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Yeast as a Model Organism to Study Transport and Homeostasis of Alkali Metal Cations

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Summary

To maintain an optimum cytoplasmic K^+/Na^+ ratio, cells employ three distinct strategies: 1) strict discrimination among alkali metal cations at the level of influx, 2) efficient efflux of toxic cations from cells, and 3) selective sequestration of cations in organelles. Cation efflux and influx are mediated in cells by systems with different substrate specificities and diverse mechanisms, e.g. ATPases, symporters, antiporters, and channels. Simple eukaryotic yeast *Saccharomyces cerevisiae* cells proved to be an excellent model for studying the transport properties and physiological function of alkali-metal-cation transporters, and the existence of mutant strains lacking their own transport systems provided an efficient tool for a molecular study of alkali-metal-cation transporters from higher eukaryotes upon their expression in yeast cells.

Key words

Alkali metal cations • Na^+/H^+ antiporter • Osmotolerance • Plasma membrane • Yeast

Introduction

In natural environments, sodium belongs to the abundant and potassium to the scarce ions. However, high internal concentrations of Na^+ (or its analogue Li^+) are generally toxic for cells. On the other hand, K^+ is required for many physiological functions (regulation of cell volume and intracellular pH, protein synthesis, enzyme activation) and this cation is accumulated in cells at a fairly high concentration. To maintain an optimum cytoplasmic concentration of potassium and a stable high intracellular K^+/Na^+ ratio, cells employ three distinct strategies: 1) strict discrimination among alkali metal cations at the level of influx (higher affinity of transporters for potassium than for sodium), 2) efficient efflux of toxic cations from cells, and 3) selective sequestration (compartmentation) of cations in organelles. Transport systems mediate cation efflux and influx with different substrate specificities and using diverse mechanisms, e.g. ATPases, symporters, antiporters, and channels. Malfunction or absence of these transporters is lethal for microorganisms that cannot cope with sudden changes in external osmolarity, causes severe diseases in mammals, and aggravates the growth capacity of crop plants in the world's increasing areas of salted soils.

For an effective Na^+ extrusion, cells of most organisms use active transport systems, mainly ATPases and Na^+/H^+ antiporters. Although the alkali-metal-cation/proton antiporters represent conserved transport systems existing in almost all types of organisms (archaea, bacteria, fungi, parasites, insects, plants,

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mammals), their structure, substrate specificity and probably cell function have diverged during evolution. While most microorganisms and plants use the inward gradient of protons created by the plasma membrane H^+-ATPase as a driving force to pump out alkali metal cations, mammalian cells usually consume the Na^+ gradient resulting from the Na^+/K^+-ATPase activity in order to force the excess of protons out and to regulate the intracellular pH.

Cells usually express several Na^+/H^+ antiporters in parallel, e.g. 8 isoforms in human cells (Orlowski and Grinstein 2004) or 3-5 isoforms in many plants (Blumwald 2000, Mansour *et al.* 2003). The proper biochemical and physiological characterization of a transport system in human or plant cells is often hampered, by the existence of other transport systems with similar transport velocity and substrate specificity in the same cell as well as by laborious knocking-out of genes encoding the other transporters. To overcome these difficulties, heterologous expression of a studied transporter in the host lacking its own transport systems for the given substrate is used. One of the model organisms employed for heterologous expression is the yeast *Saccharomyces cerevisiae*.

Yeast as a model organism

S. cerevisiae belongs to the best characterized eukaryotic organisms (Smutzer 2001). Despite its simplicity as a free-living unicellular fungus, yeast cells are similar to higher eukaryotes in the cell structure and physiological processes. The *S. cerevisiae* genome was the first completely sequenced eukaryotic genome. The sequence of 12000 kilobases defines more than 6000 genes, provides information about the higher-order organization of yeast's 16 chromosomes and allows some insight into their evolutionary history (Goffeau *et al.* 1996). The experimental tractability of yeast cells, mainly as concerns employment of methods of molecular genetics resulted in a wide application of yeast as a model in studies elucidating regulation of cell cycle, organelle biogenesis, signaling pathways and many other cell functions including transport mechanisms (Botstein and Fink 1988).

