Flocculation of hyphae is associated with a deletion in the putative *CaHK1* two-component histidine kinase gene from *Candida albicans*

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In *Candida albicans*, three putative histidine kinase genes have been described thus far, including *CaSLN1*, *CaNIK1/COS1* and *CaHK1*. The encoded proteins for *C. albicans*, *CaSLN1p* and *CaNik1p*, which are similar to *Sln1p* from *Saccharomyces cerevisiae* and *Nik-1* from *Neurospora crassa*, seem to function in osmoregulation and morphogenesis, respectively. Recently, the isolation of *CaHK1*, a putative histidine kinase gene from *C. albicans* has been reported. In addition to the histidine and aspartyl domains located at its C-terminus as previously described, it is shown here that the N-terminal domain of *Cahk1p* contains a P-loop motif and a sequence which shows significant homology with the seven C-terminal domains of serine/threonine kinases. The Ser/Thr-homologous domains of *Cahk1p* could, in fact, correspond to its sensor sequence. *CaHK1* has been mapped to chromosome 2 and gene deletion studies were undertaken to understand its function. Δ *cahk1* mutants are phenotypically different from any other histidine kinase mutants thus far described either in *C. albicans* or in any other yeast or filamentous fungus. This study demonstrates that Δ *cahk1* mutants flocculate extensively in a gene-dosage-dependent manner under conditions which induce germ-tube formation, such as growth in medium 199 (pH 7.5). The flocculation occurs by an interaction along the hyphal surfaces, probably because of the altered expression of one or more hyphal-cell-surface components in the Δ *cahk1* mutants. These results indicate that *CaHK1* could be involved in regulating their expression.

Keywords: Flocculation, histidine kinase, two-component, hyphae, *Candida albicans*

INTRODUCTION

The sensor histidine kinases and their corresponding response regulators were first described in prokaryote phosphotransfer pathways which enable cells to detect environmental changes and elicit an adaptive response (Hoch & Silhavy, 1995). More recently, homologues of sensor and response regulator genes were identified in *Saccharomyces cerevisiae* (Banuett, 1998). Since that observation was made, a systematic search for these genes has been performed on many other eukaryotic micro-organisms, including *Candida albicans* (Alex *et al.*, 1998; Calera *et al.*, 1998; Nagahashi *et al.*, 1998).

Although bacterial histidine kinases are involved in the

detection of many different stimuli (Hoch & Silhavy, 1995), in eukaryotic micro-organisms the variety of environmental signals has been limited to osmolarity (Posas *et al.*, 1996; Schuster *et al.*, 1996; Shieh *et al.*, 1997), oxidative stress (Morgan *et al.*, 1997; Shieh *et al.*, 1997), heat stress (Shieh *et al.*, 1997) and nutritional limitations (Cottarel, 1997; Shieh *et al.*, 1997). On the other hand, histidine kinases have been shown to play a key role in developmental processes of eukaryotic micro-organisms. Thus, these proteins have been implicated in the morphogenesis of *Dictyostelium discoideum* and *Neurospora crassa* (Alex *et al.*, 1996; Chang *et al.*, 1998; Wang *et al.*, 1996; Zinda & Singleton, 1998). Finally, a link between environmental stress and growth in eukaryotic micro-organisms has also been established. Thus, in *Schizosaccharomyces pombe*, the Mcs4 response regulator has been reported to be an essential component of the Sty1 pathway, which coordinates the

The GenBank accession number for the *CaHK1* sequence reported in this paper is AF013273.

Table 1. *Candida albicans* strains used in this study

Strain	Relevant genotype	Source/reference
CAF2	$\Delta ura3::imm434/URA3$	Fonzi & Irwin (1993)
CAI4	$\Delta ura3::imm434/\Delta ura3::imm434$	Fonzi & Irwin (1993)
CHK11	$\Delta ura3::imm434/\Delta ura3::imm434 \Delta cahk1::hisG-URA3-hisG/CaHK1$	This work
CHK12	$\Delta ura3::imm434/\Delta ura3::imm434 \Delta cahk1::hisG/CaHK1$	This work
CHK21	$\Delta ura3::imm434/\Delta ura3::imm434 \Delta cahk1::hisG/\Delta cahk1::hisG-URA3-hisG$	This work
CHK22	$\Delta ura3::imm434/\Delta ura3::imm434 \Delta cahk1::hisG/\Delta cahk1::hisG$	This work
CHK23	$\Delta ura3::imm434/\Delta ura3::imm434 \Delta cahk1::hisG/CaHK1::URA3-hisG$	This work

stress response with mitosis and meiosis (Cottarel, 1997; Shieh *et al.*, 1997).

C. albicans, a polymorphic fungus of increasing medical importance, can grow as a yeast or form true hyphae and an intermediate pseudohyphal shape. *In vitro*, this morphological plasticity is regulated by a number of environmental signals, mainly pH, temperature and medium composition. The yeast phase is favoured at 25 °C and an acidic pH; hyphal growth is favoured at 37 °C and a pH near neutrality. Medium composition, particularly the presence of serum or other inducing compounds, can favour growth as hyphae. The factors responsible for the virulence of *C. albicans* are still not well understood, but pseudohyphae/hyphae exhibit increased adherence to host tissues and increased invasiveness (Chaffin *et al.*, 1998). Several hyphal-cell-wall moieties mediate the interaction between the fungal cell and the host, and contribute to the initiation of the pathogenic process, including cell-wall proteins and mannoproteins which are the major mediators of adhesion. During the past several years, many candidal adhesins and host ligands that mediate the adherence of *C. albicans* have been characterized; most of the adhesins that have been detected are associated with the cell wall of germ tubes (for a detailed review see Chaffin *et al.*, 1998). However, few adhesins have been characterized at the molecular level, and although the expression of many of these components seems to be environmentally regulated, no information is available about the mechanisms that regulate their expression. Recently, Als1p (agglutinin-like sequence) has been identified as a putative candidal adhesin (Fu *et al.*, 1998a). Also, a relationship between flocculation and adherence was suggested since the expression of the *CAD1/AAF1* from *C. albicans* in *S. cerevisiae* causes cells to flocculate and exhibit enhanced adherence to endothelial cells (Fu *et al.*, 1998b). Again, the relationship of cell-surface-adhesin expression and signal pathways is unknown.

