VACUOLAR CATION/H⁺ ANTIPORTERS OF SACCHAROMYCES CEREVISIAE

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We previously demonstrated that Saccharomyces cerevisiae vnx1Δ mutant strains displayed an almost total loss of Na⁺ and K⁺/H⁺ antiporter activity in a vacuole-enriched fraction. However, using different in vitro transport conditions, we were able to reveal additional K⁺/H⁺ antiporter activity. By disrupting genes encoding transporters potentially involved in the vnx1 mutant strain, we determined that Vcx1p is responsible for this activity. This result was further confirmed by complementation of the vnx1Δvcx1Δ nhx1Δ triple mutant with Vcx1p and its inactivated mutant Vcx1p-H303A. Like the Ca²⁺/H⁺ antiporter activity catalyzed by Vcx1p, the K⁺/H⁺ antiporter activity was strongly inhibited by Cd²⁺ and to a lesser extent by Zn²⁺. Unlike as previously observed for NHX1 or VNX1, VCX1 overexpression only marginally improved the growth of yeast strain AXT3 in the presence of high concentrations of K⁺ and had no effect on hygromycin sensitivity. Subcellular localization showed that Vcx1p and Vnx1p are targeted to the vacuolar membrane, whereas Nhx1p is targeted to prevacuoles. The relative importance of Nhx1p, Vnx1p, and Vcx1p in the vacuolar accumulation of monovalent cations will be discussed.

In prokaryotes as well as in eukaryotes, the tight control of alkali metal concentrations is necessary for the maintenance of important biological processes, such as pH regulation, enzyme activity, osmoregulation, and signaling (1–3). Cation/proton antiporters play a major role in the control of cytosolic ion concentrations (4–7). In Escherichia coli, NhaA contributes to the maintenance of a low cytosolic Na⁺ concentration and also enables growth in alkaline conditions (8). Based on recent structure determination and experimental data, a detailed model of Na⁺ extrusion has been proposed for NhaA (9, 10). The E. coli genome encodes two other functional Na⁺/H⁺ homologs, NhaB and ChaA. NhaB unlike NhaA transports Na⁺ out of the cell in a pH-independent manner (11). Interestingly, ChaA, a Ca²⁺+/H⁺ antiporter, also catalyzes direct Na⁺/H⁺ (12) and K⁺/H⁺ antiport (13), conferring resistance to the presence of high concentrations of salts (Ca²⁺, Na⁺, or K⁺) in the medium.

Comparatively, in Saccharomyces cerevisiae four Na⁺(K⁺)/H⁺ exchangers have been described so far. Three belong to the monovalent cation proton antiporter superfamily (Nha1p, Nhx1p, and Kha1p) (5, 14, 15), and the fourth and last one to be characterized, Vnx1p, is a member of the type II calcium exchanger family (CAX) of the calcium cation antiporter (CaCA) superfamily (16). Despite its homology with Vcx1p, a well characterized vacuolar Ca²⁺/H⁺ antiporter (17–20), Vnx1p is unable to transport Ca²⁺ into the vacuole. We demonstrated its direct implication in the ΔpH-dependent monovalent cation transport across the yeast vacuolar membrane (16). Indeed, a vacuole-enriched fraction of vnx1Δ cells was totally devoid of exchange activity in the presence of NaCl or KCl, whereas vacuoles from nhx1Δ cells behaved like wild type cells as reported previously (21, 22).

Recently, transport of monovalent cations has been reported for three other members of the CaCA superfamily. It was shown that CAX1 from the chlorophyllian unicellular alga Chlamydomonas reinhardtii mediates vacuolar proton gradient-dependent Ca²⁺ and Na⁺ exchange activity when expressed in yeast (23). Furthermore, co-expression of AtCAX1 and AtCAX3 in yeast cells leads to the formation of “hetero-CAX” complexes able to transport Li⁺ without affecting Ca²⁺ transport rate (24). Finally, expression of AtCCX3 (formerly AtCAX9) in yeast cells induced a high capacity uptake of radioactive Rb⁺ in the vacuolar membrane-enriched fraction, which could be inhibited by an excess of cold K⁺, Na⁺, and Mn²⁺ but not Ca²⁺ (25).

