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The yeast SR protein kinase Sky1p modulates salt tolerance, membrane potential and the Trk1,2 potassium transporter

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Abstract

Protein kinases dedicated to the phosphorylation of SR proteins have been implicated in the processing and nuclear export of mRNAs. Here we demonstrate in *Saccharomyces cerevisiae* their participation in cation homeostasis. A null mutant of the single yeast SR protein kinase Sky1p is viable but exhibits increased tolerance to diverse toxic cations such as Na^+, Li^+, spermine, tetramethylammonium, hygromycin B and Mn^{2+}. This pleiotropic phenotype correlates with reduced accumulation of cations, suggesting a decrease in membrane electrical potential. Genetic analysis and Rb^+ uptake measurements indicate that Sky1p modulates Trk1,2, the high-affinity K^+ uptake system of yeast and a major determinant of membrane potential.

Keywords: *Saccharomyces cerevisiae*; Ion homeostasis; Protein kinase; Salt tolerance; Potassium transport; Membrane potential

1. Introduction

An important feature of the physiology of living cells is accumulation of K^+ and the exclusion of Na^+ from the intracellular medium. Preference for K^+ as the major intracellular cation may be explained by the capability of Na^+, but not K^+, to displace essential Mg^{2+} ions from the catalytic sites of some enzymes such as the Hal2 phosphatase [1,2]. Accordingly, cellular membranes contain diverse cation transport systems to ensure the right intracellular ionic composition. These transporters are regulated by several signal transduction pathways, which tune their activity to the changing conditions experienced by the cells [3].

The mechanisms of monovalent cation homeostasis in living cells are only starting to be elucidated. Mutants affected in salt tolerance have been valuable tools for the understanding of cation homeostasis in fungi and plants, where the primary pump is a H^+-ATPase (Pma1) and K^+ and Na^+ are transported by secondary systems, channels and antiporters, coupled to the electrochemical H^+ gradient. A Na^+-extrussion ATPase (Ena1) has also been described in fungi [3,4]. Both the pumps and the secondary transporters are regulated by different metabolic and environmental factors, but a detailed mechanism of the adjustment of cation concentrations by modulation of transport rates is not available and many regulatory components are still missing.

In the present work, we describe a novel modulator of cation homeostasis in *Saccharomyces cerevisiae*, the protein kinase Sky1p. This kinase has been implicated in the phosphorylation of SR proteins [5], proteins with domains containing alternating serine and arginine residues and which are components of the machinery for the processing [6] and nuclear export [7] of mRNAs. Recently, *sky1* mutation has also been described as able to confer resistance to polyamine toxic analogues [8] and to several anticancer drugs such as cisplatin and carboplatin and to display a mutator phenotype [9]. We report that Sky1p also regulates the Trk1,2 K^+ transporter and other determinants of cation homeostasis and salt tolerance and discuss possible mechanisms for these physiological functions.

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2. Materials and methods

2.1. Yeast strains

S. cerevisiae strains RS801 (W303-1A *MAT*a *ura3 leu2 his3 trp1 ade2 ena1-4::HIS3*), RS1187 (W303-1A *MAT*a *ura3 leu2 his3 trp1 ade2 ena1-4::HIS3 trk1::LEU2 trk2::HIS3*), JF194 and JF223 (isogenic to RS801 and RS1187, respectively, but with deletion of the *SKY1* locus) and JF219 (isogenic to RS801 but with deletion of the *HMT1* locus) were used in this study. Deletions of the loci indicated were achieved by homologous recombination with PCR-synthesized disruption cassettes containing the kan^R gene [10].

2.2. Spot tests of yeast growth

About 3 μl of serial dilutions (10^{-1}, 10^{-2} and 10^{-3}) from yeast-saturated cultures were dropped on plates containing standard yeast-rich medium (YPD) supplemented with different toxic cations. Plates were incubated at 28 °C, and growth was recorded after 2–7 days of culture.

2.3. Measurement of intracellular cation concentrations

For lithium measurements, cells were grown to exponential phase in liquid medium, and LiCl was then added. Samples were taken at several time points, and cells were washed with ice-cold 10 mM $MgCl_2$ and were finally resuspended in 10 mM $MgCl_2$ containing 0.1 M HCl to extract intracellular ions. After removal of cell debris by centrifugation, lithium concentration in the supernatant was determined with an atomic absorption spectrometer (Varian SpectrAA-10 Plus) in flame emission mode. Sample analysis and preparation of standards was performed as described by the manufacturer.

