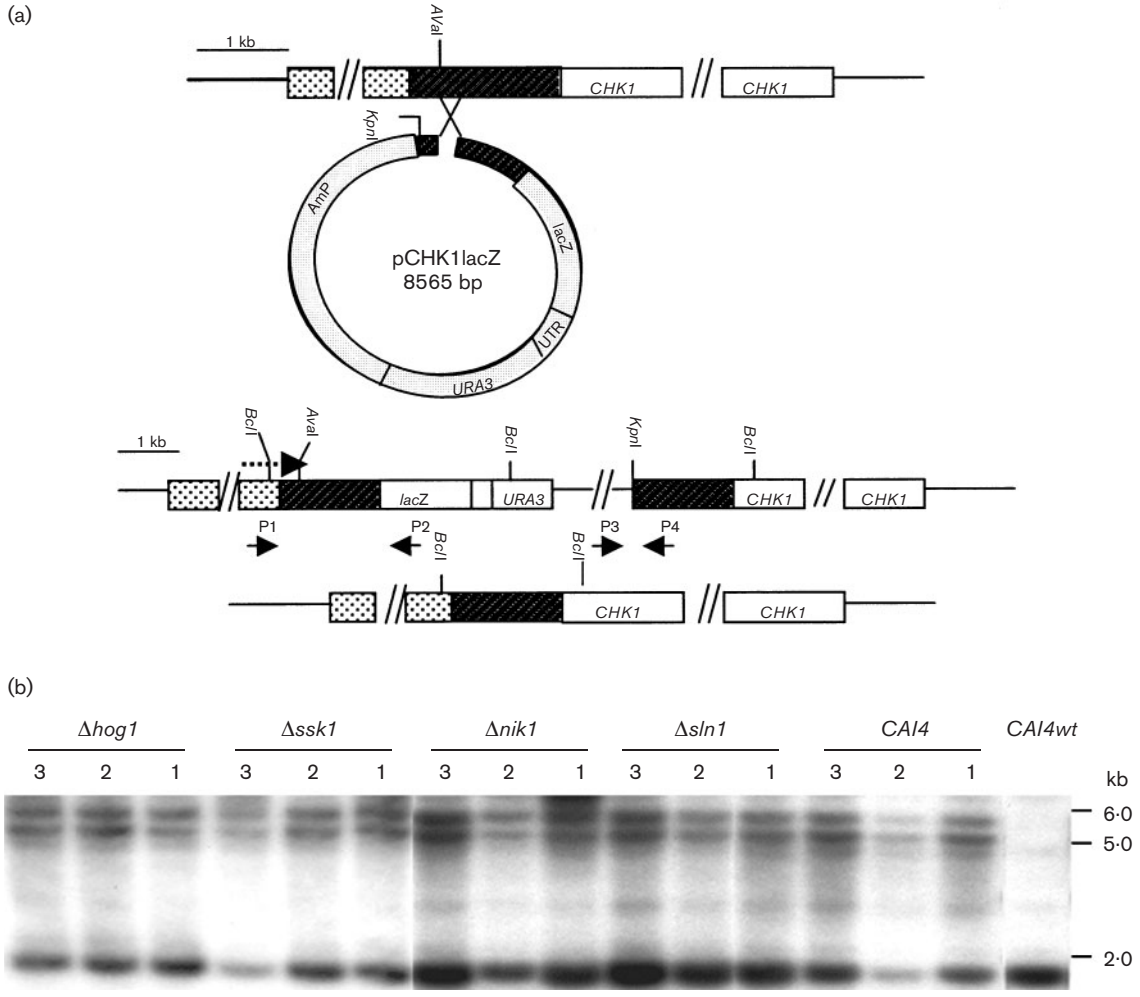

Fig. 1. (a) Top. Construction of the pChk1lacZ plasmid. The stippled (non-integrated) and dark (integrated) rectangles indicate the *CHK1* promoter. The *CHK1*, *URA3* and *lacZ* ORFs are indicated as clear rectangles. Middle. Restriction map of the p*CHK1-lacZ* cassette and primers (P1–P4) used for 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.