AtCAX9 as well as four other CAX transporters from Arabidopsis (AtCAX7–11) have been reassigned to the cation calcium exchanger family together with a mammalian K⁺/Ca²⁺ antiporter (NCX6) (26, 27), based on sequence homology and the presence of a characteristic α-repeat.

These observations would indicate that yeast CaCA evolved differently from other organisms attributing monovalent or divalent cation transport to two distinct members of the same family. However, we were able to detect a “residual” K⁺/H⁺ exchange activity in the vacuolar fraction extracted from vnx1Δ or nhx1Δnhx1Δ cells by changing the sodium and the potassium chloride, used in the transport assay, for their corresponding sulfate salts. Disruption of potential candidate genes in vnx1Δ background strains permitted us to assign this residual

The abbreviations used are: CAX, calcium exchanger; CaCA, calcium cation antiporter; ER, endoplasmic reticulum; eYFP, enhanced YFP.

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activity to Vcx1p. Overexpression of VCXI, or its inactive
mutant form VCXI-H303A, in the yeast triple mutant
vnxlΔvcx1Δnhx1Δ confirmed this newly discovered transport
activity of Vcx1p.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—The vcx1Δ yeast strains (Table 1)
were generated in the W303-1A background by replacing the
YDL128w ORF with the kanMX6, natNT2, or lph1NT1 gene by
PCR-based gene deletion method (28, 29) using the following
primers: Vcx1-F1 (5-’CGCATATCATTACGCCTGT-GATAGCAAAATAAACAGATGGGCACTAGTGAT-
CTG-3’) and Vcx1-R1 (5-’TTACGAGAGATAAAAAATA-
TAAAAAAAAAGGAATGTTGTCAGCTAGGCTCTGTCGACGC-3’). The deletion constructs contain 40 bp of homology
with the beginning and end of the YDL128w ORF. Yeast strains
were transformed with the deletion cassette using the standard
method (30). Genomic DNA from antibiotic-resistant strains was isolated as described previously (31). Insertion
of the disruption cassette into the correct locus was verified by
PCR.

By double mutant strains were obtained by crossing the strain BY a vcx1Δ with strain BY from the other disruption
mutants listed in Table 1 to generate diploid strains. Diploids
were allowed to sporulate and were then subjected to tetrad
segregation to generate haploid strains, according to Guthrie
and Fink (32). All yeast strains used in this study are listed in Table 1. Yeast cells were grown in YPD (1% yeast extract, 2% peptone,
2% glucose), YPG (1% yeast extract, 2% peptone, 2% galactose),
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 (33). The intact
cavocules were collected on top of the gradient and resuspended
in 5 mM Tris/Mes (pH 6.9). Protein concentrations were deter-
mined by the Bio-Rad DC protein assay according to the man-ﬁ;ufacturer’s protocol.

Transport Assays—The fluorescence quenching of acridine
orange was used to monitor the establishment and dissipation
of vacuolar inside acidic pH gradients (supplementary Fig. 1S).
Intact vacuoles were added to a buffer containing 50 mM tetra-
 methylammonium 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 2.5 mM Tris-ATP, and time-depen-
dent activity to Vnx1p, a Vacuolar K+/H+ Antipporter

ATGCA-3’) were used to amplify, from the commercial vector
pYES-DEST52 (Invitrogen), a DNA fragment starting from the
GAL1 promoter to the CYC1 terminator that includes the
Gateway® recombination cassette and the V5 and the His6
epitopes. This PCR product was then inserted in the SmaI
restriction site of pRS306. The Vcx1 coding sequence was
cloned by PCR using the Phusion® hot start high fidelity DNA
polymerase (Finnzymes, Espoo, Finland). The PCR was per-
formed on genomic DNA with primers Vcx1-For (5-’CAGCA-
CATGGATGCAAATACCCCCAC-3’) in combination with
Vcx1-Rev (5-’TCAAACTATTACCAATAGTC-3’) for the full
ORF or Vcx1-Tag-Rev (5-’TAACTCATTTCCAATAA-
GACT-3’) for the Vcx1-TAG fusion construct. Each resulting
PCR product was then in pENTR SD/D TOPO and then in pDEST vectors as described by the manufacturer
(Addgene). All pAG destination vectors (37) used in this study
are available via Addgene.

