Plasma-membrane Cnh1 Na^+/H^+ antiporter regulates potassium homeostasis in *Candida albicans*

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The physiological role of *Candida albicans* Cnh1, a member of the Na^+/H^+ antiporter family, was characterized. Though CaCnh1p had broad substrate specificity and mediated efflux of at least four alkali metal cations upon heterologous expression in *Saccharomyces cerevisiae*, its presence in *C. albicans* cells was important especially for potassium homeostasis. In *C. albicans*, CaCnh1p tagged with GFP was localized in the plasma membrane of cells growing as both yeasts and hyphae. Deletion of *CNH1* alleles did not affect tolerance to NaCl, LiCl or CsCl, but resulted in increased sensitivity to high external concentrations of KCl and RbCl. The potassium and rubidium tolerance of a *cnh1* homozygous mutant was fully restored by reintegration of *CNH1* into the genome. The higher sensitivity of the *cnh1/cnh1* mutant to external KCl was caused by a lower K^+ efflux from these cells. Together, the functional characterization of the CaCnh1 antiporter in *C. albicans* revealed that this antiporter plays a significant role in *C. albicans* physiology. It ensures potassium and rubidium tolerance and participates in the regulation of intracellular potassium content of *C. albicans* cells.

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INTRODUCTION

In all living cells, including fungi, the major cytoplasmic cation is potassium, and cells expend energy to accumulate large amounts of K^+ . Potassium serves as an important co-factor in many biosynthetic processes and is required for various biological functions (e.g. regulation of cell volume and intracellular pH). In contrast, sodium is a minor component whose increased internal concentration is toxic for many cells, and thus the Na^+ surplus must be efficiently eliminated from the cell cytosol (Rodriguez-Navarro, 2000). Plasma-membrane Na^+/H^+ antiporters belong to the group of transport systems that ensure the optimal intracellular level of alkali metal cations in most organisms (Orlowski & Grinstein, 1997; Padan *et al.*, 2001; Sychrová, 2004). As typical secondary transport systems, they either use the gradient of protons across the plasma membrane to mediate the efflux of alkali metal cations (in bacteria, yeasts and plants) or they employ the inward gradient of Na^+ to pump out protons (in mammalian cells) (Sychrová, 2004).

The genes encoding plasma-membrane Na^+/H^+ antiporters (*NHA1/SOD2* family) from many yeast species have been cloned and their transport properties (substrate specificity and transport activity) determined via their heterologous expression in *Saccharomyces cerevisiae*. Members of the Nha1/Sod2 family differ in their substrate specificities and cell functions. The genomes of most yeast species (e.g. *S. cerevisiae*, *Candida albicans*, *Pichia sorbithophila*,

Debaryomyces hansenii) contain only one gene encoding a Na^+/H^+ antiporter with a broad substrate specificity for Na^+ , Li^+ , K^+ and Rb^+ (Banuelos *et al.*, 1998, 2002; Kinclova *et al.*, 2001b, c; Velkova & Sychrová, 2006). Only two species (*Yarrowia lipolytica*, *Schizosaccharomyces pombe*) possess two types of plasma-membrane Nha1/Sod2 antiporter: one of them primarily transports $\text{Na}^+(\text{Li}^+)$ cations, the other mainly transports K^+ (and Rb^+) (Papouškova & Sychrová, 2006, 2007). The antiporters transporting only Na^+ and Li^+ participate especially in detoxification of cells from toxic cations, whereas members of the Nha1/Sod2 family with broad substrate specificity are supposed to be involved, besides in the elimination of toxic cations, in other cell functions (e.g. in the regulation of intracellular K^+ concentration, pH and cell volume). As most of the data concerning substrate specificity and transport capacity of yeast Na^+/H^+ antiporters were obtained for heterologously expressed proteins, their physiological role in the original yeast species can be only speculated about. The most characterized, as far as its structure and physiological function is concerned, is the plasma-membrane $\text{Na}^+,\text{K}^+/\text{H}^+$ antiporter Nha1 from *S. cerevisiae*. Besides detoxification, it exhibits complementary action with Ena1 Na^+,K^+ -ATPase in the maintenance of an intracellular steady-state concentration of K^+ (Banuelos *et al.*, 1998). Furthermore, the *S. cerevisiae* Nha1 antiporter is involved in the regulation of the cell cycle (Simon *et al.*, 2001), in the immediate cell response to osmotic shock (Kinclova

et al., 2001c; Kinclova-Zimmermannova & Sychrová, 2006; Proft & Struhl, 2004), in homeostatic regulation of plasma-membrane potential (Kinclova-Zimmermannova *et al.*, 2006), and electrogenic exchange of protons for alkali metal cations via ScNha1p (Ohgaki *et al.*, 2005) is also vital for the regulation of intracellular pH (Brett *et al.*, 2005; Sychrová *et al.*, 1999).

The genes encoding plasma-membrane Nha1/Sod2 orthologues in two *C. albicans* wild-types were isolated and their products partially characterized upon expression in *S. cerevisiae* (CNH1, Soong *et al.*, 2000; and CNH1-G23, Kinclova *et al.*, 2001b). Both CaCnh1 antiporters have broad substrate specificity for several alkali metal cations (Kinclova *et al.*, 2001b). In addition, *C. albicans* antiporters contribute to the buffering of cytoplasmic pH in *S. cerevisiae* cells (similarly to *S. cerevisiae*'s own Nha1p), since upon alkalization of the intracellular pH, they are able to mediate an immediate high efflux of potassium to draw in some protons (Kinclova *et al.*, 2001b). These results suggest that the role of Cnh1p in *C. albicans* cells is broader than simply detoxification from surplus alkali metal cations.

