Identification and Characterization of Vnx1p, a Novel Type of Vacuolar Monovalent Cation/H\(^+\) Antiporter of *Saccharomyces cerevisiae*\(^*\)

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We identified and characterized Vnx1p, a novel vacuolar monovalent cation/H\(^+\) antiporter encoded by the open reading frame YNL321w from *Saccharomyces cerevisiae*. Despite the homology of Vnx1p with other members of the CAX (calcium exchanger) family of transporters, Vnx1p is unable to mediate Ca\(^{2+}\) transport but is a low affinity Na\(^{+}\)/H\(^+\) antiporter with a \(K_m\) of 22.4 and 82.2 mM for Na\(^{+}\) and K\(^{+}\), respectively. Sequence analyses of Vnx1p revealed the absence of key amino acids shown to be essential for Ca\(^{2+}\)/H\(^+\) exchange. VNX1Δ cells displayed growth inhibition when grown in the presence of hygromycin B or NaCl. Vnx1p activity was found in the vacuoles and shown to be dependent on the electrochemical potential gradient of H\(^+\) generated by the action of the V-type H\(^+\)-ATPase. The presence of Vnx1p at the vacuolar membrane was further confirmed with cells expressing a VNX1::GFP chimeric gene. Similar to Nhx1p, the prevacuolar compartment-bound Na\(^{+}\)/H\(^+\) antiporter, the vacuole-bound Vnx1p appears to play roles in the regulation of ion homeostasis and cellular pH.

In *Saccharomyces cerevisiae* as well as in other eukaryotes and prokaryotes, K\(^{+}\) is the most abundant cation and plays key roles in cellular ionic homeostasis and osmotic regulation. Potassium uptake is mediated mainly by the plasma membrane bond Trk1p and Trk2p. Trk1p, the high affinity K\(^{+}\) transporter, seems to be predominant over Trk2p, since trk1 mutants lose the ability to grow in low K\(^{+}\) media (1, 2). Trk2p, the low affinity K\(^{+}\) transporter, is able to substitute Trk1p activity only under K\(^{+}\) limiting conditions or at low pH (3). Na\(^{+}\) and other alkali cations enter the cell through K\(^{+}\) uptake systems, and steady-state cytosolic Na\(^{+}\) concentrations are established by the balance between Na\(^{+}\) inward and Na\(^{+}\) outward fluxes among the cytosol and the external medium or the vacuole.

In *S. cerevisiae* four *Ena* genes in tandem encode Na\(^{+}\)-ATPases that are the primary pathway for Na\(^{+}\) exclusion (4). Several fungal Ena-type ATPases have been also described as K\(^{+}\) efflux enzymes. *S. cerevisiae* ena1-ena4 null mutants display lower Na\(^{+}\) tolerance at alkaline pH than at acidic pH due to the presence of Nha1p, a plasma membrane-bound Na\(^{+}\)/H\(^+\) antiporter (4, 5). Nha1p plays a role in pH regulation and also cell cycle regulation (6) and catalyzes the transport of Na\(^{+}\) and K\(^{+}\) with similar affinity (\(K_m\) ≈ 12 mM). Similar to the 1Na\(^{+}\)/2H\(^+\) bacterial antiporters (7), Nha1p activity is electrogenic and induces a net charge movement across the membrane, whereas other eukaryotic Na\(^{+}\)/H\(^+\) antiporters are electroneutral (8, 9).

Two other Nha1p homologues, Kha1p and Nhx1p, have been identified in *S. cerevisiae*. Kha1p has been described as a putative K\(^{+}\)/H\(^+\) antiporter, and its deletion induced a growth defect at high external pH and hygromycin (10). Kha1p co-Localized with Mntp1p, a Golgi-specific marker (11). Although Kha1p displays high amino acid sequence similarity to other Na\(^{+}\)/H\(^+\) antiporters, its ability to mediate K\(^{+}\)/H\(^+\) exchange has not been yet demonstrated. Nhx1p localizes to the pre-vacuolar compartment and function in the sequestration of sodium ions (\(K_m = 16\) mM) by using the electrochemical proton gradient generated by the V-type H\(^+\)-ATPase in an electroneutral manner (12–14). Nhx1p can be also localized to discrete patches at the vacuolar membrane (13, 15, 16).

Cells harboring *nhx1Δ* mutations displayed a decrease in Na\(^{+}\) tolerance only under specific conditions, such as acidic pH (pH 4.0) and low K\(^{+}\) concentrations (1 mM) (12, 14). These data suggested the predominance of Na\(^{+}\)-ATPase and Nha1p over Nhx1p in Na\(^{+}\) and K\(^{+}\) tolerance and suggested other roles for Nhx1p. Nhx1p, also known as Vps44, act at the same step in trafficking to the vacuole as other class E vacuolar protein sorting proteins that are thought to control protein trafficking out of the pre-vacuolar compartment (17). Disruption of vacuolar the H\(^{+}\)-ATPase gene induced an increase of pH in the vacuolar lumen leading to a phenotype similar to that observed in *nhx1Δ* null mutants. This phenotype displayed changes in intravesicular activities and missorting of carboxypeptidase Y (17), suggesting that Nhx1p might play a role in regulating pH to control trafficking out of the endosome, inducing a H\(^{+}\)-leak that counterbalanced the action of the V-ATPase (16).