To identify genes of environmentally regulated pathways, a search for histidine kinase homologues of *C. albicans* has also been performed and three putative

histidine kinase genes, *CaHK1*, *CaSLN1* and *CaNIK1/COS1*, have been described thus far (Alex *et al.*, 1998; Calera *et al.*, 1998; Nagahashi *et al.*, 1998). *CaSLN1* and *CaNIK1/COS1* are the homologues of *SLN1* from *Sacch. cerevisiae* and *nik-1⁺* from *N. crassa*, which are involved in osmoregulation and morphogenesis, respectively (Alex *et al.*, 1998; Nagahashi *et al.*, 1998). However, the structural homology that is found between Cahk1p and other histidine kinases is restricted to its C-terminal domain, in which the key histidine and aspartyl domains involved in phosphotransfer are located. The histidine kinases that are most likely to be related to Cahk1p, both structurally and perhaps also functionally, are two putative sensor kinases from *Schiz. pombe* (GenBank accession nos Z98978 and AL031543). These are the only histidine kinases in which the N-terminal domains show similarity with the N-terminal domain of Cahk1p. However, the role of these sensor histidine kinases in *Schiz. pombe* is unknown, and no function for Cahk1p can be inferred from their similarity. In this paper we describe the construction of a $\Delta cahk1$ null strain and its phenotype. Our data are indicative of a role for Cahk1p in regulating the expression of hyphal cell surface component(s).

METHODS

Strains and growth conditions. The *C. albicans* strains used in this work are listed in Table 1. All strains were routinely grown either in YPD complex medium (1% yeast extract, 2% peptone and 2% glucose) or YNB minimal medium [0.67% yeast nitrogen base (Gibco-BRL), 2% glucose] at 28 °C for 14–16 h. Cells were harvested from liquid medium by centrifugation at 4000 g for 10 min at 4 °C. For specific experiments, strains were also grown in YED (1% yeast extract, 2% glucose) with the pH adjusted either to pH 3.5 or 7.5, or in medium 199 containing Earle's salts and glutamine but lacking sodium bicarbonate (Gibco-BRL) and buffered with 155 mM Tris/HCl either at pH 7.5 or 3.5. These media were also supplemented with either 1 M sorbitol or 1.5 M NaCl. Cultures were inoculated to a density of 10^7 cells ml⁻¹ into prewarmed medium and incubated either at 28 °C or 37 °C with vigorous agitation. In all cases, stationary-phase cells grown at 28 °C in YPD were used as the inoculum. Uridine was added to a final concentration of 25 µg ml⁻¹ when necessary.

Fig. 1. (a) Schematic representation of the construction of the cassette used to disrupt *CaHK1* and (b) the *CaHK1-URA3-hisG* cassette used to reintroduce one wild-type *CaHK1* allele. (c) The corresponding Southern-blot analysis of representative $\Delta cahk1$ mutants obtained during the disruption process, and one revertant strain (CHK23). Genomic DNA from these strains was *Bgl*II digested, and hybridized with a 0.89 kb fragment located at the 5' end of the *CaHK1* gene as a probe. The exact size and genotype of the expected hybridizing DNA fragments are indicated on the right.

Escherichia coli strain DH5 α , which was used for transformation and plasmid amplification, was grown at 37 °C in LB broth supplemented with 0.1 mg ampicillin ml⁻¹ when necessary.

DNA manipulations. *E. coli* was routinely transformed by standard methods (Sambrook *et al.*, 1989). Plasmid DNA was extracted from *E. coli* DH5 α cells using the Plasmid Midi Kit according to the manufacturer's instructions (Qiagen). Re-

striction enzymes and T4 DNA ligase were obtained from Gibco-BRL and used with buffers provided by the supplier under the recommended conditions. Agarose gel electrophoresis of both DNA and RNA was performed according to standard protocols (Sambrook *et al.*, 1989). Construction of recombinant plasmids and selection of transformants were done by standard techniques (Sambrook *et al.*, 1989).

Southern- and Northern-blot analyses. Genomic DNA from all *C. albicans* strains was obtained according to the method described by Sherman *et al.* (1986). Four micrograms of DNA per lane was typically loaded for Southern analyses. DNA was transferred by capillarity to positively charged nylon membranes (Hybond-N+; Amersham) by standard protocols (Sambrook *et al.*, 1989). All probes used for Southern blots were digoxigenin labelled by random priming (DIG DNA Labelling Kit; Boehringer Mannheim), and hybridized and detected according to the chemiluminescent method described in the manufacturer's recommendations (DIG Nucleic Acid Detection Kit; Boehringer Mannheim).

Total RNA was isolated according to the protocol described by Sherman *et al.* (1986). Fifteen micrograms of total RNA per lane was loaded onto 1% (w/v) formaldehyde agarose gels and transferred by capillarity to nylon membranes (Hybond-N; Amersham) using $20\times$ SSC, according to standard protocols (Sambrook *et al.*, 1989). The probes used for hybridization of Northern blots were labelled by random priming using the DNA Labelling Beads (-dCTP) kit and [α - 32 P]CTP (Pharmacia Biotech). A probe for the *C. albicans* actin gene was used as a control for equal loading. Hybridization was carried out at 68 °C in a solution containing 0.5 M NaH_2PO_4 (pH 7.2), 7% SDS and 1 mM EDTA. After hybridization, the blots were washed twice with $2\times$ SSC containing 0.2% SDS at 68 °C for 20 min. The 0.24–9.5 kb RNA ladder (Gibco-BRL) was used for sizing in formaldehyde agarose gels.

