Overexpression of the sodium ATPase of *Saccharomyces cerevisiae*: conditions for phosphorylation from ATP and P_i

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Abstract

The *ENA1* gene of *Saccharomyces cerevisiae* encodes a putative ATPase necessary for Na^+ efflux. Plasma membranes and intracellular membranes of a yeast strain overexpressing the *ENA1* gene contain significant amounts of ENA1 protein. Consequences of the overexpression with reference to the wild-type strain are: (1) a 5-fold higher content of the ENA1-protein in plasma membranes; (2) lower Na^+ and Li^+ effluxes; (3) slightly higher Na^+ tolerance; and (4) much higher Li^+ tolerance. The ENA1-specific ATPase activity in plasma membrane preparations of the overexpressing strain was low, but an ENA1 phosphoprotein was clearly detected when the plasma membranes were exposed to ATP in the presence of Na^+ or to P_i in the absence of Na^+. The characteristics of this phosphoprotein, which correspond to the acyl phosphate intermediaries of P-type ATPases, the absolute requirement of Na^+ or other alkali cations for phosphorylation, and the Na^+ and pH dependence of phosphorylation from ATP and P_i suggest that the product of the *ENA1* gene may be a Na,H-ATPase, which can also pump other alkali cations. The role of the intracellular membranes structures produced with the overexpression of ENA1 in Na^+ and Li^+ tolerances and the existence of a β-subunit of the ENA1 ATPase are discussed. © 1997 Elsevier Science B.V.

1. Introduction

In 1954, Conway and coworkers [1] described active transport of sodium ions from yeast cells. Since then, many reports on the biochemical charac-

teristics of this transport system [2,3] failed to unambiguously establish the mechanism involved. Using techniques of molecular genetics, a tandem of four genes, *ENA1* (*PMR2*) to *ENA4*, in *Saccharomyces cerevisiae* [4,5], and several repeats of the *sod2* gene in *Schizosaccharomyces pombe* [6] were found necessary for the existence of Na^+ efflux in these species [7]. According to the deduced amino acid sequences of the proteins encoded by these genes and based on studies with *S. pombe* expressing the *ENA1* gene [8] and *S. cerevisiae* expressing *sod2* gene [9], it has been proposed that Na^+ efflux is mediated by P-type ATPases in *S. cerevisiae* and by a Na^+/H^+ an-

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Abbreviations: DTT, dithiothreitol; ER, endoplasmic reticulum; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanethanesulfonic acid; TAPS, *N*-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid; CAPS, 3-(cyclohexylamino)1-propanesulfonic acid; DMSO, dimethyl sulfoxide; TCA, trichloroacetic acid

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tiporter in *S. pombe*. Since the first report on the putative Na-ATPase of *S. cerevisiae*, the regulation of expression and function of the product of the *ENA1* gene has been studied [10–17]. However, a biochemical clue demonstrating that ENA ATPases are Na-ATPases has not been reported because these ATPases show very low activity in vitro [8].

Cation-transporting P-type ATPases form a well-defined class of enzymes, including bacterial, fungus, and plant ATPases as well as the animal Na,K-ATPase of cell membranes, Ca-ATPases of plasma membranes and sarcoplasmic reticulum, and the H,K-ATPase of gastric mucosa [18,19]. The reaction cycles and transport characteristics for most of the P-type ATPases have been established [18,19] and when a new member of the group is discovered there is substantial information for comparative studies. We report here the physiological consequences of over expressing the ENA1 ATPase and the conditions for the formation of the ENA1 phosphoenzyme. We report that ATP in presence of Na^+ and P_i in its absence phosphorylates the ENA1 protein, forming a phosphoprotein which fulfils the characteristics of phosphorylated intermediates of P-type ATPases. The effects of Na^+ and H^+ on phosphorylation give biochemical support to the previous proposal [8] that ENA1 is a Na,H-ATPase.

2. Materials and methods

2.1. Yeast strain and antibodies

Yeast *S. cerevisiae* strains DBY746 (*Mat α ura3 his3 trp1 leu2*) and its derivative RH16.6 carrying a disruption of the four *ENA* genes (*Mat α ura3 his3 trp1 leu2 ena1Δ::LEU2::ena4Δ*) [4,5] were used for this study. Cells were grown in either YPD [20] or in arginine phosphate mineral medium [21]. Purified antibodies against the fragment of the ENA1 protein comprised between amino acids 27 and 386 were prepared as described elsewhere [8].

2.2. Recombinant DNA methods

Manipulation of DNA was by standard protocols [22] or, when appropriate, by following the reagent manufacturer's instructions. Yeast cells were trans-

formed after lithium acetate treatment [23]. Plasmid pJQ10 was constructed inserting the coding region of the *ENA1* gene into plasmid YEp91, between the 5′- and the 3′-non-coding regions of the *PGK1* gene. To construct YEp91 the *Hin*dIII fragment of plasmid pMA91 [24], containing the 5′- and the 3′-non-coding regions of *PGK1*, was moved into vector YEp24 after removing the 2.1-kb *Pvu*II–*Sma*I fragment. The coding region of the *ENA1* gene was then inserted into plasmid YEp91 as described previously for a similar construct in plasmid pMA91 [8]. Cells transformed with pJQ10 or YEp91 were maintained in minimal medium [20] without uracil.

To test the activity of two putative defective ENA1 ATPases with C-terminal tails truncated at Lys^{1052} or Tyr^{1075}, 3′-truncated *ENA1* coding regions were inserted in the correct orientation on the *Bgl*II site existing between the 5′- and 3′-non-coding regions of the *PGK1* gene in plasmid YEp91. The two truncated coding regions were synthesized by PCR using a common 19-mer oligonucleotide, containing a *Bgl*II site, the ATG encoding the first methionine, and five more nucleotides downstream of the coding region, and two 30-mer oligonucleotides, containing a *Bgl*II site, a CTA stop codon, a glutamine codon, to maintain the last amino acid of the normal gene, and 18 nucleotides of the coding region upstream of the codon corresponding to Lys^{1052} or Tyr^{1075}. PCR products were sequenced.

