The *Candida albicans* antiporter gene *CNH1* has a role in Na⁺ and H⁺ transport, salt tolerance, and morphogenesis

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The isolation and functional characterization of a *Candida albicans* Na⁺/H⁺ antiporter gene, *CNH1*, is reported here. The gene encodes a protein of 840 amino acids that exhibits high levels of similarity in sequence, size, and structural and functional domains to a group of known Na⁺/H⁺ antiporters of fungi. The *CNH1* gene is able to functionally complement the salt-sensitivity of a *Saccharomyces cerevisiae* *ena1 nha1* mutant, and mutations of two conserved aspartate residues to asparagines in the putative Na⁺-binding site abolish this activity. Deletion of *CNH1* results in retardation of growth and a highly elongated morphology in a significant fraction of cells under conditions that normally support yeast growth. These results indicate that *CNH1* has a role in Na⁺ and H⁺ transport, salt-tolerance, and morphogenesis.

Keywords: Na⁺/H⁺ antiporter, *Candida albicans*, salt tolerance, morphogenesis, gene disruption

INTRODUCTION

The fungus *Candida albicans* is an opportunistic human pathogen that causes a large proportion of systemic and disseminated fungal infections in immunocompromised patients (Beck-Spague & Jarvis, 1993). One feature of this organism that is important for its pathogenicity is its ability to grow in, and switch between, several distinct morphological forms, namely ellipsoidal yeast cells, pseudohyphae and hyphae (Soll, 1996; Gow, 1997). Mutants defective in such growth-mode transitions exhibit attenuated virulence (Braun & Johnson, 1997; Lo *et al.*, 1997). The transition between different morphological forms can be triggered by a host of environmental signals including pH, temperature and nutritional status (Gow, 1997).

How pH change leads to *C. albicans* growth-mode transition is largely unknown. Stewart *et al.* (1988) reported a dramatic transient alkalization of cytoplasm preceding the appearance of germ tubes. Strains defective in germ-tube formation did not show such a rise of cytoplasmic pH under conditions favourable for

filamentous growth (Kaur & Mishra, 1994). Inactivation of plasma-membrane H⁺-extruding ATPase inhibited both cytoplasmic alkalization and germ-tube formation (Monk *et al.*, 1991, 1993). These results show that the induction of morphological transition in *C. albicans* is accompanied by a steep rise of cytoplasmic pH probably due to an elevated activity of plasma membrane H⁺-ATPase, though it is not clear whether the fluctuation of pH is the cause or consequence of morphogenesis. Na⁺ at above 50 mM also has an inhibitory effect on germ-tube formation (Northrop *et al.*, 1997). At alkaline pH, as little as 1 mM Na⁺ was effective in reducing acid-extrusion rate in *C. albicans* (Northrop *et al.*, 1997). Thus, high concentrations of Na⁺ might inhibit the germ-tube formation by limiting the alkalization of cytoplasm. In addition to H⁺-ATPase, Na⁺/H⁺ antiporters are known to play an important role in the maintenance and regulation of intracellular H⁺ and Na⁺ homeostasis (Padan & Schuldiner, 1994; Utsugi *et al.*, 1998). This antiporter carries out cross-membrane electroneutral exchange of Na⁺ and H⁺ (Jia *et al.*, 1992; Watanabe *et al.*, 1995; Hahnenberger *et al.*, 1997; Iwaki *et al.*, 1998). The observed effect of Na⁺ on the extrusion of H⁺ and germ-tube formation in *C. albicans* indicates the existence of a Na⁺/H⁺ antiporter(s) and their possible role in influencing the growth-mode transition.

Abbreviation: GFP, green fluorescent protein.

The GenBank accession number for the sequence reported in this work is AF128238.

Na^+/H^+ antiporter genes have been cloned and characterized from several fungi including *Saccharomyces cerevisiae* (NHA1; Prior *et al.*, 1996), *Schizosaccharomyces pombe* (*sod2*; Jia *et al.*, 1992) and *Zygosaccharomyces rouxii* (*ZSOD2* and *ZSOD22*; Watanabe *et al.*, 1995; Iwaki *et al.*, 1998). However, no Na^+/H^+ antiporter has so far been reported in *C. albicans*. This group of proteins are characterized by the presence of 12 putative transmembrane regions and, except for *sod2p*, a large cytoplasmic C terminus. Sites implicated in Na^+ binding and pH sensing have been identified in *Schiz. pombe* *sod2p* and *Escherichia coli* NhaAp (Padan & Schuldiner, 1996; Dibrov & Fliegel, 1998; Dibrov *et al.*, 1997, 1998). Gene deletion and heterologous-expression studies demonstrate that these proteins are involved in pH regulation of the cell and in salt tolerance.

In a screen designed to identify genes that may play a role in *C. albicans* growth-mode transition, we cloned a *C. albicans* Na^+/H^+ antiporter gene, *CNH1*. Due to the potential roles of this gene in the regulation of cellular response to pH changes and pH-induced morphogenesis, we investigated the functions of this gene in both *Sacch. cerevisiae* and *C. albicans*.