C. albicans is a major opportunistic fungal pathogen. Its pathogenicity is believed to be linked to its ability to grow in distinct morphological forms. *C. albicans* can grow as yeasts, pseudohyphae or hyphae in response to various environmental factors, including presence of alkali metal cations and changes in pH (Odds, 1985; Sudbery *et al.*, 2004). The induction of germ-tube formation is associated with a steep rise in internal pH (Stewart *et al.*, 1988), probably due to an increased activity of plasma-membrane H⁺-extruding ATPase (Kaur & Mishra, 1991; Monk *et al.*, 1993). Previous studies of the effect of alkali metal cations on *C. albicans* cells revealed that (1) high external concentrations of alkali metal cations (Na⁺, Li⁺, K⁺) decrease the growth capacity of *C. albicans* cells (Hermann *et al.*, 2003), (2) an increased extracellular sodium

concentration inhibits germ-tube formation (Biswas *et al.*, 2000; Northrop *et al.*, 1997), (3) preincubation with alkali metal cations negatively influences certain *C. albicans* virulence traits, such as adhesion, cell-surface hydrophobicity and germinating ability (Hermann *et al.*, 2003), and (4) hyphal cells contain a higher concentration of K⁺ than yeast cells (Watanabe *et al.*, 2006). Thus, it is obvious that transport systems mediating fluxes of protons and alkali metal cations across the plasma membrane might play an important role in controlling *C. albicans* morphology and virulence. A previous study of the role of CaCnh1p in *C. albicans* showed that the deletion of CaCNH1 alleles did not change cell tolerance to high NaCl or LiCl concentrations (Soong *et al.*, 2000). However, the role of CaCnh1p in K⁺ homeostasis and the antiporter transport activity in *C. albicans* cells were not examined.

This study reports detailed characterization of the transport properties of CaCnh1p and its importance for *C. albicans* tolerance to alkali metal cations. The results presented clearly demonstrate that CaCnh1p ensures the potassium and rubidium tolerance of *C. albicans* cells and participates in the regulation of intracellular potassium concentration.

METHODS

Strains and media. Strains (listed in Table 1) were routinely grown at 30 °C in standard media (either YPD or YNB with appropriate auxotrophic supplements when necessary) containing 2% (w/v) glucose. Uridine was added to a final concentration of 50 µg ml⁻¹, other auxotrophic supplements to a final concentration of 20 µg ml⁻¹. Solid media were prepared by adding 2% (w/v) agar. Before each functional assay, *C. albicans* cells were refreshed from a 20% (v/v) glycerol stock at -80 °C on YPD plates.

Transformation of yeast cells. *S. cerevisiae* cells were transformed by electroporation, and *C. albicans* cells by the lithium acetate/PEG method (Walther & Wendland, 2003).

Table 1. Yeast strains used in this study

Strain	Relevant genotype	Source or reference
<i>S. cerevisiae</i>		
BW31	<i>MATa leu2-3/112 ura3-1 trp1-1 his3-11/15 ade 2-1 can1-100 GAL SUC2 mal10 ena1-4::HIS3 nha1::LEU2</i>	Kinclova-Zimmermannova <i>et al.</i> (2005)
<i>C. albicans</i>		
BWP17	<i>ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>	Wilson <i>et al.</i> (1999)
BWP17-S1	BWP17 with Clp10 integrated into RP1 locus	This study
BWOZ 1	BWP17, except for <i>CNH1/cnh1Δ::hisG-URA3-hisG</i>	This study
BWOZ 13	BWP17, except for <i>CNH1/cnh1Δ::hisG</i>	This study
BWOZ 13-8	BWP17, except for <i>cnh1Δ::hisG-URA3-hisG/cnh1Δ::hisG</i>	This study
BWOZ 13-81	BWP17, except for <i>cnh1::hisG/cnh1Δ::hisG</i>	This study
BWOZ 13-81Clp10	BWOZ13-81 with Clp10 integrated into RP1 locus	This study
BWOZ 13-81N17	BWOZ13-81 with CNH1-Clp10 integrated into RP1 locus	This study
BWOZ 13M2GFP	BWP17, except for <i>CNH1-MoGFP-URA3/cnh1Δ::hisG</i>	This study

Plasmids. The multi-copy plasmid pCNH1-M18 containing the *CNH1* gene from *C. albicans* SC5314 behind the *S. cerevisiae* *NHA1* promoter (Kinclova *et al.*, 2001b) was used for the heterologous expression of CaCnh1p in *S. cerevisiae* cells. For constructing the deletion cassette, PCR fragments corresponding to the *CNH1* upstream and downstream flanking sequences were cloned into plasmid KB986 (provided by D. Kornitzer, Rappaport Faculty of Medicine, Technion, Haifa, Israel) containing the *hisG-URA3-hisG* sequence (Fonzi & Irwin, 1993). To amplify the flanking regions, *C. albicans* BWP17 genomic DNA was used as template. A 407 bp DNA fragment (the sequence from –490 to –83 upstream of the *CaCNH1* ORF) was synthesized using oligonucleotides CaSaI (5'-GTAGTTGAGCTCACATGTAACCGACTTTTC-3') and CaBamHI (5'-GCGGGATCCAAGAAATTGTATCACCTG-3'), in which SaI and BamHI sites were introduced (underlined). The PCR product digested with SaI and BamHI was cloned into KB986 at the 5' end of the deletion cassette, resulting in pCNH1-5'. A 477 bp DNA fragment (the sequence from +12 to +489 behind the *CaCNH1* STOP codon) was synthesized using CaPstI (5'-AAACTGCAGTTATGCGAACCTCGGTAG-3') and CaHindIII (5'-AACTTAAGCTTGACTTGTCGTTGTTTGG-3'), introducing PstI and HindIII sites (underlined). The PCR product digested with PstI and HindIII was cloned into pCNH1-5' at the 3' end of the deletion cassette, resulting in pCNH1-5'-3'. For reintegration of the *CaCNH1* gene into the genome, the *CaCNH1* ORF with a 496 nt promoter region was amplified with oligonucleotides CaCNH1_BamHIF (5'-TAGGGGGATCCTTCACCTGGTAGTTG-3') and CaCNH1_BamHIR (5'-CGCGGATCCAAGGTTTGGGAGATGGTG-3'), introducing a BamHI site at both ends (underlined). The PCR fragment obtained (3.1 kb) was digested with BamHI and cloned into the BamHI site of the Clp10 integrative vector (provided by A. J. P. Brown, University of Aberdeen, UK) (Murad *et al.*, 2000), resulting in plasmid CNH1-Clp10.