2.3. Na^+ and Li^+ effluxes

Cells loaded with Na^+ or Li^+ were suspended in 10 mM MES brought to pH 6.0 with $Ca(OH)_2$, 10 mM KCl, 0.1 mM $MgCl_2$, and 2% glucose (efflux buffer). Then, at intervals, the decrease in Na^+ or Li^+ contents were determined in samples of cells removed by filtration, acid extracted, and analyzed by atomic emission spectrophotometry, as described previously [25]. Results are referred to the dry weight of the cells.

2.4. Isolation of membranes

Cells of RH16.6(YEp91), RH16.6(pJQ10), and DBY746 strains were prepared as follows: (1) growth overnight in YPD medium [20]; (2) incubation for 3 h in arginine phosphate medium, pH 7.5 [21], supple-

mented with 3 mM KCl, 0.4 M NaCl, 20 mg/l histidine, 20 mg/l tryptophan; and (3) incubation for 5–10 min in 0.1 M Tris-HCl (pH 8.0), 0.4 M NaCl. This procedure, which did not result in an appreciable loss of plasmids, inactivated the H-ATPase during the incubation in the absence of glucose [26] and produced a modest activation of the ENA1 ATPase. 'Total membranes' of these cells were prepared as described previously [27,28], except that 25 mM DTT was included in all buffers and the membranes were maintained for 60 min in ice-cold buffer (containing DTT) before purification in sucrose gradients, as reported for activation of the ATPase of sugar beet roots [29].

For the analysis of the subcellular distribution of the ENA1 ATPase, total membranes were centrifuged in a continuous gradient of 20–53% (w/w) sucrose, as described for localization of the plant plasma membrane H-ATPase expressed in yeast [28]. Fractions of the gradient of 0.75 ml were collected and analyzed by Western blot with antibodies against the ENA1 protein. 'Purified plasma membranes' and 'purified ER membranes' were recovered at the interface of discontinuous gradients of 43/53% (w/w) and 27/34% (w/w) sucrose, respectively [28].

2.5. ATPase activity and protein determination

ATP hydrolysis was assayed at 30°C in buffer containing 50 mM MES, HEPES, TAPS or CAPS (as appropriate for the selected pH value), the required amount of Tris to adjust the pH, 5 mM $MgSO_4$, 50 mM KNO_3 (to inhibit vacuolar ATPase), 5 mM sodium azide (to inhibit mitochondrial ATPase), 0.2 mM ammonium molibdate (to inhibit acid phosphatase), different concentrations of NaCl, and 2 mM Tris-ATP. Experiments in which some or all of the inhibitors were withdrawn were also performed. Inorganic phosphate was measured by the method of Fiske and Subbarow [27].

Protein concentration was determined by the bicinchoninic acid method, using bovine serum albumin as standard [30].

2.6. Electrophoretic and immunological methods

The presence of the ENA1 protein was investigated by standard SDS–PAGE (8% acrylamide). The proteins were transferred to Immobilon polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA, USA) at pH 11.0 [31], exposed to the anti-ENA1-protein antibodies, and detected with alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad, Richmont, CA, USA) [32].

Electrophoresis for the detection of ^{32}P-phosphorylated intermediates was carried out at pH 2.4 in lithium dodecyl sulfate-polyacrylamide gels (6.0% acrylamide) [33] at 40 V for 4 h at 4°C. The ENA1 protein in these gels was immunodetected following the procedure as described above, except that the gels were soaked for 30 min in transfer buffer, for pH equilibration, before starting the transference to the membranes.

2.7. Phosphorylation from ATP and dephosphorylation experiments

Phosphorylation from ATP was assayed with 'purified plasma membranes' (30 μg of protein) in buffer (20 μl) containing 50 mM MES, HEPES, TAPS or CAPS (as appropriate for the selected pH value), the required amount of Tris to adjust the pH, 5 mM $MgCl_2$, 200 μM ATP containing [γ-^{32}P]ATP (final activity, 75 MBq μmol^{-1}), and the amount of NaCl, LiCl, KCl, RbCl, CsCl or choline chloride is indicated in each experiment. The glycerol added with the sample of membranes resulted in a final 10% glycerol content in phosphorylation buffer. In some experiments, 100 μM vanadate was also included. The membranes were incubated for 15 s at 0°C and then the reaction was stopped with 200 μl of an ice-cold solution containing 15% TCA, 2 mM KH_2PO_4 and 1 mM ATP. The precipitated protein was centrifuged; washed twice with cold water; suspended in 12 μl of a solution at pH 9.3 [33], containing 1% lithium dodecyl sulfate, 10 mM NaOH, 10 mM DTT, and 0.2 M sucrose; and electrophoresed as described above.

In dephosphorylation experiments, the enzyme was phosphorylated using 50 μM [γ-^{32}P]ATP (75 MBq μmol^{-1}) instead of 200 μM [γ-^{32}P]ATP. After phosphorylation for 15 s the release of ^{32}P$_i$ was started by adding 20 μl of a buffer containing 100 mM MES, HEPES or CAPS, depending on the pH of each experiment, 250 mM NaCl, and 2 mM ATP. The pH of the buffer added in each experiment was

adjusted with Tris 0.5–1.0 pH unit above or below the pH selected for the experiment, in such a way that the mix of the buffer used during phosphorylation and the buffer added afterwards gave the selected pH value. After 4 s, the reaction was stopped with TCA and the protein was centrifuged, washed, and prepared for electrophoresis as described in phosphorylation experiments.

2.8. Phosphorylation from P_i and dephosphorylation experiments

Phosphorylation was assayed in buffers as described for phosphorylation experiments from ATP, except that Tris-phosphate containing $^{32}P_i$ substituted for ATP. Standard experiments were performed at 0.2 mM P_i with a radioactivity of 75 MBq μmol^{-1} and 20°C. Under many conditions, parallel experiments were performed in the absence and in the presence of 20% DMSO. Preliminary experiments carried out for different times, from 30 s to 30 min, demonstrated that phosphorylation was complete in less than 30 s and that the amount of phosphoenzyme did not decrease in 30 min. In the experiments reported in this report, the incubation time was 5 min. The reaction was stopped with 200 μl of an ice-cold solution containing 15% TCA and 2 mM KH_2PO_4; then the samples were processed as described for phosphorylation from ATP.

