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The Sodium/Proton Exchanger Nhx1p Is Required for Endosomal Protein Trafficking in the Yeast *Saccharomyces cerevisiae*

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We show that the vacuolar protein sorting gene *VPS44* is identical to *NHX1*, a gene that encodes a sodium/proton exchanger. The *Saccharomyces cerevisiae* protein Nhx1p shows high homology to mammalian sodium/proton exchangers of the NHE family. Nhx1p is thought to transport sodium ions into the prevacuole compartment in exchange for protons. Pulse-chase experiments show that ~35% of the newly synthesized soluble vacuolar protein carboxypeptidase Y is missorted in *nhx1*Δ cells, and is secreted from the cell. *nhx1*Δ cells accumulate late Golgi, prevacuole, and lysosome markers in an aberrant structure next to the vacuole, and late Golgi proteins are proteolytically cleaved more rapidly than in wild-type cells. Our results show that efficient transport out of the prevacuolar compartment requires Nhx1p, and that *nhx1*Δ cells exhibit phenotypes characteristic of the "class E" group of *vps* mutants. In addition, we show that Nhx1p is required for protein trafficking even in the absence of the vacuolar ATPase. Our analysis of Nhx1p provides the first evidence that a sodium/proton exchange protein is important for correct protein sorting, and that intraorganellar ion balance may be important for endosomal function in yeast.

INTRODUCTION

Newly synthesized proteins destined for the yeast vacuole are inserted into or transported across the endoplasmic reticulum (ER) membrane. After exit from the ER, they are delivered to the Golgi complex, and it is upon leaving the late Golgi that transport pathways to the vacuole diverge (reviewed in Bryant and Stevens, 1998; Burd *et al.*, 1998; Conibear and Stevens, 1998; Figure 11Ai). Proteins such as the vacuolar protease carboxypeptidase Y (CPY), follow a route from the late Golgi via an endosomal intermediate, the prevacuolar compartment (PVC), to the vacuole. This pathway is referred to as the CPY pathway. Other vacuolar proteins, for example, alkaline phosphatase (ALP), follow a distinct route (the ALP pathway) to the vacuole that bypasses the PVC. A third route allows proteins transported to the cell surface from the Golgi to reach the vacuole after endocytosis. The route taken to the vacuole by proteins endocytosed from the plasma membrane is thought to converge with the route taken by vacuolar proteins following the CPY pathway, at the PVC (Davis *et al.*, 1993; Piper *et al.*, 1995; Rieder *et al.*, 1996).

Genetic screens in yeast have identified >50 genes required for CPY trafficking and processing (*PEP* genes, Jones 1977; *VPS* genes, Bankaitis *et al.*, 1986; Rothman and Stevens, 1986; Robinson *et al.*, 1988; Raymond *et al.*, *VAC* genes, Weisman *et al.*, 1990; 1992; and *VAM* genes, Wada *et al.*, 1992). Complementation analysis has revealed extensive overlap between the *vps*, *pep*, *vac*, and *vam* mutants. The *VPS* genes have been grouped into classes (A through F) based on the vacuolar morphology of mutant yeast cells (Banta *et al.*, 1988; Raymond *et al.*, 1992). Based on the assumption that the loss of any one of the proteins required at a certain transport step will result in a similar vacuolar morphology phenotype, this classification of *vps* mutants has been helpful in identifying proteins that act at the same step of CPY transport to the vacuole.

Previous reports have suggested that the lumenal acidic environment of endocytic organelles, generated by the proton-translocating, V-type ATPase (V-ATPase), is essential for endosomal trafficking in mammalian cells. The acidic lumenal pH of endosomes is required for ligand–receptor dissociation, binding of coat proteins to early endosomes, and for the entry of some enveloped viruses (reviewed in Stevens and Forgac, 1997). However, the role of endosomal pH and ion balance in endosomal trafficking in yeast is less clear. We have identified the first yeast Vps protein that

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Abbreviations used: ALP, alkaline phosphatase; CPY, carboxypeptidase Y; ER, endoplasmic reticulum; HA, haemagglutinin; PVC, prevacuolar compartment; V-ATPase, vacuolar proton ATPase.

shows homology to known ion transporters. *VPS44*, a previously uncloned gene identified in a screen for *vps* mutants, encodes the sodium/proton exchange protein Nhx1p. Loss of this ion transporter affects the transport of CPY to the vacuole, suggesting a role for ion balance in protein trafficking to the yeast vacuole.

By protein sequence homology, Nhx1p/Vps44p is the only member of the NHE family of sodium/proton exchangers in yeast. Mammalian sodium/proton exchangers of the NHE protein family are required for several key cellular processes, and function to regulate cell volume, intracellular pH, and sodium reabsorption across renal, intestinal, and other epithelial membranes (reviewed in Orlowski and Grinstein, 1997; Counillon and Pouyssegur, 2000). NHE proteins exchange one sodium ion for one proton across the membrane, and are passive exchangers, driven by ion gradients in the cell. All NHE proteins are predicted to have a similar structure, with 10–12 membrane-spanning domains and a large C-terminal domain. Experimental evidence suggests that the C-terminal domain of NHE1 is cytosolic (Shrode *et al.*, 1998; Wakabayashi *et al.*, 2000). However, residues within the C-terminal region of NHE3 may be extracellularly exposed (Biemesderfer *et al.*, 1998), making it difficult to draw general conclusions concerning NHE protein topology. There is some evidence that NHE proteins may exist as homodimers (Fliegel *et al.*, 1993; Fafournoux *et al.*, 1994).

By amino acid sequence alignment, and construction of a phylogenetic tree, NHE proteins fall into two subfamilies (Fukuda *et al.*, 1999). The first contains NHE proteins known to be localized to the plasma membrane, including mammalian NHE proteins 1–5. The second subfamily contains several exchangers proposed to be on the membranes of intracellular organelles, including the yeast NHE protein Nhx1p, the *Arabidopsis thaliana* vacuolar sodium/proton exchanger (AtNhx1), and human NHE6. The phylogenetic analysis has led to the hypothesis that the two subfamilies of NHE proteins have distinct cellular localizations: there is one subfamily of plasma membrane NHE proteins, and a second of NHE proteins localized to intracellular membranes (Fukuda *et al.*, 1999).

The plasma membrane class of NHE proteins has been extensively studied, and much is known about their expression and regulation (Orlowski and Grinstein, 1997; Counillon and Pouyssegur, 2000). However, much less is known about the intracellularly localized subfamily of NHE proteins. Nhx1p has been proposed to function in salt tolerance, by sequestering sodium ions from the cytosol into the PVC in exchange for protons (Nass *et al.*, 1997). Intracellular NHE proteins from other organisms may also play a role in salt tolerance, and this is supported by a recent study showing that overexpression of the *A. thaliana* AtNhx1 allowed plants to survive salt stress (Apse *et al.*, 1999).

Data reported in this article reveal a novel role for an intracellular NHE protein. Nhx1p/Vps44p is required for normal membrane traffic out of the PVC in yeast. Whereas vacuolar, prevacuolar, and Golgi membrane proteins following the CPY pathway accumulate in an exaggerated PVC in cells lacking Nhx1p, traffic along the ALP pathway is unaffected in these cells.