Construction of *C. albicans* strains. The two allelic copies of the *CaCNH1* gene were deleted using a two-step procedure (Fonzi & Irwin, 1993). In each step, the genotype of at least two independent mutants was verified by PCR and by Southern blot analysis of HindIII-digested genomic DNA with a 407 bp probe synthesized by PCR (template: *C. albicans* BWP17 genomic DNA; oligonucleotides: CaSaI and CaBamHI). The entire *CNH1*-deletion cassette was released from pCNH1-5'-3' by SaI/HindIII digestion and used for the transformation of strain BWP17. After the first transformation, two Ura⁺ clones (BWOZ 1, Table 1) with the *hisG-URA3-hisG* cassette correctly integrated into the *CaCNH1* locus were selected. The *URA3* gene was excised on media containing uridine (50 µg ml⁻¹) and 5-fluoroorotic acid (1 mg ml⁻¹), and the resulting Ura⁻ *cnh1/CNH1* heterozygous strains (BWOZ 13, Table 1) were used for deletion of the second *CaCNH1* copy, following the same procedure. In the end, two independent homozygous *cnh1/cnh1* mutant strains (BWOZ 13-81, Table 1) were obtained.

Wild-type BWP17 and the homozygous *cnh1/cnh1* mutant BWOZ 13-81 were made Ura⁺ by transformation with *StuI*-digested empty Clp10 containing *CaURA3* (Murad *et al.*, 2000). For the complementation test, the *cnh1/cnh1* mutant (BWOZ 13-81) was transformed with *StuI*-digested CNH1-Clp10. In each case, two independent transformants of BWP17-S1, BWOZ 13-81Clp10 and BWOZ 13-81N17 (Table 1), were isolated and correct integration of Clp10 or CNH1-Clp10 verified by PCR.

For CaCnh1p visualization, the *CaCNH1* gene was tagged at its 3' end with the green fluorescent protein (GFP)-encoding sequence (Gola *et al.*, 2003). To synthesize the GFP module, consisting of the last 100 nt of the *CaCNH1* ORF without a stop codon in-frame attached to the GFP sequence, *CaURA3* gene sequence and 100 nt sequence downstream of the *CaCNH1* ORF, we used 120 nt and 124 nt primers

[S1CNH1GFP (5'-GAGTGCGGACGAGGAGGAGACGGAAGTTGA-AAAGAAAAGAAGATTGCAAGCTTTGGGTACTTGCCAAAGTAG-TCGTCGCGATAGAGAAGACGAAGAGGAGGGTGTGGCGCAG-GTGCTTC-3') (forward) and S2CNH1GFP (5'-GGTTTGGGAG-ATGGTGTGGTTGGCATATTCGTTGAAACAGGAGTGAAACAAT-TCACTTGTCAACTGAACGACGCCTCAAAGTCTTATTAATGA-ATTGCTCTGATATCATCGATGAATTCGAG-3') (reverse)] and plasmid pFA-MoGFP-URA3 (Gola *et al.*, 2003) (provided by J. Wendland, University of Basel, Switzerland). The Ura⁻ *cnh1/CNH1* strain (BWOZ 13) was transformed with the resulting PCR fragment (2.3 kb), and the Ura⁺ clones were selected (BWOZ 13M2GFP, Table 1). Site-directed integration of the GFP module behind the *CaCNH1* allele was verified by PCR.

Salt-tolerance determination on solid media. The tolerance of cells to alkali metal cations was estimated by spotting 3 µl of serial 10-fold dilutions of saturated cultures on solid YNB medium supplemented with increasing amounts of salts (NaCl, 300–2300 mM; KCl, 800–1800 mM; LiCl, 10–300 mM; RbCl, 500–1600 mM; CsCl, 30–400 mM). pH 3.5, 5.5 and 7.0 plates were prepared as described previously (Kinclova *et al.*, 2001c). Growth was recorded for 4–7 days. Growth assays were repeated two to four times with similar results. Representative data are shown.

Growth assay in liquid media. YNB medium (30 ml), at pH 3.5 (adjusted with tartaric acid after autoclaving), without or supplemented with the indicated amounts of KCl was inoculated to OD₆₀₀ 0.002 from cell pre-cultures grown in 15 ml YNB at pH 3.5 for 8 h (exponential phase of growth). Cultures were incubated with vigorous shaking at 30 °C, and the growth was assessed by measuring the increase in cell suspension OD₆₀₀ for 25 h. The experiment was repeated twice and representative results are shown.

