Characterization of DhKHA1, a gene coding for a putative Na\(^+\) transporter from Debaryomyces hansenii

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Abstract

The KHA1 gene from Debaryomyces hansenii has been identified and characterized by heterologous expression in Saccharomyces cerevisiae. The gene is orthologous to ScKHA1, previously reported in S. cerevisiae, and on the basis of the deduced amino acid sequence, DhKha1p can be classified as an Na\(^+\)/H\(^+\) transporter. Reverse transcriptase (RT)-PCR experiments indicated that the expression level of DhKHA1 was not dependent on high pH or on the presence of a high salt level in the growth medium. Overexpression of DhKHA1 in a salt-sensitive S. cerevisiae mutant (ena1-4 nha1 kha1) rendered cells specifically more tolerant to Na\(^+\). In addition, internal K\(^+\) and Na\(^+\) measurements and experiments performed with green fluorescence protein (GFP)-tagged DhKha1p indicated the intracellular localization of this protein when expressed in S. cerevisiae.

Introduction

Cation homeostasis is a basic process in cell physiology. Yeasts regulate monovalent cation content, mostly K\(^+\), Na\(^+\) and H\(^+\), by the activity of different proteins that mediate cation fluxes. Most of the genes coding for those transporters have been identified in the model yeast Saccharomyces cerevisiae, and information on their activities and regulation is abundant (Serrano, 1991; García-Deblasa et al., 1993; Bert et al., 2003; Brett et al., 2005a, b). One amazing exception is the case of KHA1, as very few papers have been devoted to the study of the protein encoded by this gene. Although Kha1p was first proposed to work as a K\(^+\)/H\(^+\) antiporter localized at the plasma membrane and involved in the regulation of intracellular pH, K\(^+\) accumulation and cell volume (Ramírez et al., 1998), it was later shown that mutants lacking KHA1 exhibited impaired growth at high pH and increased sensitivity to the cationic antibiotic hygromycin B (Maresová & Sychrova, 2005). The same researchers also proposed the antiporter to be preferentially localized intracellularly. Finally, and almost at the same time, Flis et al. (2005) established that Kha1p is localized in the Golgi apparatus and that it may take part in the regulation of intracellular cation homeostasis and pH control.

Debaryomyces hansenii is an ascomycetous yeast that has been defined as halotolerant/halophilic (Prista et al., 1997, 2002; Lages et al., 1999; Breuer & Harms, 2006), and can be isolated from sea water, salty food or brines. Therefore, cation homeostasis must be very important for the cell. In fact, in the very first papers describing D. hansenii as a salt-tolerant yeast, the importance of the regulation of internal K\(^+\) and Na\(^+\) was already proposed (Norkrans, 1966; Norkrans & Kylin, 1969). Several genes coding for Na\(^+\) transporters have been studied in D. hansenii; ENA1 (Almagro et al., 2001), NHA1 (Velkova & Sychrova, 2006) and NHX1 (Montiel & Ramos, 2007) are involved in Na\(^+\) tolerance, and as the complete genome sequence is available (http://cbi.labri.fr/Genolevures/), some other monovalent cation transporters have been identified, although they have not yet been characterized.

In this article, we present the identification and characterization of DhKHA1, a gene orthologous to ScKHA1, previously reported in Sa. cerevisiae. We propose that, on the basis of the deduced amino acid sequence, DhKha1p can be classified as an Na\(^+\)/H\(^+\) transporter, and we show that overexpression of DhKHA1 in a salt-sensitive Sa. cerevisiae mutant (ena1-4 nha1 kha1) renders cells more tolerant to Na\(^+\) but not to other toxic cations or stress factors. Moreover, cation content determination and experiments performed with green fluorescence protein (GFP)-tagged DhKha1p indicated the intracellular localization of this protein when expressed in S. cerevisiae.
Materials and methods

