The two-component hybrid kinase regulator CaNIK1 of Candida albicans

Thyagarajan Srikantha, Luong Tsai, Karla Daniels, Lee Enger, Kumiko Highley and David R. Soll

Using degenerate primers of highly conserved regions of two-component response regulators for PCR amplification, a two-component response regulator was cloned from Candida albicans that is homologous to nik-1+ of Neurospora crassa. This two-component hybrid kinase, CaNIK1, also shows features of bacterial two-component response regulators, including a putative unorthodox second histidine kinase motif at the carboxy-terminal end. CaNIK1 was expressed at low levels in both the white and opaque switch phenotypes and in the bud and hyphal growth forms of C. albicans strain WO-1, but in both developmental programmes, the level of transcript was modulated (levels were higher in opaque cells and in hyphae). Partial deletion of both CaNIK1 alleles, by which the histidine autokinase- and ATP-binding domains were removed, did not inhibit either high-frequency phenotypic switching or the bud-hypha transition in high salt concentrations, but in both cases the efficiency of the developmental process was reduced.

Keywords: Candida albicans, histidine kinase, phenotypic switching, CaNIK1, two-component hybrid kinase regulator

INTRODUCTION

Candida albicans is the most successful fungal commensal in humans. It colonizes the oral cavity, gastrointestinal tract and genitalia of healthy individuals and, as an opportunistic pathogen, is well positioned to cause a subsequent infection as a result of a predisposing alteration in host physiology (Odds, 1988). In the case of vaginal candidiasis, the predisposing condition is not always obvious (Sobel, 1996), and otherwise healthy women suffer frequent recurrence. In the case of immunocompromised individuals, C. albicans can become systemic and invade a variety of tissues and organs. In a significant number of cases, the patient dies (Wey et al., 1989). C. albicans is, therefore, capable of residing as a commensal in a number of quite different anatomical locations, and, as a pathogen, is capable of invading just about every tissue in the human body. DNA fingerprinting analyses of commensal isolates from different body locations in the same individuals have demonstrated that strains and substrains rapidly adapt to specific body locations (Soll et al., 1991). In addition, individual strains of C. albicans have been demonstrated to be capable of evading drug therapies in recurrent vaginitis (Schmid et al., 1993; Lockhart et al., 1996), of becoming resistant to azole antifungals (Rex et al., 1995; Nguyen et al., 1996) and of evading the immune system. C. albicans, therefore, is highly adaptive, suggesting that it can rapidly modify its own physiology and phenotype in response to environmental changes. It has also been demonstrated that each strain of C. albicans, in addition to being capable of differentiating in a reversible fashion between a budding and a hyphal growth form (Soll, 1986), is capable of undergoing high-frequency phenotypic switching between a limited number of general phenotypes that differ in a variety of traits, including putative virulence factors (Slutsky et al., 1985; Soll, 1992). The frequencies of both these developmental programmes are influenced by environmental conditions. In the case of the bud–hypha transition, pH and temperature can be used to induce mass conversion (Lee et al., 1975; Buffo et al., 1984; Soll, 1986) and in the case of high-frequency phenotypic switching, temperature, UV irradiation, white blood cell metabolites and colony ageing affect the frequency, in some cases by several orders of magnitude (Slutsky et al., 1985, 1987; Rikkerink et al., 1988; Morrow et al., 1996).

Abbreviation: FOA, 5-fluorooorotic acid.
The GenBank accession number for the sequence reported in this paper is AF029092.
1989; Soll, 1992; Kolotila & Diamond, 1990). In addition, C. albicans hyphae are able to assess topographical changes in the supporting substrate and turn corners (Sherwood et al., 1992). C. albicans must, therefore, contain sensors that assess the environment.

Recently, Fonzi and co-workers cloned two genes that are differentially expressed in two pH ranges and are involved in sensing environmental pH in the regulation of the bud–hypha transition (Saporito et al., 1995; Muhlschlegel & Fonzi, 1997). In bacteria and lower eukaryotes, autophosphorylating histidine kinases, or two-component response regulators, have been demonstrated to sense the external environment for a variety of parameters, providing these organisms with the capacity to respond rapidly to an environmental perturbation (Saier, 1994; Alex & Simon, 1994; Loomis et al., 1997).

Because two-component response regulators have been identified in other fungi (Ota & Varshavsky, 1993; Alex et al., 1996), we examined their possible role in the developmental programmes of C. albicans. Using degenerate primers of highly conserved regions of the two-component response regulator gene, we (Brown et al., 1996) identified a response regulator homologous to the two-component hybrid kinase gene from another strain of C. albicans. This gene, CaNIK1, was first cloned (Nagahashi et al., 1998) from another strain of C. albicans. In addition, CaSLN1, a two-component response regulator homologous to the two-component response regulator gene from C. albicans, has been cloned (Nagahashi et al., 1998) from C. albicans. CaNIK1 and CaSLN1, respectively, are homologous to the two-component response regulator gene from the budding yeast S. cerevisiae (Safer, 1994; Maeda et al., 1994; Varshavsky, 1993; Simon, 1996), and the two-component hybrid kinase gene from the filamentous fungi Neurospora crassa (Alex et al., 1996). This gene, Slb1, has been recently cloned from the yeast Saccharomyces cerevisiae (Fonzi et al., 1996), var1, and in the filamentous fungi Neurospora crassa (Alex et al., 1996). This gene, Slb1, has been recently cloned from the yeast Saccharomyces cerevisiae (Fonzi et al., 1996).