PFGE. Chromosomal DNA was resolved by PFGE according to the protocol described by Wickes *et al.* (1991), in a Bio-Rad CHEF DR11 unit with a linear ramp switch time of 300–1400 s for 144 h at 13 °C. Gels were stained for 30 min with ethidium bromide (0.5 $\mu\text{g ml}^{-1}$ in water), destained in distilled water for 30 min and then photographed. The gels were blotted onto nylon membranes by alkaline transfer for 16 h. The probe used was radioactively labelled by random priming and hybridized as described above.

Isolation of the *CaHK1* gene. *CaHK1* was isolated and subcloned into three different fragments which overlapped, as previously described (Calera *et al.*, 1998). However, for further studies, an 8.4 kb *SpeI*–*NheI* DNA fragment which contains the entire ORF of *CaHK1*, was subcloned into the *SpeI*/*XbaI* restriction sites of pBSK+ (Stratagene) and used to construct the *CaHK1*–*URA3*–*hisG* cassette.

Construction of the vectors used in disruption and reconstitution experiments of *CaHK1*. To obtain Δcakh1 mutants, the pMB73 vector, which carries a cassette designed to delete most of the ORF of *CaHK1*, was constructed. A 1.25 kb *Bam*HI–*Bgl*II fragment, that includes the 3' end of *CaHK1*, was ligated to the only *Bgl*II site that flanks the 5' end of the *hisG*–*URA3*–*hisG* cassette in the pMB7 vector (Fonzi & Irwin, 1993), to generate pMB71. To complete the construction of pMB73, a 0.89 kb *Hind*III fragment located in the 5' end of *CaHK1*, 5.42 kb upstream from the 1.25 kb *Bam*HI–*Bgl*II fragment, was introduced in the appropriate orientation into the *Hind*III site that flanks the 3' end of the pMB71 cassette to generate pMB73 (Fig. 1a). The pMB73 plasmid was linearized by digestion with *SacI* which cuts once in the plasmid outside of the cassette. Approximately 15 μg trans-

forming DNA was used to transform the *Ura*[–] *C. albicans* strain CA14 according to the method described by Gietz *et al.* (1991). Transformed cells were selected as *Ura*⁺ on YNB minimal medium. One spontaneous *Ura*[–] derivative from a *Ura*⁺ independent clone was selected on YNB medium containing 5'-fluoroorotic acid (5'-FOA) and uridine, and used to delete the second allele of *CaHK1*. The disruption–transformation was repeated to generate a null mutant, and both pre- and post-5-FOA isolates were verified by Southern blot analyses. To obtain a reconstituted strain in one of the alleles, a Δcakh1 null strain was transformed with the *CaHK1*–*URA3*–*hisG* cassette as shown in Fig. 1(b).

RESULTS

Structural features of the N-terminus of Cakh1p

As we have previously reported, *CaHK1* encodes a 2471 aa hybrid histidine kinase (Cakh1p) whose C-terminal domain shows a strong homology with both the histidine and aspartyl domains from several bacterial and fungal histidine kinases (Calera *et al.*, 1998). The N-terminal domains of sensor histidine kinases are involved in the detection of specific environmental signals, and their primary structure determines the kind of stimuli they sense (Parkinson, 1993). In this regard the CaSln1p and CaNik1p histidine kinases from *C. albicans*, which show a high homology in their N-terminal domains with the proteins Sln1p from *S. cerevisiae* and Nik-1 from *N. crassa* which are involved in osmoregulation and morphogenesis, respectively, were reported to function similarly in *C. albicans* (Alex *et al.*, 1998; Nagahashi *et al.*, 1998). For this reason, we searched for similarities to the N-terminal domain of Cakh1p with other proteins in the databases, to predict the potential stimuli it could sense. This search revealed significant homology of the Cakh1p N-terminal domain with the putative PknB Ser/Thr kinase from *Mycobacterium tuberculosis* (GenBank accession no. Z80233) and less homology (but still significant) with the calcium/calmodulin-dependent kinases type I from different species. This homology lies between amino acids 463 and 640 of Cakh1p and 112 and 278 of PknB from *M. tuberculosis* (31.5% identity and 49.4% similarity). However, this homology is restricted to domains VIa–XI of PknB and other Ser/Thr kinases (Fig. 2a), which have been described to be largely responsible for binding of peptide substrates and initiating phosphotransfer (Hanks & Hunter, 1995). A P-loop motif, which could potentially be involved in ATP or GTP binding (Saraste *et al.*, 1990), was also found between amino acids 718 and 724 of Cakh1p, downstream of the homologous Ser/Thr-kinase XI-domain (Fig. 2b). On the other hand, from a recent search against the non-redundant database using the BLASTP program (Altschul *et al.*, 1990), we found a putative 2310 aa sensor histidine kinase from *S. pombe* which shows significant homology with Cakh1p (GenBank accession no. Z98978, hereafter called Sphk1 for *S. pombe* histidine kinase; the name given by the genome sequencing project is SPAC27E2.09). In addition to the similarity of the C-terminus of Sphk1 with the Cakh1p C-terminus, in which the histidine and aspartyl domains are located,

(a)

Cahk1p	463	PMKLEILTDMRVVETISVIELHGFVHNGLTSSNLLKSEKNVRDIKITGWGFASFSTENCSSQGYRNKHLAQVQDLIPYMAPEVLAITN	551
PknB	112	PKRAIEVIAD---ACQALNFSHQNGIHRDVVKPANIMISATNA--VKVMDFGIARAIADS---GNSVTQTAAVIGTAQYLSPE--QARG	190
		<div style="display: flex; justify-content: space-around; align-items: center;"> <div style="text-align: center;"> <p>Via</p> </div> <div style="text-align: center;"> <p>Vib</p> </div> <div style="text-align: center;"> <p>VII</p> </div> <div style="text-align: center;"> <p>VIII</p> </div> </div>	
Cahk1p	552	SVVDYRSDFYSLGVIMYELVLGILPFKNSNPQKLIRMHTFENPIAPALPGWISSEKLSGVIMKLEKHPHNRDCHSLLDHDLIEVKN	640
PknB	191	DSVDARSDVYSLGCVLYEVLGTGEPTTGDSPVSVAYQHVREDPIPPSARHEG--LSADLDVVLKALAKNPENRYQTAAEMRADLVRVHN	278
		<div style="display: flex; justify-content: space-around; align-items: center;"> <div style="text-align: center;"> <p>IX</p> </div> <div style="text-align: center;"> <p>X</p> </div> <div style="text-align: center;"> <p>XI</p> </div> </div>	