Strains, media and growth conditions

The type strain *D. hansenii* PYCC2968 (Prista et al., 1997, 2002) was used for *DhKHA1* cloning. Heterologous expression of *DhKHA1* was performed in the salt-sensitive *S. cerevisiae* mutant LMB 01 (ena1Δ::HIS3:ena4Δnha1-Δ::LEU2 kha1A::kanMX) (Maresova & Synchrova, 2005) obtained from the parental strain W303.1A (MATa ade-2-1 his3-11,15 ura3-1 leu2-3,112 trp1-1). Strains PR1, RK1, VP2 and VK2 were obtained from LMB 01 by transformation with the plasmids pYPGE15, pDhKHA1, PKGYCp and PK-KHA(GFP), respectively. Yeast cells were grown at 28 °C in YPD medium (1% yeast extract, 2% peptone, 2% glucose) or complete minimal medium SD (0.67% YNB with 2% glucose, adjusted to pH 5.8 except when indicated) lacking or complete minimal medium SD (0.67% YNB with 2% glucose, adjusted to pH 5.8 except when indicated) lacking uracil and NaCl. The type strain *D. hansenii* mutant LMB 01 (ena1Δ::HIS3:ena4Δnha1-Δ::LEU2 kha1A::kanMX) (Maresova & Synchrova, 2005) obtained from the parental strain W303.1A (MATa ade-2-1 his3-11,15 ura3-1 leu2-3,112 trp1-1). Strains PR1, RK1, VP2 and VK2 were obtained from LMB 01 by transformation with the plasmids pYPGE15, pDhKHA1, PKGYCp and PK-KHA(GFP), respectively. Yeast cells were grown at 28 °C in YPD medium (1% yeast extract, 2% peptone, 2% glucose) or complete minimal medium SD (0.67% YNB with 2% glucose, adjusted to pH 5.8 except when indicated) lacking the appropriate requirements for selection (Adams et al., 1997). When required, 2% agar was added to the media. *Escherichia coli* DH5α (Hanahan, 1985) was used as a host for DNA cloning experiments. Bacterial cells were grown at 37 °C in Luria Bertani medium containing, when needed, ampicillin (100 μg mL⁻¹) and 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (4 μg mL⁻¹) for plasmid selection.

Growth assays

Yeast cells at an initial OD₆₀₀nm of 0.05 and the appropriate dilutions were spotted (5 μL) on minimal medium agar plates adjusted to the appropriate pH with arginine and containing diverse compounds such as NaCl, KCl, LiCl or hygromycin B. Growth was recorded after 3 days of incubation. To test the Na⁺ tolerance of the yeast cells in liquid media, SD medium (50 mL) supplemented with different NaCl concentrations was inoculated to OD₆₀₀nm 0.02. Flasks were incubated in a rotation shaker (200 r.p.m.) at 28 °C; samples were withdrawn at various time intervals over a period of 24 h, and the OD₆₀₀nm was measured. Doubling times were calculated from the OD values obtained during the exponential phase of the growth curves (Gómez et al., 1996). The growth curves were repeated three times and the SDs calculated.

RNA preparation and reverse transcriptase (RT)-PCR

*Debaryomyces hansenii* cells were grown to an OD₆₀₀nm of 1.0 in SD medium under different conditions of pH and salinity. Cells were collected by filtration, frozen in liquid nitrogen, and ground in a mortar. The powder was resuspended in 1 mL of Tripure (Roche) and centrifuged (12 000 g, 10 min, 4 °C). The supernatant was extracted with 0.2 mL of CHCl₃, and centrifuged (12 000 g, 15 min, 4 °C), and nucleic acids were precipitated from the aqueous supernatant by addition of isopropanol (0.5 mL) followed by centrifugation (12 000 g, 10 min, 4 °C). Pellets were washed with 75% ethanol and dried. RT-PCR was performed with the RetroTools kit (Biotos) using 1 μg of RNA and the following oligonucleotides: 5'-ATGTTGAAAGCCGGTTTTCG-3' and 5'-GGGGCTTCTGAAATCTTCTGTA-3' for *DhACT1* amplification; 5'-ATGC CAGTTAATACTATCCAG-3' and 5'-CTGTAACCTGTTT GATATG-3' for *DhKHA1* amplification; and 5'-ACTCACC TACCGACGACATG-3' and 5'-TGCTATCACCCTGCTTCCA TTCACA-3' for amplification of a *DhKHA1* promoter region used as a control to detect possible DNA contamination.

Plasmid construction

The entire ORF of *DhKHA1* was amplified by PCR (nucleotides 167 to 2631 from the initiating Met), using K1 (5'-GGATCCGCTCATACATGTT-3') and K2 (5'-TTAGGGCTTT GACCGGAATCT-3') as sense and antisense primers, respectively. The gene was cloned into vector pGEM-T (Promega) and digested with EcoRI. The released insert was cloned into these same sites of vector pYPGE15 (Brunelli & Pall, 1993) to yield pDhKHA1.

