Potassium- or sodium-efflux ATPase, a key enzyme in the evolution of fungi

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INTRODUCTION

The membrane potential across the plasma membrane of most fungal and plant cells may be around $-150$ mV or even more negative (Rodríguez-Navarro, 2000). This high membrane potential is the driving force that supports the function of many uniporters, symporters and antiporters in the cell. However, it is also a source of problems with those cations whose concentrations in the external medium are approximately the same as or even higher than the maximum internal concentration that the cell can tolerate. In these cases, the difference between the electrochemical potentials of the cation across the membrane may be so high that the entrance cannot be cancelled by simply inhibiting the transporter mediating the uptake. To keep these inevitable leaks from building up an internal toxic concentration of the cation, different types of efflux systems have evolved to balance any excessive entrance. Two ions, Na$^+$ and Ca$^{2+}$, are typical examples of this situation, and for both of them a large number of efflux systems, Na$^+/H^+$ antiporters (Bañuelos et al., 1998; Padan et al., 2001; Shi et al., 2000), Na$^+$ and Ca$^{2+}$-ATPases (Carafoli, 1994; Geisler et al., 2000; Murata et al., 2001; Stein, 1995), Na$^+$ decarboxylases (Buckel, 2001; Dimroth et al., 2001) and Na$^+$ oxidoreductases (Steuber, 2001), have been described in both prokaryotes and eukaryotes. In fungi, a P-type ATPase, which extrudes Na$^+$ probably in exchange for H$^+$, was initially described in Saccharomyces cerevisiae (Haro et al., 1991) and later in other yeasts (Almagro et al., 2001; Bañuelos & Rodríguez-Navarro, 1998) and in Neurospora crassa (Benito et al., 2000).

In contrast with Na$^+$ and Ca$^{2+}$ effluxes, research on K$^+$ efflux has been scarce. This can be explained because K$^+$

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The GenBank accession numbers for the sequences reported in this paper are: Pleurotus ostreatus ENA1, AJ420741; Phycomyces blakesleeanus ENA1, AJ420742; Ph. blakesleeanus PCA1, AJ420743; Blakeslea trispora ENA1, AJ420744; B. trispora BCA1, AJ420745; B. trispora BCA2, AJ420746.
is the most abundant cation in the cells, and in most natural environments the external K$^+$ concentration is much lower than the internal concentration. In these conditions, the K$^+$ diffusion potential is not very different from the membrane potential, with the consequence that non-specific loss is insignificant. Exceptions to this rule are the cellular milieu encountered by intracellular parasites and that found by organisms growing on plant debris, whose composition is dominated by the cellular components of plant cells. Furthermore, in the latter case, the effect of drying may increase the concentration of K$^+$ well over the normal cytoplasmic concentration. In these two cases, an effective K$^+$ efflux system is required. The most representative example of intracellular life is the mitochondrion, and also it is the best example of the indispensability of K$^+$ efflux (Nicholls & Ferguson, 1997). This is mediated by an electroneutral K$^+$/H$^+$ antiporter (Garlid, 1996), whose existence was predicted by Mitchell 40 years ago (Mitchell, 1961).

Fungus–plant associations conquered the lands that emerged from the sea some 450 million years ago (Hass et al., 1994; Redecker et al., 2000; Simon et al., 1993; Taylor et al., 1994, 1999; Wilkinson, 2001). These associations include fungal growth on plant debris and fungal–plant symbioses, either mutualistic or parasitic. In mycorrhizal mutualistic associations, the membrane of the fungal cell does not contact the cytoplasm of the plant cell from which it is separated by the interface matrix (Harrison, 1999), but even in this case the fungal cells may be exposed to high K$^+$ if the plant cells lyse. Therefore, when associated with plants, the fungal cell is circumstantially or permanently exposed to high K$^+$. This suggests that, as in the case of the mitochondrion, fungi need an effective K$^+$ efflux system.