CHK5 (5′-AAATTCAAGGTACCCAATCAACTTTTCCAAG-3′) and CHK3 (5′-CTGAAAAACTATTCTGTAAAAGGTCTGTACG-3′). The *CHK1* promoter was ligated with the *lacZ* gene, including a 6 nt plasmid sequence before the ATG start codon of *lacZ*.

Transformation. Transformation methods followed those of Calera & Calderone (1999a). *Ava*I-linearized pChk1prlacZ (5 μg) was used to transform CAI4 and each of the mutant strains (*ssk1*, *nik1*, *sln1*, *hog1*) using lithium acetate. Also, 5 μg of the *Btg*I fragment obtained from linearized pAU36 that includes the *ACT1* promoter sequence in-frame with *lacZ* was introduced into CAI4 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. *Ava*I-linearized pChk1prlacZ (5 μg) or *Btg*I-linearized pAct1prlacZ (5 μg) and 100 μg denatured salmon sperm DNA were added to 100 μl competent CAI4 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 *CHK1prlacZ* and *Act1prlacZ* cassettes in all transformants of CAI4 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 *Bcl*I and probed with the 550 bp fragment that is located at approximately 175 bp upstream of the *Kpn*I 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 *Hin*dIII 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′-GACACCTCCTAATAACTCAC-3′), located at ∼80 bp upstream of the 5′ end of the *CHK1* promoter corresponding to the *Kpn*I restriction site and an StlacZ3′ primer (5′-TTCTTGAGGAACTTGAGGTG-3′) at ∼200 bp downstream of the *lacZ* start codon (Fig. 1a, bottom, indicated as primer pair P1 and P2). The second PCR was performed using primers P3 (from pBSII KS+ vector) and P4 (CHKpr3′, 5′-CTCGGCGATACTCTACTAC-3′) at about 350 bp downstream of the *Kpn*I 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 ACT1pr5′ (5′-GAGAGATTTGAAATGATCAG-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′-TAGCACACACCCACAACAAC-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 CAI4 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 CAI4 or each transformant. Cultures were incubated at 30 °C with vigorous shaking for 3 h to achieve an OD_{600} of approximately 0·3. Cultures were then supplemented with 4 mM H_2O_2, 0·1 mM menadione (ViK_3) 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, $MgSO_4$ 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 Na_2CO_3, cells were then centrifuged in a Sorvall Biofuge Pico for 5 min at 3000 r.p.m. and A_{420} was determined for each reaction. The units of β-galactosidase activity were determined by the following equation:

$$U = \frac{1000 \times A_{420}}{t \times v \times \mathrm{OD}_{600}}$$

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 (1×10^6) 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 H_2O. 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 H_2O_2 or 1·5 M NaCl as described previously (Chauhan *et al.*, 2003). At designated times following incubation (t_0–t_{60} 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-TGY^P; 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 H_2O_2, 1–1·5 M NaCl or 0·1 mM menadione in YPD agar (Chauhan *et al.*, 2003). To these media, inocula of $50–5 \times 10^5$ yeast cells of CAF2-1, CHK21 (*chk1*/*chk1*), CHK23 (*chk1*/*CHK1*) and, for comparison, two other histidine kinases, the *sln1* and *nik1* mutants, were spotted onto the agar media. Plates then were 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 $P < 0{\cdot}05$.

RESULTS

Construction of *C. albicans CHK1-lacZ* reporter gene

We used the *Str. thermophilus lacZ* gene in reporter assays with the *CHK1* promoter to determine the environmental signals that regulate transcription of *CHK1* in *C. albicans* wild-type cells (strain CAI4) as well as in strains that were deleted of other histidine kinase genes (*sln1*, *nik1*), the response regulator gene (*ssk1*) or the *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 *CHK1* was influenced by different environmental conditions. As an internal control, we used *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. *Bcl*I-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 *ACT1-lacZ* transformants were digested with *Hin*dIII 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 (CAI4). 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).

