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.

**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 cofactor 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 sorbitophila*, *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 Na\(^+\)/(Li\(^+\)) cations, the other mainly transports K\(^+\) (and Rb\(^+\)) (Papouskova & 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 Na\(^+\),K\(^+\)/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 (Kinclová...
et al., 2001c; Kinclova-Zimmermannova & Sychorova, 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 (Ogahki et al., 2005) is also vital for the regulation of intracellular pH (Brett et al., 2005; Sychorva 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 CaNha1 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 alkalination 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 CaNha1p 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 CaNha1p 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 CaNha1p and its importance for C. albicans tolerance to alkali metal cations. The results presented clearly demonstrate that CaNha1p 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. Uracil 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 resuspended 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 (Walthier & Wendland, 2003).

### Table 1. Yeast strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>S. cerevisiae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW31</td>
<td>MATα leu2-3/112 ura3-1 trp1-1 his3-11/15 ade 2-1 can1-100 GAL SUC2 mal10 ena1-4:: His3 nha1:: LEU2</td>
<td>Kinclova-Zimmermannova et al. (2005)</td>
</tr>
<tr>
<td><strong>C. albicans</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BWP17</td>
<td>ura3Δ::imm434/ura3Δ::imm434 hist:: hisG/his1::hisG arg4:: hisG/arg4:: hisG</td>
<td>Wilson et al. (1999)</td>
</tr>
<tr>
<td>BWP17-S1</td>
<td>BWP17 with Clp10 integrated into RP1 locus</td>
<td>This study</td>
</tr>
<tr>
<td>BWOZ 1</td>
<td>BWP17, except for CNH1/cnh1Δ::hisG-URA3-hisG</td>
<td>This study</td>
</tr>
<tr>
<td>BWOZ 13</td>
<td>BWP17, except for CNH1/cnh1Δ::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>BWOZ 13-8</td>
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<td>This study</td>
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<td>This study</td>
</tr>
<tr>
<td>BWOZ 13-81Clp10</td>
<td>BWOZ13-81 with Clp10 integrated into RP1 locus</td>
<td>This study</td>
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<td>BWOZ 13-81N17</td>
<td>BWOZ13-81 with CNH1-Clp10 integrated into RP1 locus</td>
<td>This study</td>
</tr>
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<td>BWOZ 13M2GFP</td>
<td>BWP17, except for CNH1-MoGFP-URA3/cnh1Δ::hisG</td>
<td>This study</td>
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</table>
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 *C. albicans* cells. For constructing the deletion cassette, PCR fragments corresponding to the CNH1 upstream and downstream flanking sequences were cloned into plasmid KB896 (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 CaSacI (5'-GCTTTAGGATCCATGATCAGGATCTTCTTC-3') and CaBamHI (5'-GGGGATCCAGCTTATCGGAC- CTCGGTAGA-3'), which SacI and BamHI sites were introduced (underlined). The PCR product digested with SacI and BamHI was cloned into KB896 at the 3' 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 oligonucleotides CaSalI (5'-AAAACTCAGTATATGCGAAC- CTCGGTAGA-3') and CaHindIII (5'-AAAGTTAAGCGTACTTT- GCTGTTTGGTG-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 CaSacI_BamHI (5'-TACCAG- GGCCATCTCTCCTCCTCGTGTAGTT-3') and CaCNH1 (5'-GGGGATCCAGCTTATCGGACCTCAGTCTG-3'), producing 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 CNH1 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* BWPI7 genomic DNA; oligonucleotides: CaSacI and CaBamHI). The entire CNH1-deletion cassette was released from pCNH1-5'-3' by SacI/HindIII digestion and used for the transformation of strain BWPI7. After the first transformation, two Ura<sup>+</sup> 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<sup>-1</sup>) and 5-fluoroorotic acid (1 mg ml<sup>-1</sup>), and the resulting Ura<sup>-</sup> 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 BWPI7 and the homozygous cnh1/cnh1 mutant BWOZ 13-81 were made Ura<sup>+</sup> 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/CNH1-Clp10. In each case, two independent transformants of BWPI7-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'-GAGTGGGAGCAAGGACAGAATCGTTGA- AAAAAAAGAAGTTGGAAGGAGGAGGAAATGCTG- CTCGCCGATGAAGAGAGGAGGAGGAGGCTCTG-3'), in which SacII and BamHI sites were introduced (underlined). The PCR product digested with SacII and BamHI was cloned into pCNH1-5' at the 3' end of the deletion cassette, resulting in pCNH1-5'. For reintegration of the CaCNH1 gene into the genome, the CaCNH1 ORF with a 496 nt promoter region was amplified with oligonucleotides CaSacI_BamHI (5'-TACCAGGGGATCTCTCCTCCTCGTGTAGTT-3') and CaCNH1 (5'-GGGGATCCAGCTTATCGGACCTCAGTCTG-3'), producing 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 J. Wendland, University of Basel, Switzerland). The Ura<sup>-</sup> cnh1/CNH1 strain (BWOZ 13) was transformed with the resulting PCR fragment (2.3 kb), and the Ura<sup>-</sup> 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 OD<sub>600</sub> 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 OD<sub>600</sub> 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 OD<sub>600</sub> ≤ 0.2, harvested and washed. To preload with Na<sup>+</sup> or Li<sup>+</sup>, cells were incubated in YNB medium (pH 7.0) and supplemented with 100 mM NaCl or 50 mM LiCl for 60 min. For K<sup>+</sup> 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)<sub>2</sub>] or in 10 mM Tris buffer [C. albicans cells; pH adjusted to 4.4 with citric acid and Ca(OH)<sub>2</sub>] that was added to the cell suspension to bring the pH up to 4.5. Both buffers contained 0.1 mM MgCl<sub>2</sub> and 2% (v/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<sup>+</sup>, Li<sup>+</sup> or K<sup>+</sup> 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

CaCnh1p 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

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**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 ± 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)\(^{-1}\).
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 Ca\(\text{Cnh1p}\) 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.

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**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/cnh1::hisG-URA3-hisG; BWOZ 13, CNH1/cnh1::hisG) or both (BWOZ 13-8, cnh1::hisG-URA3-hisG/cnh1::hisG; BWOZ 13-81, cnh1::hisG/cnh1::hisG) CNH1 alleles. Data presented are from a representative experiment of four independent assays.

**Fig. 3.** Growth of C. albicans wild-type strain (BWP17, squares) or homozygous cnh1::hisG/cnh1::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 OD\(\text{600}\) 0.002, incubated with vigorous shaking at 30 °C, and growth was assessed by measuring the increase in OD\(\text{600}\) 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 motive force 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 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 CIP10 into the same RP1 locus, resulting in strains BWP17-S1 and BWOZ13-81CIP10 (Table 1). The growth of BWP17-S1, BWOZ 13-81CIP10 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 13-81CIP10) 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. 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.](image-url)
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 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

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**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.

**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 13-81Cp10 (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, to some other members of the Nha1/Sod2 family (e.g. Zygosaccharomycetes 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 reintroduction 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 that 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; Garcia-deblas 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; Sychrova et al., 1999), whereas ScNha1p over-expression is associated with a decrease in intracellular pH (Sychrova 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 (Biswa et al., 2000; Watanabe et al., 2006). Our finding that CaCnh1p, essential 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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