Biochemical and functional analyses of several fungal Na$^+$ ATPases have identified some of these ATPases as bifunctional enzymes for Na$^+$ and K$^+$ (Bañuelos & Rodriguez-Navarro, 1998; Benito et al., 1997; Haro et al., 1991), suggesting that these ATPases may be the predicted K$^+$ efflux system of fungi. These ATPases form a phylogenetic cluster, named IID (Axelsen & Palmgren, 1998) or ENA (Benito et al., 1992), which surprisingly includes the CTA3 ATPase of Schizosaccharomyces pombe, originally described as a Ca$^{2+}$-ATPase (Ghislain et al., 1990; Halachmi et al., 1992). The function of CTA3 as a Ca$^{2+}$-ATPase and not as a Na$^+$-ATPase was supported by the conservation in CTA3 of several amino acids typical of Ca$^{2+}$-ATPases and was consistent with the functional absence of a Na$^+$-ATPase in Schiz. pombe, where Na$^+$ tolerance and Na$^+$ efflux is dominated by the Na$^+$/H$^+$ antiporter SOD2 (Jia et al., 1992; Hahnenberger et al., 1996). However, with the increasing number of P-ATPases isolated and studied, the phylogenetic isolation of CTA3 from other Ca$^{2+}$-ATPases also increased, and therefore so did the doubts about the real function of CTA3.

We here report that the Schiz. pombe CTA3 ATPase is a K$^+$-efflux ATPase and that the capacity of ENA ATPases to pump Na$^+$ has evolved recently as an adaptation of fungi to salinity. We also discuss the existence and function of ENA ATPases in the parasites Leishmania and Trypanosoma, which in some cases have to overcome a problem of K$^+$ stress similar to that existing in fungi.

**METHODS**

**Strains, plasmids, media and growth conditions.** The yeast (Saccharomyces cerevisiae) strains W303.1B (MATa tyr3 his3 leu2 ade2 trp1), and its derivatives B31 (ena1A::HIS3:: ena4 nha1A::LEU2) (Bañuelos et al., 1998) and K609 (pmr1A::HIS3) (Cunningham & Fink, 1994) were used in this study. The yeast expression vector pDR195 (Rentsch et al., 1995) was used to express the ATPases, except for the Sacch. cerevisiae ATPases, which were expressed from YEp91, as described previously (Benito et al., 1997), and the Debaryomyces hansenii ATPases, which were expressed from pYPGE15 (Almagro et al., 2001). The Sacch. cerevisiae strains were grown in either the complex medium YPD (1% yeast extract, 2% peptone, 2% glucose) or in the minimal arginine phosphate medium (Rodriguez-Navarro & Ramos, 1984). Media were supplemented with KCl and NaCl as recorded in each case. Growth rates were determined by following the optical density of the culture in liquid media. Lag times, which are long in high-salt media, were not recorded. The Schiz. pombe strain 556 was grown in complex medium as described previously (Bañuelos et al., 1995). In some tests, this medium was supplemented with 10 mM TAPS or 10 mM TAPS plus 50 mM NH$_4$Cl, and adjusted to pH 7.8 with KOH. For Northern analyses, Schiz. pombe was incubated in YPD supplemented as described in Fig. 1. Genomic DNA samples from Phycocyanax blakesleeanus strain NRR1555 (−) and Blakeslea trispora strain F921 (−), were a generous gift of E. Cerdá-Olmedo, Departamento de Genética, Universidad de Sevilla, Spain. Pleurotus ostreatus DNA was obtained from a commercial sample, by using the DNeasy Plant Kit (Qiagen).

**Recombinant DNA techniques.** Manipulation of nucleic acids was carried out using standard protocols (Sambrook et al., 1989).
Fungal K\(^+\) or Na\(^+\)-ATPases

1989) or, where appropriate, according to the manufacturer’s instructions.

