The *Candida albicans* Na^+/H^+ antiporter exports potassium and rubidium

Olga Kinclová[a,b], Serge Potier[b], Hana Sychrová[a,*]

[a] *Department of Membrane Transport, Institute of Physiology CzAcadSci, Videnska 1083, 14220 Prague 4, Czech Republic*
[b] *Laboratory of Microbiology and Genetics, UPRES A-7010-CNRS, Université Louis Pasteur, 28 rue Goethe, 67083 Strasbourg, France*

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Abstract The *Candida albicans* Cnh1p belongs to the family of Na^+/H^+ antiporters (TC 2.A.36) but it transports besides toxic sodium and lithium also rubidium and potassium. Upon heterologous expression in a *Saccharomyces cerevisiae* salt-sensitive strain, the Cnh1p is targeted to the plasma membrane and its transport activity results in increased tolerance of cells to external alkali metal cations. The cation efflux activity of Cnh1p in *S. cerevisiae* depends on the gradient of protons across the plasma membrane, and a Cnh1p-mediated K^+ efflux is involved in a cell response to sudden rise of cytoplasmic pH. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Na^+/H^+ antiporter; Potassium efflux; Salt tolerance; *Candida albicans*

1. Introduction

The diploid fungus *Candida albicans* is an important human pathogen existing in two principal forms, budding and mycelial. The transition between budding and hyphal growth is reversible and is regulated by a number of environmental factors, including ambient pH, nutritional status and temperature [1]. Transport systems mediating the fluxes of protons across the plasma membrane are believed to play an important role in the regulation of intracellular pH and in the switching of cell dimorphism [2–4].

The *CNH1* gene encoding a Na^+/H^+ antiporter (TC 2.A.36) was cloned from the *C. albicans* SC5314 strain [5]. Deletion of *CNH1* leads in *C. albicans* to retardation of growth and highly elongated morphology of cells, but it has little effect on the sensitivity of cells to high concentrations of sodium or lithium. On the other hand, overexpression of *CNH1* complements the sodium sensitivity of a *Saccharomyces cerevisiae* strain defective in sodium extrusion [5]. The transport activity of Cnh1p has not been tested in either *C. albicans* or *S. cerevisiae*.

By its sequence, the Cnh1 antiporter belongs to the family of yeast plasma membrane Na^+/H^+ antiporters identified in three other yeast species, *Schizosaccharomyces pombe* [6], *Zygosaccharomyces rouxii* [7] and *S. cerevisiae* [8]. The main physiological function of *Z. rouxii* and *S. pombe* antiporters is supposed to be the efficient elimination of toxic sodium and lithium cations from cells [6,9]. The *S. cerevisiae* Nha1p was shown to be a transport system with broad substrate specificity mediating the efflux not only of toxic Na^+ and Li^+, but also of K^+ and Rb^+ [10,11]. In addition to its detoxication function, the Nha1 antiporter is probably involved in the regulation of intracellular pH [12] and intracellular potassium content [10,11].

Intracellular potassium concentration is assumed to be important in controlling the morphology of *C. albicans* [13,14]. To find out if the activity of the *C. albicans* Na^+/H^+ antiporter contributes to the potassium homeostasis in *C. albicans*, we expressed the *CNH1* gene in a *S. cerevisiae* alkali metal-sensitive mutant and compared its transport properties with *S. cerevisiae* Nha1p.

2. Materials and methods

2.1. Yeast strains and media

The alkali metal cation-sensitive *S. cerevisiae* B31 strain (*ena1Δ::HIS3::ena4Δ nha1Δ::LEU2*) [10] was used in this work for *CNH1* expression and phenotype characterization. The *C. albicans* wild type strain MEN [15] was used to prepare genomic DNA for PCR amplification of the *CNH1* gene. Yeast cells were grown aerobically at 30°C in standard YP or YNB medium with 2% glucose and appropriate supplements. To estimate the cell tolerance to alkali metal cations, media were supplemented with LiCl, NaCl, KCl and RbCl. To adjust the pH of the media to 3.5, tartaric acid was added after autoclaving. Media with buffered pH were supplemented either with 20 mM MES (pH 5.5) or 20 mM MOPS (pH 7.0), and the required pH was adjusted with NaOH.