Fluorescence microscopy. Stationary-phase cells (incubated overnight at 30 °C in water) were used for the inoculation of 2.5 ml YPD or YPD containing 20% (v/v) fetal bovine serum (FBS) and incubated at 30 °C or 37 °C, respectively. After 4 h, cells were viewed with an Olympus BX60 microscope with an F-view II digital camera microscope. For whole-cell pictures, Nomarski optics was used. A U-MGFPHQ GFP filter block with excitation from 460 to 480 nm and emission from 495 to 540 nm was used for GFP visualization. Images were processed with Jasc Paint Shop Pro 7.04.

Alkali-metal-cation loss measurements. For cation loss measurements, *S. cerevisiae* or *C. albicans* cells were grown in YNB medium to OD₆₀₀ ≈ 0.2, harvested and washed. To pre-load with Na⁺ or Li⁺, cells were incubated in YNB medium (pH 7.0) and supplemented with 100 mM NaCl or 50 mM LiCl for 60 min. For K⁺ efflux measurements, no preloading was necessary. Cation efflux was followed in 20 mM MES buffer [*S. cerevisiae* cells; pH adjusted to 5.5 with Ca(OH)₂] or in 10 mM Tris buffer [*C. albicans* cells; pH adjusted to 4.4 with citric acid and Ca(OH)₂ then added to bring the pH up to 4.5]. Both buffers contained 0.1 mM MgCl₂ and 2% (w/v) glucose, and were supplemented with KCl or RbCl as indicated in the text. Samples of cells were withdrawn at regular time intervals, collected on Millipore membrane filters, washed and the intracellular concentration of Na⁺, Li⁺ or K⁺ was estimated by atomic absorption spectrophotometry (Camacho *et al.*, 1981; Kinclova *et al.*, 2001c). Data shown are the means ± SD of at least three replicate values.

RESULTS

CaCnh1 antiporter has broad substrate specificity

To verify the transport properties of CaCnh1p, a series of experiments in *S. cerevisiae* cells were performed. The

CaCNH1 gene (in pCNH1-M18) was expressed in the *S. cerevisiae* mutant strain BW31 (*ena1-4Δ nha1Δ*), which is very sensitive to alkali metal cations due to the absence of its main sodium and potassium extrusion systems, Na^+ -ATPases and the Nha1 antiporter. The salt tolerance of the transformants was tested in drop tests on plates containing increasing amounts of NaCl, LiCl, KCl and RbCl. As expected, expression of the *CaCnh1* antiporter in BW31 cells considerably increased the tolerance of cells to higher concentrations of sodium, lithium, potassium, and rubidium cations (Fig. 1a). The transport activity of *CaCnh1p* was also confirmed by measurements of the efflux of sodium, lithium and potassium. Significantly higher Na^+ , Li^+ and K^+ loss was observed from BW31 cells expressing *CaCnh1p* than from cells with the empty vector (Fig. 1b). These data confirmed (1) that the *CaCnh1* antiporter recognizes and transports at least four alkali metal cations (Na^+ , Li^+ , K^+ and Rb^+), and (2) that its activity can be monitored as cell tolerance to increased amounts of alkali-metal-cation salts, and suggested that the physiological role

of *CaCnh1p* in *C. albicans* cells could be much broader than simple detoxification from surplus Na^+ and Li^+ .

CNH1* is important for alkali-metal-cation tolerance in *C. albicans

Previous characterization of *Cnh1p*'s function in the *C. albicans* strain CAI-4 revealed that deletion of both copies of *CNH1* did not decrease the tolerance of *C. albicans* cells to NaCl and LiCl at pH 5.5 or 7.5 (Soong *et al.*, 2000). Unfortunately, CAI-4-derived strains with *cnh1* deletion(s) are no longer available (Y. Wang, personal communication). Thus, to extend our knowledge of the role of the *Cnh1* antiporter in *C. albicans* cells physiology, we created a new set of isogenic *C. albicans* strains lacking either one or both chromosomal copies of the *CNH1* gene (Table 1). The two *CNH1* alleles were sequentially deleted from the *ura3 his1 arg4 C. albicans* strain BWP17 (a derivative of wild-type SC5314; Wilson *et al.*, 1999). First, we examined the extent to which *cnh1* mutations affected the growth of

Fig. 1. Expression of *CaCnh1p* in *S. cerevisiae* cells. (a) Tolerance of *S. cerevisiae* BW31 cells expressing the *CaCnh1* antiporter to alkali-metal-cation salts. Serial 10-fold dilutions of saturated cultures were spotted on YNB medium supplemented with NaCl, LiCl, KCl and RbCl as indicated; plates were incubated for 2 (YNB) or 5 (YNB + salts) days at 30 °C. Dilution series correspond to BW31 cells containing empty YEp352 or expressing the *CaCnh1* antiporter from pCNH1-M18. Very similar results were observed in three independent experiments. (b) Loss of alkali metal cations from *S. cerevisiae* BW31 cells expressing *CaCnh1p*. BW31 cells containing the empty vector or expressing *CaCnh1p* from pCNH1-M18, grown in YNB medium, were preincubated with 100 mM NaCl or 50 mM LiCl at pH 7.0 for 60 min, transferred to the incubation buffer (pH 5.5) containing 10 mM KCl and the internal Na^+ or Li^+ content was followed for 40 min. Loss of K^+ was followed for 60 min in the incubation buffer at pH 5.5 containing 10 mM RbCl (no preloading necessary). Columns represent the amount of particular cation lost from cells within the indicated time. Data shown are the mean $\pm$ SD of three replicate values. The mean initial cation concentrations in both strains were: Na^+ , 143 ± 10 nmol (mg dry wt)⁻¹; Li^+ , 68 ± 13 nmol (mg dry wt)⁻¹; K^+ , 612 ± 33 nmol (mg dry wt)⁻¹.