For *DhKHA1*-GFP expression, the 2.6-kbp fragment containing the gene *DhKHA1* (nucleotides 1–2631 from the ATG codon) was amplified by PCR using P1 (5'GGATCCGCTCATACATGTT-3') and K2 (5'-TTAGGGCTTT GACCGGAATCT-3') as sense and antisense primers, respectively. The ORF was cloned into vector pCR2.1-TOPO (Invitrogen), digested with restriction enzymes BamHI and EcoRI, and cloned into plasmid PKGYCp (Gietz & Sugino, 1988), under PLAI promoter control, to yield PK-KHA(GFP). All DNA fragments cloned by PCR were sequenced to verify their accuracy and correct in-frame tagging.

Control of plasmid loss using 5-fluoro-orotic acid

The origin of the phenotype observed was confirmed by curing the clones for the plasmid using 5-fluoro-orotic acid following the procedure described by Boeke et al. (1987). RK1 cells were plated on SD medium supplemented with uracil (40 mg L⁻¹) and 5-fluoro-orotic acid (0.1%), and immediately incubated for 3 days at 28 °C. Loss of the plasmid was checked on SD medium lacking uracil. The Na⁺ sensitivity of isolated colonies was confirmed by transferring them to minimal media supplemented with uracil and NaCl.

Determination of K⁺ and Na⁺ content

Yeast cells were grown for 24 h in liquid SD medium containing different NaCl concentrations in order to test
their K⁺ and Na⁺ content. After 24 h, samples of cells were collected on Millipore filters, which were rapidly washed with 20 mM MgCl₂. The cells were then extracted with acid, and the extracts were analyzed by atomic emission spectrophotometry (Ramos et al., 1990). The experiments were repeated three times, and the SDs calculated.

**Microscopy analysis**

Cells expressing DhKHA1 alleles tagged with the GFP sequence were viewed with an Olympus AX70 microscope using a U-MWB cube with a 450–480-nm excitation filter and a 515-nm barrier filter. The micrographs were recorded with a DP70 digital camera.

**Results**

**Identification, cloning and characterization of KHA1 from D. hansenii**

Several cation transporters involved in cation homeostasis have been identified in yeast. One of the least studied and characterized is Kha1p, and the scarce information available is not consistent with regard to its cellular localization. A search in the Genolevures database (http://cbi.labri.fr/Genolevures/) revealed different genes coding for cation transporters in *D. hansenii*, one of them being orthologous to ScKHA1. We cloned the gene from *D. hansenii* as described in ‘Materials and methods’. Sequence comparison of this ORF (accession number AM410633) with the available entire sequence of *D. hansenii* showed complete identity with ORF DEHA0F28985g. This sequence encodes a predicted 822-residue protein (estimated molecular mass of 91.6 kDa) containing 12 putative hydrophobic regions, similar to the family of Na⁺/H⁺ exchangers (NHEs) (Brett et al., 2005a, b; Pribylova et al., 2006) and a large hydrophilic C-terminal domain. Figure 1a shows the amino acid sequence corresponding to the 12 transmembrane segments present in the exchanger domain of DhKha1p. Sequence comparison of this protein with that deduced from ScKHA1 reveals a high number of conserved residues (gray boxes). Recent research (Kinclova-Zimmermannova et al., 2005, 2006) has reported the existence of Thr141 and Pro146 in the fifth transmembrane domain of the Sod2-22 antiporter from *Zygosaccharomyces rouxii*, and it was proposed that these residues are important for the recognition and transport of substrates. A search in the database confirmed the existence of the corresponding residues in KHA and NHA genes from different organisms, including *D. hansenii*. Interestingly, we found a CITA/CVTA consensus sequence in the same region of ScNHA, CgNHA1, DhNHA1, KINHA1 and DhKHA (Fig. 1a, underlined), but not in other KHA genes.

The deduced amino acid sequence of DhKHA1 is 37%, 36% and 26% identical to those of *Candida glabrata*, *Sa. cerevisiae* and *Schizosaccharomyces pombe*, respectively. Figure 1b shows a phylogenetic tree of DhKha1p and other fungal Kha1p transporters. The tree confirms that DhKha1p is most closely related to CgKha1p and ScKha1p transporters and less related to *Neurospora crassa* and *Sc. pombe* Kha1 proteins.