METHODS

Strains and growth conditions. *E. coli* XL1-Blue (*supE44 hsdR17 recA1 endA1 gyrA46 thi-1 relA1 lac* [F' *proAB lacI*^qZAM15 Tn10]) (Statagene) was used for cloning experiments. *C. albicans* strain SC5314 was used as the wild-type strain in growth analysis and pathogenicity tests (Fonzi & Irwin, 1993). CAI-4 (*URA3::imm434/URA3::imm434*), derived from SC5314 was used as the parental strain for gene disruption. *Sacch. cerevisiae* B31 (MATa *ade2-1 can1-100 his-11,15 leu2-3,112 trp1-1 ura3-1 ena1-4::HIS3 nha1::LEU2*) was used for functional expression of *CNH1* (Prior *et al.*, 1996). All the strains were grown routinely at 30 °C in YPD medium (0.5%, w/v, yeast extract; 1%, w/v, peptone and 0.5%, w/v, glucose). For growth assays, *C. albicans* cells were cultured in glucose minimal medium, GMM, which consists of 2% (w/v) glucose, 1 × yeast nitrogen base (Difco) buffered either at pH 5.5 or pH 7.5 by addition of 0.1 M MES or 0.1 M HEPES, respectively. Cell counts were determined directly using a haemocytometer or indirectly by measuring OD₆₀₀.

Isolation of *CNH1*. The coding region of the green fluorescent protein (GFP) gene *yEGFP3* (Cormack *et al.*, 1997) was PCR amplified and spliced into the *Sma*I site of the autonomously-replicating vector pRC2312 (Cannon *et al.*, 1992). A clone, pRCGFP, was chosen in which the 5' end of *yEGFP3* gene was proximal to the *Bam*HI site of the vector. *C. albicans* genomic DNA was partially digested by *Sau*3AI. DNA fragments ranging from 500 bp to 1000 bp were purified from a low-melting-point agarose gel after electrophoresis and spliced into the *Bam*HI site of pRCGFP to construct a library. The library plasmids were transformed into CAI-4 and transformants selected on GMM plates. Each transformant was grown at 30 °C in two media, GMM and GMM + 5% (v/v) foetal calf serum, supporting yeast and hyphal growth, respectively. GFP expression was examined by using a fluorescence microscope. Clones showing enhanced GFP expression in the hyphal form were selected. The plasmid was

recovered by lysing the transformants and transforming *E. coli* cells with the lysate. For the isolation of the full-length gene, a *C. albicans* genomic library was constructed. Genomic DNA was partially digested using *Sau*3AI. DNA fragments ranging from 4 to 6 kb were gel purified, ligated to pBKCMV and packaged using Stratagene's Gigapack III Goldsystem. The phage library was screened by following the manufacturer's instructions. A 4 kb DNA fragment was isolated containing the full length *CNH1* gene with 5' and 3' flanking sequences. DNA was sequenced by using a model 377 DNA sequencer (Applied Biosystems). The DNASTAR program was used to determine the sequence identity and hydrophilicity plots.

Southern and Northern analyses. *C. albicans* genomic DNA was isolated by the method of Cannon *et al.* (1992). Genomic DNA (10 µg) of each strain was cleaved by a restriction enzyme, resolved on 1% (w/v) agarose gel by electrophoresis and capillary-transferred to a Hybond-N nylon membrane (Amersham). For Northern hybridization, SC5314 was grown to exponential phase in GMM in the presence of different concentrations of NaCl, and total RNA was extracted by the method of Chirgwin *et al.* (1979). RNA (20 µg each well) was electrophoresed through a 1.2% (w/v) agarose gel containing 0.22 M formaldehyde and transferred to a Hybond-N nylon membrane. DNA probes were labelled with ³²P by using the High Prime DNA Labelling Kit (Boehringer Mannheim). Hybridizations and washes of the blots were done according to standard procedures (Maniatis *et al.*, 1982).

Functional complementation of *CNH1* in *Sacch. cerevisiae*

B31. The DNA fragment containing the coding region and termination signal of *CNH1* was cloned into a *Sacch. cerevisiae* centromeric expression vector, pUS234 (gift from Uttam Surana, Institute of Molecular and Cell Biology, Singapore), under the control of the *GAL1-10* promoter to give pUSCNH1 and transformed into *Sacch. cerevisiae* B31 cells by the method of Becker & Lundblad (1994). Multiple colonies selected on GMM agar plates were grown separately to saturation in GMM liquid medium at 30 °C, serially diluted and spotted onto galactose MM (GalMM) agar plates containing different concentrations of NaCl.

Site-directed mutagenesis. The Quikchange Site-Directed *In Vitro* Mutagenesis kit (Stratagene) was used for site-directed mutagenesis. Two aspartate residues (D310 and D311) of the *CNH1* gene in pUSCNH1 were mutated to asparagines to generate a new plasmid, pUScnh1. The mutated clones were verified by sequencing and transformed into *Sacch. cerevisiae* B31.

Disruption of *CNH1*. We used the *hisG*-*CaURA3*-*hisG* cassette to generate a gene-disruption construct for sequential deletion of both copies of *CNH1* gene (see Fig. 4). The genotype of each mutant was verified by Southern analysis. A 340 bp DNA fragment immediately upstream of the coding sequence was obtained by PCR in which a *Pst*I site was introduced into the 3' end. The PCR primers used were 5'-CTCGCCAATCAAACCTCAAAT-3', -283 to -264 (the first base of the ATG start codon is +1), and 5'-GGCTGCAGC-CAAGAAATTGTATCACCTG-3', +25 to +44 (*Pst*I site is underlined). The PCR product was cloned into pGEMTeasy (Promega) and a clone, pCNH1-5', was selected in which the *Pst*I end of the insert is proximal to the *Sac*I site in the vector. A 470 bp DNA fragment downstream of the coding sequence was similarly obtained with a *Bam*HI site added to the 5' end of the PCR fragment, and cloned into pGEMTeasy. The PCR primers were 5'-CCGGATCCTGTTATGCGAACCTCGG-TAG-3', +2542 to +2562, and 5'-TTGGCTTGTAAGTTCGTTG-3', +3013 to +2994 (*Bam*HI site is underlined).

