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 antipporter in *C. albicans* revealed that this antipporter 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 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 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 antipporters: one of them primarily transports Na\(^+\)/(Li\(^+)\) cations, the other mainly transports K\(^+\) (and Rb\(^+\)) (Papouskova & Sychrová, 2006, 2007). The antipporters 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 antipporter 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 & Sychrova, 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 (Ogaki 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 \textit{C. albicans} wild-types were isolated and their products partially characterized upon expression in \textit{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, \textit{C. albicans} antiporters contribute to the buffering of cytoplasmic pH in \textit{S. cerevisiae} cells (similarly to \textit{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 Nha1p in \textit{C. albicans} cells is broader than simply detoxification from surplus alkali metal cations.

\textit{C. albicans} is a major opportunistic fungal pathogen. Its pathogenicity is believed to be linked to its ability to grow in distinct morphological forms. \textit{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 \textit{C. albicans} cells revealed that (1) high external concentrations of alkali metal cations (\( Na^+ \), \( Li^+ \), \( K^+ \)) decrease the growth capacity of \textit{C. albicans} cells (Hermann et al., 2003), (2) an increased extracellular sodium concentration inhibits germ-tube formation (Biswa et al., 2000; Northrop et al., 1997), (3) preincubation with alkali metal cations negatively influences certain \textit{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 \textit{C. albicans} morphology and virulence. A previous study of the role of CaCnh1p in \textit{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 \textit{C. albicans} cells were not examined.

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

\section*{METHODS}

\textbf{Strains and media.} Strains (listed in Table 1) were routinely grown at 30 \(^\circ\)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 \( \mu \)g ml\(^{-1}\), other auxotrophic supplements to a final concentration of 20 \( \mu \)g ml\(^{-1}\). Solid media were prepared by adding 2 \% (w/v) agar. Before each functional assay, \textit{C. albicans} cells were refreshed from a 20 \% (v/v) glycerol stock at \(-80\) \(^\circ\)C on YPD plates.

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

\begin{table}[h]
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\caption{Yeast strains used in this study}
\begin{tabular}{|l|l|l|}
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\textbf{Strain} & \textbf{Relevant genotype} & \textbf{Source or reference} \\
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\textit{S. cerevisiae} & & \\
BW31 & MAT\( \alpha \) leu2-3/112 ura3-1 trp1-1 his3-11/15 ade 2-1 can1-100 GAL \text{SUC2} mal10 ena1-4: HIS3 nha1:: LEU2 & Kinclova-Zimmermannova et al. (2005) \\
\hline
\textit{C. albicans} & & \\
BWP17 & ura3A:::imm434/ura3A:::imm434 \text{his}:hisG\text:g:\text{his}G & Wilson et al. (1999) \\
BWP17-S1 & BWP17 with Clp10 integrated into RP1 locus & This study \\
BWOZ 1 & BWP17, except for CNH1/cnh1A:::hisG-URA3-hisG & This study \\
BWOZ 13 & BWP17, except for CNH1/cnh1A:::hisG & This study \\
BWOZ 13-8 & BWP17, except for cnh1A:::hisG-URA3-hisG/cnh1A:::hisG & This study \\
BWOZ 13-81 & BWP17, except for cnh1:::hisG/cnh1A:::hisG & This study \\
BWOZ 13-81Clp10 & BWOZ13-81 with Clp10 integrated into RP1 locus & This study \\
BWOZ 13-81N17 & BWP17, except for CNH1-\text{MoGFP}-URA3/cnh1A:::hisG & This study \\
BWOZ 13M2GFP & BWP17, except for CNH1-MoGFP-URA3/cnh1A:::hisG & This study \\
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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 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'-GTCGTTGTTTGG-3') and CaBamHI (5'-GGGATCCATGATGCAGGCTGAAAGTTGAGAGCGGAGGAGGAGACGGAAGTTGA-AAAAGAAAAGAAGATTGCAAGCTTTGGGTTACTTGCCAAGTAGTGCAGTGATAGATGAA-ATTGCTGATATCAGGATATTGGG-3') (reverse) and plasmid pFA-MGF-P-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 NaCl was inoculated to OD_600_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_600 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-MGFP HQ 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_600 0.2, harvested and washed. To prelood 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 MgCl_2 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+, 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 antipporter 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)\(^{-1}\).](attachment:image)
cells in the presence of various alkali metal cations. The growth of the \( \text{CNH1/CNH1}, \text{CNH1/cnh1} \) and \( \text{cnh1/cnh1} \) strains was tested in drop tests on plates containing increasing amounts of five salts (Fig. 2). As the transport activity of the \( \text{Na}^+/\text{H}^+ \) 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 \( \text{CNH1} \) allele(s). All strains grew equally well on YNB without salts (Fig. 2) suggesting that deletion of the \( \text{CNH1} \) gene(s) in the \( \text{C. albicans} \) BWP17 strain background did not affect the growth capacity of cells. Deletion of one of the two \( \text{CNH1} \) alleles caused no phenotype: the heterozygous strains \( \text{CNH1/cnh1} \) exhibited the same robust growth as the \( \text{CNH1/CNH1} \) wild-type on plates with all five alkali-metal-cation salts. No difference in salt sensitivity was observed between the wild-type and homozygous \( \text{cnh1} \) mutants on plates with \( \text{NaCl, LiCl and CsCl} \) (Fig. 2), though \( \text{CaCnh1p} \) is able to efficiently transport \( \text{Na}^+ \) and \( \text{Li}^+ \) 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 \( \text{CNH1} \) alleles were sensitive to potassium and rubidium cations compared to wild-type cells or heterozygous mutants with one functional \( \text{CNH1} \) 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 (\( \text{CNH1/CNH1} \)) and homozygous \( \text{cnh1} \) mutant (\( \text{cnh1/cnh1} \)) 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 \( \text{CNH1} \) 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 \( \text{C. albicans} \), the deletion of both \( \text{CNH1} \) 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 (\( \text{Na}^+, \text{Li}^+, \text{Cs}^+ \)) in \( \text{C. albicans} \) cells under the conditions tested in our experiments.
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 proton motive 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 Clp10 into the same RP1 locus, resulting in strains BWP17-S1 and BWOZ13-81Clp10 (Table 1). The growth of BWP17-S1, BWOZ 13-81Clp10 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 13-81Clp10) 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.