Transport of alkali-metal cations in yeast

Two active transporters (Trk1p and Trk2p) and two channels (Tok1p and Nsc1p) ensure the uptake of potassium in yeast cells (Fig. 1) (Ko and Gaber 1991, Bertl *et al.* 1998, Bihler *et al.* 1998). The optimum intracellular K^+ content is 200-300 mM, whereas the K^+ concentration in many environments is in the micromolar range (Rodríguez-Navarro 2000). It is mainly the Trk1p's activity and its high affinity for potassium that ensures sufficient potassium accumulation necessary for cell growth and division (Haro and Rodríguez-Navarro 2002, Bertl *et al.* 2003).

When yeast cells are exposed to a salt stress, Na^+ enters the cells as a low-affinity substrate through several cation-transporting systems, mainly those involved in potassium uptake, and cells must cope with the increased cytoplasmic concentration of toxic cations. In *S. cerevisiae*, three mechanisms function cooperatively to prevent sodium accumulation: restriction of influx, active efflux and compartmentation of Na^+ in the vacuole. Like in other organisms, two different types of transport systems mediating active sodium efflux exist in yeast plasma membranes: Na^+-ATPases and Na^+/H^+ antiporters (Fig. 1).

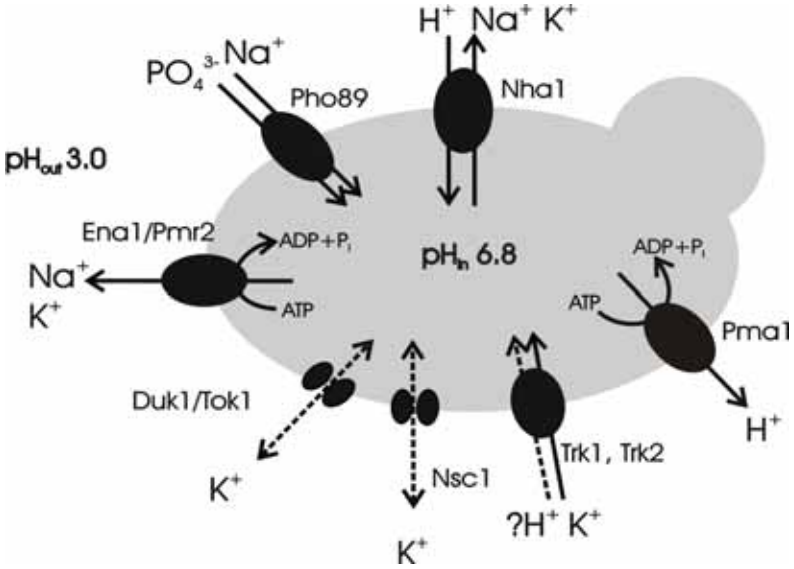

Fig. 1. Transport systems involved in the uptake and efflux of potassium and sodium in the plasma membrane of *S. cerevisiae*. Pma1, H^+-ATPase; Trk1, Trk2, potassium uptake systems; Nsc1, nonspecific cationic channel; Duk1/Tok1, potassium channel; Ena1/Pmr2, Na^+-ATPase; Pho89, P_i-Na^+ cotransporter; Nha1, Na^+/H^+ antiporter.