For sodium measurements, the same protocol as for lithium was used except for cells being twice washed with ice-cold 10 mM $MgCl_2$ and isoosmotic sorbitol and boiled 15 min to extract intracellular ions.

For rubidium and potassium measurements, cells were inoculated in AP medium (2% glucose, 0.2 mg/l $FeCl_3$, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, buffered with 80 mM H_3PO_4 taken to pH 6.5 with Arg-base and supplemented with 20 ng/ml biotine, 500 ng/ml calcium pantotenate, 500 ng/ml nicotinic acid, 2 μg/ml thyamine clorhydrate, 2 μg/ml piridoxine clorhidrate, 100 μg/ml inositol, 20 ng/ml folic acid, 200 ng/ml riboflavine, 200 ng/ml *p*-aminobenzoic, 500 ng/ml H_3BO_4, 50 ng/ml $CuSO_4$, 100 ng/ml KI, 400 ng/ml $MnSO_4$, 200 ng/ml Na_2MoO_4 and 200 ng/ml $ZnSO_4$) containing 10 mM KCl and were grown until $o.d._{660} = 0.4$. Aliquots were then reserved for internal K^+ measurements and the remaining cells were washed twice with 20 mM $MgCl_2$ and resuspended in AP medium without potassium. After 30 min of potassium starvation, 10-ml aliquots were taken, washed with 20 mM $MgCl_2$ and resuspended in 0.2 ml of buffer containing 2% glucose, 50 mM succinic acid and 20 mM $MgCl_2$. After 5 min, RbCl was added, and aliquots were taken at several time points. These aliquots and the ones reserved for the K^+ measurements were washed twice with 20 mM $MgCl_2$, resuspended in 0.5 ml of MilliQ water and boiled to extract the intracellular ions. After removal of cell debris by centrifugation, 1/50 dilutions of the supernatant were used for HPLC analysis in an equipment (Waters) with a IC-PAK CM/D column and a Waters 432 conductivity detector. Elution was made in an isocratic flux, using as a mobil phase 0.1 mM EDTA, 3 mM HNO_3. Sample analysis and preparation of rubidium and potassium standards was performed as described by the manufacturer.

For methylammonium measurements, cells were grown and starved for potassium as described before for K^+ and Rb^+ measurements. After 30 min of potassium starvation, cells were centrifuged, washed and resuspended with water, then cells were diluted to a final absorbance at 660 nm of about 10 in a reaction medium containing 2% glucose and 50 mM MES ((2-[*N*-Morpholino]ethanesulfonic acid) adjusted to pH 6 with Tris. After 5 min of preincubation, [^{14}C]Methylammonium (0.2 mM and 0.25 μCi/ml final concentration) was added from a concentrated stock solution. At the indicated times, the transport reaction was stopped by diluting samples of 100 μl with 10 ml of ice-cold water. Cells were collected by vacuum filtration through a GF/C 25-mm grass microfibre filters from Whatman and were washed three times in the filter with ice-cold water. Moist filters were transferred to scintillation cocktail (Optifase "HiSafe" 3 from Perkin Elmer), and radioactivity was monitored using a Pharmacia Wallac 1410 liquid scintillation counter.

3. Results and discussion

In a screening for Arabidopsis genes capable of conferring salt tolerance in yeast, we identified several SR-like splicing proteins [6,11]. The yeast SR protein kinase Sky1p [5] proved to be essential for the observed salt tolerance phenotype, and in the course of these studies, we made the observation that a null mutation of the *SKY1* gene resulted in salt (Li^+ and Na^+) tolerance. A similar observation has recently been reported [8], although in this work, the original connection with Sky1p came from polyamine transport and the transport of other cations was not investigated.

As indicated in Fig. 1A, the *sky1* mutation conferred a pleiotropic phenotype of tolerance to diverse toxic cations such as spermine, Li^+, Na^+, tetramethylammonium, hygromycin B and Mn^{2+}. As our experiments have been performed in a strain with a full deletion of the *ENA* locus, the *sky1* phenotype cannot be explained by modulation of this cation extrussion pump [3]. We also deleted the *sky* locus in a W3031-A wild type strain, obtaining the same results that in an *ena* strain (data not shown). Pleiotropic