Site-directed Mutagenesis—The single point mutant Vcx1p-
H303A was generated using the QuikChange® II site-directed
mutagenesis kit (Stratagene) according to modiﬁcations
described by Wang and Malcom (38). The complementary
primers used to create Vcx1-H303A were as follows: Vcx1-
H(303)A-For (5-’GGGTATAGCGGCAGACCTCTT-
CATGCCAG-3’) and Vcx1-H(303)A-Rev (5-’CCAAGACTG-
AGTGCACGTTTGCCAGATTACC-3’). Underlined
letters indicate the changes in nucleotide sequence, and bold-
face letters indicate the introduced silent restriction site BglII.

Growth Tests—Yeasts were grown in YPD medium to satu-
ration 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. 5 μl of each dilution were spotted onto YPD, or
YPG, media supplemented with salts or hygromycin B. Growth
was assessed at 30 °C after 2–5 days.

Fluorescence Microscopy—A Nikon Eclipse 50i ﬂuorescent
microscope was used to visualize the transformed cells. GFP-
and eYFP fused proteins were visualized by using B-2A filters
(excitation filter, 450–490 nm; Dichromatic Mirror cut-off
wavelength, 500 nm; Barrier filter wavelength, 515-nm long
pass filter). All images were taken at ×100 magnification, with a
Nikon DS-Fi1 CCD camera.

RESULTS

Detection of an Additional Vacuolar Cation/H+ Exchange
Component—We previously showed that Vnx1p accounts for the
monovalent cation/H+ antipporter activity from isolated yeast
vacuoles as vnx1Δ mutants displayed a total loss of Na+ (K+)/H+
antipporter activity (16). By replacing chloride salts (Fig. 1A)
with sulfate salts (Fig. 1B), or gluconate salts, we were able to
reveal another type of K+ /H+ antipporter activity in the vacuolar
fraction extracted from vnx1Δnhx1Δ cells (Fig. 1B) as well as
from vnx1Δ single mutant cells (data not shown). A similar
background activity, using the OC02 strain (vnx1Δnhx1Δ), can
be seen in the study of Hernandez et al. (39). Compared with the
activity that is dependent on the presence of Vnx1p, the
exchange activity was much lower. Also in the wild type strain
W303-1A, the relative magnitude of K+ /H+ antipporter
is dependent on the presence of chloride or sulfate salts. In the
presence of chloride salts, antipporter activity is higher with Na+
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FIGURE 1. Salt-dependent cation/H\textsuperscript{+} transport across the vacuolar membrane. Proton movements were monitored by following the fluorescence quenching of acridine orange as described under “Experimental Procedures” and supplemental Fig. 1S. At the indicated times, vacuole acidification was initiated by the addition of ATP. After a steady-state acidic inside pH gradient was attained, the activity of the H\textsuperscript{-}ATPase was partially inhibited by the addition of bafilomycin (Baf). A and B, cation/proton antiport using isolated vacuoles of the OC02 yeast strain (nxi1\textDelta, vnx1\textDelta) was monitored as the recovery of fluorescence quench upon addition of 100 mM of Na\textsuperscript{+} or K\textsuperscript{+} (white arrows). At the indicated time, 20 mM of (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} was added to collapse the \DeltapH across the vacuolar membrane to recover 100% of the fluorescence (black arrows). Traces are representative of at least three independent experiments. C, effect of the counter anion on the transport rate has been measured in isolated vacuoles from the wild type strain W303. The initial rates of cation-dependent H\textsuperscript{+} movement were assayed by measuring the initial rates of fluorescence quench recovery after addition of 100 mM chloride salts or 50 mM sulfate salts as indicated in the figure. Data represent the means \pm S.D. of six measurements from three independent preparations. Bars with different letters indicate values that are significantly different when compared with the other three conditions tested as determined by a two-tailed t test (p < 0.009) (GraphPad software).