This fragment was recovered by *Bam*HI/*Sac*I digestion and spliced together with the *Bam*HI-*Pst*I fragment of the *hisG*-*CaURA3*-*hisG* cassette into *Pst*I/*Sac*I-digested pCNH1-5' to generate pCNH1URA3. The *hisG*-*CaURA3*-*hisG* *Bam*HI-*Bgl*II fragment from pCUB-6 (Fonzi & Irwin, 1993) was initially subcloned into pGEMTeasy to obtain the *Bam*HI-*Pst*I fragment of the cassette. The entire disruption cassette was released from pCNH1URA3 by *Aat*II/*Sac*I digestion and introduced into CAI-4 by electroporation (Kohler *et al.*, 1997). The transformants were selected on GMM plates. The *CaURA3* gene was excised by growing the transformants in YPD overnight and plating 20 µl of the culture onto GMM plates containing 1 mg 5-fluoroorotic acid ml⁻¹ and 50 µg uridine ml⁻¹. The ura⁻ transformants were used for the deletion of the second copy of *CNH1*. We obtained two heterozygous mutants, CaTW1a and CaTW1b (*CNH1/cnh1Δ::hisG*-*CaURA3*-*hisG*), from independent experiments and the subsequent deletion of *CaURA3* gave CaTW2a and CaTW2b (*CNH1/cnh1Δ::hisG*). From CaTW2a and CaTW2b, we obtained two homozygous mutants, CaTW3a and CaTW3b (*cnh1Δ::hisG/cnh1Δ::hisG*-*CaURA3*-*hisG*). The two independent clones of the same genotype exhibited the same phenotype.

Morphological characteristics. SC5314, CaTW1 and CaTW3 cells were grown in GMM buffered either at pH 5.5 (0.1 M MES) or pH 7.5 (0.1 M HEPES) overnight at 30 °C. The cells were diluted to OD₆₀₀ ~ 0.2, grown to exponential phase and examined under the microscope.

Growth assays. SC5314, CaTW1 and CaTW3 cells were grown in GMM to saturation. Growth curves were obtained by seeding 1 × 10⁴ cells in 50 ml Falcon tubes containing 20 ml GMM buffered at either pH 5.5 or pH 7.5 and containing 0, 25, 50 or 100 mM LiCl. The cultures were grown at 30 °C with shaking and OD₆₀₀ values were determined at 1.5 h intervals. These experiments were conducted three times.

Animal experiment. SC5314, CaTW1 and CaTW3 cells were grown at 30 °C in GMM to saturation. The cells were washed twice in PBS and resuspended in PBS to a density of 5 × 10⁶ cells ml⁻¹. For each strain, five male Balb/c mice (Jackson Labs) of 4–6 weeks old were injected with 1 × 10⁶ cells through the lateral tail vein. Survival curves were calculated according to the Kaplan–Meier method using the PRISM program (version 2.01; GraphPad Software) and compared using the log-rank test. A *P* value less than 0.05 was considered significant. The kidneys of dead mice were removed for counting c.f.u. and microscopic examination of infected tissue. One kidney was cut into two coronal pieces and the other cut into two sagittal pieces. One piece of each kidney was put into 10% (v/v) formalin for periodic acid–Schiff staining, and the other two pieces were placed in 10 ml PBS and homogenized, and 200 µl of the solution was plated onto YPD agar for colony counting.

RESULTS

Cloning of a *C. albicans* Na⁺/H⁺ antiporter gene

To identify genes that may play a role in the growth-mode transition, we screened for short genomic DNA fragments that when inserted in front of a promoterless GFP gene in an autonomously replicating plasmid may lead to a hypha-specific GFP expression in *C. albicans* cells. This screen resulted in the isolation of a 3974 bp genomic DNA fragment containing a 2519 bp ORF, and 498 bp 5' and a 957 bp 3' flanking sequences. The ORF

encodes a putative protein of 840 aa (Fig. 1a), sharing 44.1, 41.7 and 36.2% identities with the Nha1p of *Sacch. cerevisiae*, and Zsod22p and Zsod2p of *Z. rouxii*, respectively; a family of Na⁺/H⁺ antiporters, (Fig. 1b). The identity values rose to 64, 59.1 and 49.6% when the N-terminal halves of the proteins were compared. The hydrophilicity plots of these proteins are very similar, predicting multiple transmembrane domains in the N-terminal half and a long hydrophilic C-terminal tail (Fig. 1b). The sequence comparison suggests that the gene is a member of the Na⁺/H⁺ antiporter family and we named it *CNH1*. Several putative sites for N-linked glycosylation and protein kinase C phosphorylation exist in the C-terminal half.