Contrary to a previous report (14), two independent studies showed that *nhx1Δ* null mutants did not display reduced vacuolar Na\(^{+}\) transport (18, 19) and that vacuolar preparations from different Na\(^{+}\)/H\(^+\) antiporter yeast strain mutants, including KTA 40-2 (ena1-ena4Δ, nha1Δ, nhx1Δ, kha1Δ), did not display any reduction of Na\(^{+}\) or K\(^{+}\) transport. These observations supported the notion that a vacuolar transporter, not encoded

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2 O. Cagnac, unpublished data.
by the NHX1 gene, could be responsible for the vacuolar cation/H+ exchange activity seen in the yeast vacuoles (19). Because no other monovalent cation/H+ homologues could be found in the databases, we decided to functionally screen a wide range of yeast cation/H+ antiporter-like mutants from the Euroscarf collection to isolate the gene(s) coding the transporter responsible for the vacuolar cation/H+ exchange activity. This reversed genetic approach allowed us to identify a single ORF3 YNL321w, coding for a vacuolar Na+/H+ exchanger (Vnx1p). vnx1Δ mutants displayed a total loss of Na+ and K+/H+ antiporter activity on vacuolar-enriched fraction.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Media**—The vnx1Δ yeast strain (Table 1) was generated by replacing the YNL321w ORF with the kanMX6 gene by PCR-based gene deletion method (20) using the following primers: YNL31w-F1 (5'-AAGTGAAATAACT-GCTAGCTAGAAAGAGGCGTAAAGGACCGGATCCCCGGGTTAATTTA-3') and YNL31w-R1 (5'-AAAAAGGTGATTGCCAGGATGTTACCTCCCCGCTGTTTAAAC-3'). The deletion constructs contain 40 bp of homology (underlined) to the beginning and to the end of the YNL321w ORF. Yeast strains were transformed by the deletion cassette using the standard lithium acetate method (21). Genomic DNA from Geneticin-resistant strains was isolated as described by Ohsumi and Anraku (23). The intact vacuoles were resuspended in 5 mM Tris-MES (pH 7.5). Protein concentrations were determined by the Bio-Rad DC protein assay according to the manufacturer’s protocol.

**Transport Assays**—The fluorescence quenching of acridine orange was used to monitor the establishment and dissipation of vacuolar-inside acidic pH gradients as described before (24, 25). Intact vacuoles or vesicles (25 µg of protein) were used for each assay. Vacuoles were added to a buffer containing 50 mM tetramethyl ammonium chloride, 5 µM acridine orange, 5 mM Tris-MES (pH 7.5), and 3.125 mM MgSO4. The vacuolar ATPase was activated by the addition of 5 mM Tris-ATP, and time-dependent fluorescence changes were monitored using a fluorescence spectrophotometer (PerkinElmer Life Sciences) with excitation and emission wavelengths of 495 and 540 nm, respectively, and a slit width of 5 nm with a 1% transmittance filter. When a steady-state pH gradient was established, the vacuolar ATPase was partially inhibited with the addition of 5–10 nM bafilomycin to obtain no net H+ movements (i.e. equal rates of H+ pump and H+ leak (≈3 min) (see below); chloride salts were added as indicated. Initial rates were measured as the slope of the relaxation of the quench over a period of 30s. Rates were reported as % quench/min/mg of protein. All curves were normalized to 100% quench before quantification. Curves were fitted to the mean values of rates at each concentration measured by using KALEIDOGRAPH (Synergy Software, Reading, PA).

**Plasmid Construction and Yeast Transformation**—The yeast expression vector, pYOC003, used in this study is a modification of the pDR196 (26). 3 x Myc tag was amplified by PCR (Myc-For: 5’-CGCTCTGACGAAAGTCGAC-3’ and Myc-Rev 5’-GACGCGGCGCCTACTATT-3’) and then inserted in EcoRV of the pDR196 polylinker. The Gateway® cassette was amplified from the commercial vector pYES-DEST52 (Invitrogen) (attR1, 5’-CAAGTTTGTACAAAAAAGCTG-3’; attL2, 5’-ACCACCTTTGTACAAGAAACG-3’) and then inserted in Smal restriction site. The Vnx1 coding sequence (ORF: YNL321w) was cloned as PCR fragment in the Gateway® manual (Invitrogen) using the Expand high fidelity polymerase (Roche Applied Science). The PCR were performed on genomic DNA with primers attB1-Vnx1 (5’-GGGGGACCACTTTGTACAGAAGTACGAGGATGCTGTTTAAAC-3’). The deletion constructs contain 40 bp of homology (underlined) to the beginning and to the end of the YNL321w ORF. Yeast strains were transformed by the deletion cassette using the standard lithium acetate method (21). Genomic DNA from Geneticin-resistant strains was isolated as described by Sambrook and Russell (22). Insertion of the disruption cassette into the correct locus was verified by PCR. Then, for each mutant strain three independent colonies were tested for their lack of vacuolar Na+/H+ and K+/H+ transport activity. All yeast strains used in this study are listed in Table 1. Yeast cells were grown in yeast 1% extract, 2% peptone, 2% glucose or SD media (0.67% yeast nitrogen base, 2% glucose) with appropriate amino acid supplements as indicated.

