Novel Localization of a Na\(^+\)/H\(^+\) Exchanger in a Late Endosomal Compartment of Yeast

IMPLICATIONS FOR VACUOLE BIOGENESIS

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Na\(^+\)/H\(^+\) exchangers catalyze the electrically silent countertransport of Na\(^+\) and H\(^+\), controlling the transmembrane movement of salt, water, and acid-base equivalents, and are therefore critical for Na\(^+\) tolerance, cell volume control, and pH regulation. In contrast to numerous well studied plasma membrane isoforms (NHE1–4), much less is known about intracellular Na\(^+\)/H\(^+\) exchangers, and thus far no vertebrate isoform has shown to have an exclusively endosomal distribution. In this context, we show that the yeast NHE homologue, Nhxl (Nass, R., Cunningham, K. W., and Rao, R. (1997) J. Biol. Chem. 272, 26145–26152), localizes uniquely to prevacuolar compartments, equivalent to late endosomes of animal cells. In living yeast, we show that these compartments closely abut the vacuolar membrane in a striking bipolar distribution, suggesting that vacuole biogenesis occurs at distinct sites. Nhxl is the founding member of a newly emergent cluster of exchanger homologues, from yeasts, worms, and humans that may share a common intracellular localization. By compartmentalizing Na\(^+\), intracellular exchangers play an important role in halotolerance; furthermore, we hypothesize that salt and water movement into vesicles may regulate vesicle volume and pH and thus contribute to vacuole biogenesis.

Na\(^+\)/H\(^+\) exchangers of eukaryotic cells comprise a family of membrane proteins catalyzing the electroneutral countertransport of Na\(^+\) and H\(^+\) (1–3). At the plasma membrane of animal cells, the prevailing Na\(^+\) gradient generated by the Na\(^+\)/K\(^+\) ATPase is used to drive H\(^+\) equivalents from the cell. As such, these exchangers are involved in the regulation of intracellular pH, cell volume control, and transcellular Na\(^+\) movements in epithelial tissue. These functions are closely related to physiological and pathophysiological cellular events, including fertilization, cell cycle control, differentiation, essential hypertension, gastric and kidney disease, and epilepsies. Na\(^+\)/H\(^+\) exchange activity has been detected in virtually every cell type and are therefore critical for Na\(^+\) tolerance, cell volume control, and pH regulation.

However, it is the C-terminal domain that carries multiple prolines and is sensitive to inhibition by amiloride and its derivatives (5). Nevertheless, there has been biochemical documentation of Na\(^+\)/H\(^+\) exchange activity in endosomal preparations from kidney, liver, zymogen granules of pancreatic acinar cells, and chromaffin granules of adrenal glands (6–11). In each case, the exchange activity was reported to coexist with a distinct subset (~20%) of vesicles containing the vacuolar H\(^+\)-ATPase and to exhibit kinetic similarity with the plasma membrane exchangers with respect to reversibility, simple hyperbolic response to Na\(^+\), and allosteric activation by H\(^+\). However, amiloride did not inhibit the endocytic exchange activity, Li\(^+\) was a poor substrate but a good inhibitor of Na\(^+\)/H\(^+\) exchange, and the K\(_m\) for Na\(^+\) was somewhat lower than that seen for plasma membrane isoforms (4.7–10 mM versus 15–18 mM), suggesting that the endocytic exchanger is a distinct molecular isoform.

In earlier work, we have shown that the NHXI gene of Saccharomyces cerevisiae mediates sequestration of Na\(^+\) within an intracellular compartment, suggestive of a novel intracellular localization (12). Here, we provide direct evidence that Nhxl localizes exclusively to a unique late endosomal compartment, thus providing a starting point to explore the molecular, cellular, and physiological functioning of a completely novel member of this family of transporters. We have also observed the emergence of new exchanger homologues in other organisms, as a result of systematic sequencing efforts worldwide, that share greater homology with yeast Nhxl than to the plasma membrane isoforms. We suggest that the sequence similarities among these newly discovered isoforms is indicative of a common intracellular, possibly endosomal localization.