(b)

Sphk1	90	LLLNETLGAIIYD-DPGPNILD--EVLGNPFLDLKFLKLFALGVSVLCLFHEKKIVHGEIRLDTFHYDLNAPIHAKLLTIGSSVSPI	175
Cahk1p	432	LMQNHLIGATSTNGDPFHSKLAKFEYGVSKSEPMKLEILTDMRVVETISVIELHGFVHNGLTSSNLLKSEKNVRDIKITGWGFASF	520
Sphk1	176	RFTLSSLNWKRLYQVONICHKLQFFSPEQIGNVGRPLDSRDIYSLGILFYVILTQYVWGGQSMRIVQSIHMRFPFVLPFRPDFA--	262
Cahk1p	521	ENCSSQGYRNKHLAQVQDL---IPYMAPEVLAITNSVVDYRSDFYSLGVIMYELVLGILPFKNSNPQKLIRMHTFENPIAPALPGWIS	606
Sphk1	263	PALDQLIQKMTAKSMNSRISSATDLCTIVELMGEF--STITSSPLLDQKLLSINKPQEKLFKPKLLLTNSSDYVRIFHELVAFFSKR	349
Cahk1p	607	EKLSGVIMKLEKHPHNRDCHSLLDHDLIEVKNMYISKLLDSGETIPNSNLNLS-DRQYYLTKENLLHPEKMGITPVLGLKESFIGRR	694
Sphk1	350	DLTSAKRVKDKLPQHFLKYPVDNEATYQVVTITGEKSGKSNL---LNAVADEARKFGYFAMSSFKGHHFSPYSAIFKCVSLIMQQ	435
Cahk1p	695	DFLQNVTEV-----YNNKNGIDLLEISGESGRGRTIILQDLRAAAVLKQDFYYSWKFSFFGADTHVYRFLVEGVQKIITQ	770
		P-loop	
Sphk1	436	TLREKQLVTDYFTSLWEFLGFQLIYMGEFVPELNSLL	476
Cahk1p	771	ILNSSEEIQNTWRDVILTHIPIDL---SILFYLIPELKVLL	808

(c)

Sphk2	121	NFA---EFVRFCAICKCIEYLHSGMVGHEIRLDSFIPISYDNVYMLTV---GSGASYFHNCLOAHNWRKYSEDESMSRILFISPE	203
Cahk1p	462	SPMKLEILTDMRVVETISVIELHGFVHNGLTSSNLL--KSEKNVRDIKITGWGFASFSTENCSSQGYRNKHLAQVQDL---IPYMAPE	545
Sphk2	204	QTGRTSYSVGYRTDIYSLGVLFHYLSDCSPYTGFSVQIRISILT--EPLPDISKSCPKLPHLIFIKIEKMRKNPDERYTSCSGIVND	290
Cahk1p	546	VLAITNSVVDYRSDFYSLGVIMYELVLGILPFKNSNPQKLIRMHTFENPIAPALPGWISSEKLSGVIMKLEKHPHNRDCHSLLDH	634
Sphk2	291	LEACLDIDKGLILNDHVL---EKTGRTSLFYLPCSIYGREHEIKLIRKILRNSPRAINHQDKKDLTFN	357
Cahk1p	635	LEVKNMYISKLLDSGETIPNSNLNLSDRQYYLTKENLLHPEKMGITPVLGLKESFIGRRDFLQNVTEVYNNKNGID-----	712
Sphk2	358	PYYLNAIESSESSQSLSLSQRASEVMPLVILITGCEGIGESSLIQTI---CDRREGYMAITKFEV--SQSIVYSAIVSAVAEFIRQILA	441
Cahk1p	713	LLFISGESGRGRTIILQDLRAAAVLKQDFYYSWKFSFFGADTHVYRFLVEGVQKIITQILN	773
Sphk2	442	EDQLLLNNFFFEELKNKLESDDLVSVDLVEPIRSLL	479
Cahk1p	774	SSEEIQNTWRDVILTHIPIDL---SILFYLIPELKVLL	808

(d)

Fig. 2. (a) Comparison of Cahk1p and the putative PknB Ser/Thr kinase from *M. tuberculosis*. Only the Via–XI domains of PknB, which have homology with Cahk1p, are shown. The conserved residues in all Ser/Thr kinases are indicated with an asterisk, and those residues also conserved in Cahk1p are shown in bold and indicated by bold asteriks. (b, c) Comparison of the N-terminal domains of Cahk1p and Sphk1 (b) and the N-terminus of Cahk1p and Sphk2 (c). The exact location of the homologous Ser/Thr-kinase domains are bracketed, and the conserved P-loop motifs are boxed. The asterisks indicate those residues conserved in all of the Ser/Thr kinases. (d) Comparison of the *C. albicans* histidine kinases (Cahk1p, CaSn1p and CaNik1p) with the putative sensor kinases from *S. pombe* (Sphk1 and Sphk2). In Cahk1p, Sphk1 and Sphk2, the positions of the Ser/Thr-homologous domains (hatched boxes), and the adjacent P-loop motifs in Cahk1p and Sphk1 (small black boxes) are shown. Also the histidine (white boxes) and the aspartyl domains (large black box) of these proteins, and from CaSn1p and CaNik1p are indicated. The proteins were aligned with the putative autophosphorylated histidine residue as a reference point.