**Isolation of Intact Vacuoles from Yeast**—Intact vacuoles were isolated as described by Ohsumi and Anraku (23). The intact vacuoles were collected at the top of the gradient and resuspended in 5 mM Tris-MES (pH 7.5). For vesicle preparation, 10% of glycerol and 1% of protease inhibitor mixture were added to resuspended vacuoles using a Dounce homogenizer. After centrifugation for 20 min at 100,000 × g, the vesicles were resuspended in 5 mM Tris-MES (pH 7.5). Protein concentrations were determined by the Bio-Rad DC protein assay according to the manufacturer’s protocol.

3 The abbreviations used are: ORF, open reading frame; MES, 4-morpholineethanesulfonic acid; GFP, green fluorescent protein; ER, endoplasmic reticulum.
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**TABLE 1**
List of strains used in this study

| Strain   | Genotype                          | Source          |
|----------|-----------------------------------|-----------------|
| W303-1B  | Mata, ura3-1, leu2-3, trp1-1, his3-11,15, ade2-1, can1-100, maal10, ena1–4::HIS3, nha1 Δ::LEU2, nhs1Δ::TRP1, trk1-khal Δ::KanMX | Ref. 10         |
| KTA40-2  | Mata, ura3-1, leu2-3, trp1-1, his3-11,15, ade2-1, can1-100, nhx1 Δ::HIS3 | Ref. 36         |
| OC01     | Mata, ura3-1, leu2-3, trp1-1, his3-11,15, ade2-1, can1-100, ynl321w::KanMX2 | Euroscarf       |
| OC02     | Mata, ura3-1, leu2-3, trp1-1, his3-11,15, can1-100, nhx1 Δ::HIS3, ynl321w::KanMX4 | This study      |
| RGY73    | Mata, ura3-1, leu2-3, trp1-1, his3-11,15, ade2-1, can1-100, ena1–2Δ::HIS3 | Ref. 36         |
| OC03     | Mata, ura3-1, leu2-3, trp1-1, his3-11,15, ade2-1, can1-100, ena1–2Δ::HIS3, nhx1 Δ::TRP1 | This study      |
| OC04     | Mata, ura3-1, leu2-3, trp1-1, his3-11,15, ade2-1, can1-100, ena1–2Δ::HIS3, ynl321w::KanMX6 | This study      |
| YJR106w  | Mata, ura3–52, his3Δ1, leu2-3, 112, trp1-289, yjr106w Δ::HIS3 | Euroscarf       |
| YDL128w  | Mata, ura3–52, trp1Δ63, vnx1Δ::KanMX4 | Euroscarf       |
| YDL206w  | Mata, ura3–52, his3Δ200, trp1Δ63, ydl206Δ::KanMX4 | Euroscarf       |
| YLR220w  | Mata, his3Δ1, leu2Δ0, met15Δ6, ura3Δ0, ylr220w Δ::KanMX4 | Euroscarf       |
| YOR316c  | Mata, his3Δ1, leu2Δ0, met15Δ6, ura3Δ0, yor316c Δ::KanMX4 | Euroscarf       |
| YMR243c  | Mata, his3Δ1, leu2Δ0, met15Δ6, ura3Δ0, ymr243Δ Δ::KanMX4 | Euroscarf       |

Using primers with attB2-Vnx1 (see above) and Vnx1-For (reverse complement of GFP-Rev) primers. All PCR products were combined and used as template for a nested PCR using attB1 and attB2 adaptors (5′-GGGGCACAGTTTGGTACAAA-AAGCAGGCT-3′; 5′-GGGGACACCTTTTGCATAAGATGGT-3′). Vnx1-GFP was cloned in a pYOC003 as previously described.

**Growth Tests**—Yeasts were grown in yeast extract/peptone/dextrose medium to saturation and washed twice in sterile water. All cell suspensions were adjusted to an optical density of 0.2, 0.02, and 0.002 in sterile water. 10 μl of each dilution were spotted onto yeast extract/peptone/dextrose media supplemented with 300 mM NaCl or 30 μg/ml hygromycin B. Growth was assessed at 30 °C after 2 days and 5 days for hygromycin B and NaCl, respectively.