EXPERIMENTAL PROCEDURES

Yeast Strains and Recombinant DNA Techniques—Strains K601 (wild type) and R100 (Δnhx1) used in this study are isogenic to W303 and have been described (12). A 4.5-kilobase pair (kb) SalI insert containing the intact NHXI gene was recovered from cosmid C9410

† The abbreviations used are: kbp, kilobase pair; PCR, polymerase chain reaction; HA, hemagglutinin; GFP, green fluorescent protein; PVC, prevacuolar compartment.
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Epitope-tagged and Plasmid-encoded Nhx1 Is Fully Functional and Induced by NaCl—Targeted disruption of the S. cerevisiae NHX1 (YDR456w) gene leads to loss of sodium tolerance in acidic (Fig. 1a) but not neutral or alkaline medium (12), consistent with the expected properties of a H\(^{+}\) driven Na\(^{-}\) transporter. The NHX1 gene was recovered from a 40-kilobase pair genomic insert in cosmID C9410 (see “Experimental Procedures”), and the open reading frame was tagged at the C terminus with either an HA tag or the GFP tag. Expression of the tagged constructs was directed from the endogenous NHX1 promoter in a Δnhx1 strain of yeast. Both tagged constructs appeared to be fully functional, effectively complementing the Na\(^{-}\)-sensitive phenotype of the Δnhx1 mutant in the single copy (CEN) as well as multicopy (2\(\mu\)) plasmid versions (Fig. 1a). Like other members of the NHE family, yeast Nhx1 is predicted to be an integral membrane protein, with an N-terminal domain of 12 transmembrane helices, followed by a C-terminal cytoplasmic tail (12). Differential centrifugation of yeast lysates results in a substantial enrichment of Nhx1:HA (molecular mass, 73.5 kDa) in low speed membrane pellets, whereas it clearly fractionated away from markers (Fig. 1b). In the absence of further fractionation, the Nhx1 polypeptide characteristically migrates as multiple bands on SDS-polyacrylamide gel electrophoresis of cell lysates, indicating post-translational modifications such as phosphorylation or glycosylation. Further evidence of the involvement of Nhx1 in halotolerance comes from salt induction of expression (Fig. 1b).

**Biochemical Methods—Assays of Protein, α-Mannosidase Activity, and Azide-Sensitive ATPase Activity** have been described in earlier publications (14, 15) and in references therein. Differential centrifugation of yeast lysates and nonequilibrium fractionation of yeast lysates, as described (14, 15), was performed as described previously (14). Antibodies were used as follows: mouse anti-HA antibody, 12C5 (Boehringer Mannheim); anti-Vph1 monoclonal antibody 10D7-A7-B2 (Boehringer Mannheim); mouse anti-Vph1 antibody, 12CA5 (Boehringer Mannheim); goat anti-Kex2 antibody, gift of Robert Fuller, University of Michigan; rabbit anti-Pep12 antibody (gift of Robert Piper, University of Iowa); rabbit anti-Pma1 antibody (gift of Carolyn Slayman, Yale University); rabbit anti-His antibody (Invitrogen); rabbit anti-HA monoclonal antibody (gift of Susan Michaelis, Johns Hopkins University) containing the triple HA epitope as the template. The resulting 1.9-kbp product was digested with 

\[
5\text{'-CACGAGCTCGTCTTCATCCATGACGGAAG-3'}
\]

and 

\[
5\text{'-AGAAATCTGCAGG-3'}
\]

The final PCR product was digested with the same sites in pRin72. To complete the full-length Nhx1::HA with the 1.9-kbp upstream sequence from the C9410 as template: 5\text{'-GACGTTCCAGATTACGCTGCTGAGTGCTAG-3'} (sense) and 5\text{'-GG-CACGAGCTCGTCTTCATCCATGACGGAAG-3'} (antisense). The final PCR product was used to digest the 1.3-kbp plasmid pSM491 (gift of Susan Michaelis, Johns Hopkins University) containing the triple HA epitope as the template. The resulting 1.9-kbp product was digested with SpeI and SacI and cloned into pRin72 to give the full-length Nhx1::HA with the 1.9-kbp upstream sequence from the initiating codon ATG and the 1-kbp downstream sequence from the termination codon (pRin73). The Nhx1::GFP construct was created by digesting pEGFP-N3 (CLONTECH) with BamHI and NotI to release the 0.7-kilobase GFP and ligating to the same sites in pRin72. To complete the Nhx1 open reading frame, a 1.3-kbp BamHI fragment from pRin73 was inserted in the correct orientation into this plasmid, creating the full-length fusion.