In dephosphorylation experiments, the enzyme was first phosphorylated for 5 min at pH 7.0, as described, and then the experiments were started with the addition of 20 μl of buffer pH 7.0 containing 20 mM non-radioactive P_i. Samples were maintained at 20°C for 4–30 s and the reaction was stopped with 200 μl of ice-cold 15% TCA. The protein was prepared for electrophoresis as in phosphorylation experiments.

2.9. Determination of the phosphoenzyme

Radiolabeled phosphoenzyme was detected in autoradiographs of dried gels. The radioactivity was quantified by volume integration of the signal given by the phosphoenzyme in a GS-250 Molecular Imager system (Bio-Rad, Richmont, CA, USA) and a Phosphor Analyst™/Macintosh data analysis software version 1.1. The response of this procedure was calibrated integrating the signal given by samples

with different amounts of $^{32}P_i$. All gels contained a control lane with the protein of a standard phosphorylation experiment (500 mM NaCl, pH 7.5, for phosphorylation from ATP; pH 7.0 in the absence of NaCl for phosphorylation from P_i) and a lane with a null phosphorylation experiment (pH 7.5 in the absence of NaCl for phosphorylation from ATP; 500 mM NaCl, pH 7.5 for phosphorylation from P_i). Volume integration of the signals obtained under different conditions was compared to those in the standard phosphorylation experiment in the same gel and referred to as a percentage. All experiments were repeated with at least two different batches of membranes prepared in different days and at least two independent experiments were performed with each batch of membranes. Reported data are the means ± S.E.

2.10. Stability of the phosphoenzyme at acid pH and in presence of hydroxylamine

The TCA-treated phosphoenzyme, as described above, was washed and suspended in 100 mM acetate buffer (pH 4.0). Two samples were run in parallel; in one of them, the phosphoenzyme was suspended in 200 μl of buffer, and in the other, it was suspended in 180 μl of buffer and 20 μl of 5 M hydroxylamine, pH 4.0, prepared just before use by adding NaOH to hydroxylamine hydrochloride. After 2 min at 37°C the reaction was stopped with 200 μl of ice-cold 15% TCA containing 2 mM KH_2PO_4. The protein was then prepared for electrophoresis as described in phosphorylation experiments.

3. Results

3.1. Presence of the ENA1-ATPase in plasma membranes

Analysis of 'purified plasma membrane' preparations from RH16.6 (*ena1Δ::LEU2::ena4Δ*) strain transformed with plasmid pJQ10 (carrying the chimerical gene $P_{PGK1}::ENA1$) by SDS–PAGE and Coomassie blue stain showed the presence of a protein of approximately 120 kDa, which could be ENA1 because it was absent in plasma membrane preparations from RH16.6 transformed with YEp91 (plasmid without insert). Western blot analysis confirmed the identity of the 120-kDa protein because it was im-
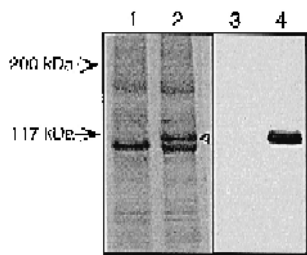
Fig. 1. Detection of the ENA1 protein in plasma membranes of RH16.6(pJQ10) strain. Purified plasma membranes of RH16.6 strain transformed with YEp91 (lanes 1 and 3) or pJQ10 (lanes 2 and 4) were prepared as described in Section 2: Materials and methods and analyzed (20 μg of protein per lane) by SDS–PAGE (left panel) and by Western blot using antibodies against ENA1 protein (right panel).

munodecorated with ENA1-specific antibodies (Fig. 1).

Densitometric analysis of the Coomassie blue-stained gels and comparison of the signal of the ENA1 protein to that of the H^+-pump PMA1 protein (the major, 100-kDa, non-immunoreactive band in Fig. 1), which amounts 0.1 mg mg^{-1} of total protein in this type of preparations [34], allowed to estimate that purified plasma membrane preparations of RH16.6(pJQ10) strain contained 0.07 mg of ENA1 protein per mg of total protein. The same analysis with the wild-type strain (DBY746) grown at 400 mM NaCl, pH 7.0, to enhance the expression of ENA1 [5], allowed to estimate a content of 0.015 mg of the ENA1, ENA2, ENA3, and ENA4 proteins per mg of total protein.

Overexpression of homologous [35] and heterologous [28] H-ATPases in yeast cells leads to their accumulation in intracellular structures, probably belonging to the endoplasmic reticulum. To test whether overexpressed ENA1 protein accumulated in internal structures, total plasma membranes of RH16.6(pJQ10) were centrifuged in a linear sucrose gradient (20–53%) and the gradient fractions were Western blot analyzed with the antibodies against the ENA1 protein. The results of these analyses showed that the ENA1 protein appeared mainly in two peaks of similar sizes at 32 and 47% sucrose and at much smaller amounts at intermediate concentrations of sucrose. According to the distribution of membranes of different origins in this sucrose gradient [28], it was estimated that approximately half of the total ENA1 protein was in the plasma membrane and the other

half in internal structures, probably belonging to the endoplasmic reticulum.

3.2. Functional consequences of overexpression

To test the functional consequences of ENA1 overexpression, it was necessary to test the effectiveness of the efflux mediated by the overexpressed protein and this was difficult if the ENA1 ATPase was functional in internal membranes structures and removed Na^+ from the cytoplasm pumping it into the internal space of the membrane structures. Therefore, we investigated Na^+ and Li^+ tolerances and Na^+ and Li^+ effluxes in parallel. These two cations are transported with the same efficiency by ENA1 [5], but whereas Li^+ is toxic at low levels, Na^+ toxicity occurs only at high concentrations [36]. As expected, the functional consequences of ENA1 overexpression were complex, the tolerance to Na^+ increased insignificantly with reference to the wild-type strain (500 vs. 400 mM, in mineral medium with 0.5 mM K^+), whereas the tolerance to Li^+ increased 7-fold (200 vs. 30 mM, in mineral medium with 1 mM K^+). Consisting with the idea that, in the overexpressing strain, Na^+ and Li^+ were removed from the cytoplasm and transported into the internal structures, Na^+ and Li^+ effluxes were slower in the overexpressing strain than in the wild-type strain. The effect of overexpression was apparently more important in
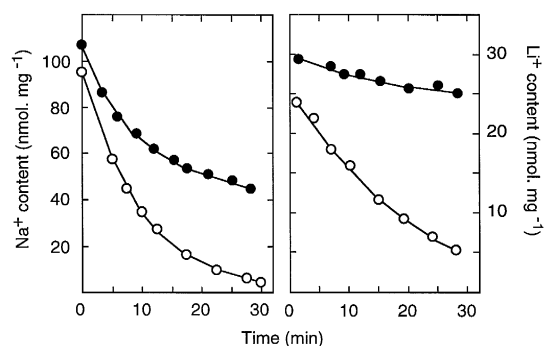