Fig. 2. Growth of *C. albicans* cells with *cnh1* deletion(s) in the presence of alkali metal cations. Serial 10-fold dilutions of saturated cultures were spotted on YNB medium (pH 3.5) supplemented with NaCl, LiCl, CsCl, KCl and RbCl as indicated; plates were incubated for 2 (YNB) or 4 (YNB + salts) days at 30 °C. Dilution series correspond to *C. albicans* wild-type strain (BWP17) and its derivatives lacking one (BWOZ 1, *CNH1/cnh1Δ::hisG-URA3-hisG*; BWOZ 13, *CNH1/cnh1Δ::hisG*) or both (BWOZ 13-8, *cnh1Δ::hisG-URA3-hisG/cnh1Δ::hisG*; BWOZ 13-81, *cnh1Δ::hisG/cnh1Δ::hisG*) *CNH1* alleles. Data presented are from a representative experiment of four independent assays.

cells in the presence of various alkali metal cations. The growth of the *CNH1/CNH1*, *CNH1/cnh1* and *cnh1/cnh1* strains was tested in drop tests on plates containing increasing amounts of five salts (Fig. 2). As the transport activity of the Na⁺/H⁺ antiporter is governed by the electrochemical gradient of protons across the plasma membrane, i.e. increases with a decrease in extracellular pH, the pH of the medium was adjusted to 3.5 to better observe the potential effect of the absence of *CNH1* allele(s). All strains grew equally well on YNB without salts (Fig. 2) suggesting that deletion of the *CNH1* gene(s) in the *C. albicans* BWP17 strain background did not affect the growth capacity of cells. Deletion of one of the two *CNH1* alleles caused no phenotype: the heterozygous strains *CNH1/cnh1* exhibited the same robust growth as the *CNH1/CNH1* wild-type on plates with all five alkali-metal-cation salts. No difference in salt sensitivity was observed between the wild-type and homozygous *cnh1* mutants on plates with NaCl, LiCl and CsCl (Fig. 2), though *CaCnh1p* is able to efficiently transport Na⁺ and Li⁺ cations (Fig. 1). No difference in growth was observed even if the concentration was increased to 2300 mM NaCl, 300 mM LiCl or 400 mM CsCl, or when growth was tested in liquid media at pH 3.5 containing 1000 mM NaCl (data not shown). On the other hand, cells lacking both *CNH1* alleles were sensitive to potassium and rubidium cations compared to wild-type cells or heterozygous mutants with one functional *CNH1* allele (Fig. 2). The results observed in drop tests on solid media supplemented with KCl were further confirmed by growth assays in liquid media containing KCl. As shown in Fig. 3, the wild-type (*CNH1/CNH1*) and homozygous *cnh1* mutant (*cnh1/cnh1*) grew at similar rates in the absence of added KCl (the generation time was about 2 h for both strains). However, cells lacking both copies of the *CNH1* gene grew much more slowly in the presence of 1000 mM KCl compared to the wild-type (generation time 4 h vs 2.25 h, respectively). Overall, our data revealed that in *C. albicans*,

the deletion of both *CNH1* alleles results in a clear phenotype of sensitivity to high external concentrations of KCl and RbCl. It also suggests that other transport system(s) provide efficient elimination of toxic alkali metal cations (Na⁺, Li⁺, Cs⁺) in *C. albicans* cells under the conditions tested in our experiments.

Fig. 3. Growth of *C. albicans* wild-type strain (BWP17, squares) or homozygous *cnh1Δ::hisG/cnh1Δ::hisG* null mutant strain (BWOZ 13-81, triangles) in liquid YNB medium (pH 3.5) without (filled symbols) or supplemented with 1000 mM KCl (empty symbols). Cells were inoculated from pre-cultures to OD₆₀₀ 0.002, incubated with vigorous shaking at 30 °C, and growth was assessed by measuring the increase in OD₆₀₀ for 25 h. The experiment was repeated twice and representative results are shown.

Potassium sensitivity of homozygous *cnh1/cnh1* strain is dependent on extracellular pH

As mentioned above, the transport activity of Na^+/H^+ antiporters is dependent on the proton gradient across the plasma membrane, i.e. the protonmotive force. In the next series of experiments, the dependence of the potassium sensitivity of the *cnh1/cnh1* strain on extracellular pH was tested. Drop test experiments of *CNH1/CNH1* (BWP17), *CNH1/cnh1* (BWOZ 13) and *cnh1/cnh1* (BWOZ 13-81) growth were performed on YNB medium buffered to pH 3.5, 5.5 and 7.0 without or supplemented with 1400 mM or 1800 mM KCl. At all three pHs, no difference in growth was observed on plates without salts (Fig. 4), again indicating that the deletion of the *CNH1* gene does not change the growth capacity of the strain. In the presence of 1400 mM KCl, growth inhibition of the *cnh1* homozygous mutant was only observed at pH 3.5, whilst at pH 5.5 and 7.0, all three strains grew equally (data not shown). The high potassium sensitivity of cells lacking both *CNH1* alleles at all three pHs could be observed on plates with 1800 mM KCl (Fig. 4). Compared to wild-type or heterozygous *CNH1/cnh1* strains, the potassium sensitivity of *cnh1/cnh1* cells was highest at pH 3.5 and lowest at pH 7.0 (Fig. 4). This observation is in accordance with the nature of the substrate/ H^+ antiport mechanism, and