2.2. Genetic and molecular methods, DNA sequencing and sequence analysis

Standard protocols for nucleic acid manipulation and yeast transformation were used [16,17]. DNA sequencing was carried out using the Thermo Sequenase radiolabelled terminator cycle sequencing kit (Amersham Life Science Inc., USA). Both strands of the DNA inserts were completely sequenced, and the Lasergene99 (DNASTAR Inc., Madison, WI, USA) was used for DNA and protein sequence analyses.

2.3. Construction of plasmids

The *CNH1* genes were amplified by PCR (Peltier Thermal Cycler, PTC-200 MJ Research, USA; *Taq* DNA polymerase, Promega, USA) using the oligonucleotides corresponding to the 5′ and 3′ ends of the *CNH1* (accession No. AF128238). Oligonucleotides 5′-GTTCCCCGGGCTTTGCCATGGCTTGGAG-3′ and 5′-GGGGTACCTAGCATGCGAGACCAAGCGTTTTTGATAGCGC-3′ introduced a *Sma*I site in the upstream end, and a *Sph*I-STOP-*Sac*I sequence in the downstream end of the *CNH1* gene. Either the genomic DNA from the *C. albicans* MEN strain or the pBKCMV plasmid (a gift from Dr. Y. Wang; harbouring the *CNH1* gene from the *C. albicans* SC5314 strain [5]) was used as template. The amplified DNA fragments (2.4 kb) were digested with restriction enzymes *Sma*I and *Sac*I and cloned into the YEp352 vector [18] behind the *NHA1* promoter

*Corresponding author. Fax: (420)-2-475 24 88.
E-mail address: sychrova@biomed.cas.cz (H. Sychrová).

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A

aa	Cnh1p-G23	Cnh1p	aa
311	S	A	311
473	A	T	473
498	T	I	498
503	T	A	503
513	R	K	513
785	S	L	781

C

Cnh1p-G23	765	D E E S A D E E E	773
Cnh1p	766	D E - - - - - E E	770

B

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547 T S G L S A F G E K P P T K A E K A P V P T V A 570
CNH1-G23 1639 A C A T C - A G G G T T A T C T G C A T T T G G A G A G A A A C C A C C - T A C A A A A G C C G A A A A G G C A C C I G T C C C A A C C G - T T G C T 1710

CNH1 1639 A C A T C C A G G G T T A T C T G C A T T T G G A G A G A A A C C A C C C T A C A A A A G C C G A A A A G G C A C C A G T G C C A A C C G G T T G C T 1713
547 T S R V I C I W R E T T L Q K P K R H Q C Q P V A 571
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Fig. 1. Comparison of Cnh1p sequences isolated from *C. albicans* strains MEN (Cnh1-G23p) and SC5314 (Cnh1p). A: Different amino acid residues within the sequences of Cnh1-G23p and Cnh1; B: sequence of the *CNH1*-G23 part aligned to the originally published *CNH1* sequence containing three frameshifts (two silent base substitutions are underlined); C: extra amino acid residues in Cnh1-G23p C-terminus aligned to the corresponding part of Cnh1p.

resulting in plasmids pCNH1-G23 (from the *C. albicans* MEN strain) and pCNH1-M18 (SC5314 strain).

Plasmids pCNH1-G23GFP and pCNH1-M18GFP were constructed by cloning the 3.1 kb *Xba*I-*Sph*I fragments from pCNH1-G23 and pCNH1-M18, respectively, in frame into the corresponding sites preceding the GFP sequence in the pGRU1 plasmid (a gift from Dr. B. Daignan-Fornier).

2.4. Fluorescence microscopy

Living cells were examined with the Olympus BX60 microscope using the filter U-MWIB. Micrographs were recorded with Lucia G/F software (Laboratory Imaging s.r.o., Czech Republic) and processed for display with Adobe Photoshop.

2.5. Cation efflux experiments

To determine the antiporter activity, cells were grown in YNB medium to $OD_{600} \cong 0.3$, harvested and washed. Measurement of alkali cation efflux measurements was described earlier [11]. Each experiment was repeated at least three times; the deviation among parallel results was always $< 5\%$.