Fig. 2. Time courses of Na^+ and Li^+ contents of Na^+ or Li^+ loaded cells of DBY746 (open circles) and RH16.6(pJQ10) (close circles) strains when transferred to Na^+ and Li^+-free buffer. For Na^+, cells were grown overnight in 0.5 mM K^+, 100 mM Na^+, arginine phosphate medium, and then transferred to efflux buffer. For Li^+, cells were grown in 1.0 mM K^+, arginine phosphate medium, adding LiCl to make the medium 50 mM, when they were in the mid exponential phase; after 30 min of incubation, cells were transferred to efflux buffer.

Li^+ efflux than in Na^+ efflux (Fig. 2), but probably it only reflects that when the content of the cation in the cell is low, the proportion of the cation in the internal structures is higher than when the cation content is high. Two interesting observations can be made from the data in Fig. 2. The first is that accumulation of the cation into the internal structures is very rapid, because the overexpressing strain exhibited a low efflux even when it was measured immediately after a rapid loading of the cells (compare Li^+ and Na^+ in Fig. 2). The second is that it returns very slowly to the cytoplasm. Otherwise the differences between effluxes in the two strains had been less important.

3.3. ATPase activity

Because sequence analysis and physiological observations in different type of mutants suggested that the product of the *ENA1* gene was a P-type ATPase [4,5,8,37], we determined the ATPase activity in 'purified plasma membranes' and in 'purified ER membranes' preparations recovered at the interfaces of discontinuous 43/53% and 27/34% sucrose gradients, respectively. Comparing the vanadate-sensitive activities in both type of membranes in RH16.6(pJQ10) and RH16.6(YEp91) strains, it was clear that, in the presence of Na^+ and at high pH (> 6.0), the expression of the ENA1 protein in the strain transformed with pJQ10 was associated to an increase in the ATPase activity. In plasma membranes, where the results were more consistently reproduced, in the absence of Na^+ and at pH 5.5–6.0, the activities in both strains did not show statistically

significant differences. However, whereas the activity in RH16.6(YEp91) was fairly constant at pH values between 5.5 and 8.5 and NaCl concentrations from 0 to 200 mM, the activity in RH16.6(pJQ10) increased with the pH from 5.5 to 8.0, if NaCl was present (50–200 mM), and increased with the concentration of NaCl, if measured at high pH values (from 7.0 to 8.0). Therefore, the activities showed great differences when they were tested at high pH (7.5–8.5) and in the presence of 50–200 mM NaCl (Table 1).

Although these experiments showed the pH and NaCl dependence of the ENA1-specific ATPase activity, a complete biochemical study of the specific activity of this ATPase, carried out by subtracting the vanadate-sensitive activities measured in RH16.6(pJQ10) and RH16.6(YEp91) strains, proved to be statistically complex. At any set of conditions, the results obtained using the same plasma membrane preparation were reasonably constant, but with different preparations they showed a notable variability, making the subtraction procedure somewhat inexact (Table 1). This problem applied to all conditions, because the activity in RH16.6 was always an appreciable fraction of the activity in RH16.6(pJQ10) (Table 1), but especially to the control activity at pH 7.5–8.0 in the absence of Na^+, because, in this case, the activities of both strains were very similar, but with significant variability. As a consequence, by the subtraction procedure, it was impossible to decide if Na^+ was an absolute requirement for the ENA1 ATPase activity or a mere enhancer of this activity.

We tested if the ENA1 ATPase activity was inhibited in the conditions of the assay, withdrawing from the testing medium the inhibitors normally used,

Table 1

Effect of NaCl on vanadate-sensitive ATPase activities in purified plasma membranes of RH16.6(pJQ10) and RH16.6(YEp91) strains

Strain	Membrane preparation	NaCl (mM)			
		0	50	100	200
RH16.6(pJQ10)	1	0.19 ± 0.015	0.25 ± 0.090	0.32 ± 0.020	0.42 ± 0.030
	2	0.35 ± 0.005	0.44 ± 0.010	0.62 ± 0.010	0.80 ± 0.015
RH16.6(YEp91)	1	0.08 ± 0.005	0.10 ± 0.010	0.08 ± 0.005	0.10 ± 0.005
	2	0.15 ± 0.010	0.16 ± 0.010	0.13 ± 0.010	0.15 ± 0.030

Purified plasma membranes were obtained from overnight cultures, which were treated to activate the ENA1 ATPase and to inactivate the H^+ ATPase before breaking the cells. The ATPase activity was assayed at pH 7.5, in standard conditions, but withdrawing KNO_3, in presence and in absence of vanadate. The table summarizes the vanadate-sensitive activities in two different membrane preparations in each strain. The vanadate-insensitive activity was always a small fraction (approximately 10%) of the total activity. Data represent the mean $\pm$ S.E. (P_i μmol min^{-1} mg protein^{-1}) of three or four different experiments.

sodium azide (added to inhibit mitochondrial ATPase), ammonium molibdate (added to inhibit acid phosphatase), and potassium nitrate (added to inhibit vacuolar ATPase), finding no differences, except for an enhancement of the activity by potassium nitrate. We also tested the effect of including phosphatase inhibitors during the preparation of the membranes or adding calmodulin and Ca^{2+} in the testing medium [15], but the results did not change. Substitution of Na_2SO_4 or Na^+ acetate for NaCl did not affect the results.

Autoinhibition of the ENA1 ATPase was also tested. In plasma membrane Ca-ATPases the C-terminal tail, in which a calmodulin binding domain is situated, acts as a repressor of the enzyme [38] and in the yeast H-ATPase the C-terminal tail regulates the activity of the enzyme [39]. To release a possible autoinhibition by the C-terminal tail, we used two mutant genes with truncated coding regions in the 3'-end. In the translated proteins, the first truncation extended up to Tyr^{1075}, eliminating the last sixteen amino acids, in which there is a putative phosphorylation site; in the second, Lys^{1052} was reached, leaving only thirteen amino acids beyond the last transmembrane fragment. Transformants of RH16.6 with the mutant genes substituting for *ENA1* in pJQ10 were tested for Li^+ tolerance and ENA1 ATPase activity, finding that the mutations did not show significant effects.