MATERIALS AND METHODS

Plasmid Construction and Site-directed Mutagenesis

The plasmids used in this study are shown in Table 1. Enzymes used in DNA manipulations were purchased from New England Biolabs (Beverly, MA), or Boehringer Mannheim Biochemicals (Indianapolis, IN). Oligonucleotides were synthesized by Keystone Laboratories (Camarillo, CA). All site-directed mutagenesis was performed by QuikChange mutagenesis (Stratagene, La Jolla, CA). The *NHX1* gene was generated as a polymerase chain reaction (PCR) product from a yeast genomic DNA template by using Pfu polymerase (Stratagene) and the oligonucleotides 5′-CCGATGAGTACG**GTCGAC**ATTAGC-3′ (incorporating a *Sal*I site, shown in bold type) and 5′-GCTTATCGATAGCGGCGAGTTTCTC-3′. This 2.9-kb PCR product included 500 bp upstream of the ATG and 450 bp downstream of the stop codon of *NHX1*, and was cloned first into pCR-Blunt (Invitrogen, Carlsbad, CA), and then subcloned by using *Sal*I and *Not*I (from the polylinker of pCR-Blunt) into pBluescript II KS$^+$ (Stratagene) to give pKEB24, or pRS316 (Sikorski and Hieter, 1989) to produce pKEB37. Knockout constructs were generated by digesting pKEB24 with *Hpa*I and *Stu*I removing bp 45–1682 of the *NHX1* ORF, and replacing this by blunt end ligation with either the *TRP1* gene (the 948-bp *Pvu*II-*Stu*I fragment from pJJ246; Jones and Prakash, 1990) or the kanamycin resistance gene (Kan^r; the 1-kb *Sal*I-*Eco*RV fragment from pFA6-kanMX2; Wach *et al.*, 1994) generating pKEB32 and pKEB43, respectively. From pKEB37, the ATG of ORF YDR455C was mutated to ATT (confirmed by sequencing) to give pKEB36. This mutation leaves the predicted amino acid sequence of Nhx1p unchanged. A *Sna*BI site was inserted in-frame before the stop codon of *NHX1* (changing AAC to TAC, and CAC to GTA) in pKEB36 to allow the C-terminal tagging of Nhx1p by using a triple hemagglutinin (HA) epitope fragment from pKEB35, generating pKEB38. *NHX1-HA* from pKEB38 was subcloned into pRS306 (pKEB39), and pRS315 (pKEB53). pKEB38 was used as a template to generate mutations GAC to AAC (D201N; pKEB44, pFP1, and pFP2), GAA to CAA (E225Q; pKEB45 and pFP1), GAT to AAT (D230N; pKEB46 and pFP2), and GAG to CAG (E355Q; pKEB47), which were confirmed by sequencing. The *GEF1* gene was generated as a PCR product from a yeast genomic DNA template by using Pfu polymerase and the oligonucleotides 5′-GC**GAATTC**GCTTCCTCGATCCTCA-3′ and 5′-CG**GGATCC**TGCGAGCCTTATAATG-3′, incorporating *Eco*RI and *Bam*HI sites (shown in bold type). This 3.3-kb PCR product included 500 bp upstream of the ATG and 470 bp downstream of the stop codon of *GEF1*, and was cloned first into pCR-Blunt and then subcloned by using *Eco*RI and *Bam*HI into pBluescript II KS$^+$ to produce pBL2. A deletion construct, pBL20, was generated by digesting pBL2 with *Hpa*I and *Nar*I (removing bp 302-1558 of the *GEF1* ORF), and replacing this by blunt-end ligation with a DNA fragment containing *LEU2*. pKEB58 was generated by subcloning the 4.2-kb *Bam*HI-*Sal*I fragment containing *PEP4* from pTS18 into the same sites of pRS315.

Yeast Strains

Yeast strains used in this study are shown in Table 2. Strains were constructed by standard genetic techniques and grown in rich medium (1% yeast extract, 1% peptone, 2% dextrose; YEPD) or synthetic dextrose minimal medium with appropriate supplements (SD; Kaiser *et al.*, 1994). YEPD buffered to pH 5.0, and YEPD with 100 mM $CaCl_2$, were also used for the selection and growth of Vma$^-$ strains (Yamashiro *et al.*, 1990). All strains were derived from SF838–9D (Rothman and Stevens, 1986) with the exception of KEBY10, which was derived from SEY6210 (Robinson *et al.*, 1988). Strain KEBY11 was generated by transforming SF838–9Dα with *Bgl*II digested pKEB39. Ura$^+$ colonies were plated on 5-fluoroorotic acid-containing minimal medium to select for Ura$^-$ loopouts, and colonies that contained Nhx1p-HA identified by Western blot. KEBY12 was derived from KEBY11 by transformation with *Bam*HI and *Pst*I digested pKJH2. Leu$^+$ col-

Table 1. Plasmids used in this study

Plasmid	Description	Source
pKEB24	*NHX1* in pBluescript II KS$^+$	This study
pKEB32	*nhx1*Δ::*TRP*::*TRP1* construct in pBluescript II KS$^+$	This study
pKEB35	123 bp triple HA epitope tag sequence in pCR-Blunt	This study
pKEB36	CEN-*URA3* plasmid with *NHX1* gene (pKEB37) with ATG of YDR455C mutated to ATT	This study
pKEB37	CEN-*URA3* plasmid with *NHX1* gene	This study
pKEB38	pKEB36 with a triple HA epitope tag inserted in-frame immediately before the stop codon of *NHX1*	This study
pKEB39	Integrating plasmid for *NHX1*-HA allele (loop in/loop out)	This study
pKEB43	*nhx1*Δ::*Kan*^{r} construct in pBluescript II KS$^+$	This study
pKEB44	CEN-*URA3* plasmid with *NHX1*-HA D201N	This study
pKEB45	CEN-*URA3* plasmid with *NHX1*-HA E225Q	This study
pKEB46	CEN-*URA3* plasmid with *NHX1*-HA D230N	This study
pKEB47	CEN-*URA3* plasmid with *NHX1*-HA E355Q	This study
pKEB53	CEN-*LEU2* plasmid with *NHX1*-HA	This study
pKEB58	CEN-*LEU2* plasmid containing *PEP4*	This study
pFP1	CEN-*URA3* plasmid with *NHX1*-HA D201N E225Q	This study
pFP2	CEN-*URA3* plasmid with *NHX1*-HA D201N D230N	This study
pBL2	*GEF1* in pBluescript II KS$^+$	This study
pBL20	*gef1*Δ::*LEU2* construct in pBluescript II KS$^+$	This study
pKH105	pQE16 (Qiagen, Valencia, CA) with four copies of the triple HA epitope	This study
pKJH2	*vps27*Δ::*LEU2* construct	Piper *et al*. (1995)
pSN55	CEN-*URA3* plasmid encoding A-ALP	Nothwehr *et al*. (1993)
pCY40	*vma2*Δ::*LEU2* construct	Yamashiro *et al*. (1990)
pMK10	*vma3*Δ::*URA3* construct	Kane *et al.* (1992)
pTS18	CEN-*URA3* plasmid containing *PEP4*	Ammerer *et al.* (1986)
pJLU34	CEN-*URA3* plasmid encoding Ste3p-GFP	Urbanowski and Piper (1999)
pSL1417	Integrating plasmid for *PEP4* (loop in/loop out)	Dr. G. F. Sprague, Jr.