also the same structural features in their N-terminal domains were observed, including the homologous Ser/Thr-kinase domains and the P-loop motif (20.7% identity, 40.0% similarity) (Fig. 2b). In addition, we have recently found a second putative 2344 aa histidine kinase from *S. pombe* which also exhibits similarity with both the N- and C-termini of Cahk1p [GenBank accession no. AL031543, hereafter called Sphk2; the name given by the genome sequencing project is SPCC74.06]. The homology between the N-terminal domains of Cahk1p and Sphk2 was restricted to their Ser/Thr-kinase domains (24.1% identity, 46.0% similarity) (Fig. 2c). However, contrary to Sphk1 and Cahk1p, Sphk2 does not have a P-loop motif. Also, in these putative sensor kinases from *S. pombe*, the Ser/Thr-kinase domains and the P-loop (in Sphk1) are arranged in the same relative positions as in Cahk1p (Fig. 2d). Thus, in Cahk1p and Sphk1, the conserved arginine residues in the Ser/Thr homologous XI domains are separated from their corresponding P-loops by 94 and 106 aa respectively, and the P-loops from Cahk1p and Sphk1 are separated from the corresponding putative autophosphorylated histidine residues by 1289 and 1395 aa, respectively. Similarly, the conserved arginine residue in the Ser/Thr homologous XI-domain of Sphk2 is separated from its corresponding putative autophosphorylated histidine residues by 1515 aa.

Construction of a *CaHK1* null mutant

To characterize the function of *CaHK1* in the growth and morphogenesis of *C. albicans*, we used the urablaster technique (Fonzi & Irwin, 1993) for obtaining a Δ *cahk1* null mutant. We also constructed a strain reconstituted for one allele to ensure that the resulting phenotype was not due to extraneous mutations that could happen during transformation. Thus, we used a cassette (see Methods) to replace a 5.42 kb fragment of *CaHK1* which includes the coding regions for the conserved Ser/Thr-kinase domains, the P-loop motif and putative autophosphorylated histidine (Fig. 1a). After the first round of transformation, Ura⁺ transformants were selected on YNB minimal medium and 15 isolates were tested by Southern blotting to confirm this replacement. The results showed that all transformants had incorporated the *hisG*-*URA3*-*hisG* cassette in the predicted site. DNA from a representative isolate (strain CHK11) exhibited two *CaHK1* hybridizing bands: a 3.3 kb *Bgl*II fragment characteristic of the parental strain and an additional fragment of 7.07 kb, consistent with the replacement in one allele of *CaHK1* with the *hisG*-*URA3*-*hisG* cassette (Fig. 1c). Ura⁻ segregants of strain CHK11 were selected on 5'-FOA plates and examined by Southern blotting. Strain CHK11 is a representative intrachromosomal recombinant (Fig. 1c). The 7.07 kb *Bgl*II fragment seen in strain CHK11 which had contained the Δ *cahk1*::*hisG*-*URA3*-*hisG* disruption was absent in CHK12, and a new 4.34 kb hybridizing fragment was present. The size of this fragment is consistent with the desired event, loss of *URA3* and one copy of *hisG*. Following the same protocol, a second

Fig. 3. CHEF gel analysis of the Δ *cahk1* null (CHK21) and revertant (CHK23) strains. CAI4 and CAF2 were used as controls. The chromosome bands were visualized by ethidium bromide staining (left panel), transferred to a nylon membrane and probed with a 0.89 kb fragment located at the 5' end of the *CaHK1* gene as a probe (right panel). A hybridizing band corresponding to chromosome 2 was detected. The chromosomal designations are indicated on the left, according to Wickes *et al.* (1991).

round of transformation was performed and the remaining allele was disrupted. In Fig. 1(c) a representative Ura⁺ isolate (strain CHK21) and a Ura⁻ segregant (strain CHK22) are shown. A strain reconstituted for one allele was constructed after transformation of CHK22 with the *CaHK1*-*URA3*-*hisG* cassette. This cassette allowed the integration event to occur between the remaining 5' end of *CaHK1* and the *hisG* that had replaced 5.42 kb of the *CaHK1* sequence in CHK22 (Fig. 1b). Ura⁺ transformants were selected on YNB medium, and the integration of the transforming DNA in the Δ *cahk1*::*hisG* locus was verified by Southern-blot analysis from several isolates. Southern-blot analysis of one representative isolate (CHK23) is shown in Fig. 1(c). The 3.3 kb *Bgl*II fragment that was detected is consistent with the replacement of one disrupted allele (Δ *cahk1*::*hisG*) of the CHK22 strain with the *CaHK1*-*URA3*-*hisG* cassette, restoring one *CaHK1* allele.

Chromosomal location of *CaHK1*

To show that in the null and revertant strains, the *CaHK1* locus had not undergone extraneous chromosomal rearrangements during their manipulation, chromosomes from these and the control strains CAF2 and CAI4 were separated by PFGE and probed with a 0.89 kb *Hind*III fragment from the 5' end of *CaHK1*. Results showed that *CaHK1* is located on chromosome 2 in the four strains (Fig. 3). Further, additional sequencing of the 3' end of *CaHK1* also contributed to the mapping studies, in that the stop codon of *CaHK1* is located only 136 bp from the stop codon of the *CaHOG1* gene, which also is located on chromosome 2 (San Jose *et al.*, 1996).

Phenotypic analysis of the $\Delta cahk1$ mutants

The effect of the *CaHK1* deletion on the phenotype of the $\Delta cahk1$ mutants was studied under conditions that promote both yeast and hyphal growth in *C. albicans*. Thus, the Ura^+ $\Delta cahk1$ mutants (CHK11 and CHK21) and the control strain (CAF2) were grown in a rich medium (YED at pH 3.5 and pH 7.5) and a synthetic medium (medium 199 at pH 3.5 and pH 7.5) at 28 °C and 37 °C. Also, because many of the eukaryotic histidine kinases act as osmosensors, each medium was supplemented with 1.5 M NaCl or 1 M sorbitol to determine if *CaHK1* was also involved in osmoregulation. In both liquid media at pH 3.5, we observed that the disrupted strains grew like CAF2 but at pH 7.5 the disrupted strains showed extensive aggregation that we defined as a flocculation-like phenotype (Fig. 4a). The flocculation was more dramatic in medium 199 at pH 7.5 than in complex media at the same pH. In addition, flocculation was more extensive in the null strain than in the single-allele-disrupted strain, indicating a gene-dosage-dependent phenotype (Fig. 4a). Also, it was observed that flocculation of CHK21 was much less extensive in the presence of osmostressing agents and under these conditions of growth the appearance of the culture in liquid media was similar to that of CAF2 at pH 7.5 (Fig. 4a).