**Total Protein Extract and Western Blots**—Yeast cells (A₆₀₀ = 1) were washed and resuspended in water. To break the cells, the samples were incubated on ice for 10 min after the addition of 185 mM NaOH and 0.35% β-mercaptoethanol (0.35%). Total proteins were precipitated with 5% trichloroacetic acid on ice for 10 min. After centrifugation trichloroacetic acid-precipitated pellets were resuspended in 20 μl of 1 M Tris. Samples were denatured by adding an equivalent volume of 2× Laemmli buffer. Samples (20 μl) were subjected to a 6% SDS-PAGE followed by Western blot analysis. Anti-β-Myc-epitope monoclonal antibody (GeneTex, Inc. San Antonio, TX) and anti-mouse IgG antibody-horseradish peroxidase conjugate (Molecular Probes) were used at a dilution of 1:3,000 and 1:10,000, respectively.

**Fluorescence Microscopy**—A Leica DMR series fluorescent microscope equipped with a Chroma 86013 filter set (Chroma Technology, Rockville, VT) and CoolSNAP-HQ (Roper Scientific, Tucson, AZ) was used to visualize the transformed cells. GFP was visualized by using filters S484/15 for excitation and S517/30 for emission. All images were taken at ×100 magnification. Images were pseudocolored with METAMORPH software (Universal Imaging, Downingtown, PA).

**Gene Expression**—Total RNA was isolated from yeasts with an RNAeasy kit according to the manufacturer’s protocol (Qia-gene). Samples where then treated by DNase RNase-free (Roche Applied Science) to degrade the potential DNA contamination. Five micrograms of the total RNA were reverse transcribed with iScript cDNA synthesis kit (Bio-Rad). PCR-specific primers to Nhx1 (forward, 5′-ACATGTCAAGAAGATCAGCAG 3′; reverse, 5′-TCGGCGTGTGAGTAAGAGAATG-3′), to Vnx1 (forward, 5′-ATGGCCAAAAAATAACCACAT-3′; reverse, 5′-ATTGGAACAGTCACCTCCC-3′), and Kha1 (forward, 5′-ATGGCAAAACACTGAGG-3′; reverse, 5′-CAAATGCGGTATCTTCCG-3′). One microliter of each cDNA sample was used to perform the gene specific PCR (35 cycles). The specificity of the PCR product was checked by sequencing.

**RESULTS**

**Identification of YNL321w**—A functional screening of yeast antipporter mutants from the Euroscarf collection was performed (Table 1). To select for putative vacuolar cation/H⁺ antipporter-like proteins, we used the yeast transport protein database (YTPdb) to choose only proteins that were predicted to be localized at the vacuole or at endosomes. Vacuoles were isolated from each of the Euroscarf knock-out mutants of putative cation/H⁺ antiporters and knock-out mutants of the KHA1 encoding a putative K⁺/H⁺ antiporter and NHX1 encoding a pre-vacuolar-bound Na⁺/H⁺ antiporter (Table 1). The measurement of Na⁺/H⁺ and K⁺/H⁺ exchange activity in vacuoles isolated from each of the above-mentioned antiporters revealed insignificant changes to the transport (Na⁺/H⁺, K⁺/H⁺) seen in vacuoles isolated from wild-type yeast (Fig. 1, A–C), with exception of ynl321Δ (Fig. 2B). Vacuoles isolated from the YNL321w ORF knock-out showed the absence of a cation/H⁺ exchange activity (Fig. 2B). To further confirm YNL321 function(s), the transport activity of ynl321Δ knock-outs that were complemented with the YNL321 gene was studied. VNX1 was cloned into a high expression vector downstream of the PMA1 promoter. Transformation of the ynl321Δ disruptants with this construct restored Na⁺/H⁺, K⁺/H⁺, and Li⁺/H⁺ transport of the vacuoles (Fig. 2C), confirming YNL321 as a monovalent cation/H⁺ antipporter.

The cation/H⁺ transport activity of YNL321 was dependent on the pH gradient generated by the vacuolar H⁺-ATPase. It should be noted that a steady-state ΔpH gradient without net H⁺ movements was established after the addition of a relatively low bafilomycin A concentration (10 nM) that partially inhibited the H⁺-ATPase. Further inhibition of the H⁺-ATPase with increasing concentrations of bafilomycin A increased the H⁺ conductance of the vacuoles, thus impinging on the measurements of cation-dependent H⁺ movements mediated by YNL321 (Fig. 2D). The addition of 1 μM bafilomycin before the addition of ATP totally abolished the formation pH gradient.
FIGURE 1. **Vacuolar cation-dependent H\(^+\) transport.** Cation-dependent proton movements were monitored by following the fluorescence quenching of acridine orange as described under “Experimental Procedures.” At the indicated times vesicle acidification was initiated by the addition of ATP. After a steady-state acidic-inside pH gradient was attained, the activity of the H\(^+\)-ATPase was partially inhibited by the addition of 10 nM bafilomycin (Baf). At the indicated times (white arrows) 50 mM KCl was added, resulting in a cation-dependent H\(^+\) movement and the alkalinization of the vesicular lumen (recovery of the fluorescence). At the indicated times (black arrows) 5 \(\mu\)M nigericin was added to collapse the \(\Delta\)pH across the tonoplast. Assays were performed with various yeast strains as indicated (A, B, and C).