**Confocal Microscopy—Cells (0.7–1.2 x10^6 units/ml) were labeled with 53 μM FM 4-64 (N-3-triethylammoniumpropyl)-4-(6-[4-diethylaminophenoxy]ethyl)tetramethylindolenine), 13 μM DiOC(3,3'-dihexyloxacarbocyanine iodide), and 40 nM MitoTracker Red CMXROS (all from Molecular Probes) using a variable labeling period (10–60 min) followed by chase in fresh medium (30–60 min). Confocal microscopy was performed by the Noran Oz Confocal Microscope System; single label controls for each fluorophore were captured under identical double label conditions to eliminate any fluorescence bleed-through.

**RESULTS**

**Colocalization of HA-tagged Nhx1 with Vacular and Pre-vacular Markers in Subcellular Fractions**—Our measurements of steady state intracellular 22Na levels indicated that enhanced sequestration of Na\(^{+}\) via Nhx1 correlated with salt-tolerant growth (12). By analogy with observations of vacuolar compartmentation of salt in halotolerant plants (17, 18), we hypothesized that Na\(^{+}\) transport by Nhx1 was likely to be coupled to the vacuolar H\(^{+}\) pump in an acidic compartment. Here, we show that HA-tagged Nhx1 colocalizes with markers for the vacuole, prevacuolar compartment, and the late Golgi compartment on sucrose density gradients (Fig. 2a), whereas it clearly fractionated away from markers representing the endoplasmic reticulum, plasma membrane, and mitochondria, pointing to a hitherto novel cellular loca-
tion for a Na\(^{+}/H^{+}\) exchanger. To distinguish between pre-vacuolar, vacuolar, and Golgi distributions, subcellular fractions from sequential centrifugation of yeast lysates were analyzed by gel electrophoresis (Fig. 2b). Fractionation of Nhx1 closely followed that of the vacuolar marker, Vph1 (a subunit of the vacuolar H\(^{+}\)-ATPase), and that of Pep12 (a prevacuolar syntaxin), but it was clearly different from that of Kex2.

Confocal Microscopy of GFP-tagged Nhx1 Shows Localization to Unique, Bipolar Perivacuolar Compartments—To further define the cellular location of this novel exchanger, we used laser scanning confocal microscopy to examine the distribution of GFP-tagged Nhx1 in conjunction with the vacuolar stain FM 4-64 in exponentially growing, unfixed cells (Fig. 3). Fluorescence from Nhx1::GFP appears as 1–2 intensely fluorescent spots per cell, immediately abutting the vacuolar membrane, usually with a striking bipolar distribution. The number and size of the spots typically increase in salt-containing me-
dia, consistent with the observed induction of Nhx1 expression levels. The distinct perivacuolar location of the signal is highly reminiscent of the prevacuolar compartment (19, 20), at which the biosynthetic, autophagic, and endosomal pathways converge for sorting of cargo before final delivery to the vacuole. Indeed, we show that the syntaxin Pep12, which defines the identity of the prevacuolar compartment (21), colocalizes with Nhx1::GFP in fixed and permeabilized cells. Importantly, we were able to show by Western blotting using anti-GFP antibodies that the distribution of Nhx1::GFP was identical to that of Nhx1::HA on sucrose density gradients (data not shown). Together with the functionality of the tagged constructs (Fig. 1a) and the modest levels of expression achieved from the endogenous NHX1 promoter, the observations argue against a potential mislocalization because of the large GFP tag.

The frequent distribution of Nhx1::GFP to opposing ends or poles of the vacuole is particularly intriguing and was clearly observed in three-dimensional reconstructions of the vacuole from sequential confocal planes (not shown). Such a bipolar pattern recalls the similar “patched” distribution of vacuolar assembly proteins, Vam3 and Vam6, on the vacuolar membranes (34, 35) and suggests that fusion of vacuolar precursors occurs at discrete sites. We note that this orientation is apparently lost upon fixation of cells.