as compared with K\textsuperscript{+}, although in the presence of sulfate salts this is inverted (Fig. 1C). Together, these data suggest the presence of one or several other vacuolar membrane-bound transporter(s), distinct from Nhx1p and Vnx1p, and are able to drive the transport of K\textsuperscript{+} in a pH-dependent manner. However, it is not clear why the detection of this activity is dependent on the counter anion used during the transport assay. It is possible that the relatively permeant Cl\textsuperscript{-} and H\textsuperscript{+} ions induce passive leaks that mask directly coupled K\textsuperscript{+}/H\textsuperscript{+} antiport. In this sense, addition of the more permeant monovalent Cl\textsuperscript{-} ions compared with SO\textsubscript{4}\textsuperscript{2-} ions would lead to a dissipation of the inside positive membrane potential created by the H\textsuperscript{-}ATPase, which would counteract H\textsuperscript{+} efflux from the vesicles, and thus mask antiporter activity.

Identification of Vcx1 as K\textsuperscript{+}/H\textsuperscript{+} Antiporter—Based on the same strategy used to identify Vnx1p, where we screened single disruption mutants, we screened double disruption mutants to identify the gene(s) coding for the transporter responsible for the residual K\textsuperscript{+}/H\textsuperscript{+} activity in the vacuolar membrane preparation of the VNX1 disruption mutant. We tested three candidate genes, KHA1, a monovalent/cation antiporter of the CPA2 family localized in Golgi membranes (15), and Vcx1 and YDL206w, two of the remaining CaCA family proteins besides Vnx1. Each disruption mutant of candidate genes was crossed with the BY Mat a vnx1\textDelta strain. The resulting diploids were then sporulated and micro-dissected to collect the spores. The progeny of G418-resistant spores presenting a 2:2 segregation pattern was then tested for mating type assignment. To be consistent with the previous transport experiments, only mating type a strains were conserved. Freshly extracted vacuoles were isolated from the above mentioned double mutant strains and tested for their ability to mediate vacuolar alkalinization upon addition of 50 mM K\textsubscript{2}SO\textsubscript{4}. Surprisingly, the K\textsuperscript{+}/H\textsuperscript{+} antiporter activity was abolished only in the strain harboring the vnx1\textDelta disruption (Fig. 2). Vcx1p has been characterized before as a low affinity Ca\textsuperscript{2+}/H\textsuperscript{+} antiporter (40–42), but to our knowledge nothing has been mentioned about the role of Vcx1p in K\textsuperscript{+} transport against protons at the vacuolar membrane. Therefore, to ensure that the transport activity was dependent on Vcx1 expression, this gene was disrupted in the OC02 W303-1A background strain to generate a triple mutant OC05 (Table 1), using a PCR-based method (28, 29). The disruption was confirmed, first by PCR on genomic DNA to ensure the correct integration of the selection marker hphNT1 and then by testing the Ca\textsuperscript{2+}/H\textsuperscript{+} exchange across the vacuolar membrane.
of isolated vacuoles compared with controls, W303 and OC02, respectively. Disruption of VCX1 completely abolished the exchange of Ca\(^{2+}\) in the enriched vacuolar fraction confirming what had been described before (Fig. 3A) (16, 18), and it also abolished the residual K\(^{+}/H^{+}\) exchange activity observed in the presence of sulfate salts, confirming the results obtained using the BY strains (Fig. 3B). No transport is observed using NaCl and KCl in isolated vacuoles from \(\text{nhx1}/\Delta\;vnx1/\Delta\;vcx1/\Delta\) mutant strains (Fig. 3C). Overexpression of VCX1 under the control of the GAL1 promoter in this yeast strain restores all exchange activities in isolated vacuoles only when grown in the presence of galactose as a carbon source (Fig. 4A). Overexpression of VCX1 not only restores transport, but Ca\(^{2+}\) and K\(^{+}\) transport rates are even higher when compared with the OC2 strain (\(\text{nhx1}/\Delta\;vnx1/\Delta\)) (Fig. 4A). This clearly indicates that monovalent and divalent transport activities are catalyzed by Vcx1p.