In N. crassa, nik-1 + is expressed in the vegetative hyphal stage, but not in the sexual protoperithecial stage (Alex et al., 1996). Deletion of the gene resulted in an aberrant hyphal morphology that was accentuated in high salt concentration (Alex et al., 1996). Here, we demonstrate that CaNIK1 is transcribed in both budding cells and hyphae of C. albicans and in both phases of the white–opaque transition in C. albicans strain WO-1. However, during opaque-phase cell growth, the level of CaNIK1 transcript reaches a level twice that reached during white-phase cell growth. Because there is no effective gene knockout system for strain WO-1, which undergoes the highly characterized white–opaque transition, CaNIK1 was partially deleted in C. albicans strain CA18 (Fonzi & Irwin, 1993). Deletion of the histidine autokinase- and ATP-binding domains of the two alleles of CaNIK1 did not inhibit the bud–hypha transition, nor did it affect the hyphal phenotype, even at high ionic strength. It also did not inhibit high-frequency phenotypic switching. It did, however, slow hyphal development at high ionic strength and reduce the frequency of UV-induced switching. Partial deletion of CaNIK1 had no significant effect on growth at high osmotic strength, nor did it significantly increase sensitivity to calcifluor, caffeine, hygromycin, echinocandin or polymyxin B. The precise physiological role of CaNIK1 in C. albicans, therefore, remains to be elucidated.

**METHODS**

**Strains and growth conditions.** C. albicans strains WO-1 (Slutsky et al., 1987) and 3153A (Slutsky et al., 1985) were grown on modified Lee's medium (Bedell & Soll, 1979). Strain CA18, an ade2 ura3 auxotroph, was maintained on modified Lee's medium supplemented with adenine (40 µg ml⁻¹) and uridine (25 µg ml⁻¹). Other mutant derivatives of CA18 were maintained on media supplemented with those ingredients necessary for growth according to their genotypes (Table 1).

**PCR amplification of a two-component hybrid kinase gene homologue in C. albicans.** The three following deoxynosine-containing degenerate primers were designed that encompassed the highly conserved regions of the two-component response regulators lemA (Hrabak & Willis, 1992), barA (Nagashawa et al., 1992) and SLN1 (Ota & Varshavsky, 1993) for PCR amplification, we (Brown et al., 1998) cloned a gene from C. albicans strain WO-1 that encodes a protein homologous to the two-component response regulator gene, nik-1 + of Neurospora crassa (Alex et al., 1996). This gene, CaNIK1, has also been recently cloned (Nagahashi et al., 1998) from another strain of C. albicans. In addition, CaSLN1, a two-component response regulator homologous to the two-component response regulator gene from C. albicans, has been cloned (Nagahashi et al., 1998) from C. albicans. CaNIK1 and CaSLN1, respectively, are homologous to the two-component response regulator gene from the budding yeast S. cerevisiae (Safer, 1994; Maeda et al., 1994; Varshavsky, 1993; Simon, 1996), and the two-component hybrid kinase gene from the filamentous fungi Neurospora crassa (Alex et al., 1996). This gene, Slb1, has been recently cloned from the yeast Saccharomyces cerevisiae (Fonzi et al., 1996).

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**Nucleotide sequence of PCR products.** Plasmid DNA was isolated using the CsCl/ethidium bromide protocol described earlier (Sambrook et al., 1989). The nucleotide sequence of each plasmid insert was determined in both directions by using the ABI model 373A Auto Sequence system (Perkin Elmer/Applied Biosystems), the PCR cycle sequencing protocol and fluorescent dye terminator dideoxynucleotides (Perkin Elmer/Applied Biosystems). The electropherograms were edited with Edit View software (Perkin Elmer/Applied Biosystems). Homology and alignment of nucleotide sequences with those in gene and protein databases were performed with DNAsis software (Hitachi Software Engineering).

**Cloning and sequence analysis of the CaNIK1 gene.** To isolate a full-length gene, approximately 8 × 10⁴ plaques of a C. albicans genomic library (Srikantha & Soll, 1993) were screened using a 1.2 kb DNA fragment isolated from...
Table 1. C. albicans strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>CA18</td>
<td>ade2::hisG/ade2::hisG/Δura3::imm434/ura3::imm434</td>
<td>Fonzi &amp; Irwin (1993)</td>
</tr>
<tr>
<td>CA18U5</td>
<td>ade2::hisG/ade2::hisG/ura3::imm434/URA3</td>
<td>This study</td>
</tr>
<tr>
<td>NNL6</td>
<td>ade2::hisG/ade2::hisG/Δura3::imm434/Δura3::imm434/nik1::hisG–URA3–hisG/NIK1</td>
<td>This study</td>
</tr>
<tr>
<td>NNS7</td>
<td>ade2::hisG/ade2::hisG/Δura3::imm434/Δura3::imm434/nik1::hisG–URA3–hisG/NIK1</td>
<td>This study</td>
</tr>
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<td>This study</td>
</tr>
<tr>
<td>HH80</td>
<td>ade2::hisG/ade2::hisG/Δura3::imm434/Δura3::imm434/nik1::hisG/Δura3::imm434/hisG–URA3–hisG</td>
<td>This study</td>
</tr>
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</table>

pCN1.3/13, which spanned the histidine autokinase- (H1) and aspartyl receiver (D1) domains. Lambda DNA from 20 positive clones was extracted and Southern blots probed with pCN1.3/13. Using combinations of primer pairs for the arms of the lambda DNA and either the degenerate primers for the histidine autokinase domain (Slb1) or the response regulator domain (Slb3), lambda clones containing inserts larger than 4 kb were identified. The screen was performed with a high-fidelity long PCR protocol (Boehringer Mannheim). Three lambda clones contained DNA fragments larger than 3 kb that flanked the upstream region of the histidine autokinase domain and the downstream region of the aspartyl receiver domain. One of these clones, ΔSA15.1, was chosen to determine the complete nucleotide sequence of the gene in both directions using the ABI automated sequencing system and fluorescent dideoxynucleotides as described earlier. The alignment of nucleotide sequences and comparisons with sequences in the databases were performed with the DNASIS software program (Hitachi Software Engineering). The deduced amino acid sequences were aligned, and significant motifs, such as membrane-spanning domains and potential glycosylation sites, were searched for with the GCG program (Devereux et al., 1984).