The coding region of the Schiz. pombe cta3 gene was cloned by PCR and inserted into plasmid pDR195. Fragments of the Ph. blakesleeanus and B. trispora ENA genes were amplified from genomic DNA using the degenerate primers described elsewhere (Benito et al., 2000). The PCR products were cloned into the PCR2.1-Topo vector using the TOPO TA cloning kit (Invitrogen). Plasmid pDR195 with the cta3 insert is toxic in bacteria and was constructed directly in yeast. The correct sequence of the construct transformed into the yeast strain was checked by sequencing PCR amplifications of two fragments. Sequencing was done with an automated ABI PRISM 377 DNA sequencer (Perkin-Elmer). For Southern blot hybridizations, genomic DNA was digested with restriction enzymes. Then, after electrophoresis and blotting to a nylon membrane, it was hybridized in the presence of 50% formamide. For Northern blots, total RNA was extracted (Carlson & Botstein, 1982), fractionated through formaldehyde-agarose gels and blotted onto a nylon membrane. Membranes were hybridized with DNA probes that were labelled with \([\alpha-\text{P}]\text{dATP}\) by the random priming method (Feinberg & Vogelstein, 1983).

**Cation losses.** Yeast cells with different K\(^+\) and Na\(^+\) contents were obtained by growing and preincubating the cells in the arginine phosphate medium supplemented with KCl or NaCl as described in each case. To perform the experiments, the cells were suspended in 10 mM Ca\(^{2+}\)-MES buffer, pH 6.0, 2% glucose, supplemented with RbCl as recorded in each case. At intervals, the cells were collected on Millipore membrane filters, rapidly washed with 20 mM MgCl\(_2\), acid-extracted, and analysed by atomic emission spectrophotometry (Rodríguez-Navarro & Ramos, 1984). Flux experiments were repeated three times. The initial values of the cation contents varied slightly among experiments (sd < 10% of the mean), but the rates of the losses were almost identical.

**RESULTS**

**CTA3 is a K\(^+\)-ATPase**

In a recent paper we reported the cloning of a cDNA from N. crassa, ph-7 (thereafter named NeENA2), which could encode a Ca\(^{2+}\)\,- or Na\(^+\)-ATPase and for which we could not get functional expression in yeast (Benito et al., 2000). Although this has not been attained so far, several results obtained in the study of NeENA2 suggested that this ATPase was in some way involved in K\(^+\) metabolism. Considering that several amino acids typical of Ca\(^{2+}\)-ATPases are identical in CTA3 and NeENA2, the notion arising from the experiments with NeENA2 was that CTA3 was a K\(^+\)-ATPase.

To obtain a definitive answer on the function of CTA3, we completed a previous study on the expression of the cta3 transcript (Nishikawa et al., 1999), and cloned and expressed the cta3 gene in Sacch. cerevisiae mutants that are sensitive to high K\(^+\) or Na\(^+\), ena1-4 nha1, or to low Ca\(^{2+}\), pmr1. In the report of Nishikawa et al. (1999) the expression of cta3 was enhanced by Na\(^+\), which was very surprising, because, as we have mentioned, it is very unlikely that CTA3 pumps Na\(^+\). A likely possibility was that the Na\(^+\) added induced a rapid Na\(^+\) uptake and an increase in the cytoplasmic pH. Consistent with this possibility we found that a high external pH and especially the presence of NH\(_4\)\(^+\), which alkalinizes the cytoplasm, strongly enhanced the expression of cta3. We also found that its expression was insensitive to changes in Ca\(^{2+}\) concentration (Fig. 1). Simultaneously, we found that cta3 suppressed the sensitivity of the ena1-4 nha1 mutant to high K\(^+\) (Fig. 2), but slightly increased its Na\(^+\) tolerance, and that it had no effect at all on the sensitivity of the pmr1 mutant to low Ca\(^{2+}\) concentrations (not shown). Consistent with its capacity to increase the K\(^+\) tolerance of the ena1-4 nha1 mutant, CTA3 also induced a rapid K\(^+\) efflux (Fig. 3). Taken together, these results demonstrate that CTA3 is a K\(^+\)-ATPase.