In parallel with the studies described above, we determined the ENA1 phosphoenzyme formation from ATP and P_i, because phosphorylation of P-type ATPases constitutes another approach to study the activity of these enzymes (see references below). Contrarily to the problems derived from the subtraction procedure necessary for measuring the ATPase activity, determination of the phosphoenzyme was a fairly accurate procedure to study the biochemical characteristics of the ENA1 ATPase. Specially important for this purpose was that no phosphoenzyme was detected in RH16.6(YEp91). Experiments of phosphorylation were carried out with RH16.6(pJQ10) strain because, in comparison with the wild-type strain, it had a constitutive expression of ENA1, and the ENA2, ENA3, and ENA4 proteins were absent. Strain RH16.6(pGB34) [5] expresses only ENA1, but its ENA1-specific activity was lower than in RH16.6(pJQ10).

3.4. Phosphorylation from ATP and dephosphorylation

Analysis by acrylamide gel electrophoresis at pH 2.4 showed that incubation of plasma membranes of RH16.6(pJQ10) strain with $[\gamma\text{-}^{32}P]ATP$ and Na^+ led to the formation of a phosphorylated protein of approximately 120 kDa, which was absent in RH16.6(YEp91) strain (Fig. 3). The identity of this protein with ENA1 was confirmed by Western blot analysis. As expected for acyl phosphate, intermediates of P-type ATPases [18], phosphorylation of the protein was inhibited by 100 μM vanadate and the phosphoprotein was stable at pH 4.0, but hydrolyzed at the same pH in the presence of 0.5 M hydroxylamine (Fig. 3). Quantitative analysis of the radioactivity in the acrylamide gels showed that phosphorylation of ENA1 below 100 mM Na^+ was not significantly different from phosphorylation in the absence of Na^+ and that the process did not show complete saturation at 500 mM Na^+, which was the highest concentration that we tested (Fig. 4).

Other alkali cations substituted for Na^+ in ENA1 phosphorylation although with lower effectiveness, probably in the order $Cs^+ \geq K^+ \geq Rb^+$ (Fig. 5). Li^+ was also a good substitute, but a reliable comparison
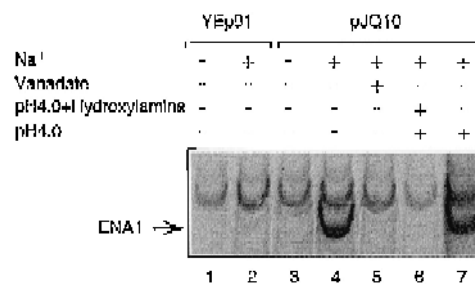
Fig. 3. Phosphorylation from ATP of the ENA1 ATPase. Purified plasma membranes of RH16.6(YEp91) (lanes 1 and 2) and RH16.6(pJQ10) (lanes 3–7) strains were incubated for 15 s with 0. 2 mM ATP labeled with $[\gamma\text{-}^{32}P]ATP$ at pH 7.5, and the phosphoproteins were detected by electrophoresis (30 μg of protein per lane) at pH 2.4 and autoradiography, as described in Section 2: Materials and methods. Experiments were performed in the following conditions: lanes 1 and 3, without Na^+; lanes 2 and 4, with 250 mM NaCl; lane 5, 250 mM NaCl, 100 μM vanadate; lane 6, the phosphoprotein obtained as in lane 4 was exposed for 2 min to 500 mM hydroxylamine pH 4.0, as described in Section 2; lane 7, the phosphoprotein obtained as in lane 4 was exposed for 2 min at pH 4.0 in the absence of hydroxylamine.

between the amount of phosphoenzyme formed with Na^+ and Li^+ could not be made because the radioactive background signal along the electrophoresis lane in experiments with Li^+ was much clearer than with any other alkali cation. K^+ and Na^+ together showed a strictly additive effect. To rule out that phosphorylation was only a salt effect, we tested phosphorylation in the presence of 250 mM choline chloride, finding that phosphorylation was less than 10% of the phosphorylation with alkali cations. Phosphorylation of the enzyme increased with the pH from pH 5.0 up to 8.5 and then decreased slowly (Fig. 6). Below pH 5.0, the phosphoenzyme was not detected. Under all conditions, the amount of phosphoenzyme reached the steady state in less than 4 s, which is the shortest time we could attain in our experimental conditions. The half-maximum amount of phosphoenzyme was observed at 1–5 μM ATP.

The high concentration of Na^+ or other alkali cations which was required for phosphorylation from ATP led us to investigate the possibility that the ENA1 ATPase was really a Ca-ATPase, as proposed by sequence analysis [37], whose phosphorylation in our conditions was activated by contaminant Ca^{2+}. Therefore, we performed experiments of phosphorylation, in the presence and in the absence of 250 mM Na^+, with 1 mM EGTA or with 20 μM, 0.2 mM, or 2 mM Ca^{2+}, finding that Ca^{2+} did not produce any significant effect, either positive or negative.

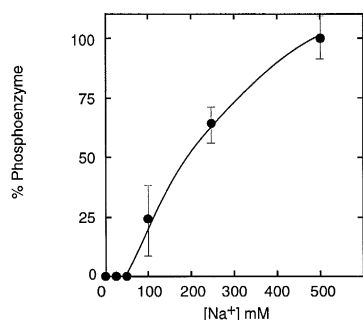Phosphorylated intermediates of P-type ATPases are dephosphorylated with the progress of the reac-

Fig. 5. Effectiveness of K^+, Rb^+, and Cs^+ to substitute for Na^+ in phosphorylation from ATP of the ENA1 ATPase. Purified plasma membranes were phosphorylated and analyzed as in Fig. 3, except that 250 mM CsCl, KCl, or RbCl substituted for NaCl, as indicated.

tion cycle. Therefore, radioactive phosphate is rapidly released from the ^{32}P-enzyme by conditions which inhibit phosphorylation or upon dilution of the radioactive ATP with non-radioactive ATP. To study the dephosphorylation of ENA1 the enzyme was phosphorylated with 50 μM [γ-^{32}P]ATP for 15 s and then the formation of the radioactive phosphoenzyme was inhibited by decreasing the pH to 6.0 or diluting the radioactive ATP with 2 mM non-radioactive ATP. In both cases dephosphorylation followed a biphasic pattern. A rapid process dephosphorylated 80% of the enzyme in 4 s, which was the shortest time we could test, whereas the remaining phosphoenzyme exhibited a half life of 15 s. When 2.5 mM ADP was added, in addition to the pH change or cold ATP, the slow process disappeared and all the enzyme was dephosphorylated in 4 s.