3. Results

3.1. Cloning of the CNH1-G23 gene

The *CNH1* gene encoding the Na^+/H^+ antiporter has already been isolated from the *C. albicans* SC5314 strain [5]. To study the Cnh1p transport properties in more detail we amplified the *CNH1* gene from the genomic DNA of the *C. albicans* MEN strain [15]. Sequencing of the amplified DNA fragment named *CNH1*-G23 (open reading frame (ORF) long 2403 nucleotides (nt) corresponding to 800 amino acids (aa), accession No. AF375984) revealed a discrepancy with the original sequence (from the SC5314 strain) in the database (accession No. AF128238). (i) There are 22 silent base substitutions at the DNA level. (ii) There are six amino acid substitutions as shown in Fig. 1A. (iii) Three nucleotides are missing and consequently three frameshifts were found changing the putative protein sequence following the ORF nucleotide 1643 (aa residue 548; Fig. 1B). Resequencing of the originally cloned genomic DNA fragment containing the *CNH1* gene from the SC5314 strain confirmed the six amino acid substitutions listed in Fig. 1A, the silent substitutions at the DNA level, but the sequence after the ORF nucleotide 1643 (aa residue 548) was the same (except for two silent base substitutions) as the nucleotide sequence of *CNH1*-G23, i.e. two C and one G missing compared with the original *CNH1* sequence (Fig. 1B). The corrected ORF length of the *CNH1* gene isolated from SC5314 is 2388 bp, corresponding to a protein of 795 aa. (iv) Unlike with the original *CNH1* gene sequence from SC5314, an insert of 15 base pairs was found close to the 3′ terminus of the *CNH1*-G23 gene (Fig. 1C).

To compare transport properties of both genes encoding *C. albicans* Na^+/H^+ antiporters, recombinant plasmids pCNH1-G23 and pCNH1-M18, respectively, were transformed to the *S. cerevisiae* alkali cation-sensitive strain B31 (*ena1-4Δ nha1Δ*) lacking Na^+-ATPases and the Na^+/H^+ antiporter.

3.2. Cnh1p expressed in S. cerevisiae is localized in the plasma membrane

To verify the localization of *C. albicans* antiporters expressed in *S. cerevisiae*, *CNH1*-G23 and *CNH1*-M18 genes were fused downstream with the sequence encoding the GFP. Both tagged antiporter genes were expressed from the *NHA1* promoter in *S. cerevisiae* B31 cells and they appeared to be fully functional since their products effectively improved the salt tolerance of *S. cerevisiae* mutant to the same level as non-tagged versions (see below). Fluorescence microscopy of *S. cerevisiae* transformants showed for both *C. albicans* antiporters a similar peripheral distribution (Fig. 2, an example of cells containing the Cnh1-G23GFPp) as was observed for Nha1p tagged with GFP [11]. These results demonstrate the presumed plasma membrane localization of heterologous *C. albicans* Na^+/H^+ antiporters expressed in *S. cerevisiae*.

3.3. Cnh1p substrate specificity

Till now, the Na^+/H^+ antiporter Cnh1 has been shown to complement only the sodium sensitivity of *S. cerevisiae* B31 cells when expressed from a centromeric plasmid under the

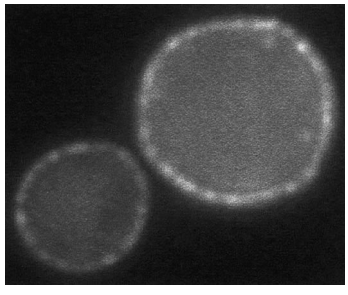

Fig. 2. Fluorescence micrograph of B31 cells expressing the Cnh1-G23p tagged with GFP.