onies were screened for deletion of *VPS27* by colony overlay assay to detect CPY secretion (Roberts *et al.*, 1991), and by immunofluorescence with anti-Vma2p to screen for class E Vps$^-$ vacuolar morphology (Raymond *et al.*, 1992). KEBY15 was generated by transformation of SF838–9Dα with *Xho*I/*Not*I digested pKEB43. Kanamycin-resistant colonies were selected on YEPD with 200 μg/ml geneticin sulfate (G418), and then screened for secretion of CPY. Insertion of the *Kan*^{r} gene into the *NHX1* open reading frame (ORF) was confirmed by PCR from genomic DNA by using oligonucleotides complementary to the DNA sequence 500 bp upstream of the *NHX1* ORF, and to a sequence within *Kan*^{r}. KEBY13, KEBY14, and KEBY10 were similarly derived from RPY10, AACY28, and SEY6210 (with pKEB32), respectively. KEBY26 and KEBY27 were generated from SF838–9Dα and RPY10, respectively, by transformation with *Sal*I/*Cla*I digested pCY40 and Leu$^+$ colonies were selected for lack of growth on YEPD with 100 mM $CaCl_2$. KEBY34 and KEBY35 were similarly derived from KEBY15 and KEBY13, respectively. KEBY29 was derived from SF838–9Dα by transformation with *Eco*RI/*Hin*dIII digested pMK10, and Ura$^+$ colonies were screened for the Vma$^-$ phenotype as described above. KEBY32 was derived from RPY10 by transformation with *Xba*I/*Xho*I digested pBL20. Leu$^+$ colonies were screened for correct insertion of *LEU2* into the *GEF1* ORF by PCR, with oligonucleotides complementary to the genomic DNA sequence 600 bp upstream of *GEF1*, and a sequence within *LEU2*. BLY1 was generated by transforming NBY72 (Bryant *et al.*, 1998) with *Sac*I-digested pSL1417. Ura$^+$ colonies were plated on 5-fluoroorotic acid-containing medium to select for Ura$^-$ loopouts, and colonies that contained proteinase A identified by APNE assay (Wolf and Fink, 1975). KEBY37 was derived from BLY1 by transformation of *Bam*HI/*Pst*I digested pKJH2 and screened for deletion of *VPS27* as described above.

Immunoblotting

Exponentially growing cells (10 OD_{600} total) were harvested and treated with 10 mM dithiothreitol for 15 min at room temperature. Cells were converted to spheroplasts by incubation with 250 μg/ml Zymolyase 100T (Seikagaku America, Ijamsville, MD) in 1.2 M sorbitol, 50 mM potassium phosphate buffer, pH 7.5, 1 mM $MgCl_2$ for 45 min at 30°C. Spheroplasts were washed once with 1.2 M sorbitol and lysed by resuspension in 0.2 M sorbitol, 50 mM Tris, pH 7.5, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin. Unbroken cells were removed by centrifugation at 500 × *g* for 5 min at 4°C. The cell lysate was assayed for total protein concentration by using the bicinchoninic acid protein assay (Pierce, Rockford, IL), and equal amounts of total protein loaded per lane on 8% SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose membranes and immunoblot analysis of Nhx1p-HA performed by using the 12CA5 anti-HA monoclonal antibody (Roche Molecular Biochemicals, Indianapolis, IN), a secondary anti-mouse horseradish peroxidase-conjugated antibody (Bio-Rad Laboratories, Hercules, CA), and chemiluminescent detection (New England Nuclear, Boston, MA).

Radiolabeling and Immunoprecipitation

Pulse-chase immunoprecipitation of radiolabeled CPY, ALP, A-ALP, and Vps10p was carried out essentially as previously described (Raymond *et al.*, 1992; Nothwehr *et al.*, 1995). For CPY immunoprecipitation, yeast cultures were grown overnight at 30°C in minimal medium without methionine to mid-log phase. Cells were then resuspended at 1 OD_{600}/ml in fresh minimal medium (without methionine) with 50 mM KPO_4, pH 5.7, and 2 mg/ml bovine serum albumin (BSA), incubated for 15 min at 30°C, and pulse-labeled with 200 μCi of Express [^{35}S] labeling mix (New

Table 2. Yeast strains used in this study

Strain	Genotype	Source
SF838-9D	*MATα ura3-52 leu2-3,112 his4-519 ade6 gal2 pep4-3*	(Rothman and Stevens, 1986)
SEY6210	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-901 lys2-801 suc2-Δ9*	(Robinson et al., 1988)
KEBY10	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-901 lys2-801 suc2-Δ9 nhx1Δ::TRP1*	This study
KEBY11	*MATα ura3-52 leu2-3,112 his4-519 ade6 gal2 pep4-3 NHX1::3XHA*	This study
KEBY12	*MATα ura3-52 leu2-3,112 his4-519 ade6 gal2 pep4-3 NHX1::3XHA vps27Δ::LEU2*	This study
KEBY13	*MATα ura3-52 leu2-3,112 his4-519 ade6 gal2 PEP4 nhx1Δ::Kan^r*	This study
KEBY14	*MATα ura3-52 leu2-3,112 his4-519 ade6 gal2 PEP4 pho8Δ::LEU2 nhx1Δ::Kan^r*	This study
KEBY15	*MATα ura3-52 leu2-3,112 his4-519 ade6 gal2 pep4-3 nhx1Δ::Kan^r*	This study
KEBY26	*MATα ura3-52 leu2-3,112 his4-519 ade6 gal2 pep4-3 vma2Δ::LEU2*	This study
KEBY27	*MATα ura3-52 leu2-3,112 his4-519 ade6 gal2 PEP4 vma2Δ::LEU2*	This study
KEBY29	*MATα ura3-52 leu2-3,112 his4-519 ade6 gal2 pep4-3 vma3Δ::URA3*	This study
KEBY32	*MATα ura3-52 leu2-3,112 his4-519 ade6 gal2 PEP4 gef1Δ::LEU2*	This study
KEBY34	*MATα ura3-52 leu2-3,112 his4-519 ade6 gal2 pep4-3 nhx1Δ::Kan^r vma2Δ::LEU2*	This study
KEBY35	*MATα ura3-52 leu2-3,112 his4-519 ade6 gal2 PEP4 nhx1Δ::Kan^r vma2Δ::LEU2*	This study
KEBY37	*MATα ura3-52 leu2-3,112 his4-519 ade6 gal2 PEP4 pho8Δ::X vps27Δ::LEU2*	This study
BLY1	*MATα ura3-52 leu2-3,112 his4-519 ade6 gal2 PEP4 pho8Δ::X*	This study
NBY72	*MATα ura3-52 leu2-3,112 his4-519 ade6 gal2 pep4-3 pho8Δ::X*	Bryant *et al.* (1998)
RPY10	*MATα ura3-52 leu2-3,112 his4-519 ade6 gal2 PEP4*	Piper *et al.* (1995)
SGY73	*MATα ura3-52 leu2-3,112 his4-519 ade6 gal2 pep4-3 vps27Δ::LEU2*	Gerrard *et al.* (2000a)
AACY5	*MATα ura3-52 leu2-3,112 his4-519 ade6 gal2 PEP4 vps27Δ::LEU2*	Piper *et al.* (1995)
AACY28	*MATα ura3-52 leu2-3,112 his4-519 ade6 gal2 PEP4 pho8Δ::LEU2*	Cooper and Stevens (1996)
vpl27-1	*MATa ura3-52 leu2-3,112 his4-519 lys2 gal2 pep4-3 vps44*	Raymond *et al.* (1992)