Functional expression of *CNH1* in *Sacch. cerevisiae* B31 mutant

To demonstrate that *CNH1* is a functional homologue of *Sacch. cerevisiae* *NHA1*, *CNH1* was cloned in a *Sacch. cerevisiae* centromeric vector under the control of a *GAL1-10* promoter. The resulting plasmid, pUSCNH1, was transformed into B31 mutant cells (Prior *et al.*, 1996). B31 cells are highly sensitive to high Na⁺ concentration, because it has deletions in both *NHA1* and Na⁺-ATPase *ENA1-4* genes (Prior *et al.*, 1996), which makes it useful in testing the function of genes controlling Na⁺ transport. The growth of B31 cells transformed with either the vector or pUSCNH1 were inhibited by 200 mM or higher NaCl concentrations when grown on glucose agar, a condition repressing *CNH1* expression from the vector (Fig. 2a). When glucose in the medium was replaced by galactose, only the pUSCNH1 transformants grew in the presence of up to 800 mM NaCl. The result indicates that expression of *CNH1* confers upon B31 cells tolerance to high Na⁺ concentrations, presumably by performing the function of the *Sacch. cerevisiae* *NHA1*. The restoration of salt tolerance to B31 by the function of Cnh1p was further confirmed by the following mutagenesis experiment. All fungal and bacterial Na⁺/H⁺ antiporter proteins contain two conserved aspartate (D) residues in the putative Na⁺-binding site (Inoue *et al.*, 1995; Dibrov *et al.*, 1998; Hiramatsu *et al.*, 1998; Utsugi *et al.*, 1998). In Cnh1p, these residues are found at positions D310 and D311. Mutation of the two aspartate residues of *Schiz. pombe* sod2p to asparagines inactivated the Na⁺-transport activity of the protein (Dibrov *et al.*, 1998). We mutated D310 and D311 of Cnh1p to asparagines and found that the B31 cells transformed with the mutated gene did not show detectable growth on medium supplemented with 400 mM NaCl (Fig. 2b). This experiment confirms the Na⁺-transport function of Cnh1p and the importance of the two conserved aspartate residues.

Southern and Northern blot analyses

Southern analysis of *C. albicans* genomic DNA cut with various restriction enzymes showed that *CNH1* is a single gene (data not shown; see also Fig. 4b). To

(a)