Using medium 199 to perform further analysis of this phenotype, it was observed that at 28 °C the flocculation became apparent in the null strain after 3 h culture but only after 4 h did the flocculation become obvious in the heterozygote. The same phenomenon was observed at 37 °C but flocculation became apparent earlier in both the null and heterozygote strains. Thus, it seems that temperature has no effect on the phenotype except to accelerate the flocculation process, most likely as a consequence of faster growth of the cells at 37 °C. These dramatic changes in the growth of the disruptants were not observed in the control strain CAF2. Microscopic observations were consistent with the macroscopic appearance of liquid cultures from CAF2 and the CHK21 $\Delta cahk1$ null strain. Thus, CHK21 formed germ tubes at pH 7.5 at the same rate as CAF2 (after 1.5 h, 100% of the cells have formed germ tubes in CAF2 and CHK21), but CHK21 formed larger aggregates that rapidly sedimented (Fig. 4a). A microscopic study (Fig. 4b) demonstrated that, even though germ-tube formation in CHK21 occurred as fast as in CAF2 (Fig. 4b, bottom left panel), flocculation in the $\Delta cahk1$ null strain primarily occurs through interactions along the hyphal surface which become clustered in stacks (Fig. 4b, bottom right panel). However, at pH 3.5 the $\Delta cahk1$ null strain, as well as CAF2 (Fig. 4b, top left panel), grew as yeasts but the CHK21 cells showed a more elongated shape (Fig. 4b, top right panel).

On the other hand, to understand the effect of osmostressing agents on flocculation we considered that increased osmolyte concentration inhibits hyphal development in a wild-type strain of *C. albicans* as previously reported (Alex *et al.*, 1998). However, the

Fig. 4. (a) Broth cultures of both Ura^+ $\Delta cahk1$ mutants (CHK11 and CHK21) and revertant strain CHK23. Stationary-phase cells from all strains were inoculated (10^7 cells ml^{-1}) in medium 199 at either pH 3.5 or pH 7.5, grown at 37 °C for 3 h and photographed. Only at pH 7.5 did the null strain flocculate, forming clumps of cells that sedimented rapidly after the cell suspension was shaken. Also, in the heterozygotes some flocculation is observed at pH 7.5. (b) Microscopic observations of CAF2 and the $\Delta cahk1$ null strain (CHK21) grown in medium 199 at pH 3.5 (top panels) and pH 7.5 (bottom panels) for 3 h at 37 °C. Flocculation occurs by an intimate interaction of hyphae along their surface forming stacks of germinated cells (bottom right panel). Bar, 6 µm.

osmolyte concentrations that we have used (1.5 M NaCl and 1 M sorbitol) did not alter the rate of germination of CAF2 and CHK21 compared to cells grown in absence of osmostressing agents (100% germination after 1.5 h in both), but did retard the elongation of hyphae in both strains. In fact, the elongation of the germ tubes of CHK21 was more retarded by osmostressing agents than CAF2 (after 1.5 h the length of the germ tubes from CHK21 was half that of CAF2). Thus, this is consistent with the reduced flocculation of the $\Delta cahk1$ null strain observed in medium 199 (pH 7.5) supplemented with 1.5 M NaCl or 1 M sorbitol. Since flocculation occurs primarily along the entire length of the hyphal surface, the reduced elongation of germ tubes in presence of

Fig. 5. Colony phenotype of the $\Delta cakh1$ mutants (CHK11 and CHK21). The CAF2 and CHK23 strains were included as controls. All strains were grown on medium 199 agar at either pH 3.5, 28 °C or pH 7.5, 37 °C for 4 d. At pH 3.5, all strains grew like yeasts and formed smooth colonies, including the $\Delta cakh1$ mutants (c, e). At pH 7.5 the CHK11 (heterozygote, panel d) and CHK23 (revertant, panel h) formed less fuzzy colonies in comparison to CAF2 (b). However, the $\Delta cakh1$ null strain (CHK21) formed non-fuzzy colonies (f). Bar, 1 mm.

osmopressing agents also reduces the amount of flocculation. Hence, the effects of osmopressure upon the flocculation of the $\Delta cakh1$ mutant appears to be indirect.

Colony phenotypes on solid media were also evaluated on medium 199 at pH 3.5 and 7.5 (Fig. 5a–f). We observed that the control and heterozygote strains at pH 7.5 produced colonies with hyphae emerging from the periphery of the colonies (Fig. 5b and d), but the null strain formed non-fuzzy colonies (Fig. 5f). Microscopic observations of the $\Delta cakh1$ null strain from non-fuzzy colonies revealed that these contained filamentous cells as well as an extensive aggregation of hyphae similar to that observed in liquid media. At pH 3.5, the $\Delta cakh1$ mutants formed smooth-edged colonies and grew similarly to CAF2 (Fig. 5a, c and e).

To study the invasiveness of agar of the $\Delta cakh1$ mutants in comparison with the control strain (CAF2), we grew these strains in SLAD medium, which promotes pseudohyphal formation, as described by Csank *et al.* (1998). However, no differences between the control strain and the $\Delta cakh1$ null strain were observed.

Fig. 6. Inhibition of flocculation by SDS and NaCl. Cells of a $\Delta cakh1$ mutant were prepared as described in the text. Cells were resuspended in a buffer (10 mM Tris/HCl, pH 8.0, 2 mM EDTA) without additives, in which they flocculate (right). The addition of 4% (w/v) SDS (final concentration) inhibits flocculation (left) but the addition of 5 M NaCl (final concentration) partially inhibits flocculation (middle). The addition of other additives such as DTT, mannose, glucose or maltose had no effect on flocculation. Cell suspensions incubated in the presence of these additives appeared as pictured on the right.