To further test the involvement of Vcx1p in K\(^{+}/H^{+}\) antiporter activity, we constructed an inactivated mutant enzyme. Several amino acid residues were identified as crucial for calcium transport in CAXs from plants (43, 44). These residues are highly conserved among the different species. Expression in yeast of a mutated \(\text{AtCAX1}\) or \(\text{OsCAX1a}\), in which histidine at position 338 or 330, respectively, has been replaced by an alanine did not rescue yeast Ca\(^{2+}\) sensitivity or vacuolar transport activity. Using PCR-based site-directed mutagenesis, we changed the corresponding conserved His\(^{303}\) in Vcx1p for Ala (supplemental Fig. 2S). Overexpression of this mutant enzyme did not restore Ca\(^{2+}\) and K\(^{+}\) cation transport in the \(\text{nhx1}/\Delta\;vnx1/\Delta\) yeast strain, indicating that this substitution found in the C-2-specific domain is essential for general functioning (Fig. 4B). Similarly to what has been described previously, expression of wild type VCX1 in the K667 calcium-sensitive strain did confer calcium tolerance, whereas VCX1-H303A did not (Fig. 5A).
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FIGURE 4. Transport activity measured in isolated vacuoles of the W303 triple mutant strain complemented with VCX1 genes and derivatives. The yeast strain W303 nhx1Δvnx1Δvcx1Δ was transformed with the yeast integrative vectors pRS306 Gal1 or pAG306 eYFP into which VCX1 or VCX1-H303A had been previously introduced by recombination as described under “Experimental Procedures.” The correct genomic integration of plasmids was verified by PCR. Acridine orange fluorescence recovery mediated by Vcx1p was measured in vacuoles isolated from the cell expressing VCX1 (A), Vcx1p-H303A (B), and eYFP·Vcx1p (C), by the addition of 50 mM Na₂SO₄ (---), 50 mM K₂SO₄ (---), or 12.5 μM CaCl₂ (---), was monitored after the establishment of a pH gradient and partial inhibition of the H⁺-ATPase by bafilomycin A₁ (Baf). Traces are representative of three experiments using independent membrane preparations from independent clones.

FIGURE 5. Growth of various mutant strains expressing VCX1 and VCX1-H303A in the presence of toxic concentrations of cations. Both VCX1 and VCX1-H303A were introduced into the yeast shuttle vector pYES-DEST52 using Gateway recombination as described under “Experimental Procedures.” Each construct was introduced into K667 (cnb1Δ mpc1Δ vcx1Δ Δ) and AXT3 (ena1Δ ena4Δ) mutant strains. Each strain was grown, and serial dilutions were made as described under “Experimental Procedures.” Five microliters of each dilution were spotted onto YPG medium with an addition of a high concentration of CaCl₂ (A) or K₂SO₄ (B) as indicated. Plates were incubated at 30 °C for 2–5 days.

less K⁺-sensitive yeast (ena1Δ ena4Δ) did not result in any growth delay (data not shown) indicating only a minor contribution of Vcx1p to high K⁺ tolerance. Neither the disruption of VCX1 nor its overexpression in a nhx1Δ strain resulted in an increase of sensitivity or resistance toward hygromycin (data not shown).