Noteworthy, from the members of the yeast Vcx family (comprising YDL128w, YDL206w, YJL106w, and YNL321w), only YDL128w, encoding Vcx1p, has been shown to mediate vacuolar Ca\(^{2+}\)/H\(^+\) exchange antiporters (28). Vacuoles of YNL321w displayed Ca\(^{2+}\)/H\(^+\) exchange activity that was comparable with that of vacuoles isolated from wild-type yeast (strain W303-1B). Nevertheless, the vacuolar Ca\(^{2+}\)/H\(^+\) exchange activity was completely abolished in vacuoles isolated from vnx1Δ cells (Fig. 3), indicating that the Ca\(^{2+}\)/H\(^+\) exchange was mediated exclusively by Vcx1p, as demonstrated by Pozos et al. (28), and that YNL321w did not contribute to this activity. Our results show that despite its inclusion in the Vcx family by the YTP data base, YNL321w transports monovalent and not divalent cations.

**Sequence Analysis of YNL321W**—The deduced amino acid sequence indicated that the YNL321 encodes a 908-amino acid polypeptide with a pl of 7.13 and a predicted molecular mass of 102,498 kDa that is very similar to the 106 kDa revealed by immunodetection of the Myc-tagged YNL321w (Fig. 4C). Analysis of the hydropathy profile suggests the presence of 13 putative transmembrane domains and a 242-amino-acid-long hydrophilic N terminus (Fig. 4A). YNL321w belongs to the CAX (calcium exchanger) family, that together with YRGK, NCX, NCKX, and CCX families form the cation/Ca\(^{2+}\) exchanger superfamily (29). We compared the protein sequence of YNL321w with other mem-

FIGURE 2. **Vacuolar cation-dependent H\(^+\) transport.** Cation-dependent proton movements were monitored by following the fluorescence quenching of acridine orange as described under “Experimental Procedures.” At the indicated times vesicle acidification was initiated by the addition of ATP. After a steady-state acidic-inside pH gradient was attained, the activity of the H\(^+\)-ATPase was partially inhibited by the addition of 10 nM bafilomycin (Baf). At the indicated times (white arrows) 50 mM NaCl (---), 50 mM KCl (----), or 50 mM LiCl (-----) were added, resulting in a cation-dependent H\(^+\) movement and the alkalinization of the vesicular lumen (recovery of the fluorescence). At the indicated times (black arrows) 5 \(\mu\)M nigericin (for K\(^+\)) or 5 \(\mu\)M monensin (for Na\(^+\) or Li\(^+\)) were added to collapse the \(\Delta\)pH across the tonoplast. A, assays were performed with wild-type yeast strain. B, vnx1Δ cells. C, vnx1Δ complemented with VNX1. D, inhibition of V-ATPase activity by bafilomycin A. Bafilomycin A 10 \(\mu\)M (-----), 30 \(\mu\)M (-----), or 100 \(\mu\)M (-----) was added where indicated (Baf). One \(\mu\)M bafilomycin A (-----) was added before ATPase activation. (after a steady-state pH gradient was obtained of specific inhibitor bafilomycin A (D). Traces are representative of at least 5 independent experiments.