England Nuclear) per 1 OD_{600} for 10 min at 30°C. The chase was initiated by the addition of 100 μg/ml each of unlabeled cysteine and methionine, and after incubation for the appropriate time at 30°C, 500 μl aliquots were removed and the chase terminated by addition of 50 mM sodium azide and incubation on ice. Cells were collected by centrifugation and the supernatant (extracellular fraction) reserved. After conversion to spheroplasts using oxalyticase (Enzymogenetics, Corvallis, OR), 0.5% SDS was added to lyse the cells, and the samples incubated at 100°C for 5 min. Samples were then adjusted to 0.1% SDS, 0.1% Triton X-100, 2 mM EDTA, and 90 mM Tris, pH 8.0 (1× IP buffer). Then 100 μl of 10× IP buffer was added to each extracellular fraction and the samples incubated at 100°C for 5 min before diluting 10-fold with water. Intracellular and extracellular fractions were precleared with 50 μl of a 10% slurry of fixed *Staphylococcus aureus* cells (IgGSorb; The Enzyme Center, Malden, MA) for 15 min. Anti-CPY (1 μl) serum was added and incubated for 1 h on ice, followed by a second incubation for 1 h on ice after addition of 50 μl of a 10% slurry of IgG Sorb. IgG Sorb with bound immune complexes was collected by centrifugation and washed three times in 1× IP buffer. Immunoprecipitated proteins were eluted in SDS sample buffer and separated on 7% SDS polyacrylamide gels. ALP, A-ALP, and Vps10p immunoprecipitations were carried out as described above, except extracellular fractions were discarded, and spheroplasts were lysed in 2% urea, 0.25% SDS for 5 min at 100°C.

Immunofluorescence Microscopy

Indirect immunofluorescence microscopy was performed essentially as previously described (Roberts *et al.*, 1991). Cells were grown in YEPD at 30°C to 1 OD_{600}/ml, and then fixed by the addition of 3% formaldehyde for 1 h, followed by a 16-h incubation at room temperature in 4% paraformaldehyde, 50 mM KPO_4, pH 6.5. Cells were converted to spheroplasts using Zymolyase 100T and permeabilized by treatment with 5% SDS for 5 min for visualization of ALP or Vph1p, or 1% SDS for 2 min for detection by using all other antibodies (unless otherwise described in the figure legend). Cells were allowed to adhere to poly(L-lysine) multiwell-coated slides. Nonspecific antibody binding was blocked by incubation of the cells in PBS with 5 mg/ml BSA and 1% normal goat serum. All antibodies were diluted in PBS with 5 mg/ml BSA, which was also used for all washes. Antibodies against Vph1p, ALP, Pep12p, Vps10p, and HA were preabsorbed to yeast proteins (to remove nonspecific binding) by incubation with *vph1*Δ, *pho8*Δ, *pep12*Δ, *vps10*Δ, or wild-type SF838–9Dα cells, respectively. Antibody incubations were performed at room temperature: 2 h for primary antibodies (3 h for anti-ALP), and 1 h for secondary and tertiary antibodies. Affinity-purified polyclonal antibodies against, Vma2p, and Vps10p have been described previously (Yamashiro *et al.*, 1990; Cooper and Stevens, 1996) and were used at dilutions of 1/500 and 1/400, respectively. Polyclonal serum against Vph1p (Hill and Stevens, 1994) was used at a 1/100 dilution. To produce the polyclonal anti-HA antibody, *E. coli* cells containing pKH105 were used to express a dihydrofolate reductase-12xHA fusion protein with a 6xHis tag. The antigen was purified over a nickel-agarose column, and used to inject rabbits as previously described (Vaitukaitis, 1981). The anti-HA serum was affinity-purified as described in Stevens *et al.* (1982), and used at a dilution of 1/200. Monoclonal antibodies against ALP (1D3-A10), and Pep12p (24-2C3G4) are available from Molecular Probes (Eugene, OR), and were used at concentrations of 1/3 for anti-ALP tissue culture supernatant, and 1/1000 for purified anti-Pep12p. The purified anti-HA monoclonal antibody (HA.11) was purchased from Babco (Berkeley, CA), and used at 1/500. Biotin-conjugated goat anti-mouse, biotin-conjugated goat anti-rabbit, and streptavidin-conjugated fluorescein isothiocyanate (FITC) antibodies were obtained from Jackson Immunoresearch (West Grove, PA). Alexa (A594)-conjugated goat anti-rabbit and Alexa (A594)-conjugated goat anti-mouse antibodies were obtained from Molecular Probes. Images were generated by using a Bio-Rad MRC 1024 confocal microscope, or an Axioplan 2 fluores-

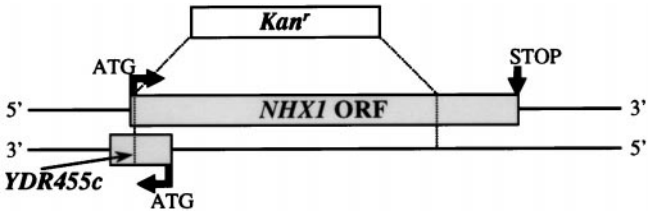


Figure 1. Deletion of the *NHX1* gene. The *NHX1* ORF is located on *S. cerevisiae* chromosome IV. YDR455C, a small yeast ORF of unknown function, overlaps *NHX1* on the other DNA strand. Replacement of *NHX1* with *Kan*^{r} by homologous recombination also disrupts YDR455C.

cence microscope (Carl Zeiss, Thornwood, NY) fitted with an Orca 100 digital camera (Hamamatsu, Bridgewater, NJ).

FM4-64 Staining

FM4-64 [*N*-(3-triethylammoniumpropyl)-4-(p-diethylaminophenylhexatrienyl, pyridinium dibromide; Molecular Probes] staining was performed as described in Vida and Emr (1995), with a 15-min incubation of the cells in YEPD with 40 μM FM4-64 at 30°C, followed by a 30-min chase in YEPD without the dye at 30°C. Cells were viewed with a 100× oil immersion lens on an Axioplan 2 fluorescence microscope and images captured with an Orca 100 digital camera.