AACAAACACATGGCAAGACGTTGTCCAATTCAGGTGATACAATTTCTTGGCGCAATGTAACTTTTCCATTACAAAAAAGAACCCAAAAATTTTTTTTCTTATCAA
 M A R T C C P I S G D T I S W R N V T F F H Y K K K R T T K N F F F L I N 37
 CTCTTCATCACCACCTTTGCCATGGCTTGGAGTCAGTTAGAATTAGAACCACACACATAGCCTACGCTGTGGGTATTTTCAGTACCCCTATTCTCATAGTCTCCCTTTTCGTCAA
 S S S P H F A M A W S Q L E L E P P H I A Y A C V G I F S T L F S L V S L F V K 77
 AGAACGTCTATACATCGGCGAGGCCACCGTCGCATCCATAGCAGGGCTTATCTTAGGCCACATGTCTAACTGGTTTGACCCCGTCAGCTGGGGTAATTCAGACTACATCACCTAGA
 E R L Y I G E A T V A S I A G L I L G P H C L N W F D P V S W G N S D Y I T L E 117
 AATCTGTCTGAATAGTATTATGTATCCAGATCGTCGCGTGGCAGTAGAGTTGCCGAAAAATATATGATGAACATTGGCTCTCAGTGACTATTTTCTGTGTGCGGTCATGACATGTGG
 I C R I V L C I Q I V A V A V E L P K K Y M M K H W L S V T I F L L P V M T C G 157
 GTGGTTGATTGTTGGGCTCTTCATCTGGTGTCTCATCCCCCATTTACATTCACAGTGGATGTCTAGTCAGTGATGTGTACAGCCACCGACCCGGTCTTGGCTGTGCTGTCTCGG
 W L I V G L F I W C L I P H F T F N D G L L V S A C V T A T D P V L A A A V V G 197
 TAAAGAAAGTTTCCGAAAGAGTCCCTGGCCATTTGCGTAATTTGTGTGTCAGCAGAAATCGGGGTGTAACGACGGTATGGCATTCCCATTTATTTTCTATCCCTAACTTATTATCCA
 K G K F A E R V P G H L R N L L S A E S G C N D G M A F P F I F L S L N L I I H 237
 TTCTGGGCACGCGCGCAATTTGTTAAAGACTGGTTTTGTTGACCATCTTTGGGAGTGATTTTTCGGCTGTTTATTTGGGACAGTCATTGGCTATGCTTTCGTAACATAGTCGCTTT
 S G H A G E I V K D W F L L T I L W E C I F G C L L G T V I G Y V L R K L V A F 277
 TGCAGAAACCAAAACCTTATCGACCGTGAATCGTTTTTGGCCATTTTCGTGTTTATCGCATTCTTCTGCTGGTATTGGGTGATGTTGGGTGGACGATTATTTGGTTTCGTTTGC
 A E N Q N L I D R E S F L A I F V F I A F I S A G I G S M L G V D D * 317
 TGCAGGTACCGCTTTGGCTGGAATGGGGATTCGCAAGAAACCGAAGAGTCACACGTCTCTGTTTATGACTGTTGTGCTAACTGTCTGTTTCTGTTCTACTTCGGGGCAATCA
 A G T A F G W N G A F A K K T E S H V S T V I D L L L N L S F F V Y F G A I I 357
 TCCTTGGCCACAATTCAATAACGCCGACTTGGGATGAATGTATGGAGACTTGTCTGTTGGCATTGTGTCATCATATTTCTTGAGAAGAATTTCCCGCGTGTGCGCCCTCAAAACATAAC
 P W P Q F N N A D L G L N V W R L L V V L A F V I I F L R R I P A V V A L K P I T 397
 CCCCAGCTCAAAACATGGAGAGGCGTATTCTGTGGCCATTTCCGGCCAAATCGGTGTGCGTGCCATATTTGCCAGTATCTTGGCCAGAAAGATTGGAAGCCCACTACACCACCGA
 P D V K T W R E A L F C G H F G P I G V G A I F A S I L A R K D L E A H Y T T E 437
 AGAAACGCGCTACACCACCTCCCAACGAGGATTTCCCTCACTACCAAGTGTCTGCGCACAAATCGGCCAATAGTCTGTTTATTTGTCATTACATCGATCATCGTCCATCTCTCAGT
 E T P L H H L P N E D F P H Y Q L L A T I W P I V C F I V I T S I I V H G S S V 477
 GGCAGTGCTAACACTAGGTAAAGACTAAACAGAATGGCCATACCATGTCTTTACGACCGACCAAGAGGTAATGGCAGTGGCGGTGGATGGCAGAGCTCCAGAACTTGACCGTAC
 A V L T L G K R L N R M A I T M S F T T D Q E G N G S G G W M Q R L Q K L D R T 517
 CACCACCTCTCTCTTACACCGTGTGGATACAATGGCACCACCGCAAACTGCAACCAAGAACTACCGGCATAAAAGTACGTCCAGCAGCGGAGCCAGAGAAAGAAGAACATAA
 T S F S L H R V D T M A P T E K S Q P E T T G I K V R P A G A K R K K N H K 557
 AGACGACAGAACCCCCAGAGCGCAGATCTTACAATAGGGGGCAACAAACGCCAGAACCCGAATCAGCTCAGACACCTTGGCATCCCAACAAACATCCAGGGTATATCGCAT
 D D R T P P E A Q I L Q L G G N K T P E P E S A S D T L A I P N K T S R V I C I 597
 TTGGAGAGAAACCCCTACAAAAGCCGAAAAGCCAGTGGCAACCGGTGCTTACCAAGATGGTGACCAAGTCATTATCGAAGACCATGTTGGTGAAGTAATGGAACCTTGAACCT
 W R E T T L Q K P K R H Q C Q P V A Y Q D G D Q V I I E D Q Y G E V M E N L K L 637
 AACCACCAACAGTACGTGCCCCGTATATCGGAAGTATCCATTCTATGGAATCATTGGAGAGACCTCACACAATACTCAACTGGGTCTGGCGAATACACAACCGACGAGGATACCCA
 T T K P V R A P D I G S I H S M E S L E R H L T Q Y S T G S G E Y T T D E D T H 677
 CGGCTTACAAGAAAATATCTCGGTCTATTCACGCGGTCTACTATAAAAAAGATGATCCAAATAAACGTAAGTATACGCCCACCGTGTGGACGATTGATTGTGATTGAAAATGA
 G L Q E K L S R S L S R R S Y Y K K D D P N K R K V Y A H R V D D L I V I E N E 717
 AGATGGTGATATCATTAGAAGATACAAAGTCAACAAACACGACCTGCACCAAGAGCAGGTGCGGTCTATCATGGGCATGGTAAAGTCTATGGTGGGGATCAAGCCACCCAGAAAT
 D G D I I R R Y K V N K H A P A P R A R S G S I M G M V K S M V G I K P P P E I 757
 CAGTGTAACCGACCTCGAGCAGCGCACCCAGCACAAGATCATACTACCGGAAGAAGAAACACCAATGAGCAGCAAAACCGAACAAGTTGGAAGACACAATAGCTAATATCCCTACA
 S V T D L E H G T Q H K I I L P E E E N T P M S S K T E Q K L E D T I A N I L Q 797
 AGAAACCCACCCCAACCCATCTCCGAAGAGAGTGGCAGGAGGAGAGCGGAAGTCGAAAGAAAGAAAGATTGCAAGCTTTGGGTACTTGGCAAGTAGTCGTCGCGATAGAGAAGA
 E N H T Q P I S E E S A D E E E T E V E K K R R L Q A L G Y L P S S R R D R E D 837
 CGAAGAGGAGTAGATAAATATATAAATTTTTTGTATTGCGAACCTCGGTAGAAAAAAACCAACAACCTCTTTCTTCTCTCCCAATGGATCACAACCTACAACTTGCTTTAAAGCAA
 E E E 840

(b)

Entire sequence

	Nha1p	Zsod22p	Zsod2p	sod2p
Cnh1p	44.1	36.2	41.7	39.3
Nha1p		35.7	50.3	37.8
Zsod22p			34.3	44.7
Zsod2p				37.8

N-terminus

	Nha1p	Zsod22p	Zsod2p	sod2p
Cnh1p	64.0	49.6	59.1	39.3
Nha1p		48.0	70.7	37.8
Zsod22p			46.1	44.4
Zsod2p				37.8

Fig. 1. (a) Nucleotide sequence of *CNH1* and the predicted amino acids of the protein. Putative transmembrane domains are underlined. Potential phosphorylation sites for protein kinases C are boxed and *N*-glycosylation sites are double underlined. Two aspartate residues (D310 and D311) important for Na^+ transport are indicated by asterisks. There is a single CTG codon which was translated as serine instead of leucine. (b) Sequence identity and structural similarity between Cnh1p and other fungal Na^+/H^+ antiporters, Nha1p of *Sacch. cerevisiae*, sod2p of *Schiz. pombe* and Zsod22p and Zsod2p of *Z. rouxii*. For comparing sequence identity, entire sequences and the N-terminus transmembrane regions corresponding to aa 1–471 of Cnh1p were analysed separately. The hydrophilicity plots were generated by using the DNASTAR program.