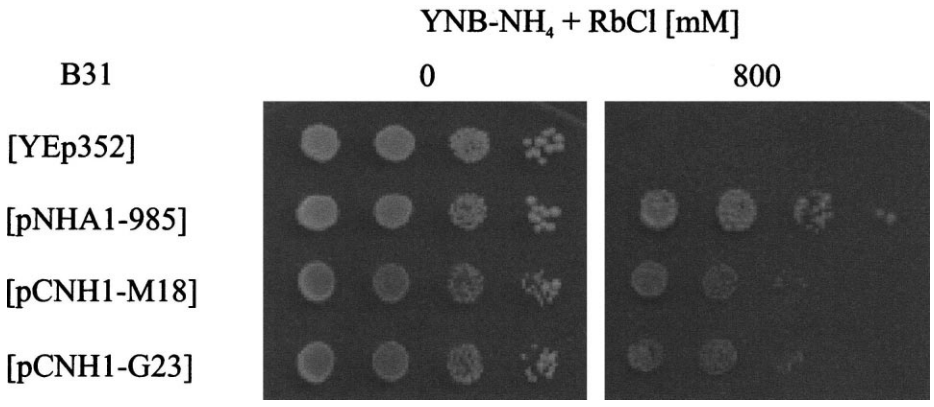

Fig. 3. Growth of B31 cells transformed either with empty vector YEp352 (control) or with plasmids pNHA1-985, pCNH1-G23 and pCNH1-M18, in the presence of a high concentration of RbCl. Serial 10-fold dilutions of saturated cultures were spotted onto YNB-glucose medium supplemented as indicated.

control of the *GAl1-10* promoter [5]. To find if Cnh1p is also involved in transport of other alkali metal cations, first the growth of B31 cells harbouring plasmids pCNH1-G23 and pCNH1-M18 was tested on plates supplemented with salts of sodium, lithium, potassium and rubidium. Expression of both Cnh1 antiporters increased significantly the cell tolerance to all four alkali metal cations. Fig. 3 shows the growth of cells expressing either Cnh1 proteins or the Nha1p (positive control) or the empty vector (negative control) in the presence of 800 mM RbCl. The maximum salt concentrations tolerated by cells harbouring the Cnh1p were on standard YNB plates: 1200 mM RbCl, 1500 mM KCl, 25–30 mM LiCl, 800–900 mM NaCl.

The driving force for a yeast cation/H^+ antiporter system is the electrochemical proton gradient across the plasma membrane, and thus its transport activity depends on external pH values. The dependence of salt tolerance on external pH was tested in drop tests with medium buffered to pH 3.5, 5.5 or 7.0, respectively. As shown in Table 1, the tolerance of cells expressing Cnh1 antiporters to high external concentrations of alkali metal cations increased with decreasing external pH, i.e. with an increase of the protonmotive force. Both Cnh1p and Nha1p were active at all pH values tested but their presence provided to cells different levels of tolerance at pH 3.5 and 5.5 (Table 1). In comparison with Nha1p, Cnh1 antiporters seem less effective in sodium and potassium tolerance at lower pH_{out} values. On the other hand, the expression of Cnh1-M18p and especially of Cnh1-G23p improved significantly the tolerance of cells to lithium (Table 1). The Cnh1-G23p provided a greater tolerance of cells than Cnh1-M18p did (Table 1).

These results showed that the *C. albicans* Na^+/H^+ antiporter has a broad substrate specificity for alkali metal cations similarly as was found for *S. cerevisiae* Nha1p [11]. Although both *C. albicans* antiporters are highly homologous (98.6% identity), the Cnh1-G23 antiporter cloned from *C. albicans* MEN seems to be more effective for sodium and lithium than the Cnh1 antiporter from the SC5314 strain.

3.4. Alkali metal cation efflux mediated by Cnh1

To verify that the different cell tolerances to alkali metal cations observed in drop tests result from the transport activity of Na^+/H^+ antiporters, the efflux of sodium and potassium was measured in B31 cells harbouring either *C. albicans* antiporters Cnh1-G23p and Cnh1-M18p or *S. cerevisiae* Nha1p or an empty vector as negative control. In the control cells lacking any antiporter, the internal concentration of sodium or potassium did not change significantly during the experiment, thus exclusively Na^+/H^+ antiporters mediated the observed loss of cations from cells (Fig. 4).

According to the phenotype observed on plates, the efflux of Na^+ from cells containing Nha1p was higher in comparison with cells harbouring *C. albicans* antiporters (Fig. 4A). The rate of sodium efflux from cells expressing the Cnh1-M18 antiporter was lower than from cells with Cnh1-G23p, corresponding to the higher sensitivity of cells with Cnh1-M18p to extracellular sodium (cf. Table 1 and Fig. 4A).