Nhx1 Does Not Colocalize with Mitochondrial Markers—The inner membrane of mitochondria in mammals has been shown to possess two distinct forms of cation/H⁺ exchange activity: one selective for Na⁺ and the other transporting all alkali cations (22, 23). Functional studies in isolated yeast mitochondria indicate an absence of selective Na⁺/H⁺ exchange, although a nonselective (K⁺/H⁺) antiporter was found (24). A very recent report (25) raised the possibility that Nhx1 localizes to mitochondria based on an overlap of signals from the DNA-binding dye 4,6-diamidino-2-phenylindole dihydrochloride and Nhx1::GFP expressed at high levels from the exogenous MET25 promoter. Evidence was also presented for low levels (1 nmol/min/mg) of Na⁺/H⁺ exchange activity in three of five crude mitochondrial preparations, although contamination by other membranes was not assessed. Here we show that fluorescence from Nhx1::GFP is distinct from that of two well characterized mitochondrial dyes, DiOC₆ and MitoTracker Red CMXRos (Fig. 4). Mitochondria appear as typically elongated snake-like forms that have no particular orientation relative to the vacuole; in contrast, Nhx1::GFP fluorescence occurs as 1–2 spots/cell that are always observed to directly abut the vacuolar membrane. Taken together with the results from subcellular fractionation (Fig. 2a), we conclusively rule out a mitochondrial localization for this exchanger.

DISCUSSION

Nhx1 Defines a Novel Cluster of Na⁺/H⁺ Exchanger Isoforms—With the ongoing success of systematic genome sequencing, genes encoding putative Na⁺/H⁺ exchangers have recently been identified in yeasts, worms, bacteria, and humans. They provide a unique opportunity to trace the phylogenetic ancestry and evolution of exchanger isoforms as well as...
provide clues to the function of newly identified homologues. In our survey of all NHE-like sequences residing in data bases, we were able to identify previously known clusters of sequences corresponding to the plasma membrane isoforms of Na+/H+ exchangers (NHE1–4), as well as a previously unreported cluster of more distantly related prokaryotic sequences. We show here that Nhx1 defines a completely novel cluster of exchanger sequences derived from such evolutionary divergent organisms as yeast, nematodes, and humana (Fig. 5a). In addition, pairwise comparisons between NHE polypeptides using global alignment methods (26) reveal significantly higher scores for members within this newly identified cluster: average identity, 34%; global score, 1000 (Box I, Fig. 5b). Comparable scores were observed among members outside this cluster: average identity, 36%; global score, 1722 (Box II, Fig. 5b). In contrast, scores for sequence pairs between the two groups were uniformly low: average identity, 23%; global score, 373 (Box III, Fig. 5b). It should be noted that to avoid bias, we chose representative sequences from widely divergent phyla in both groups and that the overall length of the polypeptides varied in both groups: 540–666 (Box I) and 660–820 (Box II).

All Na+/H+ exchanger sequences share the highest homology within predicted transmembrane segments of the N-terminal transporter domain. The conserved regions are presumably important for common transport functions, whereas the C-terminal hydrophilic domain is significantly shorter in members of the Nhx1-like cluster relative to the plasma membrane isoforms, resulting in shorter polypeptide lengths overall: 541–666 residues versus 717–832 residues. Thus, although separated from one another by a billion years or so of evolution, members of the newly identified Nhx1-like cluster are all recognizably related to each other. Taken together, these observations suggest a common intracellular, possibly endosomal location for these novel homologues.

**Fig. 4. Nhx1 does not colocalize with mitochondrial markers.** Confocal microscopy of exponentially growing wild type (panels a–d) or Δnhx1/Δnhx1::GFP-2p (panels e–h) cells in the absence of fixation. In panels a–d, mitochondria stained with DiOC6 (green, panel a) are seen in relation to the vacuole (FM 4-64; red, panel b) in the overlay (panel c). Nhx1::GFP fluorescence (panel g) is distinct from that of mitochondria (MitoTracker Red CMXRos; red, panel f) as seen in the overlay (panel h). Note that mitochondria lack the distinct perivacuolar, bipolar distribution seen in the structures containing Nhx1::GFP. Bar, 10 μm; inset bar, 5 μm.