β-Galactosidase activity in CAI4 and mutant strains

β-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 β-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 CAI4) 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 *ACT1-lacZ* in mutant strains and CAI4 under all growth conditions to determine the changes in gene expression relative to *CHK1-lacZ* (data not shown). Thus, all data shown in Fig. 2(a–d) are normalized to the expression of *ACT1-lacZ*. Expression of *ACT1-lacZ* was similar for all strains under all growth conditions ($P=0·76$), although *ACT1-lacZ* expression was higher in all strains when cells were incubated in 10 % serum or 4 mM H_2O_2 ($P<0·05$) (data not shown). We measured the temporal expression of *lacZ* under all growth conditions and in each mutant at 0, 10, 30, 60 and 120 min and found that for all strains, *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), *lacZ* expression of CAI4 is compared to the *sln1* and *nik1* mutants. At 10 min, the expression of *lacZ* in the *sln1* mutant is lower than that of CAI4 under all growth conditions ($P<0·05$). However, expression of *lacZ* was not changed under any growth condition in CAI4 or either mutant. After 120 min under stress, expression of *lacZ* varied according to the strain and environmental growth conditions (Fig. 2b). We observed that the expression of *lacZ* in CAI4 increased when cells were grown in 0·1 M menadione, 10 % serum, 4 mM H_2O_2, 1·5 M NaCl, pH 3·5, and at 37 °C ($P<0·05$). In the *sln1* mutant, expression of *lacZ* was lower than in CAI4, again under all growth conditions (Fig. 2b) ($P<0·05$). This observation indicates that Sln1p positively affects *lacZ* expression. On the other hand, *lacZ* expression in the *nik1* mutant was similar to CAI4, except when cells were grown in the presence of 4 mM H_2O_2 or 1·5 M NaCl for 120 min (Fig. 2b) ($P<0·05$).

When compared to strain CAI4, we found that *lacZ* expression increased in the *ssk1* and *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 *HOG1* negatively regulate expression of *CHK1*.

Phosphorylation of Hog1p in parental cells and the *chk1* mutant

The *lacZ* reporter assays indicated that Ssk1p, Hog1p and Sln1p affect expression of *CHK1-lacZ*. To further define this interaction, we determined the phosphorylation of Hog1p in both CAF2-1 and the *chk1* mutant (strain CHK21), since Hog1p is a MAP kinase that is downstream of Sln1p and Ssk1p in *Sac. cerevisiae* and, presumably, *C. albicans*. Both strains were stressed with either 10 mM H_2O_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 *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 *chk1* mutant compared to wild-type cells.