The first yeast gene encoding a Na^+/ATPase (*ENA1*) was cloned, based on its ability to increase the lithium tolerance in a lithium-sensitive strain (Haro *et al.* 1991). The high similarity of the predicted Ena1 protein with P-ATPases suggested that the system was a cation pump, a Na^+ -ATPase. The *ENA1* gene (allelic to *PMR2*) is the first unit of a tandem array of at least four (*ENA1* to *ENA4*) or five (*PMR2a* to *PMR2e*) genes, depending on the strain (Garciadeblás *et al.* 1993, Wieland *et al.* 1995). Whereas *ENA2, ENA3* and *ENA4* are expressed constitutively and at a low level, expression of the *ENA1* gene can be induced by Na^+, Li^+ or high pH values. Under low-salt conditions the activation of *ENA1/PMR2* transcription is mediated by the HOG-MAP kinase pathway (Márquez and Serrano 1996) and under high-salt conditions by calcineurin which antagonizes the negative regulator, cAMP-dependent protein kinase (Hirata *et al.* 1995). Genes encoding Na^+-ATPases have been isolated and their products characterized also in other yeast species, e.g. osmotolerant *Zygosaccharomyces rouxii* (Watanabe *et al.* 1999) and *Debaryomyces hansenii* (Almagro *et al.* 2001).

As the Ena1 ATPase is active mainly at alkaline pH values, the existence of another efflux system, possibly a H^+/cation antiporter, operating at acidic external pH values was predicted for *S. cerevisiae* (Ortega and Rodríguez-Navarro 1985). This prediction was confirmed by cloning the *NHA1* gene (Prior *et al.* 1996).

Yeast plasma membrane Na^+/H^+ antiporter

The *NHA1* gene (YLR138w) was selected from a multicopy *S. cerevisiae* genome library on the basis of its ability to improve the growth of a salt-sensitive strain on a medium supplemented with a high concentration of NaCl (Prior *et al.* 1996). The gene is 2958 nucleotides long and is located on the right arm of chromosome XII between *TIS11* and *SLS1* genes. So far, genes encoding specific plasma membrane Na^+/H^+ antiporters have been identified and characterized in five yeast species – *S. cerevisiae, Schizosaccharomyces pombe* (Jia *et al.* 1992), *Z. rouxii* (Watanabe *et al.* 1995), *Candida albicans* (Soong *et al.* 2000) and *Pichia sorbitophila* (Bañuelos *et al.* 2002a). Systematic genome sequencing of other yeast species revealed the existence of conserved sequences with high similarity to *S. cerevisiae NHA1* in all of them. The functionality of these putative genes and their products remains to be established.

The protein encoded by the *NHA1* gene contributes to the cell cation homeostasis by mediating the efflux not only of toxic Na^+ and Li^+ cations, but also K^+ and Rb^+ (Bañuelos *et al.* 1998, Kinclová *et al.* 2001c). Construction of a mutant strain with deletions of genes encoding both Na^+-ATPase and Na^+/H^+ antiporter (*ena1-4Δ nha1Δ*) confirmed that both transport systems are necessary for cell tolerance to high external concentrations of salts. The plasmid-based overexpression of the *ENA1* or the *NHA1* gene in the double-deleted strain showed complementary action of both systems in the maintenance of intracellular steady-state concentrations of K^+ and Na^+. The Nha1 antiporter is responsible for cell growth on high concentrations of KCl and NaCl at acidic external pH values, and the Ena1 ATPase is necessary at higher pH values (Bañuelos *et al.* 1998). In contrast to the complementary action on the protein activity level, the expression of both systems is different. The *ENA1* expression is highly regulated, as mentioned above, whereas the *NHA1* expression does not seem to be inducible by salts, pH changes or osmotic shocks, and the *NHA1* transcription is constitutive and very low (Bañuelos *et al.* 1998). The $H^+/K^+(Na^+)$ exchange mechanism of Nha1p was confirmed in reconstituted vesicles prepared from plasma membranes of strains without or with Nha1p (Sychrová *et al.* 1999). The localization of Nha1p in the plasma membrane was verified by GFP tagging and fluorescence microscopy (Kinclová *et al.* 2001c).