Fig. 4. Dependence of potassium sensitivity of homozygous *cnh1/cnh1* strain on extracellular pH. Serial 10-fold dilutions of saturated cultures were spotted on YNB medium adjusted to pH 3.5, 5.5 or 7.0 without or supplemented with 1800 mM KCl as indicated. Plates were incubated for 2 (YNB) or 3 days (KCl) at 30 °C. Dilution series correspond to the *C. albicans* wild-type strain (BWP17) and its derivatives lacking one (BWOZ 1, *CNH1/cnh1*Δ::hisG-URA3-hisG) or both *CNH1* alleles (BWOZ 13-8, *cnh1*Δ::hisG-URA3-hisG/*cnh1*Δ::hisG) as indicated. Data presented are from a representative experiment of two independent assays.

suggests that at acidic pH, the *CaCnh1* antiporter is the major transport system responsible for eliminating surplus K^+ from cells, whereas at neutral pH some other system(s) participate(s) in the ability of *C. albicans* cells to survive hyperosmotic conditions brought about by KCl.

Reintegration of the *CNH1* gene into the *cnh1* null mutant restores its potassium and rubidium tolerance

To determine whether the increased potassium and rubidium sensitivity of *cnh1* homozygous mutant cells is due to the absence of the *CaCnh1* antiporter, the *CNH1* gene was reintroduced into the *cnh1/cnh1* background (BWOZ 13-81N17, Table 1). In contrast to strain BWOZ 13-81, the resulting strain BWOZ 13-81N17 was *URA3*. As the *Ura* status of otherwise isogenic *C. albicans* mutants can affect their physiological properties (Bain *et al.*, 2001), for the comparison of BWOZ 13-81N17 phenotypes with those of *CNH1/CNH1* and *cnh1/cnh1*, the wild-type strain (BWP17) and homozygous *cnh1* null mutant (BWOZ 13-81) were made *URA3* by the integration of empty *Clp10* into the same *RP1* locus, resulting in strains BWP17-S1 and BWOZ13-81*Clp10* (Table 1). The growth of BWP17-S1, BWOZ 13-81*Clp10* and BWOZ 13-81N17 in the presence of high concentrations of KCl, RbCl and NaCl is shown in Fig. 5. All three strains grew similarly on plates without salts and on plates supplemented with NaCl. Both the wild-type and strain BWOZ 13-81N17 with a reintegrated *CNH1* gene exhibited the same robust growth on plates containing 1800 mM KCl or 1600 mM RbCl, whereas the *cnh1* null mutant (BWOZ 13-81*Clp10*) did not grow or grew more slowly under these conditions (Fig. 5). Reintegration of the *CaCnh1* gene fully complemented the potassium and rubidium sensitivity of the *cnh1* null mutant, and confirmed the importance of the *CaCnh1* antiporter for potassium (and rubidium) homeostasis in *C. albicans* cells.

CaCnh1p is localized in the plasma membrane in *C. albicans* cells

CaCnh1p heterologously expressed in *S. cerevisiae* was targeted to the plasma membrane (Kinclova *et al.*, 2001b). To verify the same localization of *CaCnh1p* in *C. albicans* cells, the antiporter was tagged with GFP at its C terminus (BWOZ 13M2GFP, Table 1). GFP tagging did not influence either the growth phenotype of cells or their tolerance to alkali metal cations (i.e. the activity of *CaCnh1p*; not shown). The localization of the *CaCnh1* antiporter was estimated by fluorescence microscopy of BWOZ 13M2GFP cells growing at 30 °C in YPD medium or at 37 °C in YPD containing FBS to induce the formation of hyphae. As shown in Fig. 6(a), *CaCnh1p* appeared to be clearly localized peripherally, corresponding to its presumed plasma-membrane occurrence in cells growing as yeasts and pseudohyphae. The same peripheral distribution was also observed in hyphal cells grown for 4 h in YPD

Fig. 5. Alkali-metal-cation salt tolerance of *C. albicans* *cnh1* null mutant with reintegrated *CNH1* gene. Serial 10-fold dilutions of saturated cultures were spotted on YNB medium at pH 3.5 without or supplemented with KCl, RbCl or NaCl as indicated. Plates were incubated for 2 (YNB) or 7 days (YNB+salts) at 30 °C. Dilution series correspond to Ura⁺ *C. albicans* strains BWP17-S1 (*CNH1/CNH1*), BWOZ 13-81C1p10 (*cnh1/cnh1*), and BWOZ 13-81N17 (*cnh1/cnh1* with *CNH1* gene reintroduced into the *RP1* locus). The growth assay was repeated three times and a representative experiment is shown.

supplemented with serum (Fig. 6b), though, under these conditions, fluorescence was also observed inside the cells, most probably in vacuoles, which suggested an increased degradation of CaCnh1p.