RESULTS

Identification of VPS44 as NHX1

VPS44 is a previously uncloned gene identified in a genetic screen for mutants that affect protein trafficking to the yeast vacuole (*vps* mutants; Rothman and Stevens, 1986; Rothman *et al.*, 1989; Raymond *et al.*, 1992). More recently, we have used a transposon-based mutagenic procedure to screen for yeast mutants that have a Golgi retention deficient (Grd$^-$) phenotype, and thus mislocalize late Golgi proteins to the vacuole (Nothwehr *et al.*, 1996; Voos and Stevens, 1998). Mutants identified in this screen were tested by complementation analysis with all *vps* and *grd* mutant strains. One mutant failed to complement a *vps44* mutant yeast strain for secretion of CPY, and sequence analysis showed that the transposon had inserted into the genome within the ORFs YDR456W and YDR455C. The 2-kb YDR456W ORF encodes the sodium/proton exchanger Nhx1p (Nass *et al.*, 1997). YDR455C, which overlaps *NHX1*, is present on the opposite strand (Figure 1), and is a small yeast ORF of unknown function (Cherry *et al.*, 1997). The transposon-based screen therefore suggests that the *VPS44* gene is either *NHX1* or YDR455C.

We have replaced bp 45–1682 of *NHX1* in the yeast genome with the gene conferring kanamycin resistance (*Kan*^{r}), to produce the mutant yeast strain *nhx1*Δ, as shown in Figure 1. This deletion strain also has a disruption of the 5′ terminus of the YDR455C ORF. The *nhx1*Δ cells were assayed for secretion of CPY by pulse-chase immunoprecipitation. Figure 2 shows that after a 10-min pulse of radiolabel and no chase, three intracellular forms of CPY were seen in wild-type cells. These three forms represent the newly synthesized ER form of CPY (p1), the slightly larger Golgi-modified form (p2), and the proteolytically cleaved, mature vacuolar form (m). After a 40-min chase, 96 ± 2% of newly synthesized CPY was intracellular and mature in wild-type cells, consistent with correct localization of CPY to the vacuole. In contrast, *nhx1*Δ cells secreted 34 ± 7% of newly synthesized CPY into the extracellular medium in the Golgi-modified p2 form, suggesting that CPY trafficking to the vacuole was disrupted. Transformation of *nhx1*Δ cells with centromere-based (CEN) plasmids containing either *NHX1* and YDR455C, or *NHX1* alone (*NHX1*-HA; the ATG of the YDR455C ORF was mutated to ATT) reduced the CPY secretion to wild-type levels. Thus, *NHX1* expressed from its endogenous promoter on a CEN plasmid was able to complement the CPY secretion phenotype of *nhx1*Δ cells. The secretion of CPY by *nhx1*Δ cells was therefore due to the loss of Nhx1p rather than the loss of a protein encoded by the YDR455C ORF. As well as a disruption of YDR455C, the

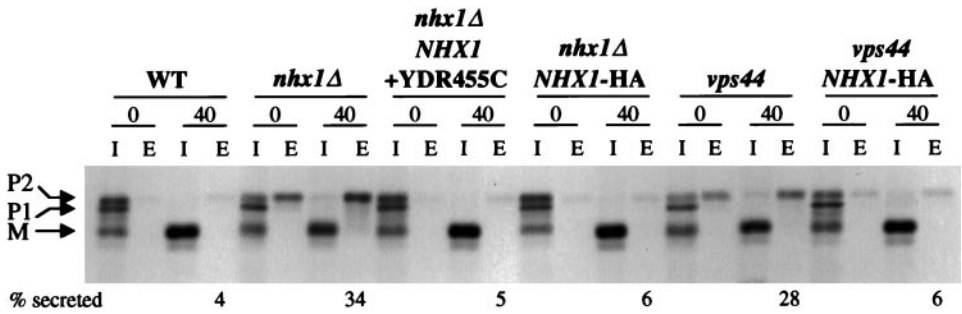


Figure 2. Secretion of CPY in wild-type (WT), *nhx1*Δ::*Kan*^{r}, and *vps44* mutant strains, and complementation by *NHX1* and *NHX1-HA*. Cells were labeled with [^{35}S]methionine and cysteine for 10 min, and then chased for 0 or 40 min at 30°C. CPY was immunoprecipitated from intracellular (I) and extracellular (E) fractions for each time point. The positions of ER and Golgi precursor forms of CPY (p1 and p2, respectively), and mature vacuolar CPY (m) are indicated. *nhx1*Δ cells were transformed with *NHX1* and YDR455C on a CEN plasmid (*NHX1*+YDR455C, pKEB37), or *NHX1* alone with an HA epitope tag (*NHX1-HA*, pKEB38). *vps44* cells were transformed with a CEN plasmid containing *NHX1* alone with an HA-epitope tag (*NHX1-HA*, pKEB53). WT and *nhx1*Δ strains have a wild-type *PEP4* gene, encoding proteinase A (required for proteolytic processing). *vps44* (*vpl27-1*) has the *pep4-3* mutation, and was transformed with *PEP4* on a CEN plasmid (pTS18) for this experiment. The percentage of CPY secreted into the extracellular medium after a 40-min chase was calculated by phosphoimager analysis, and the numbers shown are averages over several separate experiments.