FIGURE 3. **Cation-dependent H\(^+\) transport in across vacuolar vesicles.** Proton movements were monitored by following the fluorescence quenching of acridine orange as described under “Experimental Procedures” and Fig. 1. At the indicated times 10 \(\mu\)M bafilomycin (Baf) and 6.25 \(\mu\)M CaCl\(_2\) (white arrows) and 5 \(\mu\)M nigericin plus 5 \(\mu\)M KCl were added. Assays were performed with different mutants of the Vcx family: (A) ynl321wΔ, ydl128w (vcx1Δ), the wild-type (W303-1B); (B) ylr106wΔ and ydl128wΔ. Traces are representative of at least three independent experiments.
FIGURE 4. Sequence analysis of YNL321W. A, topological model based on predictions using TMHMM 2.0 program (49). M1–M11 indicate the 11 transmembrane domains similar to other CAXs; Ma and Mb are the two additional transmembrane domains found in the first half of the protein. The gray squares represent the conserved domains (DUF307 and Na_Ca_Ex) identified by Pfam. B, alignment of deduced amino acid sequences of YNL321W (M1–11), ScVCX1, and type-I CAXs from *A. thaliana*. Alignments were performed using ClustalW in the MEGA3 program. Consensus amino acid residues are boxed in black (identical) or light gray (similar). In the acidic domain (AD), acidic residues are represented by bold-faced letters and dark gray boxes. Residues important for calcium transport are indicated by stars. C, immunodetection of c-Myc-tagged YNL321w. Shown are proteins isolated from W303 transformed with empty plasmid (1), W303 expressing c-Myc-tagged YNL321w (2), and ynl321wΔ cells expressing c-Myc-tagged YNL321w (3).
members of this superfamily (Fig. 5). The closest homolog to YNL321W is ScVCX1, a S. cerevisiae vacuolar Ca\(^{2+}\)/H\(^{+}\) antiporter (30). Despite their similarity, ScVCX1 and YNL321W belong to two distinct groups of the CAX family (31). ScVCX1 and the Arabidopsis thaliana AtCAX1–6 form the type I of CAX antiporters, whereas YNL321W belongs to the type II of CAXs that are only present in fungi, Dictyostelium, and lower vertebrates. YNL321W contains some of the special structural features characteristic of the type II CAXs (31); (i) the first half of its protein sequence (amino acids 1–524) contains a conserved domain of unknown function, DUF307, and two predicted transmembrane domains (Fig. 4A); (ii) the second half of the protein (amino acids 525–908) is highly similar to the type I CAXs with 11 predicted transmembrane domains and the conserved domain Na\(_{\text{Ca}}\) exchanger (pfam PF01699) repeated twice, as in all the members of the cation/Ca\(^{2+}\) exchanger superfamily. However, YNL321W has some unique features. First, the acidic motif, a stretch of negatively charged amino acids in the loop between the two Na\(_{\text{Ca}}\)_Ex conserved domains (32), is absent in YNL321W (Fig. 4B). This acidic motif, found in several Ca\(^{2+}\)-binding proteins such as calsequestrin, calreticulin, and the cation/Ca\(^{2+}\) exchangers, is believed to be indicative of Ca\(^{2+}\) transport activity (32). Moreover, several amino acid residues (marked with the asterisk in Fig. 4B) that were identified as crucial for calcium transport in CAXs from Arabidopsis (i.e. Leu-87, Gly-137, Asn-138, and His-

338) and rice are different in YNL321W. These residues in regions named c-1 and c-2 in the CAX family are highly conserved (33), and mutations at some of these specific sites induced loss of Ca\(^{2+}\) transport activity (34).

**Functional Characterization**—Altogether, the transport data and sequence analysis strongly suggested that the protein encoded by YNL321W is a tonoplast-bound monovalent cation/H\(^{+}\) antiporter. We could not designate the YNL321W ORF Vcx (c for cation), this name being already attributed to vacuolar H\(^{+}\)/Ca\(^{2+}\) exchanger 1 or NhX (Na\(^{+}\)/H\(^{+}\) exchanger) due to the lack of similarity between YNL321W and NHX1p. Therefore, we termed YNL321W vacuolar Na\(^{+}\)/H\(^{+}\) exchanger 1 (Vnx1).

To characterize the kinetic characteristics of cation/H\(^{+}\) exchange mediated by Vnx1p, we used the wild-type strain. Given that the disruption of VNX1 induced the total loss of vacuolar cation/H\(^{+}\) exchange activity, then the measurement of this activity in vacuoles of the wild-type strain

![Phylogenetic tree of cation/Ca\(^{2+}\) exchanger superfamily members of S. cerevisiae (Sc), A. thaliana (At), Homo sapiens (Hs), and Escherichia coli (Ec). Appurtenance to one of the three subgroup of CAX family is symbolized by the presence of one, two, or three stars after the name. The tree was inferred with MEGA3 using the UPGMA method. Numbers indicated at the branch nodes are the bootstrap values from 1000 replicates.](image-url)

*Vacuolar Cation/H\(^{+}\) Antipporter*
Vacuolar Cation/H\(^{+}\) Antiporter

**FIGURE 6. Expression of monovalent cation/H\(^{+}\) antiporters in vnx1\(\Delta\) cells.** Proton movements were monitored by following the fluorescence quenching of acridine orange as described under “Experimental Procedures” and Fig. 1. At the indicated times (white arrows) 50 mM NaCl (———), 50 mM KCl (———), or 50 mM LiCl (…………) were added to collapse the ΔpH across the tonoplast, resulting in a cation-dependent H\(^{+}\) movement and the alkalization of the vesicular lumen (recovery of the fluorescence). At the indicated times (black arrows) 5 μM nigericin (for K\(^{+}\)) or 5 μM monensin (for Na\(^{+}\) or Li\(^{+}\)) were added to collapse the ΔpH across the tonoplast. Assays were performed with vnx1\(\Delta\) cells over expressing NHX1 (A) or AtNHX1 (B). Baf, bafilomycin.