Fig. 4. Na^+ concentration dependency of phosphorylation from ATP of the ENA1 ATPase. Purified plasma membranes of RH16.6(pJQ10) strain were incubated for 15 s with 0. 2 mM ATP labeled with [γ-^{32}P]ATP at pH 7.5 and different concentrations of NaCl. After electrophoresis at pH 2.4, the phosphoprotein was quantified as described in Section 2: Materials and methods.
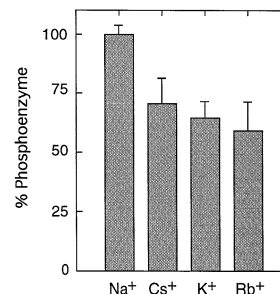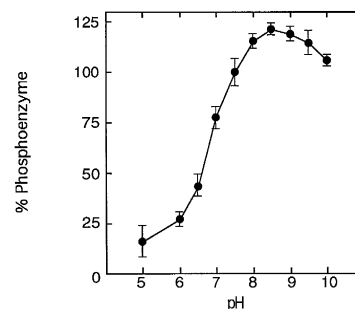

Fig. 6. pH dependency of phosphorylation from ATP of the ENA1 ATPase at 250 mM NaCl. Experimental conditions as in Fig. 3, with 250 mM NaCl in all cases. Buffers as described in Section 2: Materials and methods.

To test if a high pH inhibited dephosphorylation, we performed experiments of dephosphorylation by diluting the $[\gamma\text{-}^{32}P]ATP$ with non-radioactive ATP, as described above, but changing the pH from 7.5 to 9.0 or 10.0 simultaneously with the addition of non-radioactive ATP. In both cases, dephosphorylation was not prevented, but the amount of the enzyme dephosphorylated in the slow process increased 20–30% with reference to the experiments at pH 7.5.

3.5. Phosphorylation from P_i

Phosphorylated intermediates of P-type ATPases are also formed from P_i in the absence of the cation that is transported outward ('back door phosphorylation') [40–43]. Plasma membrane preparations of RH16.6(pJQ10) strain incubated with 0.2 mM $^{32}P_i$ in the presence of Mg^{2+} and in the absence of Na^+ produced a radioactive phosphoprotein of approximately 120 kDa, which was not found in RH16.6(YEp91). As in phosphorylation from ATP, Western blot analysis with antibodies against the ENA1 protein confirmed that the 120 kDa phosphoprotein was ENA1. The ENA1 ^{32}P-phosphoprotein was not formed in the presence of vanadate and was hydrolyzed at pH 4.0 in the presence of hydroxyl-
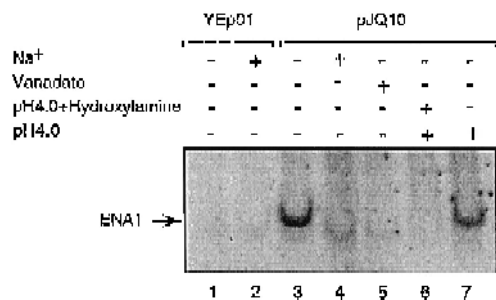

Fig. 7. Phosphorylation from P_i of the ENA1 ATPase. Purified plasma membranes of RH16.6(YEp91) (lanes 1 and 2) and RH16.6(pJQ10) (lanes 3–7) strains were incubated for 5 min with 0.2 mM P_i labeled with $^{32}P_i$ at pH 7.5 and the phosphoproteins were detected by electrophoresis (30 μg of protein per lane) at pH 2.4 and autoradiography, as described in Section 2: Materials and methods. Experiments were performed in the following conditions: lanes 1 and 3, without Na^+; lanes 2 and 4, with 250 mM NaCl; lane 5, 100 μM vanadate; lane 6, the phosphoprotein obtained as in lane 3 was exposed for 2 min at 500 mM hydroxylamine pH 4.0, as described in Section 2; lane 7, the phosphoprotein obtained as in lane 3 was exposed for 2 min at pH 4.0 in the absence of hydroxylamine.
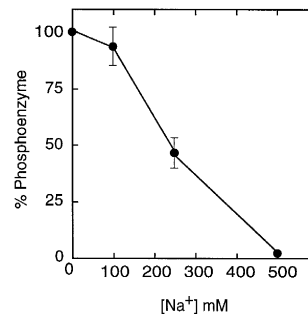

Fig. 8. Na^+ concentration dependency of phosphorylation from P_i of the ENA1 ATPase. Purified plasma membranes of RH16.6(pJQ10) strain were incubated for 5 min with 0.2 mM P_i labeled with $^{32}P_i$ at pH 7.5 and different concentrations of NaCl. After electrophoresis at pH 2.4 the phosphoprotein was quantified as described in Section 2: Materials and methods.

amine (Fig. 7). As reported for other P-type ATPases [41–43], the level of phosphoenzyme increased approximately two-fold by including 20% DMSO in the testing medium. Addition of 20 mM non-radioactive P_i to the phosphoenzyme formed from $^{32}P_i$ (0.2 mM) resulted in an undetectable level of radioactive phosphoenzyme in 4 s.