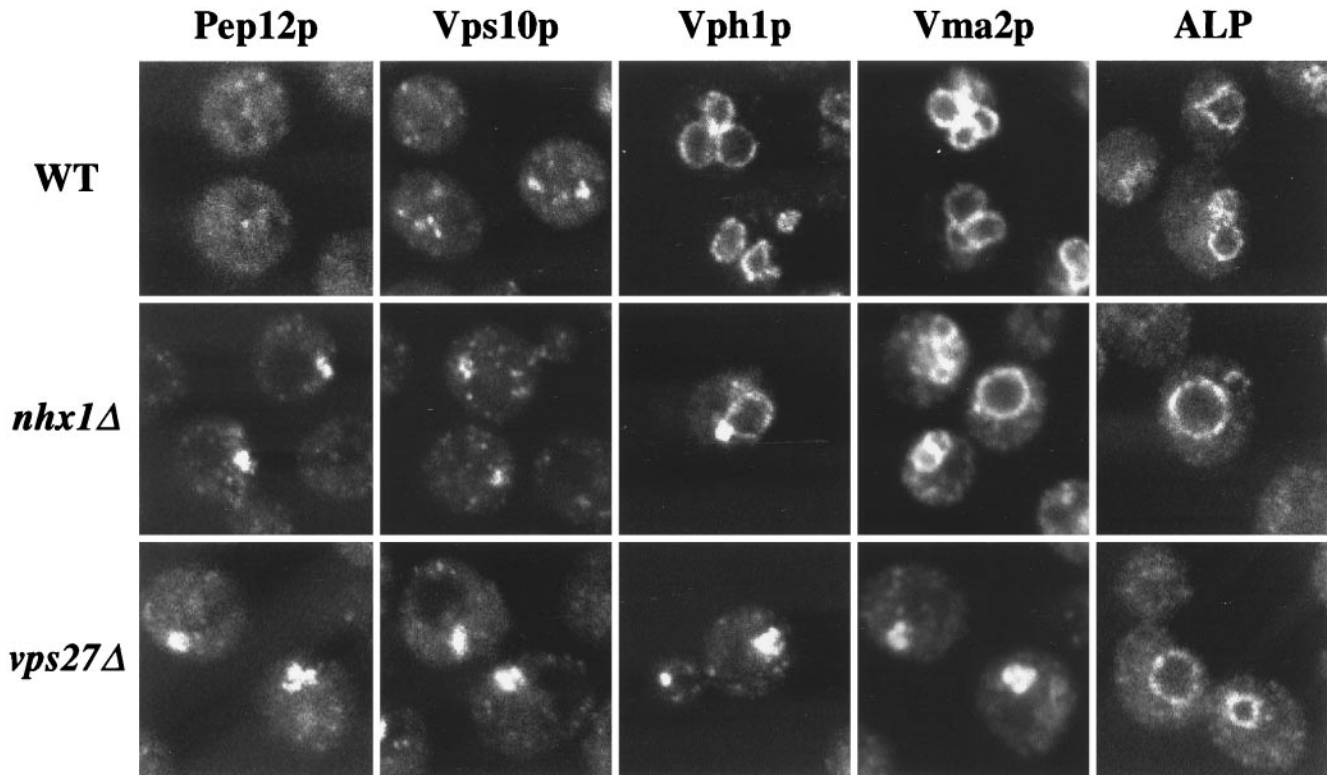


Figure 3. *nhx1*Δ cells have a similar morphological phenotype to *vps27*Δ cells. Immunofluorescence was performed as described in MATERIALS AND METHODS in wild-type (WT; SF838–9Dα), *nhx1*Δ (KEBY15), and *vps27*Δ cells (KEBY12), by using monoclonal antibodies to ALP or Pep12p, and polyclonal antibodies to Vps10p, Vph1p, or Vma2p. Staining for Vps10p, Pep12p, and ALP was enhanced by using secondary anti-mouse or anti-rabbit biotin-conjugated antibodies followed by a tertiary streptavidin-FITC conjugated antibody. Vph1p and Vma2p were detected by using a secondary Alexa-conjugated anti-rabbit antibody.

NHX1-HA construct also has an epitope tag sequence introduced into *NHX1* before the stop codon. *NHX1*-HA was able to fully complement the CPY secretion phenotype of *nhx1*Δ cells (Figure 2), showing that the addition of an HA tag to the extreme C terminus of Nhx1p did not affect the function of the protein in CPY trafficking. Examination of CPY secretion levels by immunoprecipitation also revealed that *NHX1*-HA was able to fully complement a *vps44* mutant strain for CPY secretion (Figure 2), adding further evidence that *NHX1* and *VPS44* are identical.

Nhx1p Is Required for Traffic Out of the PVC

The classification of *VPS* genes into six groups (A–F) based on vacuolar morphology allows the identification of genes that may act at distinct steps in the trafficking pathway to the vacuole (Raymond *et al.*, 1992). Although *VPS44* (*NHX1*) was originally designated a class A *VPS* gene (on the basis that *vps44* mutant cells exhibited wild-type vacuolar morphology; Raymond *et al.*, 1992), we reexamined *nhx1*Δ cells by using a variety of organelle markers that are now available. We have used Pep12p as a marker for the prevacuole (Becherer *et al.*, 1996; Gerrard *et al.*, 2000b). In wild-type cells, Pep12p is localized to small punctate structures throughout the cytoplasm (Figure 3 top row). Vps10p/Pep1p, the CPY receptor, binds CPY in the late Golgi and is transported to the PVC where CPY is released. From the PVC, CPY travels to the vacuole, whereas Vps10p is recycled back to the late Golgi where it captures more CPY (Marcusson *et al.*, 1994; Cereghino *et al.*, 1995; Cooper and Stevens, 1996). In wild-type cells Vps10p is localized by immunofluorescence to punctate structures typical of the Golgi in yeast (Figure 3, top row). Vph1p, the 100-kDa transmembrane subunit of the V-ATPase follows the CPY pathway to the vacuole and is localized to the vacuole membrane in wild-type cells (Piper *et al.*, 1997; Figure 3, top row). Unlike their distribution in wild-type cells, Pep12p, Vps10p, and Vph1p were all accumulated in an aberrant structure next to the vacuole in *nhx1*Δ cells (Figure 3, middle row).

VPS27 is characterized as a class E *VPS* gene, and cells lacking Vps27p have a large, aberrant prevacuole structure next to the vacuole (the class E compartment; Raymond *et al.*, 1992; Piper *et al.*, 1995). Proteins that normally reside in the PVC, those that normally cycle via the PVC back to the late Golgi, and proteins that travel via the PVC to the vacuole accumulate in the class E compartment of *vps27*Δ cells (Piper *et al.*, 1995). Significantly, the aberrant structure observed in cells lacking Nhx1p closely resembles the class E compartment seen in *vps27*Δ cells (compare Figure 3, middle and bottom rows). In *vps27*Δ cells Vps10p and Vph1p were seen almost exclusively in the class E compartment. How-

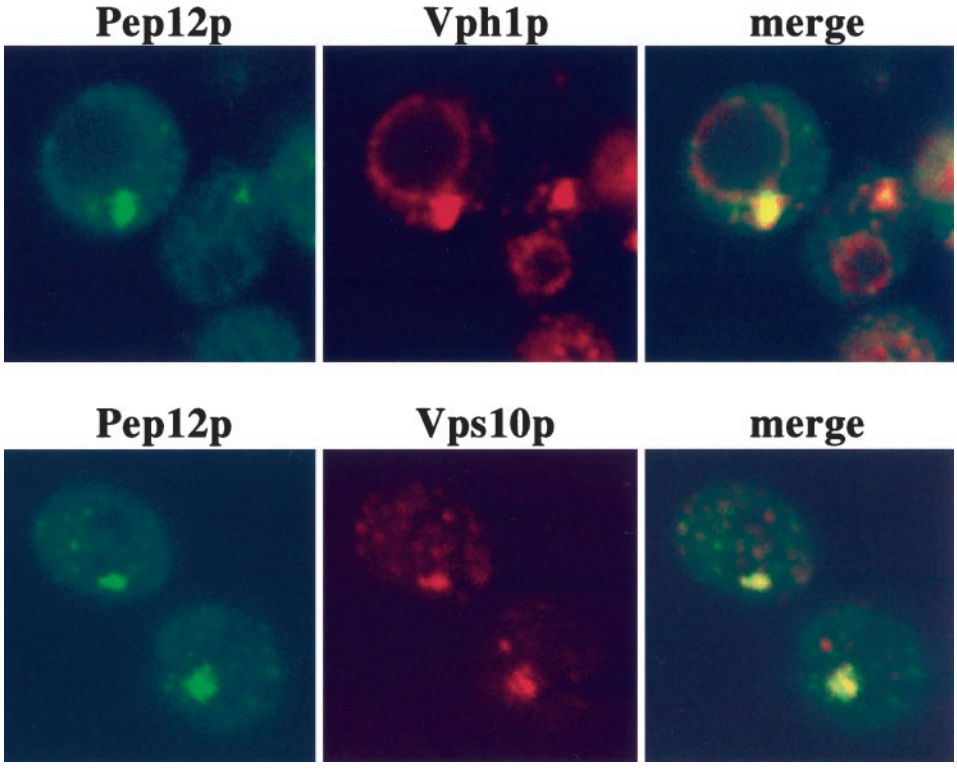


Figure 4. Colocalization of Pep12p, Vph1p, and Vps10p in *nhx1*Δ cells. Immunofluorescence was performed as described in MATERIALS AND METHODS, except cells were permeablized with 5% SDS for 5 min. The strain used was KEBY15 (*nhx1*Δ). Pep12p was visualized by using a monoclonal antibody against Pep12p followed by anti-mouse biotin and streptavidin-FITC. The same cells were also stained for Vps10p, or Vph1p by using polyclonal antibodies and a secondary Alexa-anti-rabbit antibody. Confocal micrographs were taken simultaneously of the red and green fluorescence channels and overlapped to produce the merged image.

ever, unlike *vps27*Δ, *nhx1*Δ cells appeared to possess a somewhat weaker morphological phenotype because some Vps10p and Vph1p was seen in the Golgi and vacuole, respectively.