Fig. 2. (a) Functional expression of *CNH1* in *Sacch. cerevisiae* *NHA1* mutant B31. The growth of three independent B31 clones transformed with pUSCNH1 was assayed on GMM-ura plates containing different concentrations of NaCl in the presence of either glucose or galactose. (b) Mutation of two aspartate residues (D310 and D311) to asparagines abolishes Cnh1p activity. B31 cells transformed with pUScnh1, which contains the mutated gene, were examined for Na⁺ tolerance. Four independent pUScnh1 transformants (sectors 2, 3, 4 and 5) together with B31 transformed with the vector (sector 1) and with pUSCNH1 (sector 6) are shown. Cells were grown on GMM-ura plates in the presence of 0 or 400 mM NaCl and galactose for 3 d.

Fig. 3. Northern blot analysis of *CNH1* expression in response to the change in NaCl concentration. Total RNA was isolated from exponential-phase SC5314 cells grown in GMM containing different amounts of NaCl. The blot was probed using a 2.5 kb *XhoI* DNA fragment of *CNH1*. A PCR-amplified DNA fragment of the *C. albicans* actin gene (*ACT*) was used as a control.

determine whether *CNH1* expression is regulated in response to the change of Na⁺ concentration, *C. albicans* cells were grown in medium containing different amounts of NaCl. Northern analysis (Fig. 3) of the total

Fig. 4. Deletion of *CNH1* genes. (a) Construct for deletion of the entire *CNH1* coding region using the *hisG*-*CaURA3*-*hisG* cassette. After gene replacement, a recombination event between the *hisG* repeats will loop out the *CaURA3* gene and results in *ura*⁻ phenotype. (b) Southern analysis of the genotype of *CNH1* deletion mutants. The genomic DNAs were digested with *XhoI* and the blot probed by using a 200 bp 5' fragment (shown by a bar). Strain names are shown along the top of the blot and the status of *CNH1* alleles are shown on the right.

RNA detected a transcript of ~2.7 kb, a size sufficiently large to encode the predicted protein. The levels of *CNH1* mRNA were not significantly different in cells grown at various NaCl concentrations, indicating that *CNH1* gene expression may not be regulated in response to the change of NaCl concentration.

Chromosomal gene disruption of *CNH1* in *C. albicans* CAI-4

To elucidate the function of the *CNH1* gene in *C. albicans*, gene deletion mutants were generated by using the *hisG*-*CaURA3*-*hisG* gene deletion cassette (Fig. 4a). To verify the genotype of each mutant, genomic DNA of each strain was cleaved with *XhoI* for Southern blot analysis (Fig. 4b). The wild-type *CNH1* gene contains two *XhoI* sites ~2.5 kb apart. One of the *XhoI* sites was removed when the coding sequence was replaced. The heterozygous mutants CaTW1 (*CNH1/cnh1Δ::hisG-CaURA3-hisG*) and CaTW2 (*CNH1/cnh1Δ::hisG*) gave an expected band of 6.5 kb and 3.7 kb, respectively. The homozygous deletion mutant, CaTW3 (*cnh1Δ::hisG/cnh1Δ::hisG-CaURA3-hisG*) gave two bands of 3.7 and 6.5 kb.

Fig. 5. A fraction of *cnh1* null mutant cells develop a highly elongated morphology. SC5314 and CaTW3 were grown on YPD agar plates and in liquid GMM medium (pH 5.5 and 7.5) at 30 °C. The cells on agar plates were allowed to grow for up to 10 d. One colony representative of those of SC5314 and CaTW1 (top left) and one of CaTW3 cells (top right), respectively, are shown. The liquid cultures were examined under a microscope at timed intervals and cells of different morphologies were scored. The morphology of SC5314 and CaTW1 (bottom left) and CaTW3 (bottom right) cells after 7 h growth in GMM at pH 7.5 are shown.

***CNH1* null mutant cells exhibit elongated morphology under conditions for yeast growth**

We observed that on YPD agar plates the homozygous *cnh1* mutant CaTW3 produced wrinkled colonies, an indicator of filamentous growth (Radford *et al.*, 1994). The number of wrinkled colonies increased with the time of culture, and after 10 d nearly all colonies became wrinkled. Under the same conditions, almost all colonies of SC5314 and the heterozygous mutant CaTW1 had smooth surfaces (Fig. 5). Microscopic examination of cells from the wrinkled colonies revealed a mixture of mostly yeast cells and a significant number of highly elongated cells. This result shows that the *cnh1* null mutant cells have a tendency to grow in an elongated form under a condition that normally supports yeast growth.

As the antiporter protein has a role in regulating intracellular pH, and pH changes may influence the growth modes of *C. albicans*, we studied how the *cnh1* mutants respond to pH changes. Both SC5314 and CaTW1 grew exclusively as yeast in GMM at 30 °C, pH 5.5 and about 8% of the cells grew as hyphae at pH 7.5. However, a substantial number of cells of the homozygous mutant CaTW3 displayed highly elongated morphology. Approximately 5% of the cells grown at pH 5.5, and 31% at pH 7.5, exhibit various degrees of elongation (Fig. 5). The elongated cells are also quite enlarged, a morphology different from that of the typical hyphal and pseudohyphal growth. To investigate

whether the deletion of *CNH1* has any effect on the response of cells to some standard germ-tube-inducing conditions, we transferred exponentially growing yeast cells to either GMM medium containing 5% foetal calf serum or Lee's medium (Lee *et al.*, 1975). We found that the mutant cells were indistinguishable from the wild-type cells in either the timing or the morphology of hyphal growth, indicating that the deletion of *CNH1* gene affects cell morphogenesis through a mechanism independent of the pathways for typical filamentous growth.