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**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.
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\(^{-1}\) (120 min\(^{-1}\)). 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\(^{-1}\))]), and only low potassium loss [35 ± 9 nmol K\(^+\) (mg dry wt\(^{-1}\))]) 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 \textit{cnh1} null strain, significantly higher K\(^+\) loss was found in cells with a reintegrated \textit{CNH1} gene \([63 \pm 10 \text{ nmol K}^+ \text{ (mg dry wt)}^{-1} \text{ (120 min)}^{-1}]\). However, the K\(^+\) efflux activity observed in cells with the \textit{CNH1} gene reintegrated at the RP1 locus was not as high as in BWOZ 1 cells, with the original \textit{CNH1} allele \([63 \pm 10 \text{ nmol K}^+ \text{ (mg dry wt)}^{-1} \text{ (120 min)}^{-1} \text{ vs } 129 \pm 11 \text{ nmol K}^+ \text{ (mg dry wt)}^{-1} \text{ (120 min)}^{-1}]\). This difference could result either from a different level of expression (the promoter region used for \textit{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 \textit{C. albicans} cells, and that the higher sensitivity of BWOZ 13-81Clp10 (\textit{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 \textit{CNH1} allele were lower than in the \textit{CNH1/CNH1} strain, they were sufficient to enable the long-term growth of \textit{C. albicans} cells in the presence of high KCl concentrations (Figs 2, 3 and 5).

**DISCUSSION**

In general, \textit{C. albicans} is more halotolerant than the most studied yeast \textit{S. cerevisiae} (Silva-Graca et al., 2003). In our experience, \textit{C. albicans} cells grew in the presence of 2300 mM salts whereas \textit{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 \textit{C. albicans} cells, mainly to confirm or refute its role in Na\(^+\) detoxification and to find out its importance in intracellular K\(^+\) homeostasis in \textit{C. albicans} cells. Upon heterologous expression in \textit{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, 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 \textit{CNH1} alleles did not affect \textit{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 \textit{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 \textit{C. albicans} to ensure survival in the presence of an excess of Na\(^+\) and Li\(^+\). This \textit{C. albicans} system should be more active under acidic conditions than Ena Na\(^+\)-ATPases in \textit{S. cerevisiae}. Expression of the \textit{ENA1} gene in \textit{S. cerevisiae} cells is low at acidic pH and thus the deletion of the \textit{ScNHA1} gene (encoding the ScNha1 antiporter) significantly decreases their tolerance to both NaCl and KCl (Banuels et al., 1998; Garcia-Deblas et al., 1993). Sequencing of the \textit{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 \textit{C. albicans} cells remains to be established. It is possible that higher expression of CaENA genes at acidic pH (compared to \textit{S. cerevisiae}) ensures the observed high Na\(^+\) tolerance of \textit{C. albicans} cells in the absence of CaCnh1p. Another difference in the regulation of the CAI-4 background (Soong et al., 2000). However, Soong et al. (2006) compared strains with different levels of expression of \textit{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 \textit{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 \textit{cnh1} null cells could be restored by reintroduction of \textit{CNH1} into the genome, and the loss of K\(^+\) from the \textit{cnh1/cnh1} mutant was much lower than from cells expressing the \textit{CaCnh1} antiporter. It is evident from all these observations that CaCnh1p is important for the maintenance of potassium homeostasis in \textit{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 antiporter 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 \textit{CNH1} alleles did not affect \textit{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 \textit{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 \textit{C. albicans} to ensure survival in the presence of an excess of Na\(^+\) and Li\(^+\). This \textit{C. albicans} system should be more active under acidic conditions than Ena Na\(^+\)-ATPases in \textit{S. cerevisiae}. Expression of the \textit{ENA1} gene in \textit{S. cerevisiae} cells is low at acidic pH and thus the deletion of the \textit{ScNHA1} gene (encoding the ScNha1 antiporter) significantly decreases their tolerance to both NaCl and KCl (Banuels et al., 1998; Garcia-Deblas et al., 1993). Sequencing of the \textit{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 \textit{C. albicans} cells remains to be established. It is possible that higher expression of CaENA genes at acidic pH (compared to \textit{S. cerevisiae}) ensures the observed high Na\(^+\) tolerance of \textit{C. albicans} cells in the absence of CaCnh1p. Another difference in the regulation of

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O. Kinclova-Zimmermannova and H. Sychrová
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 hyphal 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 CaCnh1p acts as a potassium efflux system in *C. albicans* is consistent with a role for Cnh1p in the regulation of intracellular potassium concentration and in the pathogenicity of *C. albicans*. The yeast Nha1p 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).

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