RT-PCR experiments indicated that neither growth at high pH nor growth in the presence of NaCl significantly affected the expression of DhKHA1 (Fig. 2).

**Overexpression of DhKHA1 in Sa. cerevisiae results in Na⁺ tolerance**

A salt-sensitive *Sa. cerevisiae* mutant (strain LMB 01) lacking the two Na⁺ efflux systems and its endogenous Kha1p antiporter (ena1-4 nha1 kha1) was transformed with a plasmid containing DhKHA1 in order to evaluate the possible functional role of this gene. We observed that, in
solid media, overexpression of *DhKHA1* conferred increased capacity to grow in the presence of Na\(^+\) (Fig. 3), but it did not affect either K\(^+\) or Li\(^+\) tolerance (Fig. 3 and results not shown). The origin of the phenotype observed in the transformant (RK1 strain) was confirmed by curing the clone for the plasmid using the 5-fluoro-orotic acid procedure previously described (Boeke *et al*., 1987). We then decided to test the effect of the expression of *DhKHA1* on tolerance to high pH and to the cationic antibiotic hygromycin B, as it has been reported that KHA1 deletion in *S. cerevisiae* renders cells sensitive to both stress factors (Maresova & Sychrova, 2005). The results shown in Fig. 3 indicated that overexpression of *DhKHA1* did not affect growth at alkaline pH or in the presence of hygromycin B.

To further characterize how the expression of *DhKHA1* affected Na\(^+\) tolerance, we performed growth experiments in SD liquid media containing different NaCl concentrations and determined both growth curves and the cation content of the cells (Fig. 4). In the absence of Na\(^+\), the doubling times of the RP1 strain (control) and the RK1 strain (pDhKHA1) were identical (2.8 h), indicating that *DhKHA1* did not influence the growth rate under normal conditions. However, when cells were exposed to increasing concentra-
tions of Na\(^+\), the toxic effect of the cation was more clearly visible in the RP1 strain than in the RK1 strain (4.9 vs. 3.8 h in 0.15 M NaCl, and 13 vs. 9.3 h in 0.20 M NaCl) (Fig. 4a), which is in accordance with results obtained in plates and shown in Fig. 3. Internal cation measurements showed that cells growing in the presence of increasing concentrations of NaCl maintained higher intracellular Na\(^+\) levels and lower intracellular K\(^+\) levels. Interestingly, similar values for Na\(^+\) and K\(^+\) were determined in both strains (Fig. 4b), which suggests that DhKha1p is not located at the plasma membrane level.

**Cellular localization of DhKha1p**

The cellular location of Kha1p in *S. cerevisiae* has been controversial since the protein was first proposed to be a plasma membrane protein (Ramírez *et al*., 1998), but Maresova & Sychrova (2005) later showed an intracellular localization of the transporter, and at the same time it was reported that the protein localized in the Golgi apparatus (Flis *et al*., 2005). In our case, the *DhKha1–GFP* fusion protein was expressed in the salt-sensitive *S. cerevisiae* mutant strain LMB 01 (*ena1*-*4 nha1 kha1*). Cells were grown

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**Fig. 2.** Expression profile of *DhKHA1*. *Debaryomyces hansenii* cells were grown in SD medium adjusted to pH 7.5 or supplemented with NaCl. RT-PCR was performed as described in ‘Materials and methods’, and the product was visualized by agarose gel electrophoresis and ethidium bromide staining. Pr indicates amplification of a *DhKHA1* promoter region used as a control to detect possible DNA contamination.

**Fig. 3.** Effect of Na\(^+\), K\(^+\), hygromycin B and high pH on the growth of *Saccharomyces cerevisiae* sensitive mutants overexpressing *DhKHA1*. Serial dilutions (10-fold) of strains RP1 (carrying plasmid pYPGE15) and RK1 (carrying plasmid pDhKHA1) were spotted on SD plates (pH 5.8) containing the indicated concentrations of NaCl, KCl or hygromycin B, or adjusted to high pH. Growth was monitored after 3 days at 28 °C.
in liquid SD medium to early exponential phase, and both Na\(^+\) tolerance and the localization of the protein were analyzed. On the one hand, the VK2 strain was more tolerant to NaCl than the VP2 strain, showing that DhKha1p-GFP was able to complement the Na\(^+\)-sensitive phenotype of the recipient strain in a similar way to what is shown in Fig. 3. On the other hand, the results presented in Fig. 5 show that the localization of labeled DhKha1 protein is intracellular.