**FIGURE 7. Vnx1p-mediated cation/H\(^{+}\) exchange kinetics.** Initial rates of cation-dependent H\(^{+}\) movement were assayed by measuring the initial rates of fluorescence quench recovery after the addition of NaCl or KCl as described under “Experimental Procedures.” Solid lines with filled circles are fitted curves for rates of K\(^{+}\) transport, whereas dashed lines with open filled squares are fitted curves for Na\(^{+}\) transport. Each data point is the mean ± S.D. (n = 3).

pH was generated by the activation of the V-type H\(^{+}\)-ATPase as described under “Experimental Procedures.” Once a steady-state pH difference was attained, the H\(^{+}\) pump activity was stopped by the addition of the specific inhibitor bafilomycin A (35), and the initial rates of cation (Na\(^{+}\), K\(^{+}\) or Li\(^{+}\))-dependent H\(^{+}\) movements were measured. The cation-dependent H\(^{+}\) fluxes displayed a Michaelis-Menten-type saturation kinetics (Fig. 7) with apparent \(K_m\) of 22.4 ± 2.5 and 82.2 ± 16 mM for Na\(^{+}\) and K\(^{+}\), respectively.

**Growth Comparison of vnx1\(\Delta\) and nhx1\(\Delta\) Mutant—**Because of the apparent functional similarity between Nhx1p and Vnx1p, we compared the sensitivities of vnx1\(\Delta\) and nhx1\(\Delta\) mutant strains to hygromycin B and alkali salts (Fig. 8). It was shown previously that yeast lacking genes encoding Nhx1p displayed sensitivity to hygromycin B (36). The high sensitivity of nhx1\(\Delta\) cells to hygromycin B appears to be due to a defective sequestration of toxic cations into intracellular compartments (37), and the mechanism by which Nhx1p confers tolerance to hygromycin B appears to be associated with the role of Nhx1p in intravesicular pH regulation (16). vnx1\(\Delta\) cells also displayed sensitivity to hygromycin B, and the disruption of VNX1 in the nhx1\(\Delta\) strain resulted in the total growth inhibition of the double mutant nhx1\(\Delta\) vnx1\(\Delta\) cells. No growth defect was observed in the single mutant nhx1\(\Delta\) or vnx1\(\Delta\) cells in the presence of increasing concentrations of NaCl (results not shown). Because of the dominant role of the plasma membrane-bound Na\(^{+}\)-ATPases in Na\(^{+}\) extrusion in S. cerevisiae (5), we generated vnx1\(\Delta\) mutants based on W303 1-B ena1-ena4Δ sodium sensitive strain and tested the tolerance of the double mutant to NaCl and LiCl. To minimize the effect of plasma membrane-bound Na+/H\(^{+}\) antiporter, the drop tests were performed at neutral rather than acidic pH (5). After 5 days of incubation at 30 °C in the presence of NaCl, the growth of vnx1\(\Delta\) and nhx1\(\Delta\) disruptants was inhibited similarly, whereas LiCl had a much more inhibitory effect on nhx1\(\Delta\) than on vnx1\(\Delta\) cells. Reverse transcription-PCR showed that the expression of VNX1 correlated well with the respective phenotypes (Fig. 8C). These results would indicate that although Ena1-Ena4 are the main contributors to Na\(^{+}\) homeostasis in S. cerevisiae, there is a contribution of Vnx1p mediating Na\(^{+}\) (and Li\(^{+}\)) transport into the yeast vacuole.

**GFP Localization—**The cation/H\(^{+}\) exchange activity mediated by Vnx1p in isolated vacuoles and tonoplast vesicles and the dependence of Vnx1p activity on the proton gradients built by the V-ATPase (sensitive to bafilomycin A) indicated the vacuolar localization of Vnx1p (Fig. 2). In absence of available antibodies raised against Vnx1p, we expressed the chimera Vnx1p::GFP with the GFP fused at the C terminus of Vnx1p. Under these conditions Vnx1p::GFP fluorescence was detected in the endoplasmic reticulum (ER) (Fig. 9, A and B). This observation was confirmed by the fluorescence of Erg1-GFP, a specific marker of the ER and lipid droplets (38) (Fig. 9, C and D). It has been shown previously that C terminus-tagged proteins can remain trapped in the ER (39), since the presence of a tag (GFP or Myc) at the C terminus often leads to the misfolding of the protein, its retention in the ER, and eventually its degradation by the ER-associated degradation machinery (40). A database of the global analysis of protein localization in S. cerevisiae using GFP::C terminus-tagged proteins (41) showed that Vcx1p, a known vacuolar cation/proton antiporter, remained trapped in the ER. Similar results were reported when Nhxl1p-HA was overexpressed using a 2-μm plasmid using the endogenous NHX1 promoter (17). A search in the database for other vacuolar GFP-tagged proteins showed a partial or full retention of the protein in the ER compartment. Froissard et al. (39) showed that when the tag was introduced inside a central loop of the transport protein, the ER retention was only partial. We constructed a new chimeric gene encoding Vnx1p with a GFP fused to the hydro-
philic loop between the predicted transmembrane domains 7 and 8. Our results showed that although there was an ER signal, indicative of a partial retention of the protein, a vacuolar signal of Vnx1::GFP was detected (Fig. 9, G–L). When GFP was expressed alone, the typical fluorescence in the cytosol was seen (Fig. 9, E and F).