**Northern blot analysis of CaNlKl transcription.** Total cellular RNA was isolated by methods previously described (Morrow et al., 1992; Srikantha & Soll, 1993), with the following minor modifications: pellets of washed cells were frozen, mixed with an equal volume of acid-washed glass beads (400 μm size) and 450 μl RNA extraction buffer from a RNAeasy Mini Kit (Qiagen), and agitated with a bead beater device (Biospec). Approximately 10 μg total RNA extracted by the RNAeasy protocol (Qiagen) was used to isolate poly(A)⁺ mRNA using the Oligotex spin column kit according to the manufacturer's specifications (Qiagen). To assess the level of the CaNlKl transcript, Northern blots containing poly(A)⁺ mRNA isolated from 10 μg total RNA were probed with a 1.17 kb PFM–XhoI fragment spanning the histidine autokinase- and the ATP-binding domains of the CaNlKl gene.

**Southern blot analysis.** To test whether reorganization at the CaNlKl locus accompanied switching in strain WO-1, and whether a polymorphism existed between the two alleles in the auxotrophic strain CA18, approximately 30 μg of total cellular DNA was digested individually with a variety of restriction enzymes and then separated in 0.8% agarose gels. The DNA was transferred to Zetabind Nylon membrane (CUNO) and probed with the entire AfIII–XhoI fragment as described for Northern blot analysis.

**Deletion of CaNlKl in C. albicans strain CA18.** To generate a CaNlKl deletion cassette, a DNA fragment of approximately 2.1 kb containing both the histidine autokinase- and aspartyl response regulator domains was amplified by PCR using ΔSA15.1 as the template (Fig. 1a), which contained the 545 bp sequence upstream of the histidine autokinase domain. The PCR fragment was gel-purified and cloned into the pGEM-T Easy vector (Promega). The DNA insert was again excised from the recombinant plasmid with EcoRI and subcloned into a PUC18 vector (Life Technologies) at the EcoRI site. The resultant recombinant plasmid was designated pCNK12.1. A deletion construct, pCNH35, was generated that spanned the histidine autokinase- and ATP-binding domains. To construct pCNH35, pUNIK12.1 plasmid DNA was digested with AfIII and XhoI, and blunt-end repaired with Klenow DNA polymerase I. The resultant plasmid DNA fragment was then gel-purified and dephosphorylated with shrimp alkaline phosphatase (US Biochemical). A hisG–URA3–hisG cassette of 3.8 kb from pMB9 was then ligated to derive the disruption cassette containing plasmid pCNH35 (Fonzi & Irwin, 1993). To isolate the CaNlKl disruption cassette from pCNH35, plasmid DNA was digested with PstI and the digested DNA extracted with phenol/chloroform (1:1, v/v). Approximately 25 μg of the digestion mixture was used to transform strain CA18, an ade2 ura3 derivative of wild-type strain SC5314, by the lithium acetate protocol (Schiestl & Gietz, 1989). Heterozygotes were selected for growth in minimal medium in the absence of uridine. Transformants were initially tested for the heterozygosity of one of the two CaNlKl alleles by Southern blot hybridization of genomic DNA digested with PstI. Positive heterozygotes were further confirmed by digesting genomic DNA with XhoI and by performing Southern blot hybridization. Since the genomic Southerns revealed polymorphism between the two CaNlKl alleles, two distinct heterozygotes, NNL6 (L stands for large allele) and NNS7 (S stands for small allele) were selected. The heterozygote NNS7 was chosen to generate the knock-out for the second copy of the CaNlKl gene. Prior to the knock-out of the second copy, NNS7 was subjected to the 5-fluoroorotic acid (FOA) selection protocol to convert it from uridine prototrophy to auxotrophy. Loss of the URA3 gene was again confirmed by digestion with XhoI and Southern blot analysis. In the final step, a single clone, NNS7.1.1, which was heterozygous for the L allele of the CaNlKl locus and ura3, was subjected to a second round of transformation with pCNH35 and selected for growth on defined minimal medium lacking uridine. Transformants which had lost the second copy of CaNlKl were selected by Southern blot hybridization. One of the 125 transformants obtained with the pCNH35-based cassette, HH80, contained a homozygous deletion.

**Functional analysis of the CaNlKl-minus strain.** The bud-hypha transition was tested by the experimental regime of pH-regulated dimorphism (Butto et al., 1984; Soll, 1986) in 0, 10 and 15 M NaCl. The proportions of cells forming buds or hyphae were scored at 400 x using Kohler illumination. To measure the frequency of switching after UV induction, cells
from a single colony were grown to mid-exponential phase in modified Lee's medium (Lee et al., 1975; Bedell & Soll, 1979), then UV-irradiated for 0, 5, 10, 20 and 40 s by a previously described protocol (Morrow et al., 1989). Cells were then plated on agar containing modified Lee's medium at a density of 60 c.f.u. per plate, incubated for 7 d at 25°C, then scored for the proportion of smooth, variant and sectored colonies. Cells of select variants were in turn plated to demonstrate heritability and reversible switching. Sensitivity to osmotic stress was tested by plating cells at a density of 60 c.f.u. per plate on both modified Lee's medium and minimal medium (Sherman et al., 1986) containing either 0.8 M NaCl, 1 M sorbitol, 0.8 M KCl or 0.5 M MgSO₄. Sensitivity to a number of metabolic inhibitors were tested on both modified Lee's medium and minimal medium. These included 20 and 40 μg calcifluor ml⁻¹ (Lussier et al., 1997), 1, 2 and 4 μg caffeine ml⁻¹ (Parsons et al., 1988), 10 and 20 μg hygromycin ml⁻¹ (Dean, 1995), 0.002 and 0.004 μg echinocandin ml⁻¹ (Ram et al., 1994), and 0.2 and 0.4 mM polymyxin B (Boguslawski & Polazzi, 1987). The sensitivity of growth to temperature was tested at 25 and 37°C.