Although the *C. albicans* antiporters provided B31 cells with lower potassium tolerance on solid medium than did Nha1p, the potassium loss measurements showed that all three antiporters mediate the efflux of potassium with a similar rate (Fig. 4B). However, the intracellular concentration of

Table 1
Maximum sodium, lithium and potassium concentrations tolerated by *S. cerevisiae* B31 cells expressing *S. cerevisiae NHA1* or *C. albicans CNH1*-M18 and *CNH1*-G23 genes

B31 (*ena1-4Δ nha1Δ*)	NaCl (mM)			LiCl (mM)			KCl (mM)		
	pH 3.5	pH 5.5	pH 7.0	pH 3.5	pH 5.5	pH 7.0	pH 3.5	pH 5.5	pH 7.0
[YEp352]	300	300	50	25	15	< 5	800	800	300
[pNHA1]	1200	800	150	30	20	5	1800	1600	800
[pCNH1-M18]	1000	700	150	40	25	10	1500	1300	800
[pCNH1-G23]	1100	750	150	50	30	10	1500	1300	800

Serial 10-fold dilutions of saturated cultures were spotted onto YNB-Glc plates (pH 3.5, 5.5 and 7.0) supplemented with different concentrations of NaCl, LiCl and KCl. Growth was recorded during 5 days.

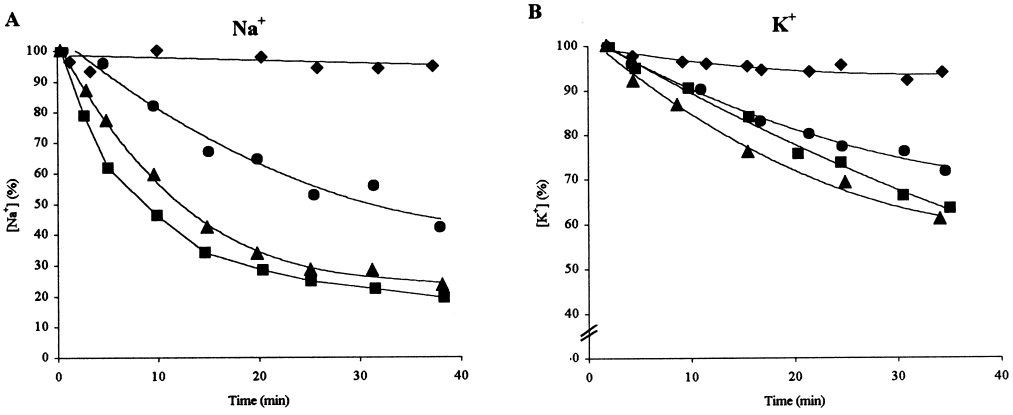

Fig. 4. Cation loss from B31 cells at pH_{out} 5.5. Cells transformed with YEp352 as control (♦), pNHA1-985 (■), pCNH1-G23 (▲) and pCNH1-M18 (●) were (A) preincubated with 100 mM NaCl at pH 7.0 for 60 min, transferred to the incubation buffer at pH 5.5 and the internal content of Na^+ was followed for 40 min; (B) directly transferred to the incubation buffer at pH 5.5 and the internal content of K^+ was followed for 35 min.

potassium was slightly higher in cells containing the Cnh1-M18p at the end of all experiments (Fig. 4B).

In *S. cerevisiae*, the Nha1p seems to play a role in the regulation of intracellular pH. Upon alkalinization of internal pH, the Nha1p mediates immediate high efflux of potassium which contributes to buffering of cytoplasmic pH (use of an outward K^+ gradient can drive in some protons) [10,11]. To estimate whether the *C. albicans* Na^+/H^+ antiporters can also be involved in this function, the efflux of potassium from cells upon a sudden increase of internal pH was measured. Only slight efflux of K^+ from cells with Nha1p was observed during the first 20 min at pH 8.0, while no significant potassium loss was found in control cells or in cells containing the *C. albicans* antiporters (Fig. 5A). Potassium does not leave the cells significantly at pH 8.0 although a high gradient across the plasma membrane is maintained (intracellular concentration of potassium, approx. 280 mM, and potassium-free incubation buffer). When NH_4Cl was added to the incubation buffer at pH 8.0, the presence of all three antiporters resulted in a loss of potassium from cells. However, the *C. albicans* antiporters did not mediate the K^+ efflux as effectively as Nha1p did (Fig. 5B).