The Nha1 antiporter is also involved in the regulation of intracellular pH. Deletion of the *NHA1* gene brought about an increase of cytoplasmic pH in cells and the addition of KCl to starved cells resulted in a much higher alkalinization of cytoplasmic pH in a strain lacking Nha1p compared to the wild type or Nha1p-overexpressing strains (Sychrová *et al.* 1999). As is obvious from Nha1p's dependence on the gradient of protons across the plasma membrane, the highest sodium or potassium efflux mediated by this transporter occurs at acidic external pH values. If the cation-preloaded cells are incubated in a cation-free buffer, at pH_{out} 8.0, the efflux of sodium or potassium in the direction of their gradients is very low. On the other hand, when the cytoplasmic pH of cells resuspended at pH_{out} 8.0 increases, Nha1p mediates a huge and immediate efflux of potassium. These observations led us to the conclusion that Nha1p, besides its function in detoxification, could act as a short-term safety valve to contribute to the buffering of cytosolic pH by using the outward gradient

of K^+ which can drive in some protons (Bañuelos *et al.* 1998, Kinclová *et al.* 2001c).

The high potassium content in yeast cells corresponds to the steady state between simultaneous influx and efflux across the plasma membrane and this continuous circulation is believed to be necessary for maintenance of potassium homeostasis, constant intracellular pH and regulation of cell volume (Ortega and Rodríguez-Navarro 1985, Lapathitis and Kotyk 1998). The role of Nha1 in the regulation of potassium homeostasis was proved by its ability to influence the transport activity of the main potassium uptake system Trk1p (Bañuelos *et al.* 2002b).

The Nha1p (985 amino acids long) is a typical hydrophobic membrane protein with a short hydrophilic N-terminus (12 amino acids), 12 transmembrane segments with short connecting loops, and an extremely long hydrophilic C-terminus (554 amino acids, i.e. 56.2 % of the whole protein). In order to study the role of the C-terminus in the Nha1p function we constructed a series of 13 truncated *NHA1* versions ranging from the complete one (2958 nucleotides, 985 amino acids) up to the shortest version (1416 nucleotides, 472 amino acids) ending with only 41 amino acid residues after the last putative transmembrane domain. Truncated *NHA1* versions were expressed in a *Sacharomyces cerevisiae* strain lacking its own alkali-metal-cation exporters (*ena1-4Δ nha1Δ*). The entire Nha1p C-terminus domain was necessary neither for proper localization of the antiporter in the plasma membrane nor for transport of all four substrates. Partial truncation of the C-terminus of about 70 last amino acids improved the tolerance of cells to Na^+, Li^+ and Rb^+ compared to cells expressing the complete Nha1p. The presence of the neighbor part of the C-terminus (aminoacids 883-928), rich in aspartate and glutamate residues, was necessary for the maintenance of maximum Nha1p activity toward sodium and lithium. In the case of potassium, we could demonstrate the participation of the long C-terminus in the regulation of intracellular potassium content (Kinclová *et al.* 2001c).

Besides the role of the C-terminus in the Nha1p transport activity, its participation in several other cell processes was clearly demonstrated. The observed importance of Nha1p C-terminus for cell survival upon a hyperosmotic shock caused by solutes other than salts (e.g. sorbitol) implied its role as a part of the rapid rescue mechanism in the immediate cell response to osmotic shock (Kinclová *et al.* 2001c). Nha1p and its long C-terminus may play an important role in the regulation of the cell cycle, as high-copy expression of the *NHA1* gene was able to rescue the blockage at the G_1/S transition of cells with conditional *sit4 hal3* mutations (Simón *et al.* 2001). This function was independent of the role of the antiporter in improving tolerance to sodium cations, and it required the integrity of a relatively large region (amino acids 800-948) of its C-terminus. A screening for loss-of-function mutations at the C-terminus revealed a number of residues required for the Nha1p function in the cell cycle, most of them clustering in two regions, from residues 869 to 876 and 918 to 927 (Simón *et al.* 2003). The importance of different regions of the Nha1p C-terminus for its function was also confirmed in a study comparing the structure and activity of four Na^+/H^+ antiporters (Kamauchi *et al.* 2002).