Photomicroscopy of budding and hypha-forming cells. Cells were fixed in Pen-Fix (Richard Allen Medical), then placed on poly-L-lysine-coated 25 mm glass coverslips. Cells were allowed to settle in a humidified chamber, the supernatant decanted and the coverslip placed in 70% ethanol for 10 min. Samples were then dehydrated through graded ethanol and mounted with Consul-Mount (Shandon). Differential interference contrast micrographs were taken with a Zeiss Axioshot microscope.

RESULTS

A PCR-based strategy for the isolation of a two-component hybrid kinase from C. albicans

Based on homology comparisons between two-component response regulator kinases containing hybrid-kinase-type configurations, the three deoxyinosine-containing degenerate primers Slb1, Slb2 and Slb3 were designed that encompassed the histidine autokinase domain (H1), the ATP-binding domain (G2) and the aspartyl response regulator kinase domain (D) (Fig. 1a). Three independent recombinant plasmids containing PCR inserts generated from the primers Slb1 and Slb2 were 550 bp in length, and the deduced primary amino acid sequences revealed a conserved histidine kinase H1 domain, a nucleotide-binding N domain and two ATP-binding domains, G1 and G2 (Fig. 1a). The deduced amino acid sequence exhibited high similarity to the bacterial hybrid kinase proteins barA (Nagasawa et al., 1992), lemA (Hrabak & Willis, 1992) and rscC, (Gottesman & Stout, 1991) and to the N. crassa hybrid kinase nik-1+ (Alex et al., 1996), and lower similarity to the S. cerevisiae hybrid kinase SLN1 (Ota & Varshavsky, 1993). The nucleotide sequences of the three PCR fragments spanning the H1 and D domains generated by the primers Slb1 and Slb3 also revealed identity in amino acid sequence to the PCR products generated by the primer pairs Slb1 and Slb2 which spanned the H1 and G2 domains (Fig. 1a).

Comparison of the deduced amino acid sequence with sequences in the SWISS-PROT database revealed that the C. albicans protein is highly similar to both the bacterial two-component hybrid kinases and the N. crassa two-component hybrid regulator nik-1+, with overall homologies of 40 and 50%, respectively. We therefore referred to the C. albicans gene as CaNIK1 in our preliminary report (Brown et al., 1998).

Isolation and characterization of the full-length C. albicans CaNIK1 gene

The DNA fragment generated by Slb1/Slb3 (Fig. 1a) was used as a probe to screen a C. albicans EMBL3a lambda genomic library (Srikantha & Soll, 1993) to identify clones containing the full-length gene. Of 10⁶ p.f.u. tested, 20 positive clones were identified. Clone zSA115.1, which contained a genomic fragment of approximately 4.8 kb with DNA flanking both the H1 and the D domains, was chosen for further characterization. The nucleotide sequence of the DNA insert was determined in both directions. The initiation codon ATG was surrounded by an atypical Kokaz consensus sequence CTGAAATGA, with cytosine at the −3 position (Kozak, 1984). The presence of cytosine at the −3 position and the composition of nucleotides surrounding the ATG codon suggested that the derived transcript of this gene was probably translated at very low levels in the cell (Kozak, 1984). When total genomic DNA of C. albicans strain WO-1 was digested with a variety of restriction enzymes and the resulting Southern blot hybridized under conditions of high stringency (Church & Gilbert, 1984) with the 1.2-kb probe spanning the 800 bp upstream of the gene, the banding pattern suggested that CaNIK1 is encoded by a single-copy gene (data not shown). A comparison of the CaNIK1 sequence published recently by Nagahashi et al. (1998) for strain IPO1060 and the sequence we obtained for strain WO-1 revealed differences at seven nucleotide positions in the ORF of 3243 bp.

The CaNIK1 protein exhibits features of the N. crassa Nik-1+ protein and bacterial two-component response regulators

Analysis of the nucleotide sequence of the CaNIK1 gene revealed a putative protein of 1081 amino acids with an estimated molecular mass of 119 kDa in the unmodified form and an isoelectric point of 5.7, suggesting that it is an acidic protein. In Fig. 2, the sequence of amino acids 454–1081 is presented, which contains the H1 box, N box, G1 box, G2 box, D box and CH box. In Fig. 3(a), the amino-terminal 516 amino acids of CaNIK1-1p are presented. A computer analysis with the SWISS-PROT database using the FASTA program of GCG (Devereux et al., 1984) indicated that CaNIK1 encodes a protein with a significantly high overall homology of 58% to the N. crassa Nik-1p, and a lower overall homology of 28% to Snl1p of S. cerevisiae. The overall homologies of the amino acid sequence spanning the histidine autokinase- and the aspartyl response regulator domains between amino acids 491 and 1081 (Fig. 2) of CaNIK1p and the bacterial hybrid kinases BarA, LemA and RscC ranges from 38.6 to 43.1%, whilst the overall homology between the amino acid sequence of the same region of
the \textit{S. cerevisiae} Sl1p and the bacterial hybrid kinases is approximately 28\%. In addition, the CaNik1p shows an amino-terminal repeat-type organization similar to that of Nik-1p of \textit{N. crassa} (Figs 1b, c, 3a). CaNik1p does not contain the Sl1p-type insertion sequence between the N and G1 domains (Fig. 2). This insertion sequence is also absent in bacterial hybrid kinases and Nik-1p of \textit{N. crassa} (Fig. 2).