Does the Point Mutation in Vcx1p Change Its Subcellular Localization?—Failure of the mutant enzyme to confer resistance to high K⁺ concentrations or to catalyze K⁺/H⁺ antiport in vacuolar fractions could also be due to a mislocalization of the enzyme. In yeast, a single point mutation can lead to a retention of the mutant protein in the ER (45), where it will be degraded (46). In addition to the potential mislocalization related to the mutation, accumulation of membrane proteins in the ER due to overexpression using high copy shuttle vectors or to the fusion of GFP to the C terminus of the protein has been reported (16, 47, 48). According to the Yeast GFP Fusion Localization Database, the chromosomal C-terminal Vcx1p·GFP-tagged fusion protein was predicted to be localized in the ER (Fig. 6A) (48). Therefore, we checked the correct localization of Vcx1p and Vcx1p·H303A by fusing it to a fluorescent protein. Fusion of eYFP to the N terminus of Vnx1 (eYFP·Vnx1p) or Vcx1 (eYFP·Vcx1p) gave us a vacuolar membrane-bound localization without any residual signal trapped in the ER (Fig. 6, B and C). Yeast cells (OC05) expressing the chimeric construct eYFP·VCX1-H303A (Fig. 6D) displayed a vacuolar membrane localization identical to controls. The exact pattern of localization was observed in all the yeast strains used in this study (data not shown). The addition of the GFP at the N terminus of Vcx1p did not affect its ability to mediate cation accumulation in the vacuolar lumen (Fig. 4C). Typical localizations in the cytosol, the vacuolar membrane, the PVC, and vacuolar lumen were observed by expression of eYFP alone, Vph1p-GFP, Nhx1p-GFP, and CPY-GFP respectively (Fig. 6, E–H). Altogether, these data confirm that the absence of Ca²⁺ and K⁺ tolerance observed in yeast cells expressing the mutated version of Vcx1p was due to inactivation and not mislocalization or degradation.

Inhibition of Vcx1p K⁺/H⁺ Exchange Activity by Cd²⁺ and Zn²⁺—Vcx1p is a Ca²⁺/H⁺ antiporter whose activity is strongly inhibited by Cd²⁺ and to a lesser extent by Zn²⁺ (19). Therefore, the effect of these ions on K⁺/H⁺ exchange mediated by Vcx1p was tested. Use of 200 μM Zn²⁺ or Cd²⁺ induced considerable leakage of H⁺ similar to what can be observed when bafilomycin A₁, a specific inhibitor of the V-ATPase, is added in excess (supplemental Fig. 25) (16). These results are supported by previously published data showing a strong non-competitive inhibition of the V-ATPase by Zn²⁺ (49). Therefore, we used a lower concentration of inhibitors (100 μM) to allow for the measurement of transport activity related to the addition of CaCl₂ or K₂SO₄. We also ensured that the integrity of the vacuoles was not affected by the high concentration of toxic heavy metal (supplemental Fig. 25). For more convenience, the inhibition experiments were conducted with vacuoles isolated from the vnx1Δ mutant strain (BY background). The alkalination of vacuoles isolated from cells expressing Vcx1 induced by the addition of 10 μM Ca²⁺ was completely inhibited in the presence of 100 μM Cd²⁺ (Fig. 7, A and B) and strongly inhibited when equal amounts of both cations were used (Fig. 7C) as compared with control conditions (Fig. 7D). Interestingly, the K⁺/H⁺ exchange reaction induced by the addition of 100 mM K⁺ was completely inhibited in the pres-
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Although the influx and the efflux of K⁺ across the plasma membrane have been well documented in the last few decades (for review see Refs. 2, 52), little is known about the mechanisms controlling the vacuolar pool of K⁺.

Until recently, monovalent cation/H⁺ exchange was ascribed to transporters of the cation proton antiporter superfamily. The E. coli ChaA antiporter was the only example of a CaCA family member able to promote movement of Ca²⁺, Na⁺, and K⁺ (12, 13). Despite this example, the sequence comparison and homology analysis, as well as experimental data, attributed distinct ion selectivity to each superfamily. However, within the last 3 years several other members of the CaCA superfamily have been shown to be involved in the vacuolar compartmentalization of alkali ions (16, 23, 53). To our knowledge, Vnx1p was the first eukaryotic CaCA antiporter homolog to be characterized as a monovalent/H⁺ exchanger (16). In this previous work, Vnx1p was described as the only antiporter responsible for the accumulation of alkali cations against protons in the vacuolar lumen. However, by using sulfate instead of chloride salts, it is now shown that the monovalent cation transport in vacuoles is a composite of several activities (Fig. 1C). A reverse genetic approach allowed us to identify a double mutant vnx1Δvcx1Δ strain apparently devoid of all vacuolar K⁺/H⁺ antiporter activity, indicating that Vcx1p was catalyzing K⁺/H⁺ exchange. The role of Vcx1p was further confirmed by overexpression of Vcx1 and Vcx1-H303A, an inactivated version, in various yeast strains. Unlike the wild type gene, the His303 substituted Vcx1 mutant did not complement the Ca²⁺- or K⁺-sensitive strain K667 or AXT3, respectively. In accordance with these complementation data, transport assays performed with isolated vacuoles of the mutant strains OC5 overexpressing both versions of the gene (Fig. 4) showed that the wild type protein displayed Ca²⁺/H⁺ and K⁺/H⁺ antiporter activity, although the mutant protein was inactive. Moreover, both Ca²⁺ and K⁺ transport activities mediated by Vcx1p are strongly affected by the presence of Cd²⁺ and to a lesser extent by Zn²⁺.