**Functional Implications of the Prevacuolar/Endosomal Localization of Nhx1**—There is emerging evidence that plasma membrane-derived endosomes and Golgi-derived transport vesicles converge at a prevacuolar compartment (PVC) equivalent to late endosomes, where cargo is sorted prior to final delivery to the vacuole/lysosome. Proteins en route to the vacuole may be visualized in the PVC transiently, as was observed for the α-factor receptor, Ste3, upon coordinated internalization from the plasma membrane (27), or by perturbation of vesicle traffic into or out of this compartment, as in the “Class E” family of vacuolar trafficking mutants (28). The PVC itself is a discrete, rather than transient, structure that can be isolated on density gradients from normal yeast (29) and can be shown to have a perivacuolar distribution by immunofluorescence and electron microscopy (20, 27). However, the only resident protein of the PVC described in the literature is the syntaxin homologue, Pep12, which mediates docking of this compartment with the vacuole (21). In this context, the selective localization of Nhx1 to the prevacuolar compartment has considerable functional significance. An intriguing possibility is that regulation of vesicle volume and pH by endosomal Na+/H+ exchange may be important for vacuole biogenesis. Thus, the H+ gradient established by the vacuolar H+-ATPase would drive Na+ accumulation via Na+/H+ exchange, and Cl− influx via chloride channels (Fig. 5d). As osmotically obliged water is dragged in, the vesicle swells and the hydrostatic pressure generated provides the energy for membrane destabilization and fusion. There is evidence that osmotic swelling precedes exocytosis and that, conversely, water loss from vesicles accompanies vesicle maturation and remodeling (30–32, 39). We suggest that the yeast chloride channel ScCLC/GEF1 also has a prevacuolar localization based on the appearance of GFP-tagged ScCLC as 1–3 perivacuolar dots/cell (33). Thus, colocalization of the Na+/H+ exchanger, Cl− channel, and H+ pump, together with specialized coat proteins, syntaxins, and other docking factors may be involved in the assembly of the vacuole/lysosome from the prevacuolar compartment/endosomes.

Our data do not exclude the possibility that Nhx1 also localizes to discrete patches on the vacuolar membrane. Recently, two components of a protein complex required for vacuole biogenesis, Vam3 (34) and Vam6 (35), were shown to have an unusual bipolar patched location on the vacuole, suggesting
that specialized domains of the vacuole may be involved in vesicle fusion. By analogy, it is known that Golgi-derived secretory vesicles fuse at specialized regions of the plasma membrane, resulting in oriented bud growth (36). We note the enrichment of mammalian Nhe1 at the leading edge and ruffles in fibroblast cells (37), and of the unrelated Na\(^+\)/H\(^+\) exchanger sod2 of Schizosaccharomyces pombe (38), consistent with a role for Na\(^+\)/H\(^+\) exchange at these specialized sites. Our data imply that regulation of vesicle pH and volume by endosomal Na\(^+\)/H\(^+\) exchange may be important for vesicle maturation and fusion.

Given the multifunctionality of Na\(^+\)/H\(^+\) exchangers, a variety of other cellular roles for endosomal exchangers may be envisaged: regulation of endosomal pH via Na\(^+\)/H\(^+\) exchange can provide a functional link between the operational diversity among endocytic compartments and the known variability in their internal pH, intraluminal sequestration of Na\(^+\) may serve to detoxify the cytoplasm or to drive Ca\(^{2+}\) accumulation via Na\(^+\)/Ca\(^{2+}\) exchange, and in the case of the renal proximal tubule, exocytic insertion of endosomal Na\(^+\)/H\(^+\) exchangers at the cell surface may effect rapid increases in H\(^+\) secretory capacity. We have already demonstrated that in yeast, Nhx1 makes an important contribution to halotolerance (12). The molecular characterization and functional role of Nhx1-like homologues in other organisms remains to be determined.

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Fig. 5. Sequence relations and proposed function of Nhx1-like homologues. a, phylogenetic clusters of NHE sequences were defined using Clustalw 1.5 and PHYLIP 3.5c. Representative examples are shown for each cluster, and accession numbers are included for unnamed isoforms. b, pairwise alignments between NHE sequences showing the percentage of identity computed for each pair. In parentheses are the global alignment scores, which reflect penalties for gaps. Box I contains the putative intracellular cluster, and Box II contains representative examples from the other clusters. NHX1, S. cerevisiae; SPX1, S. pombe 29720B; NHE6, human D87743; CE1 and CE2, C. elegans Z89646 and Z73888; NHE1, rat; NHE2, human; CM1, C. maenas. Asterisks denote the conserved amino acid residues. c, patterns of amino acid homology provide the basis for the definition of unique clusters of NHE sequences. Selected transmembrane segments are shown, numbered according to Ref. 1.
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