Deletion of *CNH1* causes retarded growth

To examine whether CaTW1 and CaTW3 were more sensitive to NaCl than SC5314, we inoculated cells onto GMM plates supplemented with NaCl at various concentrations up to 1.6 M at either pH 5.5 or pH 7.5. After 3 d incubation at 30 °C, CaTW1 and CaTW3 formed patches nearly as dense as SC5314 on all the plates. To determine the growth rates of SC5314, CaTW1 and CaTW3, we repeated the experiment in liquid GMM and monitored cell growth by measuring OD₆₀₀. Under both pH conditions, CaTW1 and CaTW3 grew considerably more slowly than SC5314 (Fig. 6a, e). In GMM, the doubling times for SC5314, CaTW1 and CaTW3 are 2.7, 3.6 and 4.9 h at pH 5.5, and 3, 3.9 and 5.3 h at pH 7.5, respectively. There is apparently a gene-dosage effect in that the heterozygous mutant CaTW1 grew at a rate intermediate between those of SC5314 and CaTW3. When 1 M NaCl was added to the medium, CaTW1 and CaTW3 did not appear to be more sensitive to the high salt concentration, because their growth rates decreased proportionately to that of SC5314 (data not shown). High concentrations of Na⁺ are well known to generate osmotic potential across the cell membrane. It is possible that the effect of high osmotic pressure on the cells may mask a small increase in NaCl sensitivity of CaTW1 and CaTW3. To limit the effect of high osmotic pressure of a high NaCl concentration, we redetermined the growth rates using Li⁺, an analogue of Na⁺. Li⁺ is much more toxic than Na⁺ so that it can be used at much lower concentrations without generating high osmotic potential. Li⁺ is often used as an analogue of Na⁺ in transport studies (Fleishman, 1991; Hahnenberger *et al.*, 1997; Ros *et al.*, 1998). The effect of Li⁺ on the growth rates of the three *Candida* strains was very similar to that of NaCl (Fig. 6). Again, CaTW1 and CaTW3 exhibited a similar level of sensitivity to an increase in LiCl concentration. These results show that the deletion of *CNH1* causes a retarded growth in *C. albicans* but has little effect on the sensitivity of cells to high salt concentrations.

Animal experiments

Though the *cnh1* null mutants exhibit retarded growth *in vitro*, it is not clear how the slower growth will affect pathogenesis in a mammalian host. It is important to know whether the deletion of *CNH1* will have *in vivo* consequences that are not fully accountable by the slow

Fig. 6. Growth of *SC5314*, *CaTW1* and *CaTW3* in response to different Li⁺ concentrations at pH 5.5 and 7.5. Cells were grown in GMM buffered at pH 5.5 (a–d) and pH 7.5 (e–h) in the presence of different concentrations of LiCl. Growth was monitored by measuring OD₆₀₀ at 1.5 h intervals up to 28 h. Li⁺ concentrations are specified at the upper right-hand corner of each group of curves. ◆, *SC5314*; ■, *CaTW1*; ●, *CaTW3*.

Fig. 7. Survival of Balb/c mice infected with *SC5314*, *CaTW1* and *CaTW3*. Mice were injected intravenously with cell suspensions of *SC5314* (●), *CaTW1* (△) or *CaTW3* (□). Survival was monitored over 28 d.

growth rate. To address this issue, we inoculated mice with *CaTW1* and *CaTW3*, using *SC5314* as positive control (Fig. 7). Within 4 d all mice inoculated with *SC5314* and *CaTW1* were dead. The five mice inoculated

with *CaTW3* as a whole survived better, with four mice dying on the second, fourth, twelfth and seventeenth day, and one still alive at the end of the 28 d monitoring ($P < 0.001$). The kidneys of dead mice were removed, sectioned for staining and homogenized for viable cell counts. The kidneys of the dead mice infected by the wild-type and mutant strains looked similar, heavily colonized by both yeast and hyphal cells, indicating that systemic *C. albicans* infection is the cause of death (not shown). The numbers of viable fungal cells in kidneys were about 3×10^6 c.f.u. g⁻¹ for all the dead mice and 6×10^4 c.f.u. g⁻¹ for the *CaTW3*-infected mouse that was sacrificed at the end of the 28 d.

DISCUSSION

Using GFP as a reporter in a screen for *C. albicans* genes that may be preferentially expressed in the hyphal cells led to the isolation of *C. albicans* Na⁺/H⁺ antiporter gene *CNH1*. The result of the initial screen suggested that the *CNH1* promoter was hypha-specific. However, this observation was not consistently repeated under

various conditions supporting hyphal growth (results not shown). In the screen, thousands of colonies duplicated onto both GMM and GMM + 5 % foetal calf serum plates were examined using fluorescence microscopy over a period of one week and the plates were kept at 4 °C most of the time. It is likely that some unknown factors contributed to the observed hyphal-specific expression in the screen. Nevertheless, the Na⁺/H⁺ antiporter has a role in H⁺ transport, and pH changes are known to affect *C. albicans* morphogenesis. Enhanced expression of *CNH1* in hyphal cells under certain conditions remains a possibility and needs further investigation.