**Some ENA ATPases are complex K\(^+\)- or Na\(^+\)-ATPases**

Finding the function of CTA3 changed our perception of the ENA ATPases as typical Na\(^+\) pumps, and indicated that some of them were K\(^+\) pumps. This notion, and the biochemistry of the ScENA1 ATPase, which demon-
whereas others may be Na⁺ sensitivity to K⁺. DhENA2, were equally effective in suppressing the sensitivity of Na⁺- or K⁺-efflux (Fig. 4). These results confirmed the bifunctionality of the Sacch. cerevisiae ENA ATPases for pumping K⁺ or Na⁺, but also uncovered a remarkable behaviour of the system, which, under certain conditions, extruded Na⁺ without extruding K⁺. Because the three ENA pumps do not seem to be different in function (Table 1), this finding cannot be explained only by the dominance of different pumps in the two experiments.

ENA ATPases probably exist in all fungi

The finding that many ENA ATPases are K⁺ pumps suggested that they may exist even in Na⁺-sensitive fungal strains. This hypothesis could be easily tested, because Na⁺- and Li⁺-sensitive yeast strains are abundant in the genus Saccharomyces (Rodríguez-Navarro, 1971) and in the species Sacch. cerevisiae (Sancho et al., 1986). We selected the Na⁺-sensitive Sacch. cerevisiae strain that was used for the cloning of the ScENA1 gene (Haro et al., 1991) and searched for ENA genes by PCR. We found that ScENA1 does not exist in this strain, but there was an ENA tandem, containing at least one copy of ScENA2 and one copy of ScENA4.

In phylogenetic trees, ENA ATPases form a cluster independent from Ca²⁺-ATPases (Axelsen & Palmgren, 1998; Bañuelos & Rodríguez-Navarro, 1998; Benito et
Table 2. Fungal species in which ENA ATPases have been found in databases

<table>
<thead>
<tr>
<th>Species</th>
<th>GenBank accession no.</th>
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<tr>
<td>Zygosaccharomyces rouxii</td>
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<td>Fusarium solani</td>
<td>U61840</td>
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<td>Gigaspora rosea</td>
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<td>AA965915</td>
</tr>
<tr>
<td>Pleurotus ostreatus</td>
<td>AT004276</td>
</tr>
<tr>
<td>Saccharomyces bayanus</td>
<td>AL402020</td>
</tr>
<tr>
<td>Yarrowia lipolytica</td>
<td>AL410476</td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td>AL440510</td>
</tr>
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<td>Kluyveromyces thermotolerans</td>
<td>AL420487</td>
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<td>Kluyveromyces lactis</td>
<td>AL429570</td>
</tr>
<tr>
<td>Saccharomyces exiguus</td>
<td>AL407827</td>
</tr>
<tr>
<td>Pichia angusta</td>
<td>AL435733</td>
</tr>
<tr>
<td>Neurospora crassa ENA3</td>
<td>Upd3*</td>
</tr>
<tr>
<td>Candida albicans ENA1-ENA2</td>
<td>Contigs6-2497/</td>
</tr>
</tbody>
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*http://www.mips.biochem.mpg.de/project/neurospora/
†http://www-sequence.stanford.edu/group/candida/
blakesleeanus and B. trispora, and to obtain a longer sequence in the basidiomycete Pl. ostreatus. Using a PCR approach we amplified three DNA gene fragments, one in each species, that unequivocally encoded ENA ATPases, PbENA1, BrENA1 and PoENA1. We also identified three sequences that encoded putative Ca\(^{2+}\)-ATPases, two PMCA type, PbPCA1 and BrBCA1, and one SERCA type, BrBCA2 (Fig. 5).