Phosphorylation from P_i of the ENA1 protein was inhibited by Na^+ at relatively high concentrations (> 100 mM) (Fig. 8), and by other alkali cations in the order of effectiveness $Li^+ > Na^+ > K^+ > Cs^+ > Rb^+$ (Fig. 9). In the absence (Fig. 10) or in the presence of 100 mM Na^+ (not shown), the amount of phosphoenzyme was maximal at pH 7.0 and non-detectable at pH values below 5.0 and above 9.0. The decrease of the amount of phosphoenzyme when pH
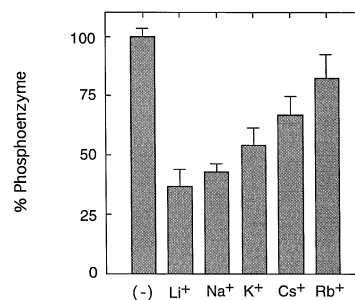

Fig. 9. Effectiveness of Li^+, K^+, Rb^+, and Cs^+ to substitute for Na^+ in phosphorylation from P_i of the ENA1 ATPase. Purified plasma membranes were phosphorylated and analyzed as in Fig. 7, except that 250 mM LiCl, KCl, CsCl, or RbCl substituted for NaCl as indicated. ($-$), control experiment without salt addition.
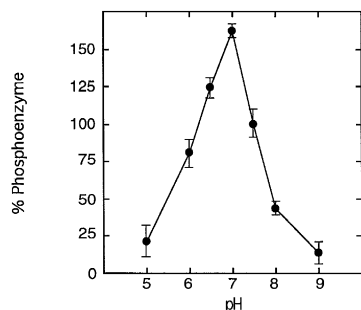
Fig. 10. pH dependency of phosphorylation from P_i of the ENA1 ATPase. Experimental conditions as in Fig. 7 in the absence of NaCl in all cases. Buffers as described in Section 2: Materials and methods.

is shifted from 7.0 to 9.0 may be explained if only primary phosphate ($H_2PO_4^-$) is able to phosphorylate the ATPase [44], because the pK_2 of P_i in 20% DMSO/10% glycerol is 7.5 (not shown). Therefore, we tested phosphorylation in the absence of DMSO where the pK_2 of P_i is 6.7 ([42] and results not shown under our conditions), finding that the response of the amount of the phosphoprotein to changes in the pH of the testing medium was identical in the presence and in the absence of DMSO.

The possibility that ENA1 is a Ca-ATPase was investigated by testing phosphorylation from P_i at pH 7.0 (other conditions as in Fig. 7) in the presence of increasing concentrations of Ca^{2+}. At 5 mM Mg^{2+}, 0.5 mM Ca^{2+} did not show effect, 5 mM inhibited by 50%, and 50 mM by 90%. However, in the presence of 50 mM Mg^{2+}, even 50 mM Ca^{2+} produced an insignificant inhibition.

4. Discussion

In an *S. cerevisiae* strain deleted of the four *ENA* genes and carrying a plasmid in which the coding region of the *ENA1* gene was under the control of the *PGK1* promoter, the ENA1 protein accumulated in plasma membranes and in internal membranes, as it has been described when other ATPases are overexpressed [28,35]. If compared to a wild-type strain, expressing the four isoforms of the ENA proteins [4], the amount of ENA1 in plasma membranes of the overexpressing strain was 5-fold the total amount of the four ENA proteins in the wild-type strain. The

functional effects of the overexpression of the ENA1 protein were complex. Na^+ and Li^+ effluxes decreased, especially in the case of Li^+ (Fig. 2), whereas the tolerances to these cations increased, very slightly in the case of Na^+ and 7-fold in the case of Li^+. A likely explanation for all this is the presence of the ENA1 ATPase in internal structures in which Na^+ and Li^+ can be accumulated. Because Na^+ is toxic, only at high internal concentrations [36], accumulation of the cation in the small volume of the internal structures has a little effect on the cytoplasmic concentration and on Na^+ toxicity, because, for osmotic reasons, Na^+ might be concentrated at 300 mM and this is only two-fold the cytoplasmic concentration. On the contrary, Li^+ is toxic at low concentrations in the cytoplasm [36], and, even if a small internal volume is available, Li^+ accumulation into it can produce a great decrease in cytoplasmic concentration and a great increase in tolerance, if it reaches a concentration of 300 mM. Because Na^+ and Li^+ effluxes mediated by the ENA1 ATPase follow first-order kinetics [5], the high difference between Li^+ effluxes in DBY746 and RH16.6(pJQ10) (Fig. 2) probably reflects a high difference between internal concentrations, as expected if Li^+ is accumulated in the internal structures.

Although the presence of the internal structures in which cations are accumulated makes it difficult to draw conclusions about the extrusion activity of the overexpressed ENA1 ATPase in the plasma membrane, the slight increase in Na^+ tolerance indicates that Na^+ efflux in this strain is not better than in wild-type strain. This is surprising, because the amount of the ENA1 protein is 5-fold higher, and suggests that something is limiting the activity of the excess of enzyme. An attractive explanation for this is the existence, as in other P-type ATPases, of a β-subunit [45], which may be required for a complete activity of the enzyme. However, if this is the case, the enzyme would keep a certain activity in the absence of the β-subunit, because the *ENA1* gene has been expressed with a measurable activity in *S. pombe*, which lacks a protein homologous to ENA1 [8], and it is unlikely that it expresses a homologous to the hypothetical β-subunit. The loss of the β-subunit during preparation of the plasma membranes might explain the low ENA1 ATPase activity found both in wild-type and overexpressing strains. How-

ever, independently of the probability of this loss, two other explanations exist for this low activity. In the first place, the activity is undetectable if the membranes are not treated with DTT, and it is possible that with this treatment, only a partial activity of the enzyme is recovered. Another possibility is that the ENA1 ATPase activity is low because cells do not require more activity. The maximum efflux of Na^+ in the most demanding conditions is not very high, perhaps one third of H^+ pumping, and, in addition, the stoichiometry of the pump, Na^+ per ATP, may be quite high because external/internal ratios of Na^+ are low and the exchange mediated by the enzyme may be electroneutral (see below).