Amino acid sequence alignment of all available Na⁺/H⁺ antiporters of fungi revealed a high degree of conservation, especially over the N-terminal half of the molecule, which contains all the similarly spaced trans-membrane segments, indicating a conserved core structure of the ion-transport machinery. Some amino acid residues are invariable in all Na⁺/H⁺ antiporters found so far from both prokaryotic and eukaryotic organisms, such as the two aspartate residues corresponding to D310 and D311 of Cnh1p, which are believed to form the Na⁺-binding site (Inoue *et al.*, 1995; Padan & Schuldiner, 1996; Dibrov *et al.*, 1998). We demonstrated that mutation of D310 and D311 to asparagines abolished the ability of *CNH1* to confer salt-tolerance upon the *Sacch. cerevisiae* *ena1 nha1* mutant. This result is in good agreement with results obtained from *Schiz. pombe* *sod2p* carrying the same mutations (Dibrov *et al.*, 1998). The C-terminal half of the Na⁺/H⁺ antiporters is less conserved and varies in length, and is speculated to play a regulatory role (Aronson *et al.*, 1982; Balcells *et al.*, 1997; Dibrov & Fliegel, 1998). Studies of the mammalian Na⁺/H⁺ antiporter family (NHE1–NHE6) also revealed the existence in the C-terminal tail of consensus sites for phosphorylation by protein kinase A and/or protein kinase C as well as sites suitable for calmodulin kinases and for proline-directed Ser/Thr kinases (Paranjape *et al.*, 1990; Padan & Schuldiner, 1994). Regulated phosphorylation of NHE1 and NHE3 have been experimentally demonstrated (Bianchini *et al.*, 1997; Wakabayashi *et al.*, 1997). In Cnh1p, several putative protein kinase C phosphorylation sites are found in the C-terminal region. Mutation of these sites would help to elucidate the function(s) of this region.

To better understand the physiological role of *CNH1*, heterozygous and homozygous gene deletion mutants were constructed. The loss of a single copy did not result in any observable changes in morphology. The wild-type and heterozygous mutants grew exclusively as yeast at pH 5.5 and ~ 8 % of the cells developed a highly elongated shape at pH 7.5 in GMM at 30 °C. In contrast, the mutants with both copies deleted grew as a mixture of yeast and elongated cells at both pH 5.5 and 7.5, the percentage of the elongated cells being 5 and 31 %, respectively. These results suggest that Cnh1p activity may influence the execution of the signalling pathways for morphogenesis, presumably by participating in

cytoplasmic pH regulation. However, since cross-membrane proton flux and regulation of intracellular pH need concerted actions of multiple transporters, it is premature to speculate on the molecular mechanism that links Cnh1p activity to cell morphology. Two pH-regulated genes, *PHR1* and *PHR2*, have been shown to be involved in morphogenesis in *C. albicans* (Saporito-Irwin *et al.*, 1995; Bernardis *et al.*, 1998).

The Na⁺/H⁺ antiporter has a role in salt tolerance in both fungi and bacteria (Jia *et al.*, 1992; Nozaki *et al.*, 1998). When heterologously expressed in a *Sacch. cerevisiae* strain severely defective in Na⁺ extrusion, *CNH1* confers resistance to 800 mM NaCl, indicating that *CNH1* may play a similar role in *C. albicans* cells. However, we found that *C. albicans* *cnh1* mutants do not exhibit higher sensitivity to an increase in either Na⁺ or Li⁺ concentration, except for a general slower growth rate in comparison with the wild-type cells. One plausible explanation for this observation is the presence of other Na⁺-extrusion mechanisms in *C. albicans* such as Na⁺-ATPases. In *Sacch. cerevisiae* there is a family of Na⁺-ATPases encoded by a tandem array of genes, *ENA1–4*, and their expression is induced by high-Na⁺ stress (Wieland *et al.*, 1995; Prior *et al.*, 1996). It has been shown that overexpression of *NHA1* significantly enhanced salt tolerance of *Sacch. cerevisiae* cells only when the four *ENA* genes were deleted (Prior *et al.*, 1996). Clearly, the Na⁺-ATPases are able to compensate for the salt sensitivity resulting from the deletion of the Na⁺/H⁺ antiporter gene. Actually, Hahnenberger *et al.* (1997) demonstrated that Na⁺/H⁺ antiporter and sodium pumps could be used interchangeably by *Sacch. cerevisiae* to regulate intracellular sodium concentration.

The animal experiment shows that the deletion of both copies of *CNH1* led to a considerable delay in the killing of infected mice. Since the numbers of viable *C. albicans* cells in kidney at the time of animal death were comparable, the delayed death of mice infected by the null mutant is probably a consequence of its slow growth. The reason why the animals infected by the heterozygous mutant did not exhibit delayed death is not clear. It could be that its rate of growth is not sufficiently slow to have an effect on the whole process of pathogenesis. Though the relevance of *CNH1* function to the virulence of the pathogen is not conclusive at the current stage of investigation, the growth defect and delayed pathogenesis as a result of *CNH1* deletion render the gene a potential target for developing anti-*Candida* drugs.

In summary, we have isolated and functionally characterized a *C. albicans* Na⁺/H⁺ antiporter gene, *CNH1*. It confers sodium tolerance when heterologously expressed in a *Sacch. cerevisiae* strain defective in sodium extrusion. Deletion of *CNH1* leads to slower growth rates in a gene-dosage dependent manner. Though deletion of the gene has no detectable effects on the response of cells to standard hypha-inducing conditions, a considerable fraction of the null mutant cells

develop a highly elongated morphology under conditions that normally support yeast growth, suggesting that *CNH1* may influence morphogenesis through different pathways.

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