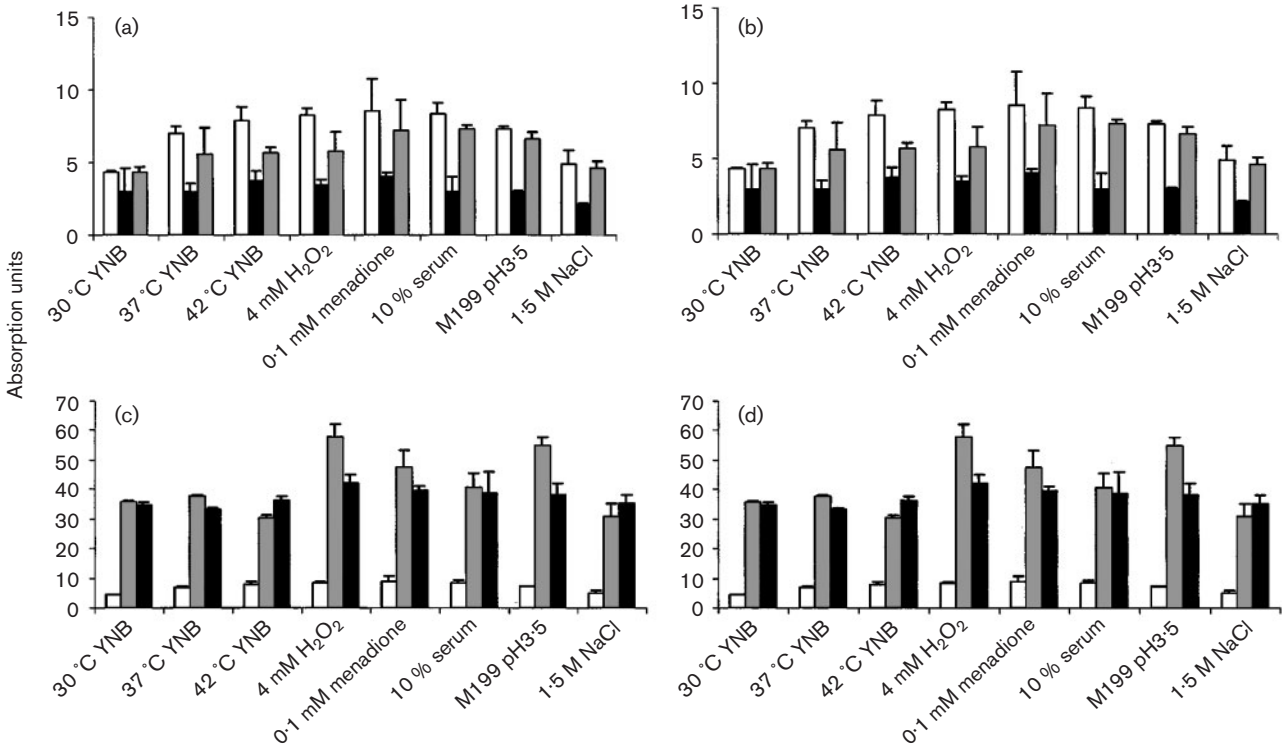

Fig. 2. β-Galactosidase activity in strains transformed with the *CHK1p-lacZ* cassette. CAI4 (white bars) is compared to the histidine kinase mutants *sln1* (black bars) and *nik1* (grey bars) after 10 (a) or 120 min (b) incubation under each of the conditions indicated. (c, d) *lacZ* expression in CAI4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

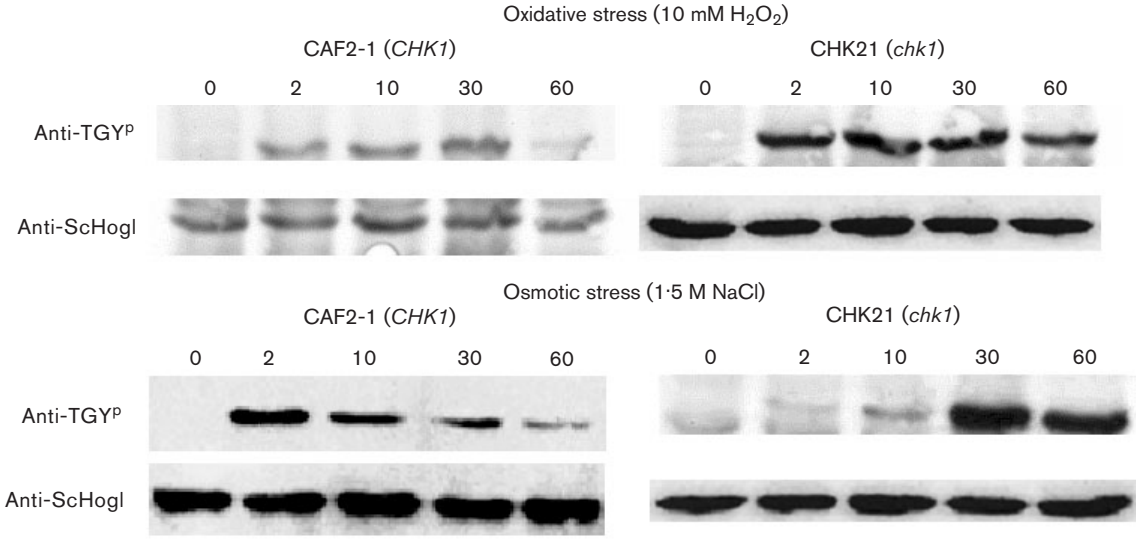

Fig. 3. Western blot analysis. Yeast cells of strains CAF2-1 and CHK21 (*chk1*/*chk1*) grown in YPD broth were collected, washed and shifted to YPD broth (0 time) containing 10 mM H_2O_2 (upper panels) or 1·5 M NaCl (lower panels) to induce oxidative or osmotic stress, respectively. At the times indicated 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 H_2O_2 (2–10 mM), NaCl (1–1·5 M) and 0·1 mM menadione. Concentrations above and below 4–5 mM H_2O_2 were either too toxic or had no effect on the strains. Thus, we evaluated the sensitivities of each strain using different inocula concentrations (5×10^5–50 cells) at 5 mM H_2O_2 (Fig. 4). Of all the strains, the *chk1* mutant (CHK21) exhibited the greatest sensitivity to H_2O_2. 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 & Sudbery (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.