Consistent with the detection of the ENA1-specific ATPase activity, a radioactive ENA1 phosphoprotein was clearly detected upon incubation of the membranes with $[\gamma\text{-}^{32}P]ATP$ or $^{32}P_i$ at definite pH values and Na^+ concentrations. This phosphoprotein showed the canonical characteristics of phosphorylated intermediates of P-type ATPases, stability at acid pH values, sensitivity to hydroxylamine, not formation in presence of vanadate, and rapid release of $^{32}P_i$ upon addition of cold ATP or P_i; all of which identify the ENA1 protein as a P-type ATPase. As in other cation-transporting ATPases, in which the cation transported outward is required for phosphorylation from ATP [19] and inhibits phosphorylation from P_i [40,41], Na^+ made phosphorylation of ENA1 from ATP possible and inhibited phosphorylation from P_i. At increasing Na^+ concentrations in the range 0–500 mM, activation of phosphorylation from ATP and inhibition of phosphorylation from P_i followed almost identical patterns (Figs. 4 and 8), suggesting that, in the presence of submolar concentrations of Na^+, the fractions of the enzyme that can be phosphorylated from ATP and from P_i are complementary. This could be expected for a Na-ATPase in which low affinity Na^+ binding is the first step in the reaction cycle of the enzyme. The capacity of the ENA1 ATPase of effective binding of alkali cations can be estimated from the capacity of these cations to activate phosphorylation from ATP and to inhibit phosphorylation from P_i. The observed order $Li^+ \geq Na^+ > K^+ = Cs^+ > Rb^+$ is consistent with known physiological functions of the ENA1 system, which mediates similar Li^+ and Na^+ effluxes [5] and a lower, although efficient, K^+ efflux (Bañuelos and

Rodríguez-Navarro, unpublished data), which might play a role in the control of the cytoplasmic pH [4,8]. Phosphorylation in the presence of choline chloride from ATP was very low, indicating that the described effects were specific of the alkali cations.

The high concentrations of alkali cations saturating or triggering a detectable phosphorylation from ATP, or inhibiting phosphorylation from P_i indicate a low affinity of the ENA1 ATPase for Na^+, and for the other alkali cations. This is entirely consistent with the kinetics of Na^+ [5] and K^+ (Bañuelos, M.A. and Rodríguez-Navarro, A., unpublished data) effluxes mediated by the product of the *ENA1* gene, which exhibit first-order kinetics. Furthermore, considering the normal concentrations of K^+ in yeast cells, which is 250 mM [46], and that Na^+ toxicity occurs above 50–100 mM [36], the concentration range of cations in which the enzyme responses in vitro is approximately the range of physiological requirements.

To address the question of whether ENA1 was a Ca-ATPase [37], we studied the effects of Ca^{2+} on phosphorylation of ENA1 from both ATP and P_i, finding that Ca^{2+} neither enhanced phosphorylation from ATP nor inhibited phosphorylation from P_i. Ca^{2+} at high concentration inhibited phosphorylation from P_i, but the inhibition was reverted by Mg^{2+}. Failure to observe Ca^{2+}-dependent phosphorylation of a Ca-ATPase from ATP may occur if a critical concentration of Ca^{2+} is not tested [47], but a similar uncertainty does not apply if Ca^{2+} does not inhibit phosphorylation from P_i. In P-type ATPases the transported cation inhibits phosphorylation from P_i [40,41] by a simple reaction of binding, which does not disappear by increasing the concentration of the cation. Therefore, our results strongly suggest that ENA1 is not a Ca-ATPase.

Phosphorylation from ATP was progressively inhibited by decreasing the pH values from 8.5 to 5.0 and slightly inhibited by increasing the pH values from 8.5 to 10.0 (Fig. 6); phosphorylation from P_i was inhibited by decreasing the pH from 7.0 to 5.0 and also by increasing it from 7.0 to 9.0 (Fig. 10). The inhibition of both types of phosphorylation by shifting the pH from 7.0 to 5.0 is consistent with the previously observed inhibition of Li^+ efflux when the pH of the cell decreases [48] (Li^+ and Na^+ effluxes are both mediated by the ENA system [5]). The physiological basis of this inhibition is probably

to avoid detrimental Na^+ loss when K^+ is limiting, because, in the absence of anion excretion, the loss of Na^+ requires an equivalent uptake of K^+ or H^+ for electrical neutrality. At low K^+, the cells present a low pH [46] and Na^+ enhances growth [36], because Na^+ uptake allows to recover the normal pH [46]. This indicates that a low pH is probably more detrimental than a high Na^+ content, and that at low pH, the loss of Na^+ must be avoided.

Unlike inhibition at low pH, inhibition of phosphorylation from P_i when the pH exceeds the value of 7.0 cannot be explained by the inhibition of the enzyme or by the instability of the phosphoenzyme, because it does not occur in phosphorylation from ATP. In sarcoplasmic reticulum Ca-ATPase, the reduction in the level of phosphoenzyme formed from P_i when the pH increases from 6.0 to 8.0 has been explained by the requirement of H^+ binding preceding phosphorylation [49] and by the preferential phosphorylation by the $H_2PO_4^-$ anion [44]. In the ENA1 ATPase, the latter cause is unlikely because the presence of 20% DMSO does not change the pH response of phosphorylation, although it changes the pK_2 of P_i from 6.7 to 7.5. Therefore, the most likely possibility to explain the inhibition of phosphorylation from P_i when the pH exceeds 7.0 is that H^+ binding precedes phosphorylation, which would occur if the ENA1 ATPase exchanged Na^+ with H^+, as proposed previously [8]. In this case, both Na^+ and H^+ would be required for a normal reaction cycle, in the same form than Na^+ and K^+ are required for the Na,K-ATPase [50].

The fungus ENA1 ATPase and the animal Na,K-ATPase are both Na^+ pumps, although with notable differences. Phosphorylation from ATP of the ENA1 ATPase did not require the simultaneous presence of Na^+ and K^+ and when they were together their effect was strictly additive. Furthermore, ENA1 mediates K^+ efflux (Bañuelos and Rodríguez-Navarro, unpublished data) and not K^+ uptake. These differences, regarding K^+ transport, are not surprising because fungi, unlike animal cells, can grow in dilute environments and have high affinity K^+ transporters [21,51,52]. The ENA1 ATPase, either a Na,H-ATPase or an electrogenic Na-ATPase, is a new type II cation transporting ATPase [45], which may exist in fungi able to thrive at high Na^+ when the external pH is higher than the cytoplasmic pH [8]. The existence of

this ATPase in other eucaryotic organisms is probably less related to the phylum they belong to than to the media where they can thrive. The alga *Heterosigma akashiwo* expresses an animal-related Na,K-ATPase [53,54], probably because this is a wall-less marine raphidophycean for which the conditions for the ionic homeostasis are closer to those facing animal cells than to those facing the organisms thriving in dilute environments.

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