Finally, the strain reconstituted for one allele (CHK23), in liquid medium 199 at pH 3.5 also grew as yeast and at pH 7.5 flocculated less than the $\Delta cakh1$ null strain (CHK21), but similar to the heterozygote strain (CHK11) (Fig. 4a). Also, the microscopic appearance and the morphology of the colonies in medium 199 at pH 3.5 and 7.5 were similar to that of the heterozygote (CHK11) (Fig. 5g and h). This indicated that the flocculation-like phenotype that was exhibited by the $\Delta cakh1$ null strain was really due to the absence of *CaHK1* and not to additional mutations that could be introduced during the disruption process.

Additional studies were carried out to quantify the rate of flocculation in the mutants compared to control strains using the method described by Miki *et al.* (1982a, b) for flocculation in *S. cerevisiae*. We used several putative inhibitors of flocculation mediated by lectin-like interactions such as 1 M mannose, 1 M glucose and 1 M maltose, and other inhibitors that disrupt protein–protein interactions such as 5 M NaCl, 50 mM DTT and 4% (w/v) SDS. To do these experiments, stationary-phase cells from each strain grown in YPD were used as inoculum in medium 199 (pH 7.5). After 3 h culture at 37 °C, the cells were collected, washed twice in 10 mM Tris/HCl pH 8.0 and 2 mM EDTA, and resuspended in the same buffer containing inhibitors of flocculation at the final concentrations described above. The addition of CaCl_2 was not required for flocculation. Unfortunately, the experiments to quantify the flocculation were unsuccessful due to the dramatic flocculation which normally occurred with the null strain. Only visual observations to determinate if flocculation was or was not inhibited could be performed. Thus, the inhibitor studies showed that only SDS totally inhibited flocculation and NaCl partially inhibited this process (Fig. 6), while other inhibitors did not have any effect, indicating that flocculation may be caused by a protein–protein interaction rather than a lectin-like interaction

Fig. 7. Northern analyses of *CaHK1* and genes which could potentially be regulated by *CaHK1*. Total RNA was obtained from the Δ *cahk1* mutants (CHK11 and CHK21) and CAF2 grown in medium 199 at pH 3.5 and 7.5 at 28 °C for 4 h. To detect *CaHK1* mRNA, a fragment of the ORF that hybridizes with the part of the gene that was deleted in the mutants was used as a probe. For the other genes, specific probes against their ORFs were used. Expression of the actin gene (*ACT1*) was used as a control.

since flocculation was totally or partially inhibited by compounds that disrupt protein–protein interactions. However, this does not preclude that additional molecular mechanisms could be responsible for flocculation. Also, the mechanisms that mediate hyphal flocculation seem to be different from those in *S. cerevisiae*. Flocculation in *S. cerevisiae* is mediated by the *FLO1* gene and is Ca^{2+} -dependent (Miki *et al.*, 1982a, b). In contrast, flocculation in the Δ *cahk1* null strain of *C. albicans* was not Ca^{2+} -dependent.

Northern analysis of *CaHK1* expression and other genes whose expression could be potentially regulated by *CaHK1*

As growth differences (flocculation) were seen when the disrupted strains were grown in medium 199 at pH 7.5, we chose this medium to study the expression of *CaHK1* and several other genes whose expression could be potentially under the control of *CaHK1*. Since flocculation appeared to be related to the pH of the culture media, we measured the expression of *CaHK1* at pH 3.5 and 7.5. Also, we measured the expression of pH-regulated genes such as *PHR1* and *PHR2* (Muhlschlegel & Fonzi, 1997; Saporito-Irwin *et al.*, 1995) in the Δ *cahk1* mutants. Likewise, since flocculation could be related to an altered expression of proteins involved in adhesion, we also tested *ALS1*, whose encoded protein (Hoyer *et al.*, 1995) has been associated with adhesion (Fu *et al.*, 1998a). Finally, we tested whether the expression of the *CAD1/AAF1* gene from *C. albicans*, which has recently been reported to confer a Flo1-like phenotype when it is expressed in *S. cerevisiae* (Fu *et al.*, 1998b), was under the control of *CaHK1*. We performed these experiments using RNA obtained from control (CAF2) and Ura⁺ disrupted strains (CHK11 and CHK21) grown in medium 199 either at pH 7.5 or pH 3.5 at both 28 and 37 °C for 4 h. The results showed that *CaHK1* is expressed in medium 199 either at pH 3.5 or pH 7.5 at both 28 and 37 °C. According to the signal on Northern blots, the heterozygote mutant showed a signal weaker than CAF2, corresponding to a smaller amount of mRNA; no expression was detected in the CHK21 null strain (Fig. 7). Those genes whose expression could be putatively altered in the Δ *cahk1* mutants showed the same pattern of expression that was previously reported for all of

them (Muhlschlegel & Fonzi, 1997; Saporito-Irwin *et al.*, 1995; Hoyer *et al.*, 1995; Fu *et al.*, 1998b), and no changes in their expression were seen in the Δ *cahk1* background (Fig. 7).

DISCUSSION

We have recently reported the isolation and cloning of the *CaHK1* histidine-kinase gene from *C. albicans* (Calera *et al.*, 1998). As with many other histidine kinases, *Cahk1p* also shows homology with the C-terminal domain of *CaSln1p* and *CaNik1p* (Alex *et al.*, 1998; Nagahashi *et al.*, 1998), however, this homology is less than that seen with several prokaryotic histidine kinases. Thus, the strongest similarity with *Cahk1p* is a 1261 aa histidine kinase from the cyanobacterium *Synechocystis* (GenBank accession no.: D90910); again, this similarity is confined to the C-terminus of this protein. Recently, we have observed that a putative 2310 aa histidine kinase from *S. pombe* (*Sphk1*) exhibited the highest overall homology with *Cahk1p*. To date, the Ser/Thr-homologous domains, the P-loop motif, the histidine and aspartyl domains, as well as their relative positions are conserved in both *Cahk1p* and *Sphk1*. Unfortunately, no information is available about the role of *Sphk1* in *S. pombe*. However, it is possible that these sequences may act as sensor domains for an unknown stimulus. On the other hand, all of the sensor histidine kinases described so far involved in the detection of environmental stimuli have hydrophobic domains which are used to anchor the protein to the plasma membrane so that the sensor domain is exposed on the outside of the membrane to sense the environmental stimulus. *Cahk1p* as well as *Sphk1* do not have, according to their amino acid sequences, any hydrophobic domains that could span the membrane. Thus, the stimuli they detect may be cytoplasmic. The way in which this stimulus is sensed is unknown, but even an autophosphorylation process in their N-terminal domains can not be precluded. In this regard, phytochromes from plants, which at their C-terminal domains look like histidine kinases, are presumably activated by autophosphorylation in a serine residue located at their N-terminal domains (Elich & Chory, 1997). In addition, although the N-terminal domain of *Cahk1p* does not show homology with the I–IV domains of Ser/Thr