The molecular mechanisms of Na^+/H^+ antiport, together with the identity of amino acids involved in binding of the transported cations are still unknown. Several polar residues specifically distributed within or immediately adjacent to the membrane-spanning regions were shown to be important for the antiporter activity (Dibrov and Fliegel 1998, Dibrov *et al.* 1998). These key amino acids are conserved in prokaryotes and in some lower eukaryotic Nha1p forms, despite their dispersion throughout the protein and despite an overall low similarity in the linear sequence of these Na^+/H^+ antiporters. Mutational analysis of the *S. pombe* antiporter showed clearly that 4 aspartate and 1 histidine residues located in transmembrane segments III, IV, VII and VIII were important for the proton translocation activity of the antiporter (Wiebe *et al.* 2003).

Na^+/H^+ antiporters of other yeast species

Though the known yeast Na^+/H^+ antiporters differ in total length, all of them contain a short hydrophilic N-terminus (11-12 amino acid residues), the hydrophobic part with twelve putative transmembrane segments and a hydrophilic C-terminus. Amino acid sequences and lengths of the N-termini, transmembrane parts and connecting loops are highly conserved but the C-terminal sequences of antiporters differ in length and show much lower similarity in amino acid sequence. To compare the impact of protein structure on its activity we compared the transport properties of four yeast Na^+/H^+ antiporters. The expression of *S. cerevisiae* Nha1p, *C. albicans* Cnh1p, *Z.* rouxii ZrSod2-22p and *S. pombe* sod2p from the same multicopy plasmid in a *S. cerevisiae* mutant strain lacking both Na^+-ATPase and Na^+/H^+

antiporter genes (*ena1-4Δ nha1Δ*) made it possible to study transport properties and contribution of all antiporters to cell salt tolerance under the same conditions. The ZrSod2-22p of the osmotolerant yeast *Z. rouxii* had the highest transport capacity for lithium and sodium but, similarly as the *S. pombe* sod2p, it did not recognize K^+ and Rb^+ as substrates. The *S. cerevisiae* Nha1p and *C. albicans* Cnh1p had a broad substrate specificity for at least four alkali metal cations (Kinclová *et al.* 2001a), but their contribution to overall cell tolerance to high external concentration of toxic Na^+ and Li^+ cations seemed to be lower compared to antiporters of *S. pombe* and especially *Z. rouxii* (Kinclová *et al.* 2001b, 2002). According to our results and to the results describing transport properties of *P. sorbitophila* Na^+/H^+ antiporters expressed in *S. cerevisiae* (Bañuelos *et al.* 2002a), the family of yeast plasma membrane Na^+/H^+ antiporters can be divided, as concerns substrate specificity and probably cell function, in two distinct subfamilies: 1) subfamily of antiporters with substrate specificity only for Na^+ and Li^+ having primary detoxification function in cells, and 2) subfamily of antiporters mediating transport of at least four alkali metal cations that, besides elimination of toxic cations, probably play a role in other cell functions (regulation of intracellular K^+ concentration, pH and cell volume).