An analysis of the carboxy-terminal 120 amino acid region of CaNik1p revealed a histidine residue (His-1000) within an unorthodox sensory kinase domain (CH box, Fig. 2) that is characteristic of some bacterial hybrid kinases, but absent in the \textit{S. cerevisiae} Sl1p and the \textit{N. crassa} Nik-1p (Fig. 2). In bacteria, this region can function as an alternative sensory kinase based on the functional suppression of second site mutations of genes involved in signal transduction (Ishige \textit{et al.}, 1994). A comparison of the amino-terminal 500 amino acid residues of CaNik1p with those of the bacterial hybrid kinases (Yao \& Spudich, 1992; Hrabak \& Willis, 1992; Nagasawa \textit{et al.}, 1992; Gottesman \& Stout, 1991) and the \textit{N. crassa} Nik-1p (Alex \textit{et al.}, 1996) showed the highest similarity between CaNik1p and \textit{N. crassa} Nik-1p. The CaNik1p contains a 92 amino acid motif that is repeated four times, followed by a truncated 72 amino acid repeat (Figs 1b, 3a). When the individual repeat units were compared to each other, a high degree of homology was revealed, ranging from 47 to 65\% (Fig. 3b). The repeat unit motif showed structural similarity to that of the bacterial histidine kinase Htr1p (Fig. 3a), with a high probability of forming a coiled-coil structure, a feature which might be important in the transduction of extracellular signals (Lupas \textit{et al.}, 1991). An analysis of the hydropathy of CaNik1p suggested that the protein does not contain any significantly long hydrophobic amino acid stretches representing a transmembrane core. Although CaNik1p contains three leucine residues, only two traditional leucine CUG codons were found, and this codon has been demonstrated to encode serine in \textit{C. albicans} (Santos \textit{et al.}, 1993; White \textit{et al.}, 1995).

\textbf{CaNIK1 transcription}

To test whether transcription of \textit{CaNIK1} was regulated by high-frequency phenotypic switching, Northern blots of poly(A)$^+$ mRNA of white- and opaque-phase cell exponential cultures of strain WO-1 were probed with
Fig. 2. Sequence comparison of the CaNiklp spanning the H, N, G1, G2, D and H2 (shown as CH box) domains with other sequence has been identified, up until now, only in bacterial hybrid kinases. Two serine residues in the sequence are (Devereux 1984). The dark-shaded boxes represent motifs with functional roles defined by biochemical or genetic criteria. A conservative replacement in at least one member is denoted by a filled circle and a conservative replacement in hybrid kinases other than Sn1p is denoted as a short vertical line. The CH box at the carboxy-terminal end of the sequence has been identified, up until now, only in bacterial hybrid kinases. Two serine residues in the sequence are translated from atypical CUG codons.
Fig. 3. Comparison of the CaNiklp amino-terminal region with NcNiklp and Htrlp, and comparison of the CaNiklp repeats with each other. (a) The deduced amino acid sequence of 600 residues of CaNiklp was analysed as described in the legend for Fig. 4. The symbols N1–N6 represent six 90 amino acid repeat units of NcNiklp. In C. albicans the repeat unit homologous to N1 is not present. N2–N5 repeat units of CaNiklp are 92 amino acids in length. Each repeat unit begins with a tryptophan-charged amino acid dipeptide signature as shown in the outlined boxes. Amino acid identities are denoted by dark shading and the lack of corresponding amino acid positions is represented by a hyphen. (b) Alignment of each of the amino-terminal repeat units of CaNiklp. The five repeat units were aligned using the DNASIS program based on identity to NcNiklp. The amino acid residues that are identical between repeat units are denoted by dark shading.
the 1.17 kb PflMI–XhoI fragment spanning the H1 and ATP-binding domains of CaNIK1. The CaNIK1 transcript was detectable at very low levels in both white-phase and opaque-phase cells throughout the exponential phase of growth and in stationary phase (Fig. 4a). The level of CaNIK1 transcript per cell remained constant throughout white-phase cell growth, but increased steadily during opaque-phase cell growth, reaching a level per cell roughly twice that of white-phase cells at stationary phase (Fig. 4b). The relative level of mRNA at each time point was determined by direct densitometric scanning of the photographic film following exposure of the radioactive blot for 2 d using a Phosphoimager (Molecular Dynamics). Maximum intensity of the hybridizing band area was derived by normalizing the signal to noise ratio to that of a non-hybridizing area of the film, using Imagequant software (Molecular Dynamics).

Fig. 4. Northern blot analysis of CaNIK1 expression during growth in the white and opaque phases in C. albicans strain WO-1. Cells in either the opaque or white phases were grown in 25 ml modified Lee’s medium and samples were taken throughout the exponential phase of growth (15–46 h) and at stationary phase (72 h). Isolation of poly(A)⁺ RNAs, Northern blotting and hybridization protocols are described in Methods. (a) Southern blots hybridized with either the PflMI–XhoI region of CaNIK1 or constitutively expressed EF1α2. (b) Plot of the level of CaNIK1 mRNA as a function of time for growth cultures of white-phase cells (○) and opaque-phase cells (●). The relative level of mRNA at each time point was determined by direct densitometric scanning of the photographic film following exposure of the radioactive blot for 2 d using a Phosphoimager (Molecular Dynamics). Maximum intensity of the hybridizing band area was derived by normalizing the signal to noise ratio to that of a non-hybridizing area of the film, using Imagequant software (Molecular Dynamics).