4. Discussion

The main task of the present work was to characterize in more detail the transport properties of *C. albicans* Na^+/H^+ antiporter encoded by the *CNH1* gene. The *C. albicans* MEN strain used in this work contains the *CNH1*-G23 gene that differs in its sequence at nucleotide and amino acid levels from the *CNH1* gene (*CNH1*-M18 in this work) isolated from the SC5314 strain (Fig. 1). This phenomenon is not unusual, e.g. sequences of a Na^+/H^+ antiporter from two different *Z. rouxii* strains (CBS732 and ATCC42981) also differ [19].

Products of both *CNH1* genes were functional in *S. cerevisiae* and fluorescence microscopy of *S. cerevisiae* cells expressing GFP-tagged antiporters showed their proper localization in the plasma membrane. Tests of salt tolerance showed the Cnh1p to be a transporter with broad substrate specificity for alkali metal cations (Na^+, Li^+, K^+, Rb^+). The hydrophobic part of Cnh1 antiporters (aa 1–430) is highly homologous with other yeast Na^+/H^+ antiporters, while the C-termini of all antiporters show only limited conservation and vary in length. Nevertheless, the alignment of the Cnh1-G23p hydrophilic C-terminus (aa 431–800) with the C-terminus of *S. cerevisiae* Nha1p (aa 432–985) revealed two conserved regions: aa 669–685 and 766–786 (corresponding to aa 800–817 and 912–932 in Nha1p). The second Nha1p region (aa 912–932), rich in aspartate and glutamate residues, was shown to be most important for maximum transport activity for sodium and lithium. This maximum activity was observed only when the very end of the Nha1p C-terminus (aa 929–985) was missing [11]. The presence of a region important for maximum tolerance in the very end of the Cnh1-G23p C-terminus could explain the higher lithium tolerance of cells expressing this

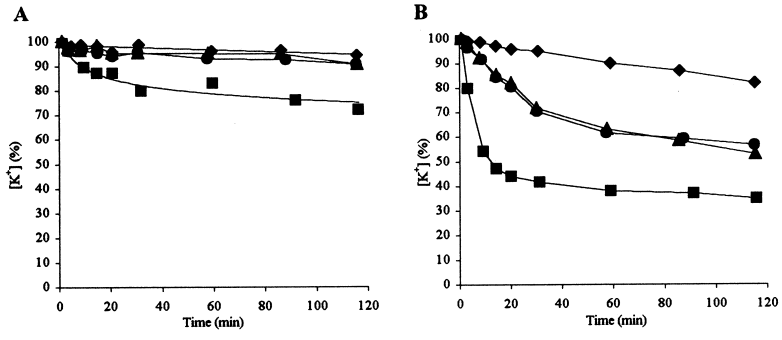

Fig. 5. K^+ loss from B31 cells at pH_{out} 8.0. Cells transformed with YEp352 as control (♦), pNHA1-985 (■), pCNH1-G23 (▲) and pCNH1-M18 (●) were transferred to the incubation buffer at pH 8.0 without (A) or with 20 mM NH_4Cl (B). Changes in the internal K^+ content were followed for 120 min.

antiporter compared with cells expressing complete Nha1p. Five amino acid residues of this region (aa 767–771, cf. Fig. 1C) are missing in Cnh1-M18p, and this antiporter provides cells with lower tolerance toward Na^+ and Li^+ than Cnh1-G23p does. Efflux measurements showed that the tolerances of cells observed in drop tests corresponded to the efflux activity of *C. albicans* antiporters. A rapid efflux of potassium was measured upon rise of cytoplasmic pH in B31 cells harbouring *C. albicans* antiporters. This suggests a putative role of Cnh1 antiporters in the regulation of intracellular pH and is in good agreement with the observation that, in general, the internal pH in *C. albicans* is not regulated as tightly as in *S. cerevisiae* and that it changes in response to environmental conditions [2].

In conclusion, we have demonstrated that the sequence of *CNH1* genes differs in two *C. albicans* strains and we have characterized transport properties of both of them upon their heterologous expression in *S. cerevisiae*. Our results revealed that the *C. albicans* Cnh1 antiporter is an effective transport system for Na^+, Li^+, K^+ and Rb^+, and that Cnh1p possibly participates in the regulation of intracellular pH. Further experiments with *C. albicans* cells are required to establish the role of Cnh1p in cation homeostasis and/or in morphogenesis of *C. albicans*.

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