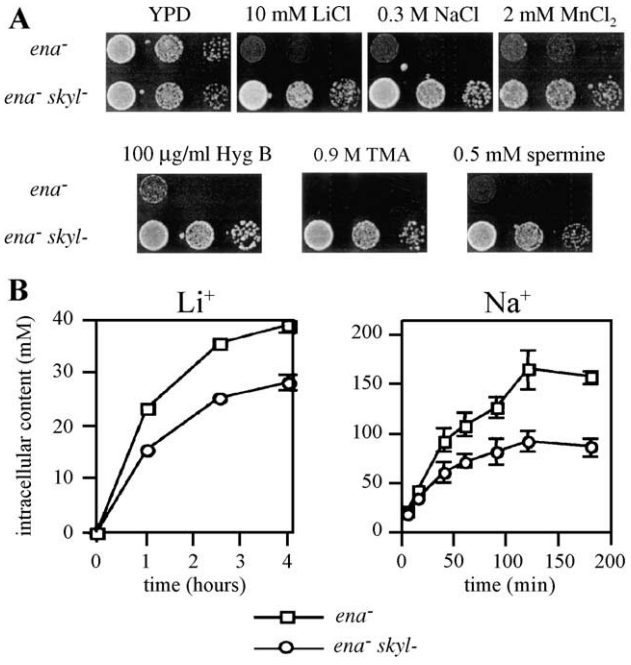

Fig. 1. (A) Spot test of growth of yeast strains RS801 (*ena1-4*) and JF194 (*ena1-4 sky1*) on YPD medium supplemented with the indicated cations. The same results were obtained with three independent disruptants. (B) Intracellular lithium and sodium content (mean ± S.D. of three replicates) of RS801 (*ena1-4*) (squares) and JF194 (*ena1-4 sky1*) (circles) yeast cells at the indicated times after a shock with 40 mM LiCl or 0.4 M NaCl in YPD medium. The experiment was repeated twice with similar results.

cation tolerance is suggestive of a decrease in membrane electrical potential, which results in reduced cation uptake [12,13]. Accordingly, the uptake of Li^+ and Na^+ by yeast cells is reduced in the *sky1* mutant (Fig. 1B). This is in accordance with the reported decrease of polyamine uptake in this mutant [8].

A decrease in membrane electrical potential and in cation uptake is associated with mutations in the genes of: (a) the Pma1 plasma membrane H^+-ATPase [14,15]; (b) the Trk high-affinity K^+ transporter (encoded by the partially redundant *TRK1* and *TRK2* genes) [16]; (c) the regulators of these systems [12,13]. The Pma1 and Trk systems are respectively the electrical generator and a major consumer of electrical potential, and we have proposed that the relative activity of these two systems sets the steady-state value of the electrical potential and in so doing modulates the activity of transporters involved in toxic cation uptake [13].

The *sky1* mutation has no effect on the "in vivo" activity of the H^+-ATPase as measured by the initial rate of acidification of the external medium (data not shown), but it increased the activity of the Trk system as measured by the increased uptake of Rb^+ in K^+-starved *sky1* cells (Table 1). Activation of the K^+ transporter by *SKY1* disruption was corroborated by an increased level of internal K^+ in growing *sky1* cells (Table 1). In addition this activation of potassium transport was found to correlate with a decrease in methylammonium uptake (Fig. 2). Methylammonium

Table 1
Effect of *SKY1* disruption on intracellular content of K^+ and uptake of Rb

Yeast strain	Rate of Rb uptake (nmol/min × mg fresh weight)	Internal K^+ (mM)
SKY1	2.2 ± 0.1	156 ± 6
sky1	3.0 ± 0.2	180 ± 9

The values are means ± S.D. of data from three independent cultures. Strains RS 801 (*SKY1*) and JF194 (*sky1*) were used.

uptake is a widely used indicator of plasma membrane electrical potential in yeast, where direct measurements by microelectrodes are not possible [17,12]. Our results indicate that plasma membrane depolarization could explain the tolerance to toxic cations of *sky1* mutants.

It was interesting to ascertain whether the pleiotropic phenotype conferred by the *sky1* mutation was dependent on a functional Trk system. As indicated in Fig. 3A, disruption of the *SKY1* gene in the *trk1 trk2* double mutant, devoid of a functional Trk system, does not confer tolerance to Na^+ and Mn^{2+}, but still confers tolerance to Li^+, spermine, tetramethylammonium and hygromycin B. In accordance to these growth phenotypes, the uptake of Na^+ by *trk1 trk2* mutant cells was not affected by disruption of the *SKY1* gene while the uptake of Li^+ was reduced in this mutant (Fig. 3B). It must be noticed that the uptake of Li^+ and Na^+ in the *trk1 trk2* mutant is much higher than in *TRK1 TRK2* cells (Fig. 1B). This is in agreement with the hyperpolarized membrane potential of the mutant [16].