Deletion of CaNIK1 in C. albicans strain CAI8

Since there is no established system for generating null mutants of a specific gene in C. albicans strain WO-1, a CaNIK1 homozygous knock-out strain was generated in strain CAI8 (Table 1) (Fonzi & Irwin, 1993). To obtain heterozygous transformants, CAI8 was initially transformed with a hisG::URA3::hisG cassette flanked by a CaNIK1 gene fragment. Southern blot analysis of the genomic DNA of 24 transformants digested with the restriction enzyme XhoI revealed three putative heterozygotes. The presence of two distinct bands in Southern blots of the parental strain (CAI8) probed with the 1.17 kb AflII–XhoI CaNIK1 fragment suggested DNA fragment length polymorphism (Fig. 5). Since the polymorphic XhoI fragment contains the repeat regions of CaNIK1 (Xh in Fig. 1a), since Southern blot analysis of PstI-digested genomic DNA hybridized with the same probe did not reveal any polymorphism (data not shown) and since the PstI site is positioned downstream from the XhoI site (P in Fig. 1a), the allelic difference is most likely to be due to a difference in the number of repeat units. Furthermore, using PCR with a pair of primers outside the repeat unit region, two distinct DNA bands were evident in Southern blots of CAI8, whilst either one or the other of the two bands were evident in Southern blots of the two heterozygotes (data not shown). The heterozygote harbouring the S allele was designated NNS6 and the heterozygote harbouring the L allele was designated NNL7. Although both of the heterozygotes were subjected to a FOA-induced pop-out protocol (Fonzi & Irwin, 1993) to convert them from uridine prototrophy to auxotrophy, a majority of the FOA-resistant clones exhibited pseudoconversion (i.e. grew on uridine-minus plates, but were still heterozygous), suggesting that they were not suitable for a second round of transformation for the disruption of the second allele. Southern blot analysis of the genomic
Hybrid kinase regulator of Candida albicans

Fig. 5. Southern blot analysis of CaN1K1 disruptants. Total genomic DNA (3 μg) from the ura3 ade2 auxotrophic strain CA18, the two heterozygote derivatives NNL6 and NNS7, a heterozygote FOA^R ura3 derivative of NNS7, 7.1.1, and a CaN1K1 homozygous mutant, HH80, were digested with Xhol. A Southern blot of digested DNA was probed with an AlfllI-Xhol fragment of the CaN1K1 gene. (b) The blot was stripped and reprobed with a Xbal-EcoRV fragment of the CaURA3 gene. The letters L and S represent the large and small alleles of the CaN1K1 gene as described in Results.

Table 2. UV-induced switching is reduced but still occurs in the CaN1K1 homozygous deletion mutant HH80

See Table 1 for the genotypes of strains CA18 and HH80.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Length of UV treatment (s)</th>
<th>No. of colonies per 10 plates</th>
<th>Percentage of colonies killed</th>
<th>No. of variant colonies</th>
<th>Percentage of variant colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA18</td>
<td>0</td>
<td>1112</td>
<td>0</td>
<td>8</td>
<td>0-7</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>987</td>
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<td>17</td>
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<td></td>
<td>10</td>
<td>562</td>
<td>57</td>
<td>28</td>
<td>9-10</td>
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<tr>
<td></td>
<td>20</td>
<td>75</td>
<td>93</td>
<td>3</td>
<td>4-9</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HH80</td>
<td>0</td>
<td>1877</td>
<td>0</td>
<td>2</td>
<td>1-2</td>
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<tr>
<td></td>
<td>5</td>
<td>1571</td>
<td>16</td>
<td>10</td>
<td>1-0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>599</td>
<td>68</td>
<td>9</td>
<td>8-4</td>
</tr>
<tr>
<td></td>
<td>20</td>
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<td>94</td>
<td>2</td>
<td>1-4</td>
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<tr>
<td></td>
<td>40</td>
<td>0</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

DNA of approximately 60 FOA-resistant pop-out clones from the two heterozygotes yielded only one true ura3 clone, which was designated NNS7.1.1. Southern blot analysis of the genomic DNA of this clone with the 1.17 kb AlfII-XhoI probe demonstrated that NNS7.1.1 was still heterozygous (Fig. 5a), and hybridization with the CaURA3 probe demonstrated that the URA3 gene was absent (Fig. 5b). To generate a homozygous deletion strain for CaN1K1, the clone NNS7.1.1 was transformed with the same hisG::URA3::hisG cassette isolated from the pCNH35 plasmid. Southern analysis of genomic DNA from 125 independent putative transformants revealed that the cassette either integrated at the already disrupted S allele or integrated at the ade2 locus in all but one transformant through homologous recombination involving the hisG cassette (data not shown). This single transformant, HH80, contained partially deleted alleles at both loci (Fig. 5a) and was used for subsequent phenotypic analyses.

Functional characterization of a partial deletion mutant of CaN1K1 in strain CA18