kinases, which are primarily involved in anchoring and orienting the nucleotide (ATP/GTP) (Hanks & Hunter, 1995), the P-loop, which is located close to the homologous VI–XI domains in Cahk1p could bind the appropriate nucleotide.

Chromosomal mapping led us to locate *CaHK1* on chromosome 2 with its stop codon near to the stop codon of *CaHOG1*. Thus, *CaHK1* and *CaHOG1* are transcribed in opposite directions, sharing the same terminator sequence. In *S. cerevisiae*, Sln1p acts as an osmosensor in the HOG pathway in which Hog1p participates as a MAPK (mitogen-activated protein kinase) involved in activating the synthesis of glycerol (Posas *et al.*, 1996). The *C. albicans* homologue of this MAPK (CaHog1p) has been identified also as a MAPK involved in the synthesis of glycerol, the major osmoprotectant in *C. albicans* against hyperosmotic conditions (San Jose *et al.*, 1996). The close proximity of these two genes in the genome of *C. albicans* led us to suppose a cluster-like arrangement of these genes. However, no effects on the growth of the Δcahk1 null strain were seen in hyperosmotic media. Thus, it seems that the close proximity of these genes on the chromosome does not have any apparent functional consequence.

The most obvious effect of the *CaHK1* deletion is that cells flocculate extensively under conditions that stimulate germ-tube formation. Microscopic observations revealed that flocculation occurs primarily because of the interaction of hyphal surfaces in the Δcahk1 null but not in the control strain. However, flocculation not only occurs in liquid media, but also when cells are grown on solid medium 199 at pH 7.5. In fact, this observation is strongly supported by the morphology of the colonies of the Δcahk1 null strain on solid medium. This strain forms non-fuzzy colonies probably because the flocculation, which occurs within the colonies, does not allow cells to grow normally and form fuzzy colonies as in the control, even though germination of the Δcahk1 null strain readily occurred.

On the other hand, in the presence of NaCl or sorbitol, the Δcahk1 null strain does not flocculate as extensively as in absence of osmopressing agents in the medium. This observation is actually what we would expect if we consider that high osmolarity inhibits hyphal development in the wild-type strains of *C. albicans* (Alex *et al.*, 1998). Thus, as reported for *CaNIK1/COS1* (Alex *et al.*, 1998), such differences between the wild-type and mutants are difficult to assess. However, even though the effect of osmopressure upon the flocculation of the Δcahk1 mutants seems to be indirect, the reduced elongation of the germ tubes from the Δcahk1 mutants in presence of osmopressing agents indicates that we can not totally preclude the possibility that *CaHK1* could have some effect in osmosensing. In fact, the phenotype observed on solid surfaces could be due to an osmotic-like effect.

Many components of the *C. albicans* cell wall have been assigned an adhesion function (Chaffin *et al.*, 1998).

Among these are the agglutinin-like proteins such as those of the ALS family or others like Aaf1p, which recently has been reported more likely to be a transcription factor (Fu *et al.*, 1998b) than a cell-wall-associated protein. Recently, experiments that involved the expression of the *ALS1* and *AAF1* in *S. cerevisiae* have reported that both genes enhance adherence of *S. cerevisiae* to epithelial and endothelial cells, respectively (Fu *et al.*, 1998a, b). Expression of *AAF1*, which is known to be an aggregation factor, stimulates endothelial cell adherence by inducing a flocculation phenotype similar to the Flo1 phenotype of *S. cerevisiae* (Fu *et al.*, 1998b). However, according to the results obtained by Northern analysis in our studies, Als1p (a cell surface protein) and Aaf1p (a potential transcription factor) do not seem to be the components associated with the flocculation-like phenotype, since their level of expression is not altered in the disrupted strains. Nevertheless, other cell-wall components have been described whose expression could potentially be regulated by *CaHK1*, such as Ala1p (Gaur & Klotz, 1997), Hwp1p (Staab *et al.*, 1996), Als3p (Hoyer *et al.*, 1998) or any other member of the ALS family. In addition, expression of *PHR1* and *PHR2*, both pH regulated (Muhlschlegel & Fonzi, 1997; Saporito-Irwin *et al.*, 1995) were also not affected by the Δcahk1 null mutation. However, other pH-regulated genes may exist in *C. albicans* which could potentially be under the control of *CaHK1*. Finally, the expression of *CaHK1* is not dependent on pH, temperature or growth morphology. However, even though *CaHK1* could be regulated at the translational level, it seems more than likely that, as with other sensor histidine kinases involved in detection of environmental changes, the activity of Cahk1p could be modulated by its state of phosphorylation rather than by the expression of *CaHK1*.

In conclusion, our results indicate that *CaHK1* may be involved in the regulation of the expression of at least one hyphal-surface component, since the Δcahk1 null strain flocculates more extensively than the control strain under those conditions which induce hyphal formation (growth in medium 199, pH 7.5). This also is an indication that changes in the development of hyphae have occurred upon the deletion of *CaHK1*. Finally, we can not preclude the possibility that Cahk1p could act as an internal pH sensor used by cells as a signal to trigger the synthesis of hyphal surface components or even play some role in osmosensing. Ongoing investigations in our lab. are focused upon the identification of genes that are differentially displayed between the control strain and the Δcahk1 null strain under those conditions that induce germ-tube formation.

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