These results indicate that the tolerance to Na^+ and Mn^{2+} conferred by the *sky1* mutation correlates with cation uptake and is fully dependent on a functional Trk system. One plausible model is that the Sky1p protein kinase, directly or indirectly, acts as a negative modulator of the Trk system. Disruption of the *SKY1* gene would then result in increased Trk-mediated K^+ uptake, and this would cause a decrease in membrane potential and a concomitant decrease in the uptake of toxic cations [12,13]. The mechanism of the Trk regulation is probably postranscriptional because no change in *TRK1* gene expression has been observed during regulation of the

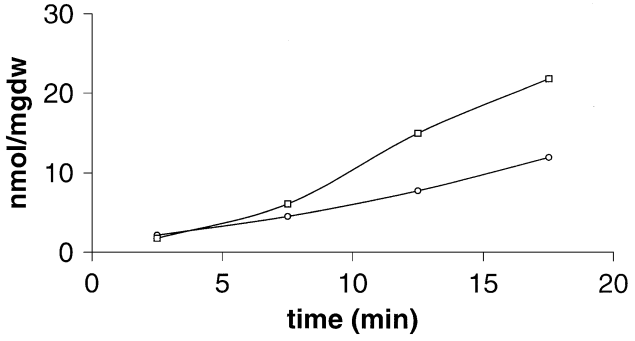

Fig. 2. [^{14}C]Methylammonium uptake by RS801 (*ena1-4*) (squares) and JF194 (*ena1-4 sky1*) (circles) yeast cells. Methylammonium uptake is expressed as nanomols per milligram of dry weight (nmol/mgdw). The experiment was repeated three times with similar results.

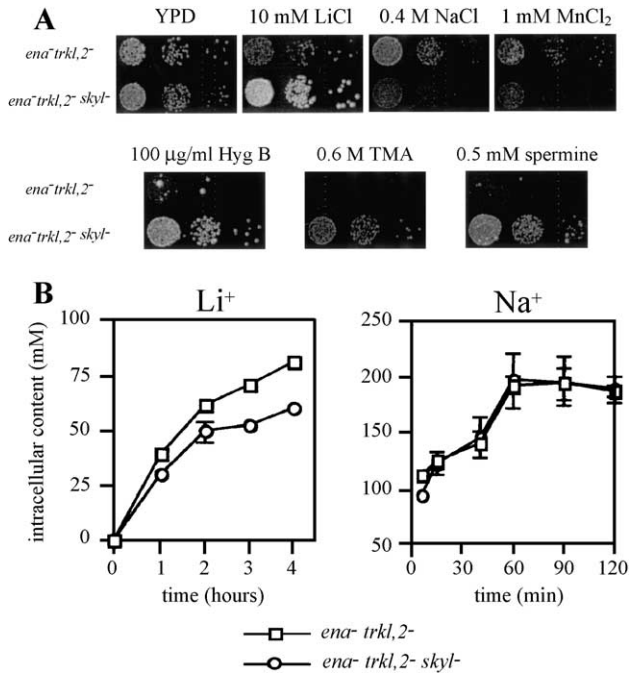

Fig. 3. (A) Spot test of growth of yeast strains RS1187 (*ena1-4 trk1,2*) and JF223 (*ena1-4 trk1,2 sky1*) on YPD medium supplemented with the indicated cations. The same results were obtained with three independent disruptants. (B) Intracellular lithium and sodium content (mean ± S.D. of three replicates) of RS1187 (*ena1-4 trk1,2*) (squares) and JF223 (*ena1-4 trk1,2 sky1*) (circles) cells at the indicated times after a shock with 40 mM LiCl or 0.4 M NaCl in YPD medium. The experiment was repeated twice with similar results.

system in other circumstances (J.M. Mulet, L. Yenush and R. Serrano, unpublished observations).

In the case of Li^+ and the other toxic cations, the tolerance conferred by the *sky1* mutation still correlates with cation uptake but is not dependent on a functional Trk system. Therefore, we must extend our model by postulating that the Sky1p kinase, in addition to inhibiting the Trk system, positively modulates membrane transporters involved in the uptake of these toxic cations and which should be different from those involved in Na^+ and Mn^{2+} uptake. Unfortunately, the nature of the transport systems mediating the uptake of toxic cations remains unknown [3,4].

The detailed mechanism of Sky1p action remains to be determined, but it is puzzling that a protein kinase designed to phosphorylate the SR domains of mRNA splicing and nuclear export factors [18] modulates cation transport. We can think of two possible mechanisms. In the first place, the processing and/or nuclear export of the mRNA for some cation transporters including Trk could be modulated by Sky1p through its known action on SR proteins [5,7]. Interestingly, nuclear mRNA export via Sky1p/Npl3p pathway is inhibited during heat shock and high salt stress [19], whereas mRNAs for heat shock proteins are exported through a distinct pathway after stress [20]. Therefore, the balance between both pathways, perhaps modulated by Sky1p, may be important for expression of stress tolerance genes.