To test whether the CaN1K1 deletion mutant HH80 underwent switching, we first had to characterize switching in this strain using a low-dose UV irradiation protocol that increases switching frequencies (Morrow et al., 1989). Cells were treated with UV irradiation for 0, 5, 10, 20 and 40 s and the percentage kill, as well as the frequency and type of switch variants, were assessed on modified Lee's medium (Bedell & Soll, 1979; Slutsky et al., 1985; Soll, 1992). The proportions of CA18 and HH80 cells killed after 5, 10, 20 and 40 s were similar (Table 2). Fig. 6(a) shows the dominant smooth colony phenotype of the parent strain CA18, and Fig. 6(b) and (c) show the two most prevalent variant phenotypes stimulated by low UV treatment (in this case, 43% killing), a wrinkled colony and a heavily myceliated-wrinkled colony, respectively. In Fig. 6(d) and (e), examples are presented of switches from the dominant smooth to the wrinkled and heavily myceliated wrinkled colony phenotypes, respectively, and in Fig. 6(f), an example is shown of a colony sector that has reverted from the heavily myceliated wrinkled to the smooth phenotype. Identical variant phenotypes were stimulated by UV in the homozygous deletion strain HH80. However, the frequency of variants induced by comparable levels of UV irradiation was consistently lower in strain HH80 (Table 2), and this was also true in a repeat experiment. For instance, 20 s of UV irradiation
resulted in 10.6% and 2.6% variants in CAI8 and HH80 cells, respectively (Table 2). These results demonstrate that the histidine autokinase- and ATP-binding domains of the CaNIK1 gene product are not essential for high-frequency phenotypic switching, but that their presence enhances switching. Since deletion of the nik-1+ gene in N. crassa affects the morphology of hyphae, especially at high osmotic strength (Alex et al., 1996), the capability of the CaNIK1-minus HH80 strain to form hyphae and the morphology of these hyphae were compared to that of the parent strain CAI8 and a URA3+ isogenic strain CAI8US (Table 1) at 0, 1.0 and 1.5 M NaCl. Under the regime of pH-regulated dimorphism, CAI8, CAI8US and HH80 cells formed buds at pH 4.5 and hyphae at pH 6.7. The kinetics of evagination for the three strains at low and high pH were similar at the three tested salt concentrations (the kinetics of evagination at 1.0 and 1.5 M NaCl are presented in Fig. 7). At 1.5 M NaCl, the proportion of cells that formed evaginations at low and high pH was dramatically reduced in all three strains. The morphology of the hyphae that formed at pH 6.7 at 0, 1.0 and 1.5 M NaCl was comparable in the three strains (Fig. 8). However, there was a significant and reproducible lag in hyphal growth at 1.5 M NaCl in HH80 after 300 min (Table 3). These results demonstrate that the histidine autokinase- and ATP-binding domains of the CaNIK1 gene product are not essential for hypha formation under the regime of pH-regulated dimorphism, but their presence enhances hypha formation at high ionic strength.

Finally, growth of the CaNIK1 deletion mutant HH80 was tested at 25 and 37 °C for differential sensitivity to osmotic strength and a variety of inhibitors. Patches of budding cells of CAI8, CAI8US and HH80 were plated on agar containing modified Lee’s medium (Bedell & Soll, 1979) alone or with one of the following ingredients: 1.0 or 1.5 M NaCl, 1 M sorbitol, 0.8 M KCl, 0.5 M MgSO4, 20 or 40 pg calcofluor ml-1, 1, 2 or 4 mg caffeine ml-1, 10 or 20 mg hygromycin ml-1, 0.002 or 0.004 mg echinocandin ml-1, and 0.2 or 0.4 M polymyxin B. In three independent experiments, no qualitative differences were observed between the growth of the control strains and the mutant strain HH80 for any of the tested conditions.

**DISCUSSION**

**Homology with other hybrid histidine kinases**

Bacteria employ two-component signal transducers to assess and rapidly respond to alterations in the environment (Hoch & Silhavy, 1995; Alex & Simon, 1994; Parkinson, 1993), and eukaryotes have followed suit (Alex & Simon, 1994; Wang et al., 1996; Otá & Varshavsky, 1993; Chang, 1993). Two-component signal transducers all contain a sensory kinase, which autophosphorylates a histidine residue in response to an environmental cue, and a response regulator, which is then phosphorylated and through a resultant conformational change effects a signal that is transduced either directly to a molecular complex, as in the case of the bacterial cheY and the flagellar motor (Welch et al., 1993), or down a signal transduction pathway, as in the case of SLN1 (Posas et al., 1996; Fassler et al., 1997). In bacteria, two-component signal transducers have been demonstrated to play key roles in pathogenesis (e.g. Uhl & Miller, 1996; Dziejman & Mekalanos, 1995; Groisman & Heffran, 1995), including phase transitions between nonpathogenic and pathogenic phenotypes (Han et al., 1997). We and others (Brown et al., 1998; Nagahashi et al., 1998; Calera et al., 1998) have therefore begun to investigate their possible roles in fungal pathogenesis.
Here, we have described the isolation and characterization of a two-component signal transducer from *C. albicans*, CaNIK1, that is a homologue of the two-component signal transducer nik-1*+* of *N. crassa* (Alex et al., 1996). Like the *N. crassa* nik-1*+*, CaNIK1 contains a unique repeat domain at the amino-terminal end. CaNIK1 contains four 92 amino acid repeats and one truncated repeat, while the *N. crassa* nik-1*+* contains five 90 amino acid repeats and one truncated repeat. This difference may not be functionally significant since the polymorphism between alleles in *C. albicans* strain CA18 is probably due to a difference in the number of these repeats. In addition, neither the *N. crassa* nik-1*+* nor the *C. albicans* NIK1 contains traditional membrane-spanning domains comparable to that contained in the *S. cerevisiae* SLN1 (Ota & Varshavsky, 1993), or in the *C. albicans* SLN1 (Nagahashi et al., 1998). CaNIK1 also exhibits a number of characteristics in common with several bacterial two-component hybrid kinases. First, the amino acid region spanning the kinase domain shares a greater number of identical amino acid residues with bacterial hybrid kinases than either *N. crassa* nik-1*+* or *S. cerevisiae* SLN1. Second, the organization of various motifs spanning the functionally mapped amino acids is more similar to bacterial hybrid kinases than eukaryotic hybrid kinases. Third, CaNik1p contains a putative unorthodox histidine kinase motif at the carboxy-terminal end found in some bacterial hybrid kinases (Ishige et al., 1994), but absent in other eukaryotic hybrid kinases.