Using all known fungal Ca\(^{2+}\)- and Na\(^{+}\)-ATPases, for which we had sequences long enough for a phylogenetic study, along with several plant Ca\(^{2+}\)-ATPases, we constructed a phylogenetic tree. Fig. 5(a) shows the tree constructed using sequences that cover almost the whole large cytoplasmic loop connecting the fourth and fifth transmembrane fragments, but the same result is obtained using the complete sequences (Benito et al., 2000). This tree produced the same clusters as previous studies (Axelsen & Palmgren, 1998; Benito et al., 2000), leaving the Leishmania sequence outside of the group of Ca\(^{2+}\)-ATPases and included in the group of ENA ATPases. However, the Leishmania sequence diverged substantially from the fungal sequences. This was expected from the studies with other proteins (Baldauf & Palmer, 1993), but made it difficult to predict its phylogenetic position. To further investigate if the Leishmania ATPase belonged to the ENA cluster, we constructed a bootstrap tree using this ATPase, some selected ENA ATPases, fungal and plant Ca\(^{2+}\)-ATPases, and the sequence of the PMA1 H\(^+\)-pump as the outgroup. The branching order supporting the notion that the Leishmania ATPase belongs to the ENA cluster is robust, supported by 97-4% of bootstraps (Fig. 5b). In the ENA-type fungal sequences, there is a hemiascomycete clade, which is consistent with evolutionary studies of fungi (Auwer & Wachter, 1996; Berbee & Taylor, 1993). The two ATPases of the two Zygomycetes Ph. blakesleeanus and B. trispora are also related each other. In contrast, the three ATPases of N. crassa are very divergent among themselves, and also with the hemiascomycete clade.

**DISCUSSION**

The CTA3 ATPase of Schiz. pombe was described as a Ca\(^{2+}\)-ATPase (Ghislain et al., 1990; Halachmi et al., 1992), but the results presented in this report demonstrate that the major activity of CTA3 is as a K\(^{+}\)-efflux ATPase. The suppression of the sensitivity of the ena1-4 nha1 strain to high K\(^{+}\) (Fig. 2) by promoting a rapid K\(^{+}\)-efflux across the plasma membrane (Fig. 3) leaves little room for doubt. Our failure to suppress the defect of the pmr1 mutant by expressing CTA3 is consistent with the lack of a Ca\(^{2+}\)-phenotype of a cta3::ura\(^+\) mutant found previously (Nishikawa et al., 1999) and suggests that the Ca\(^{2+}\)-ATPase activity of CTA3, if any, does not have physiological significance. As previously reported for ScENA1 (Benito et al., 1997), the hydrolytic activity of CTA3 was undetectable (results not described), and also no effect of Ca\(^{2+}\) or other cations on this activity could be demonstrated. We also found the same problem with NcENA1 (unpublished results), and all these failures suggest that the loss of the activity of ENA ATPases *in vitro* may be an intrinsic response of these enzymes. Perhaps, ENA ATPases are under strict metabolic control to avoid a futile cycle of K\(^{+}\) and H\(^{+}\), and the reconstitution of the activity *in vitro* requires conditions which are unknown at the moment.

The first conclusion of our results is that now ENA ATPases can be perceived not as a phylogenetic group of enzymes of variable activity, but as a group of enzymes with a common activity, the mediation of K\(^{+}\) or Na\(^{+}\) efflux. The biochemical behaviour of ScENA1 (Benito et al., 1997) and the present results (Table 1) indicate that many of these ATPases exhibit a low specificity for K\(^{+}\) or Na\(^{+}\), whereas some are more specific for K\(^{+}\) or Na\(^{+}\). All the ENA ATPases studied up to now are plasma membrane enzymes, but further studies are needed to rule out their presence in other membranes. The second conclusion is the universal presence of ENA ATPases in fungi. ENA genes have been identified in 20 species among Basidiomycetes, Ascomycetes and Zygomyccetes, and they have been found in all fungi in which their presence has been investigated, even in Na\(^{+}\)-sensitive strains.