**DISCUSSION**

To date, several transport systems operating to maintain monovalent cation homeostasis in *S. cerevisiae* have been characterized. At the plasma membrane, a Na\(^{+}\)/H\(^{+}\) ATPase (Ena1-Ena4) and a cation/H\(^{+}\) antiporter (Nha1p) mediate Na\(^{+}\)/H\(^{+}\) extrusion out of the cell (4, 5, 42). A pre-vacuolar-bound Na\(^{+}\)/H\(^{+}\) antiporter (Nhx1p) mediates cation/H\(^{+}\) exchange in prevacuoles and possibly in other intracellular compartments (12, 16). More recently, a K\(^{+}\)/H\(^{+}\) exchanger (Kha1p) at the Golgi was reported, and its role in pH regulation was postulated (10, 11). Although in *S. cerevisiae*, vacuolar Na\(^{+}\)/H\(^{+}\) (and K\(^{+}\)/H\(^{+}\)) transport has been attributed mainly to Nhx1p (14), our data and other reports (18, 19) support the notion that Nhx1p is not involved in vacuolar Na\(^{+}\)/H\(^{+}\) exchange and that another transporter is involved in vacuolar monovalent cation homeostasis.

Our strategy, the measurements of cation/H\(^{+}\) exchange activities of all of the knock-out mutants lacking putative cation/H\(^{+}\) antiporters, allowed us to identify Vnx1p, a novel vacuolar monovalent cation/H\(^{+}\) antiporter, and to functionally characterize its activity. The loss of monovalent cation/H\(^{+}\) exchange in vacuoles isolated from *vnx1* mutants, the restoration of the transport activity upon complementation of the *vnx1* mutant with a plasmid bearing *VNX1*, and the localization of Vnx1p to the vacuole provided evidence that *VNX1* encodes a vacuolar monovalent cation/H\(^{+}\) antiporter. Vnx1p belongs to the cation exchanger family (CAX), known to mediate Ca\(^{2+}\)/H\(^{+}\) exchange (31). Our work is the first to identify and functionally characterize a type II CAX transporter, showing that Vnx1p does not transport Ca\(^{2+}\) ions. Interestingly, the lack of Ca\(^{2+}\) transport is correlated with the absence of key amino acids shown to be essential for Ca\(^{2+}\) transport in other CAXs members (33, 34).
Our results clearly indicated that Vnx1 mediated Na\(^+\), K\(^+\), and Li\(^+\) transport into the vacuole and that the lack of Vnx1p increased the salt sensitivity of the cells. Nevertheless, similar to what was shown for nha1Δ and nhx1Δ strains (37), the growth of vnx1Δ single mutants in the presence of 300 mM NaCl or 10 mM LiCl was not affected. On the other hand, the growth of the ena1-ena4Δ vnx1Δ cells was affected in the presence of either NaCl or LiCl, thus indicating the predominant role of Ena1-Ena4 in Na\(^+\) extrusion. S. cerevisiae is able to accumulate different ions in the cell to up to 3–30 times the external concentration, but Na\(^+\) are largely excluded, with cells showing only 30% of the Na\(^+\) concentration of the growth medium (43). Moreover, ionomic analysis of nhx1Δ and nha1Δ cells failed to show any significant differences in ion content relative to wild type, reinforcing the notion that Na\(^+\)-ATPases are predominant in S. cerevisiae Na\(^+\) homeostasis. Vnx1p cells displayed sensitivity to hygromycin B, and the growth of the double mutant nhx1Δ vnx1Δ was halted by hygromycin B, suggesting that, similar to the role of Nhx1p in the pre-vacuolar compartment (16), Vnx1p is also involved in the intra-vacuolar pH regulation.

Although speculative, the potential involvement of Vnx1p in cell division has been postulated (44). Vnx1p was found to be the substrate of the Cdc28p/Clb2p enzymatic complex, and a cell division has been postulated (44). Vnx1p was found to be a low affinity cation/H\(^+\) antiporter encoded by YNL321w. Although YNL321w belongs to the Vcx family of putative type-II vacuolar Ca\(^{2+}\)/H\(^+\) exchangers and contrary to what it has been assumed till now, Vnx1p only mediates the transport of monovalent and not divalent cations. Vnx1p is a low affinity cation/H\(^+\) antiporter with higher affinity for Na\(^+\) than for K\(^+\). The vacuolar-bound Vnx1p appears to play roles in ion homeostasis and intracellular pH regulation, similar to the prevacuolar-bound Nhx1p. Because of its predominant role in vacuolar ion transport, vnx1Δ mutant cells can provide a new and important tool of the heterologous expression and functional characterization of endosomal monovalent cation/H\(^+\) antiporters.

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