We have also followed the immunolocalization of Vma2p/Vat2p, a peripheral membrane subunit of the V-ATPase in wild-type, *nhx1*Δ, and *vps27*Δ cells. In wild-type cells, Vma2p is localized to the vacuole membrane by immunofluorescence using an anti-Vma2p antibody (Figure 3, top row). Vma2p was predominantly in the class E compartment in *vps27*Δ cells, with little staining of the vacuole membrane (Figure 3, bottom row). In *nhx1*Δ cells, however, Vma2p clearly stained the vacuole and little could be seen in a class E-like structure (Figure 3, middle row). This result is consistent with *nhx1*Δ cells having a somewhat weaker PVC transport block than the *vps27*Δ cells. In addition, because Vma2p was used as a vacuolar marker for the original classification of *vps* mutants, this explains why *vps44* was previously designated as a class A *VPS* gene.

ALP follows a distinct pathway separate from CPY to the vacuole (Cowles *et al.*, 1997; Piper *et al.*, 1997). In wild-type cells, ALP stained the vacuole membrane by immunofluorescence with an anti-ALP antibody (Figure 3, top row). ALP was also localized to the vacuole membrane in *nhx1*Δ and *vps27*Δ cells (Figure 3, middle and bottom rows), suggesting that loss of either Nhx1p or Vps27p has no effect on the trafficking of ALP in these cells.

To determine whether PVC, late Golgi, and vacuolar proteins accumulated in the same aberrant structure in *nhx1*Δ cells, we performed colocalization immunofluorescence experiments. As shown in Figure 4, Pep12p, Vps10p, and Vph1p all colocalized in the class E-like compartment of *nhx1*Δ.

In addition to proteins that follow the CPY and ALP trafficking pathways to the vacuole, we have also examined endocytic protein trafficking by using the lipophilic fluorescent dye FM4-64, and an **a**-factor receptor-green fluorescent protein chimera (Ste3p-GFP). FM4-64 incorporates into the plasma membrane, from where it is endocytosed and transported via the endocytic pathway to the vacuole (Vida and Emr, 1995). Ste3p is a seven transmembrane receptor that is endocytosed from the plasma membrane in both a constitutive and ligand-dependent manner, and delivered to the lumen of the vacuole in wild-type cells, where it is degraded (Davis *et al.*, 1993). In wild-type cells lacking vacuolar proteases (*pep4-3*), Ste3p and Ste3p-GFP can be visualized in the vacuolar lumen (Piper *et al.*, 1995; Urbanowski and Piper, 1999; Gerrard *et al.*, 2000a; Figure 5). Cells expressing Ste3p-GFP were incubated for 15 min with FM4-64 dye, followed by a 30-min chase in fresh medium. The dye was seen in the limiting vacuole membrane of wild-type cells (Figure 5). However, in *vps27*Δ cells, both Ste3p-GFP and FM4-64 accumulated in the class E compartment. Some FM4-64 staining of the vacuole membrane was also visible (Vida and Emr, 1995; Figure 5). *nhx1*Δ cells also showed FM4-64 accumulation in the class E-like compartment, with some vacuolar staining (Figure 5). Our data indicate that *nhx1*Δ cells, similar to *vps27*Δ cells, are defective for the transport of endocytosed proteins to the vacuole.

In the experiments presented above, we have shown that Pep12p, Vps10p, Vph1p, Ste3p, and FM4-64 all accumulate in a large aberrant structure next to the vacuole in *nhx1*Δ