**Fig. 7.** Evagination kinetics of CA18, CA18U5 and HH80 cells forming either buds or hyphae under the regime of pH-regulated dimorphism at 1.0 (a-f) and 1.5 (g-I) M NaCl. Time 0 represents time of dilution of stationary-phase cells into fresh medium at pH 4.5, 37 °C to induce bud formation (a, c, e, g, i, k), or at pH 6.7, 37 °C to induce hypha formation (b, d, f, h, j, l). At time intervals, the proportion of evaginating cells in the population was determined.
Fig. 8. Micrographs of cells of strains CAI8, CAI8U5 and HH80 300 min after dilution into fresh medium at pH 6.7, 37 °C in 0 (control), 1.0 and 1.5 M NaCl. Note that although there is a decrease in the frequency and mean rate of growth of hyphae at 1.5 M NaCl in strain HH80, the hyphae that do form are qualitatively indistinguishable from those of the CAI8 and CAI8U5 strains. Micrographs were taken with a differential interference contrast microscope. Bar, 10 μm.

Table 3. Percentage occurrence of hyphae of different lengths after 300 min incubation at pH 6.7 under the regime of pH-regulated dimorphism at 1.5 M NaCl

See Table 1 for the genotypes of strains CAI8, CAI8U5 and HH80.

<table>
<thead>
<tr>
<th>Hyphal length (cell diameters)</th>
<th>Total percentage</th>
<th>Hyphae</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>CAI8</td>
<td>45</td>
<td>16</td>
</tr>
<tr>
<td>CAI8U5</td>
<td>53</td>
<td>12</td>
</tr>
<tr>
<td>HH80</td>
<td>72</td>
<td>15</td>
</tr>
</tbody>
</table>

Regulation and function of CaNIK1

Since two-component response regulators have been demonstrated to be differentially expressed in the vegetative mycelial phase of N. crassa (Alex et al., 1996) and in phase transitions in bacteria (e.g. Han et al., 1997), we first examined expression in the bud–hypha transition and then in the white–opaque transition of C. albicans strain WO-1. CaNIK1 was found to be expressed constitutively in budding and hypha-forming cells and in both phases of the white–opaque transition, but at extremely low levels, as indicated by the exposure times necessary to identify hybridization signals in Northern blots. However, the level of CaNIK1 transcript per cell increased throughout exponential growth in the opaque, but not the white phase, reaching a level at stationary phase roughly twice that in early exponential phase. There is precedence for such a change in bacteria. In Bacillus subtilis, the transcript levels of the two-component response regulators spoOA and spoOF are low during vegetative growth and increase to higher levels in stationary phase (Hoch, 1995). These increases are prerequisite for the signal for sporulation. In addition, the transcript levels of the two-component response regulator genes phoR and phoP of B. subtilis increase as cells enter stationary phase in response to the phosphate concentration in the medium (Hulett, 1995).

In the case of CaNIK1, it is not clear why the levels of transcript double as cells enter stationary phase in the opaque phase or why this increase is opaque-phase-specific. In addition to increased expression in the opaque phase, CaNIK1 transcript levels are also slightly higher in the hyphal form of growth. Interestingly, cells exhibiting the opaque-phase budding phenotype and cells exhibiting the hyphal growth form share several phenotypic characteristics, including an elongate cell shape, expression of hypha-specific antigens and the deactivation of the white-phase-specific gene WH1 (Anderson et al., 1989; Soll, 1992; Srikantha & Soll, 1993).

To assess more directly the function of CaNIK1, a partial deletion mutant was generated in C. albicans
strain CA18 (Fonzi & Irwin, 1993) that lacked the histidine autokinase- and ATP-binding domains of CaNIK1. The mutant strain HH80 was still capable of switching between the variant phenotypes in its switching repertoire, and still capable of the bud–hypha transition under the regime of pH-regulated dimorphism. However, in both cases the mutant exhibited a change in the efficiency of the process. In the case of high-frequency phenotypic switching, the mutant switched reversibly to the same variant phenotypes as the parent strain, but the frequency of variants after UV induction was approximately a third that of the parent strain. In the case of the bud–hypha transition, mutant cells formed hyphae at pH 6.7 in 1.5 M NaCl with morphologies indistinguishable from that of hyphae formed by the parent strain, but the number of cells forming hyphae and the mean length of hyphae were reduced 300 min after inoculation. These differences are ones of extent rather than quality, and for that reason have several alternative explanations. First, because HH80 still possesses the carboxy-terminal coding region of CaNIK1, a truncated polypeptide containing the response regulator domain D may still be able to establish a phosphorylation cascade via another endogenous phosphokinase or the H2 domain. Alternatively, CaSLN1 (Nagahashi et al., 1998) may substitute for CaNIK1. Finally, although CaNik1p may not be responsible for the basic bud–hypha transition or the switch event, its presence may be necessary for an efficient and maximal response in both developmental processes. Construction of a complete deletion mutant of CaNIK1 is now under way to distinguish between the above alternatives.

Finally, it should be noted that the phenotype of the CaNIK1 partial deletion mutant HH80 is really not that different from the nik-I strain in N. crassa (Alex et al., 1996). This mutant forms mycelia, but the morphology of the mycelia is altered at high ionic strength. The CaNIK1 partial deletion mutant is also not that different from the CaSLN1 deletion mutant of C. albicans (Nagahashi et al., 1998). Again, this mutant forms hyphae, but the morphology of the hyphae is slightly altered at high ionic strength. It should be stressed that in none of these three cases is the two-component hybrid kinase essential for the phenotypic transition. However, in all three cases the cellular phenotype of the deletion mutants was assessed in vitro. A far stronger mutant phenotype may emerge in a pathogenic setting involving host-derived signals. Experiments are now being performed with the homogous CaNIK1 deletion mutant in a variety of animal and tissue models to test this possibility.

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