**Discussion**

K\(^+\) and Na\(^+\) transporters play a fundamental role in cationic homeostasis in all living cells. In this work, we have cloned and heterologously expressed a gene from *D. hansenii* that is orthologous to the KHA1 gene previously found in *S. cerevisiae*. KHA1 genes have been identified in several yeast species, and although not definitively demonstrated, it is believed that they code for cation antiporters. Significant information about the gene has been reported only for *S. cerevisiae*, and even in this case, the information is scarce and somewhat contradictory. Although ScKha1p was first proposed to be a K\(^+\)/H\(^+\) antiporter located at the plasma membrane level and involved in pH regulation (Ramirez *et al.*, 1998), an intracellular location for the protein was later proposed, and, once again, involvement in cationic homeostasis and pH regulation (Maresova & Sychrova, 2005; Flis *et al.*, 2005). In fact, similar localization and functions have been proposed recently for Chx17p, a putative K\(^+\)/H\(^+\) exchanger from *Arabidopsis thaliana* (Maresova & Sychrova, 2006).

Structural analysis indicates that DhKha1p belongs to the family of NHEs (Brett *et al.*, 2005a; Pribilova *et al.*, 2006). Kinclova-Zimmermannova *et al.* (2005, 2006) found that a Thr and a Pro present in the fifth transmembrane domain of the Sod2-22 antiporter from *Z. rouxii* were very important for substrate specificity during the transport process. These two residues are also present in all Nha1 and Kha1 proteins that we have analyzed. Moreover, we found a CITA/CVTA sequence in the same transmembrane domain that is present in all Nha1 and in DhKha1 proteins but not in other Kha1 proteins.

The expression level of DhKHA1 in *Debaryomyces* was not affected by different external conditions of pH and salinity, although the *S. cerevisiae kha1* mutant is sensitive to alkaline pH and is involved in cation homeostasis (Maresova & Sychrova, 2005).
An important aspect of DhKHA1 is that it seems to have a different function than has been proposed for ScKHA1, as its overexpression in \( \text{Sa. cerevisiae} \) caused increased Na\(^{+}\) tolerance but it did not affect growth at high pH or hygromycin B tolerance. This is not very surprising, as we have reported that some other genes from \( \text{D. hansenii} \) play more specific or different functions than the corresponding genes from \( \text{Sa. cerevisiae} \). Examples are DhENA2 (Almagro et al., 2001) and the transcription factor coded by GZF3 (García-Salcedo et al., 2006). In addition, if we consider the halotolerant character of \( \text{D. hansenii} \) and the natural habitat in which this yeast can be usually be found, it seems conceivable that some genes may have evolved and acquired new functions related to Na\(^{+}\) tolerance. In any case, and in that context, it is also important to remember that a K\(^{+}\) transport function that is specifically dependent on ScKha1p has not yet been fully demonstrated.

The Kha1 protein from \( \text{Sa. cerevisiae} \) was first proposed to be located at the plasma membrane level; however, a couple of papers later reported the intracellular localization of the putative antiporter. In our case, it was not possible to link the observed increased Na\(^{+}\) tolerance due to overexpression of DhKHA1 to a decrease in the internal content of the toxic cation, which suggests a possible intracellular localization of the antiporter. This idea was also confirmed by the GFP experiments.

Much more work is required to understand the real function of Kha1 proteins. They are present in many yeasts, but only ScKha1p has been partially studied. Our results on DhKha1p pose questions about the function of Kha1 antiporters and their relationship with other genes such as the NHA1 family. Comparison of complete sequences of NHA1 and KHA1 genes indicates that DhKha1p is more closely related to the KHA1 family than to the NHA1 gene family. However, the existence of conserved regions in the Nha1 and DhKha1 proteins, such as the CITA/CVTA sequence in the fifth transmembrane domain, and their function in Na\(^{+}\) tolerance may point to a possible role of DhKha1p as an Na\(^{+}\) transporter.

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