We have performed one further experiment to test this first hypothesis. Nuclear export of yeast mRNAs via Sky1p/Npl3p pathway is also dependent on the Hmt1p arginine methylase [7] and, as indicated in Fig. 4A, loss and gain of function of *HMT1* results in increased and decreased Li^+ and Na^+ tolerance, respectively. On the basis of this result, we could favour a model where Sky1p, like Hmt1p, influences cation homeostasis by modulating the nuclear export of mRNAs encoding cation transporters such as Trk. Unfortunately, the level of expression of *TRK1* is very low (J.M. Mulet and R. Serrano, unpublished observations), and it would be technically difficult to visualize its mRNA by "in situ" hybridization of fixed cells to determine its nuclear accumulation. However, as indicated in Fig. 4B, the *hmt1* mutation does not affect the uptake of Li^+ by yeast cells. We may conclude that although components of the machinery for nuclear export of mRNA such as Hmt1p seem to modulate salt tolerance, they probably do not regulate cation transport.

A second type of mechanism is that Sky1p could participate in a signal transduction pathway modulating membrane transporters such as Trk. The only requirement is that a pathway component should contain a domain with alternating serines and arginines to be substrate of Sky1p [18]. For example, it has been noticed [8] that the amino-terminal region of the Ppz protein phosphatase contains SR-rich segments. This phosphatase is an important determinant of salt tolerance in yeast and is encoded by the partially redundant *PPZ1* and *PPZ2* catalytic subunit genes [21] and the *HAL3* inhibitory subunit gene [22]. Actually, it has been proposed that Sky1p modulates ion homeostasis through the

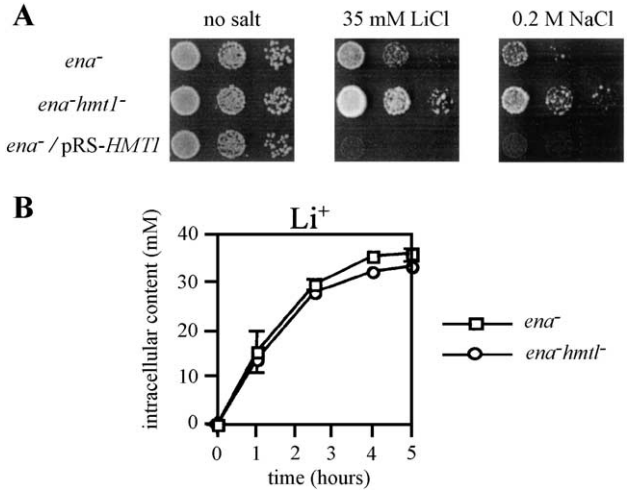

Fig. 4. (A) Spot test of growth of yeast strains RS801 (*ena1-4*), JF219 (*ena1-4 hmt1*) and RS801 overexpressing *HMT1* (*ena1-4*/pRS-*HMT1*) on YPD medium supplemented with the indicated cations. The same results were obtained with three independent disruptants and transformants. (B) Intracellular lithium content (mean ± S.D. of three replicates) of RS801 (*ena1-4*) (squares) and JF219 (*ena1-4 hmt1*) (circles) yeast cells at the indicated times after a shock with 40 mM LiCl in YPD medium. The experiment was repeated twice with similar results.

Ppz protein phosphatase [8]. Although we favour the idea that Sky1p may act in a signal transduction pathway modulating cation transporters, we do not think that it acts via Ppz because there are substantial differences in the phenotypes of *sky1* and *ppz1,2* mutants [23].

As a conclusion, we may state that the puzzle of ion homeostasis in yeast has a novel participant, the protein kinase Sky1p, previously reported to participate in the processing and nuclear export of mRNA [5,7]. Sky1p seems to regulate the Trk K^+ transporter in a way opposite to the protein kinases Hal4p and Hal5p, which are activators of this system [12]. Trk is also regulated by the calcium-dependent protein phosphatase calcineurin [24] and by the Ppz protein phosphatase [23], and, therefore, this transporter may respond to different environmental signals such a K^+ starvation [12] and Na^+ stress [24]. Future studies should determine the mechanism of regulation of Trk by Sky1p and the nature of the transporters mediating Li^+, polyamines and hygromycin B uptake and which are likely to be also modulated by Sky1p.

Acknowledgements

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