In contrast to the universal presence of ENA ATPases in fungi, they are absent in plants (Garcia-delblas et al., 2001). The strong contrast between plants and fungi regarding ENA ATPases can be understood if the original function of these ATPases were K\(^{+}\) extrusion. Life originated in the sea, and it is evident that early living cells needed an efficient Na\(^{+}\) efflux system. This requirement has been permanent for animal cells, because they have been permanently exposed to sea water, or similar fluid, and an ancestor P-ATPase evolved to the present Na\(^{+}\),K\(^{+}\)-ATPase. This is not the case for fungi and plants, which left the sea probably during the Precambrian era. They last shared a common ancestor about a billion years ago (Doolittle et al., 1996; Lee, 1999), but later evolved together in an oligotrophic medium with a low Na\(^{+}\) content (Retallack & German-Heins, 1994; Retallack, 1997). Fungi and plants adapted to these conditions using the same H\(^{+}\)-pump ATPase (Serrano, 1988) and similar K\(^{+}\) and Na\(^{+}\) transporters (Rodriguez-Navarro, 2000), but only plant-associated fungi were exposed, at least circumstantially, to the high K\(^{+}\) concentrations of the plant cells.

In cells with a very negative membrane potential, as is the case for fungal cells (Rodriguez-Navarro et al., 1986; Slayman, 1965a, b), exposure to a high K\(^{+}\) environment brings about an almost inevitable K\(^{+}\) influx which must be balanced with K\(^{+}\) efflux. In mitochondria, an electroneutral K\(^{+}\)/H\(^{+}\) antiporter mediates K\(^{+}\) efflux and volume control (Garlid, 1996), because the mitochondrion external medium is under a homeostatic control and the mitochondrial matrix pH can be maintained without exception at a pH slightly higher than cytoplasmic pH. Fungal cells are also furnished with a K\(^{+}\)/H\(^{+}\) antiporter (Bañuelos et al., 1998; Camarasa et al., 1996; Ramirez et al., 1998; Sychrová et
al., 1999), but its function cannot be permanent, because of the variability of the environmental pH. Therefore, the most reasonable explanation for the existence of the fungal K⁺-efflux ATPase is the adaptation of a P-type ATPase to mediate K⁺ efflux when the external pH is high.

Adaptation of plants to saline media occurred recently, when present plant families had already differentiated (Rozema, 1996), producing halophytes that always have close phycoyte relatives. The close evolution of plants and fungi makes it possible to predict the same recent adaptation of fungi to Na⁺. However, unlike plant cells, fungi had a K⁺-efflux ATPase with a high potential of adaptation for Na⁺ extrusion. To maintain the K⁺ efflux capacity of the cells, as well as a better adaptation to Na⁺, the ENA genes duplicated and generated pumps that were more active with Na⁺ (Table 1). So far, the only identified fungus in which this duplication did not occur and which lacks a significant Na⁺-ATPase activity is Schizosaccharomyces pombe. Although this fungus might be a singular case because it has a rather independent phylogenetic position among Ascomycetes (Gehrig et al., 1996; Keogh et al., 1998), further research may identify more cases.

The ENA gene duplication occurred very recently in the hemiascomycetes yeast Saccharomyces cerevisiae (97% identity between ScEna1p and ScEna4p), and earlier in C. albicans, Schizosaccharomyces pombe, and D. hansenii (84, 73 and 68% identity, respectively, between the Ena1p and Ena2p in the same species). The case of Neurospora, with three rather divergent ENA ATPases (42–48% identities among NcEna1p, NcEna2p and NcEna3p), may be a more refined model of adaptation to Na⁺. NcENA1 is rather Na⁺ specific (Table 1), and if either NcENA2 or NcENA3 is the K⁺-ATPase homologue to SpCtA3, there is still a third ATPase whose function is not yet clear.

The function of the Leishmania LdCA1 needs to be demonstrated, but it is possible that it fulfills the function of the fungal ENA ATPases, from which its divergence (Fig. 5) can be expected from evolutionary considerations (Baldauf & Palmer, 1993). The existence of K⁺-efflux ATPases in Leishmania and Trypanosoma is not surprising. Some of them (not T. brucei), after a lysosomal recruitment, escape from the highly acidic lysosomal vacuole into the cytoplasm, facing a neutral pH and a high K⁺ content. Assuming that the plasma membrane of the parasitic cells is hyperpolarized (negative inside) (Glaser et al., 1992), and that the cytoplasm is not at a higher pH than that of the host cells (Heyden & Docampo, 2000), the existence of K⁺-efflux ATPases in the plasma membrane can be explained as discussed for fungi.

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