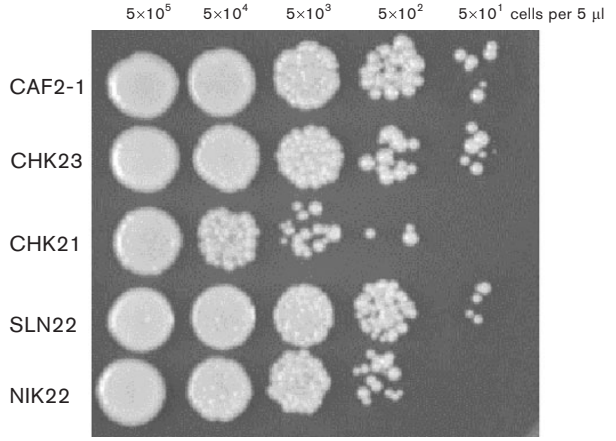

Fig. 4. Growth of CAF2-1 (wild-type), CHK23 (*chk1*/*CHK1*), CHK21 (*chk1*/*chk1*), SLN (*sln1*/*sln1*) and NIK (*nik1*/*nik1*) on YPD agar containing 5 mM H_2O_2 at 30 °C for 48 h. The medium was spotted with inocula ranging from 5×10^5 to 50 cells of each strain, in a volume of 5 µl. The plating efficiency was similar for all strains in the absence of peroxide.

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 (Hohmann, 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 *ssk1* mutant has been demonstrated in previous studies by microarray analysis, since *CHK1* transcription increases in the *ssk1* 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 *ssk1* 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 *ssk1* 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

acid-labile oligomannans were similar for each mutant (Kruppa *et al.*, 2004b), implying that the three histidine kinases may have cross-talking interactions in regard to some common activities.

Chk1p of *C. albicans*, and for that matter all fungi that have two-component signal transduction, is a functionally novel histidine kinase in that it regulates cell wall biosynthesis. We have shown that the *chk1* mutant has profound changes in the composition of its cell wall, including levels of 1,3-β-glucan (about 50 % lower) and 1,6-β-glucan (about fourfold higher), as well as a truncation in the oligosaccharide side chains of the acid-stable mannan (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 *nik1* 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.

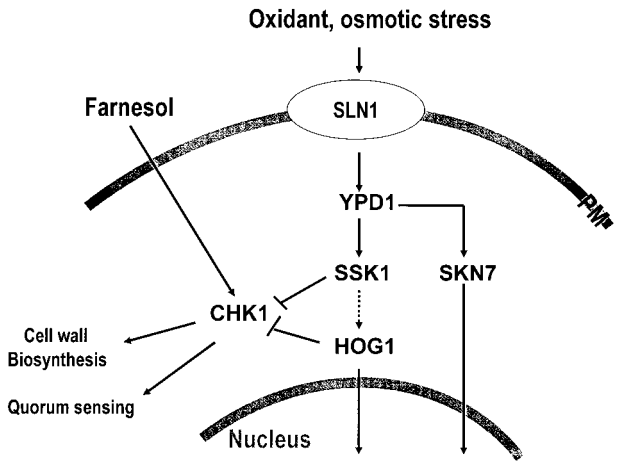

Fig. 5. Chk1p functions and its relationship to the HOG1 signal pathway of *C. albicans*. Hog1 is activated by either oxidant or osmotic stress via the Sln1p histidine kinase sensor protein. Both Ssk1p and Hog1p downregulate *CHK1* transcription, while Sln1p upregulates transcription, since in the *sln1* mutant Ssk1p is probably constituently 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.

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