The fact that the additional K⁺/H⁺ antiport can only be observed using sulfate salts can have several causes. First, it is possible that Cl⁻ or SO₄²⁻ specifically modify antiporter activity. Second, specific anion transport systems in the vacuolar membrane, for instance a Cl⁻:H⁺ symporter, could be responsible for anion-coupled H⁺ influx, thus masking H⁺/K⁺ antiporter activity. This would point to the physiological significance of the differences observed. However, using several purified plant antiporters reconstituted in liposomes, we gen-
In that sense, addition of relatively permeable Cl\(^-\) ions to the system, real antiporter activity can only be adequately measured using relatively impermeant anions like sulfate or gluconate.

**Role of Vcx1p, Vnx1p, and Nhx1p in Vacuolar Ion Compartmentalization**—The vacuole is the main calcium store and sink of yeast cells (54). Vcx1p along with Pmc1p (a high affinity Ca\(^{2+}\) ATPase) were shown to play a key role in the vacuolar translocation of Ca\(^{2+}\) by a cytosolic spike induced by various stimuli (55–57). In addition to regulating calcium homeostasis, in this paper we show that Vcx1p along with Vnx1p play a role in the sequestration of cytosolic K\(^+\) into the vacuole. Like Ca\(^{2+}\), K\(^+\) is mainly stored in the vacuoles. The ratio \([K^+]_{cyt}/[K^+]_{vac}\) is close to 1:8 during the exponential growth phase but would appear to be inferior during the stationary phase (58). A third antiporter that has been proposed to be involved in vacuolar K\(^+\) and Na\(^+\) sequestration is NhxA1p. Although, Vnx1p and Vcx1p are clearly shown to be targeted to the vacuolar membrane, the NhxA1 protein resides in the prevacuolar compartment (44, 56, 58–61). Accordingly, we could not detect an effect of NHX1 disruption on vacuolar Na\(^+\)/H\(^+\) or K\(^+\)/H\(^+\) exchange activity (16). Despite this, the biochemical characterization of several vacuolar antiporters from plants and green alga has been carried out using a yeast strain disrupted on NHX1 only. According to these studies, the enriched vacuolar fraction used for transport assays did not have any significant Na\(^+\)/H\(^+\) or K\(^+\)/H\(^+\) exchange activity despite the presence of Vnx1p and Vcx1p (23, 25, 53), although cells expressing NHX1 did have antiporter activity (23). In these studies yeast vacuolar membrane vesicles were prepared using a two-step sucrose gradient as previously described by Nakaniishi et al. (59). These results are in contradiction with data obtained with vacuoles isolated by the Ficoll (polysucrose) floating method (16, 21, 22, 39). The most plausible explanation for detection of NhxA1 activity in vacuolar membranes isolated by the two-step density gradient centrifugation is that these membranes are contaminated with many other endosomal membranes, although the Ficoll floating method could give rise to a purer vacuolar preparation. Recently, Wiederhold et al. (60) published an S. cerevisiae vacuolar membrane protein using vacuoles purified with a similar Ficoll floating method. A subtractive proteomic approach (LOPIT) with iTRAQ labeling was used to distinguish vacuolar proteins from contaminants and allowed the identifi-

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\(^5\) K. Venema, unpublished results.
This result shows that Vcx1 and Vnx1 proteins are both ubiquitous and that Vnx1 proteins are not homoplasic as suggested by De Herdtog et al. (51).