Expression of heterologous Na^+/H^+ antiporters in *S. cerevisiae*

The construction of a series of *S. cerevisiae* mutants lacking besides transport systems for sodium extrusion (*ena1-4Δ nha1Δ*), also the intracellular Na^+/H^+ antiporter (*nhx1Δ*), putative K^+/H^+ antiporter (*kha1Δ*), potassium channel (*tok1Δ*) or potassium uptake systems (*trk1Δ trk2Δ*) provided super-salt-sensitive strains suitable for elucidation of the individual contributions of different transporters to the overall cell alkali-metal-cation tolerance and homeostasis (unpublished results). These strains also serve as an apposite tool for the expression of both plasma-membrane and intracellular alkali-metal-cation transporters from higher eukaryotes. As an example, Figure 2 shows the growth of a yeast triple-deleted mutant (*ena1-4Δ nha1Δ nhx1Δ*) harboring plasmids encoding either *S. cerevisiae* own Nha1p or the rat NHE2 exchanger in a medium with a gradually increasing concentration of KCl. Growth of cells expressing the mammalian NHE2 protein in media with higher concentrations of KCl, compared to cells without any antiporter, clearly affirms that the NHE2p is functionally expressed and suggests that its substrate specificity involves, besides Na^+ and Li^+, also K^+ cations. Similarly, we could observe a functional expression of some mammalian potassium channels in our mutants that resulted in changes of cell growth in the presence of extremely high/low concentrations of potassium cations (data not shown). As concerns plant intracellular vacuolar Na^+/H^+ antiporters, we expressed the rice *OsNHX1* gene in our strains and demonstrated that it was able to substitute for the endogenous yeast *Sc*Nhx1 antiporter and had a broad substrate specificity for at least four alkali-metal cations (Na^+, Li^+, K^+ and Rb^+) (Kinclová-Zimmermannová *et al.* 2004).

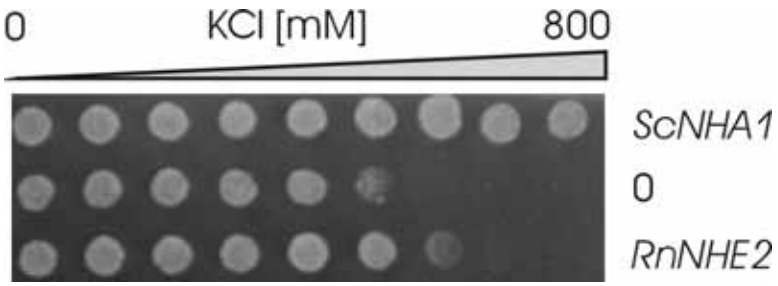

Fig. 2. Growth of *S. cerevisiae* mutant cells lacking their own Na^+/ATPase and Na^+/H^+ antiporter (*ena1-4Δ nha1Δ*) in a medium with increasing KCl concentration. ScNHA1, cells expressing Nha1p; RnNHE2, cells expressing rat NHE2p; 0, cells expressing no antiporter.

Perspectives

Our current knowledge leads the general study of yeast Na^+/H^+ antiporters in two different directions: 1) very high similarity in protein sequence but different substrate specificity of *S. cerevisiae* Nha1 and *Z. rouxii* ZrSod2-22 antiporters pose a question about the amino-acid residues responsible in the molecule of Nha1p for recognition and transport of two more substrates (K^+, Rb^+) compared to ZrSod2-22p; 2) the distribution (2:3) of known yeast Na^+/H^+ antiporters into two subfamilies differing in substrate specificity does not answer the question whether the general physiological role of Na^+/H^+ antiporters in yeast cells is only the detoxification as

shown for *Z. rouxii* and *S. pombe* or whether it could be a more complex function involving participation in the regulation of cell volume and intracellular pH as presumed for *S. cerevisiae*, *C. albicans* and *P. sorbitophila*. Systematic sequencing of several other yeast genomes revealed recently ORFs with high sequence similarity to already known yeast Na^+/H^+ antiporters. Cloning of these putative genes and their expression in the *S. cerevisiae* mutant (*ena1-4Δ nha1Δ*) will make it possible to estimate their transport properties. Consequently, a broader list of yeast Na^+/H^+ antiporters with characterized substrate specificities and cell functions will help to elucidate the evolution of this type of transport systems in phylogenetically related (or distant) yeast species.

Mutant strains with deletions of different sets of alkali-metal-cation transporters will be further used for the expression of Na^+/H^+ antiporters and K^+ channels from higher eukaryotes. Characterization of cell localization, transport activity and substrate specificity of these transporters, together with their influence on yeast cell physiology will allow a direct comparison of structure, properties and cell function of the family of Na^+/H^+ antiporters from different organisms.

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Reprint requests

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