CaCnh1 antiporter mediates potassium efflux from *C. albicans* cells

As shown in Fig. 1(b), CaCnh1p mediated efficient K⁺ efflux upon heterologous expression in *S. cerevisiae* cells. To estimate CaCnh1 antiporter activity directly in *C. albicans*, the potassium efflux from Ura⁺ strains containing none, one or both functional *CNH1* alleles was compared. Exponentially growing BWP17-S1 (*CNH1/CNH1*), BWOZ 1 (*CNH1/cnh1*), BWOZ 13-81C1p10 (*cnh1/cnh1*), and BWOZ 13-81N17 (*cnh1/cnh1* with the *CNH1* gene reintroduced) cells were transferred to a pH 4.5, K⁺-free incubation buffer, and K⁺ loss was

followed for 120 min. To prevent potassium reuptake, the incubation buffer was supplemented with RbCl. The initial intracellular concentration of K⁺ in all strains was 775 ± 58 nmol K⁺ (mg dry wt)⁻¹. Small differences in K⁺ loss among strains with low extracellular RbCl concentrations (10 mM or 20 mM usually used for K⁺ efflux measurements in *S. cerevisiae* cells; Fig. 1b) suggested a very efficient high-affinity reuptake of lost K⁺. To compensate, the amount of RbCl in the incubation buffer was increased to 50 mM. The highest amount of potassium lost from cells was observed in the wild-type strain BWP17-S1 [174 ± 16 nmol K⁺ (mg dry wt)⁻¹ (120 min)⁻¹], whereas deletion of one *CNH1* allele resulted in a 25 % decrease in K⁺ loss [129 ± 11 nmol K⁺ (mg dry wt)⁻¹ (120 min)⁻¹], and only low potassium loss [35 ± 9 nmol K⁺ (mg dry wt)⁻¹ (120 min)⁻¹] was observed from cells with both *CNH1* alleles deleted. This means that under the conditions set in our experiment, the activity of (an)other

Fig. 6. Nomarski and fluorescence micrographs of BWOZ 13M2GFP cells expressing CaCnh1p tagged with GFP. (a) Cells were grown in YPD at 30 °C (image of yeasts and pseudohyphae form taken from the same microscope sample). (b) Cells were grown in YPD+20 % (v/v) FBS at 37 °C for 4 h. Objective, 100×. The data are representative of two separate experiments.

transport system(s) was involved in K^+ efflux. In comparison with the *cnh1* null strain, significantly higher K^+ loss was found in cells with a reintegrated *CNH1* gene [63 ± 10 nmol K^+ (mg dry wt) $^{-1}$ (120 min) $^{-1}$]. However, the K^+ efflux activity observed in cells with the *CNH1* gene reintegrated at the RP1 locus was not as high as in BWOZ 1 cells, with the original *CNH1* allele [63 ± 10 nmol K^+ (mg dry wt) $^{-1}$ (120 min) $^{-1}$ vs 129 ± 11 nmol K^+ (mg dry wt) $^{-1}$ (120 min) $^{-1}$]. This difference could result either from a different level of expression (the promoter region used for *CaCNH1* reintegration was only 496 bp long) or due to the different chromosomal locus. All the data indicated that the *CaCnh1* antiporter mediates an efficient efflux of potassium in *C. albicans* cells, and that the higher sensitivity of BWOZ 13-81C1p10 (*cnh1/cnh1*) cells to KCl observed in drop tests (Fig. 5) resulted from their inability to eliminate surplus intracellular K^+ . Although the observed K^+ efflux activities in strains with one functional *CNH1* allele were lower than in the *CNH1/CNH1* strain, they were sufficient to enable the long-term growth of *C. albicans* cells in the presence of high KCl concentrations (Figs 2, 3 and 5).

DISCUSSION

In general, *C. albicans* is more halotolerant than the most studied yeast *S. cerevisiae* (Silva-Graca *et al.*, 2003). In our experience, *C. albicans* cells grew in the presence of 2300 mM salts whereas *S. cerevisiae* wild-types usually do not tolerate more than 1800 mM NaCl or 2000 mM KCl (Kinclova *et al.*, 2001a and our unpublished results). One of the reasons for an increased cell tolerance to alkali metal cations could be the existence of efficient transporters that can pump out surplus cations against their concentration gradient. The main task of this work was to extend current knowledge on one of the plasma-membrane transporters, the alkali metal cation/ H^+ antiporter *Cnh1* in *C. albicans* cells, mainly to confirm or refute its role in Na^+ detoxification and to find out its importance in intracellular K^+ homeostasis in *C. albicans* cells. Upon heterologous expression in *S. cerevisiae*, *CaCnh1p* mediates the efflux of Na^+ , Li^+ , K^+ and Rb^+ ; thus it belongs among the systems of the *Nha1/Sod2* family with broad substrate specificity (Fig. 1). However, compared to some other members of the *Nha1/Sod2* family (e.g. *Zygosaccharomyces rouxii* *Sod2-22* antiporter; Kinclova *et al.*, 2002), *CaCnh1p*'s efficiency in exporting Li^+ is rather low (Fig. 1b). Though the data from heterologous expression indicate broad substrate specificity, our data obtained directly in *C. albicans* suggests that *CaCnh1p* plays an important role in potassium and rubidium tolerance, but its contribution to the sodium and lithium tolerance of cells is rather marginal.

All strains used in our study were derived from the BWP17 wild-type strain. We did not observe an inhibitory effect of *cnh1* deletion on the growth of cells either on solid or in liquid minimal media as has been described elsewhere in

the CAI-4 background (Soong *et al.*, 2000). However, Soong *et al.* (2006) compared strains with different levels of expression of *CaURA3*-encoded orotidine-5'-monophosphate (OMP) decarboxylase (Bain *et al.*, 2001; Lay *et al.*, 1998), and thus the previously observed growth retardation of strains lacking the *CaCnh1* antiporter in their experiments could be due to the different Ura status of the tested strains.