**Two Amino Acids Might Determine Alkali Cation Selectivity**—Two highly conserved sequence repeats from CAX proteins, named c1 and c2, which were first identified in plants, were shown to be involved in cation translocation (43). The newly discovered K\(^+\)/H\(^+\) exchange activity of Vcx1p, along with data on CrCAX1 and Vnx1p transport specificity, could be related to the potential role of two amino acids of these conserved regions in ion selectivity (supplemental Fig. 2S). In the c-1 region, a serine residue conserved in CAX1 of *C. reinhardtii* and Vnx1p of *S. cerevisiae*, both known to transport Na\(^+\) (16, 23), is absent from Vcx1-like protein sequences. Moreover, the heterodimer formed between CAX1 and CAX3 from *Arabidopsis thaliana*, bearing this conserved serine, appears to promote the transport of Li\(^+\) and/or Na\(^+\) (53). Consequently, this serine residue could be important for Na\(^+\) transport. Similarly, a conserved aspartate residue in the c-2 region of Vcx1-like proteins from yeast is substituted by an asparagine at the corresponding position in CrCAX1 and CrCAX2. This repartition suggests that an aspartate or an asparagine at this position could determine the ability of the protein to transport K\(^+\) or Na\(^+\). The alignment of the c-1 and c-2 regions also reveals that one AtCAX subgroup (AtCAX1, AtCAX3, and AtCAX4) is closer to CrCAX1, and the other subgroup (AtCAX2, AtCAX5, and AtCAX6) has more similarities to Vcx1p (supplemental Fig. 2S) and could reflect their potential ability to transport Na\(^+\) or K\(^+\), respectively. The fact that these two residues are in general conserved in species belonging to different kingdoms such as plants and yeast but not in all CAXs members of *A. thaliana* reinforces the idea that they could be functionally important. Other residues have also been suggested to play a role in cation selectivity such as Met\(^{170}\) and Cys\(^{150}\) in CrCAX1 (supplemental Fig. 2S) (23). A site-directed mutagenesis approach could easily confirm whether the exchange of one or two amino acids is sufficient to change the ion selectivity of the transporter.

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**Sequence Analysis of Yeast CAX Antiporters**—The Genolevure website (50) reveals that most yeast species contain two types of Na\(^+\)/Ca\(^{2+}\) exchangers identified by the Pfam PF01699 conserved domain as follows: the Vcx1p-like (type I CAX) and the Vnx1p-like transporters (type II CAX) (supplemental Fig. 5S). One exception is the yeast *Yarrowia lipolytica*, which also has a third antiporter, quite different from the two others although it contains the PF01699 conserved domain. Interestingly, in the YETI data base, *Y. lipolytica* and *Candida glabrata* appear to contain two Vcx1-like proteins but no Vnx1p. However, a new amino acid sequence alignment revealed that for each species one of the Vcx1p is classified together with the other Vnx1 proteins (YAL10D24233g and CAGL0M04147g).

**FIGURE 9. Model for cation accumulation in the vacuole.** Vacuolar membrane-bound antiporters Vcx1p and Vnx1p use the proton gradient generated by the V-ATPase to energize the transport of cations into the lumen of the main vacuole. The contribution of the pre-vacuolar alkali cation/H\(^+\) antiporter, Nhx1p, in the accumulation of K\(^+\) and Na\(^+\) remains to be determined. Nhx1p could play a role in the loading of the PVC from which vesicles containing ions are sent to the main vacuoles and therefore contribute to the accumulation of ions in the vacuolar lumen. Knowing the importance of Nhx1p in the control of the vesicular trafficking, this antiporter could play a role in the targeting of uncharacterized cation transporters (7) to the vacuolar membrane and indirectly affect ion homeostasis of the vacuole.