The deletion of both *CNH1* alleles caused a significantly higher cell sensitivity to high external concentrations of potassium and rubidium (Rb^+ is usually used as a K^+ analogue in the kinetic characterization of K^+ uptake systems; Ramos & Rodriguez-Navarro, 1986). The increased K^+ (Rb^+) sensitivity resulted from the absence of *CaCnh1p*, as the potassium and rubidium tolerance of *cnh1* null cells could be restored by reintegration of *CNH1* into the genome, and the loss of K^+ from the *cnh1/cnh1* mutant was much lower than from cells expressing the *CaCnh1* antiporter. It is evident from all these observations that *CaCnh1p* is important for the maintenance of potassium homeostasis in *C. albicans* cells. The inhibitory growth effect of KCl on cells lacking *CaCnh1p* was highest at pH 3.5 and decreased with increasing pH, which corresponds to the nature of an antiport mechanism using the gradient of protons across the plasma membrane. It seems that at acidic pH, *CaCnh1p* is the main transport system responsible for the elimination of surplus K^+ from cells, but at neutral pH values, the growth of cells in the presence of high concentration of KCl is enabled by the activity of some other transporters.

On the other hand, *CaCnh1p* is not the major system mediating the efflux of toxic Na^+ and Li^+ cations from cells, since the deletion of both *CNH1* alleles did not affect *C. albicans* cell tolerance to high concentrations of NaCl and LiCl, even if the protonmotive force across the plasma membrane necessary for transport was increased by decreasing the extracellular pH to 3.5. This is in accord with results observed with *cnh1* mutants derived from the CAI-4 strain (Soong *et al.*, 2000), and it suggests that other efficient transport system(s) with a high affinity for Na^+ and/or Li^+ (e.g. Na^+ -ATPase) must exist in *C. albicans* to ensure survival in the presence of an excess of Na^+ and Li^+ . This *C. albicans* system should be more active under acidic conditions than *Ena Na^+-ATPases in *S. cerevisiae*. Expression of the *ENA1* gene in *S. cerevisiae* cells is low at acidic pH and thus the deletion of the *ScNHA1* gene (encoding the *ScNha1* antiporter) significantly decreases their tolerance to both NaCl and KCl (Banuelos *et al.*, 1998; Garciadeblas *et al.*, 1993). Sequencing of the *C. albicans* genome revealed the existence of two genes encoding plasma-membrane *Ena*-ATPases (*ENA2*, *ENA21*; Jones *et al.*, 2004), but their role in alkali-metal-cation homeostasis in *C. albicans* cells remains to be established. It is possible that higher expression of *CaENA* genes at acidic pH (compared to *S. cerevisiae*) ensures the observed high Na^+ tolerance of *C. albicans* cells in the absence of *CaCnh1p*. Another difference in the regulation of*

alkali-metal-cation homeostasis between *S. cerevisiae* and *C. albicans* is also evident, since they possess a different number and different types of genes encoding high-affinity K⁺ uptake systems (Benito *et al.*, 2004).

In *S. cerevisiae*, the Nha1 antiporter participates in the regulation of intracellular pH. The deletion of *ScNHA1* results in the alkalization of the cytosolic pH (Brett *et al.*, 2005; Sychrová *et al.*, 1999), whereas *ScNha1p* over-expression is associated with a decrease in internal pH (Sychrová *et al.*, 1999). The importance of the *CaCnh1* antiporter in the regulation of intracellular pH in *C. albicans* remains to be established, though a possible role in the buffering of intracellular pH was indicated upon its expression in *S. cerevisiae*. *CaCnh1p*, similarly to *ScNha1p*, mediated rapid efflux of potassium upon a rise in cytoplasmic pH. Under these conditions, the antiporter is thought to act as a short-term safety valve, which uses an outward gradient of K⁺ to drive in some protons and rapidly decrease internal pH (Kinclova *et al.*, 2001b).

The morphological switch of *C. albicans* cells from yeasts to hyphae is associated with a rise in internal pH (Stewart *et al.*, 1988), and the intracellular concentration of K⁺ is higher in cells growing in hyphal form in the presence of serum than in cells growing as yeasts (Watanabe *et al.*, 2006). Although our results showed that deletion of both *CNH1* alleles had no significant influence on K⁺ content under conditions of exponential growth of yeast cells in minimal YNB medium, the effect of the absence of *CaCnh1p* on intracellular potassium concentration in hyphal cells has not yet been estimated. It is possible that the activity of *CaCnh1p* decreases (as a consequence of enhanced protein inactivation and degradation, cf. Fig. 6b) upon transition from the budding to hyphae form in order to (1) maintain a higher intracellular concentration of potassium and (2) not transport external protons into the cells. To clarify the role of *CaCnh1p* activity in *C. albicans* hyphae, our future work will aim for a more detailed comparison of potassium content and flux as well as *CaCnh1p* expression and activity in hyphae vs budding *C. albicans* cells.

The maintenance of potassium homeostasis in fungi is complex, including cooperative action of different K⁺-transporting systems. To our knowledge this is the first work characterizing the potassium efflux activity of an active transport system directly in *C. albicans* cells. In *C. albicans*, changes in intracellular potassium concentration are supposed to influence the development of cell morphology and virulence (Biswas *et al.*, 2000; Watanabe *et al.*, 2006). Our finding that *CaCnh1* alkali metal cation/H⁺ antiporter is, in *C. albicans* cells, important for the potassium and rubidium tolerance and plays a role in the regulation of intracellular potassium concentration will certainly contribute to elucidating the nature not only of *C. albicans* high salt tolerance, but also its pathogenicity.

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