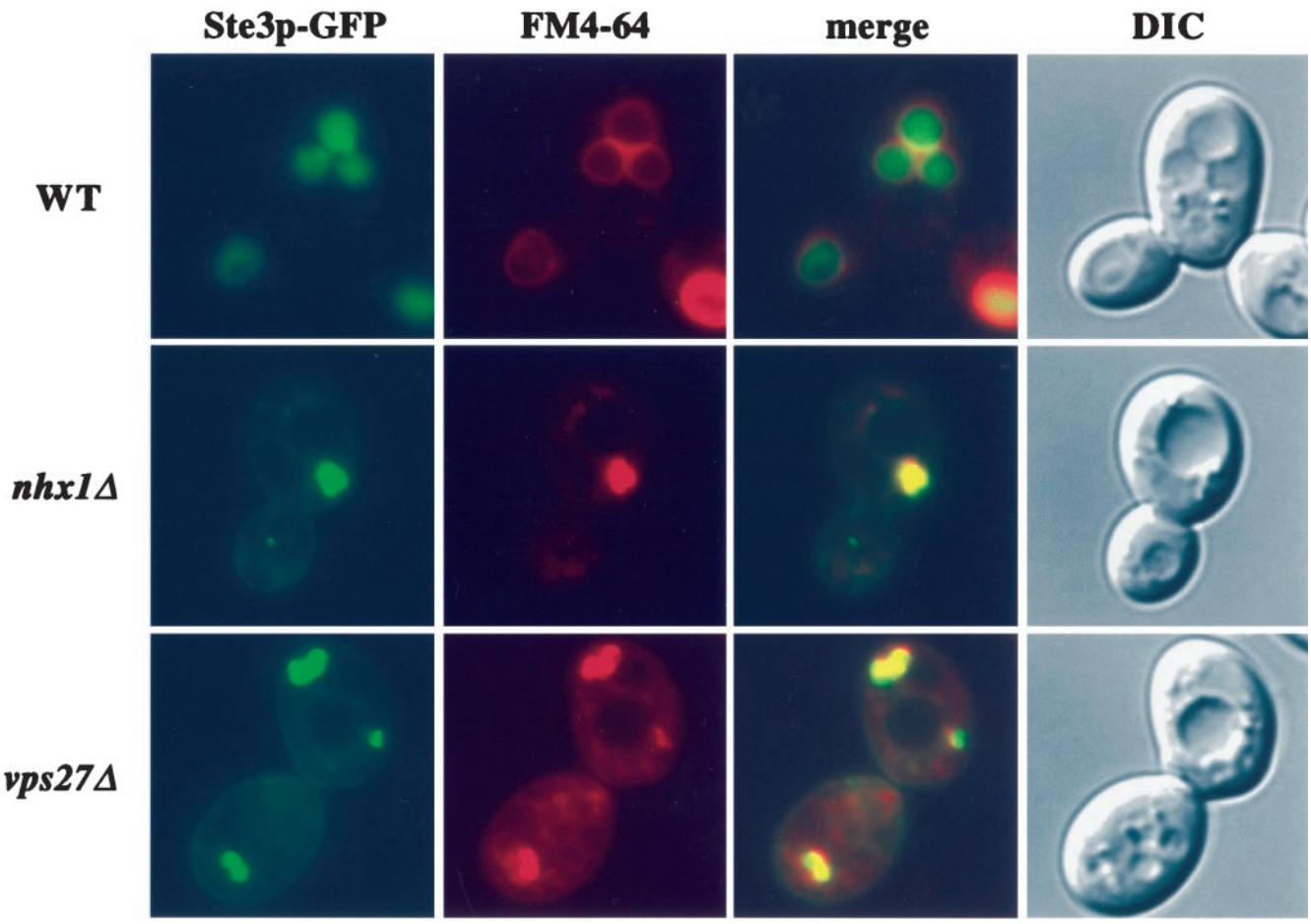


Figure 5. FM4-64 and Ste3p-GFP accumulate in the class E compartment of *nhx1*Δ cells. Wild-type (WT; SF838-9Dα), *nhx1*Δ (KEBY15), and *vps27*Δ cells (SGY73) were transformed with pJLU34. Cells were labeled in 40 μM FM4-64 for 15 min at 30°C and then chased in fresh medium for 30 min at 30°C. FM4-64 and Ste3p-GFP were photographed under the red and green fluorescence channels, respectively, and a merged image of these two channels is also shown. Differential interference contrast (DIC) images of the same cells were collected to visualize the vacuoles.

cells. This phenotype is similar to that seen for *vps27*Δ cells, and leads us to classify *NHX1/VPS44* as a class E *VPS* gene. The weaker morphological phenotype of *nhx1*Δ compared with *vps27*Δ is consistent with the observed levels of CPY secretion. *nhx1*Δ cells secrete 34 ± 7% of newly synthesized CPY (Figure 2), compared with *vps27*Δ cells that secrete 53 ± 1% (Bowers and Stevens, unpublished results; Piper *et al.*, 1995).

Previous experiments have shown that the class E compartment contains active proteases (Raymond *et al.*, 1992; Piper *et al.*, 1995; Bryant and Stevens, 1997). Thus, in a *vps27*Δ strain, Vps10p accumulates in the class E compartment and is proteolytically cleaved to a lower molecular mass form more rapidly than in wild-type cells (Piper *et al.*, 1995). To investigate the proteolytic cleavage of Vps10p in *nhx1*Δ cells, we performed pulse-chase immunoprecipitations of radiolabeled Vps10p (Figure 6, top). In wild-type cells, Vps10p was cleaved very slowly, with only 10% of Vps10p seen in the lower molecular weight form after 30 min. However, in *nhx1*Δ cells, 25% of newly synthesized Vps10p was proteolytically cleaved to the smaller form after 30 min, and in *vps27*Δ cells Vps10p was cleaved rapidly with 45% in the smaller form after 30 min.

Vps10p has signals within its cytoplasmic domain for retrieval from the prevacuole to the Golgi (Marcusson *et al.*, 1994; Cereghino *et al.*, 1995; Cooper and Stevens, 1996). Dipeptidyl aminopeptidase A/Ste13p also cycles between the late Golgi and the PVC, but unlike Vps10p is retained in the Golgi by both retrieval and retention mechanisms (Bryant and Stevens, 1997). We have investigated the proteolytic cleavage of A-ALP, a chimeric protein formed from the cytoplasmic domain (and hence including the trafficking signals) of dipeptidyl aminopeptidase A, and the membrane and lumenal domains of ALP (Nothwehr *et al.*, 1993). As shown in Figure 6 (middle), in wild-type cells, newly synthesized A-ALP is not significantly cleaved after 120 min. However, in *nhx1*Δ cells A-ALP is cleaved more rapidly, with 50% of the newly synthesized protein seen as the lower molecular weight form after 60 min. This aberrant processing of A-ALP in *nhx1*Δ cells is

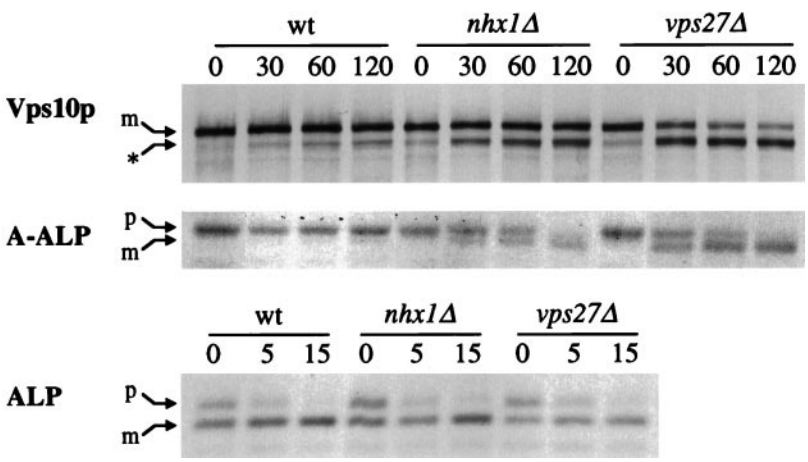


Figure 6. Pulse-chase immunoprecipitations of Vps10p, A-ALP, and ALP. Wild-type (WT; RPY10), *nhx1*Δ (KEBY13), and *vps27*Δ cells (AACY5) were pulsed for 10 min at 30°C with [^{35}S]cysteine and methionine, and chased for 0, 30, 60, or 120 min with excess unlabeled cysteine and methionine. Vps10p immunoprecipitates were separated on a 7% SDS polyacrylamide gel. The positions of mature Vps10p (m) and the smaller proteolytically cleaved form (*) are shown. A-ALP immunoprecipitations were carried out under the same conditions as used for Vps10p. The following strains were used, all of which had a deletion of the *PHO8* gene, which encodes ALP, and were transformed with pSN55 (A-ALP on a CEN-based plasmid): BLY1 (WT), KEBY14 (*nhx1*Δ), and KEBY37 (*vps27*Δ). The positions of the precursor form of A-ALP (p) and the proteolytically cleaved form (m) are indicated. ALP immunoprecipitations were carried out as for Vps10p, but by using 0-, 5-, and 15-min chase times. The positions of precursor ALP (p) and mature ALP (m) are shown.

not as rapid as in *vps27*Δ cells, which show 70% of A-ALP in the smaller form after 60 min.

Unlike Vps10p and A-ALP, which travel through a prevacuolar endosome compartment, ALP follows a distinct pathway to the vacuole that bypasses the PVC (Bryant and Stevens, 1997; Piper *et al.*, 1997). In wild-type cells, ALP is processed by vacuolar proteases to its mature form rapidly, with >50% of the protein in the mature form after a 10-min pulse of radiolabel (Klionsky and Emr, 1989; Piper *et al.*, 1997; Figure 6, bottom). A similar ALP processing rate to that in wild-type cells was observed in *nhx1*Δ and *vps27*Δ cells (Figure 6, bottom; Bryant *et al.*, 1998). Together with the immunofluorescence data presented above (Figure 3), these results indicate that Nhx1p plays no role in the trafficking of ALP to the vacuole.

The biochemical data for the proteolytic cleavage of Vps10p and A-ALP in *nhx1*Δ cells are consistent with the morphology studies presented above, and show that cells lacking Nhx1p have an aberrant PVC structure next to the vacuole that is proteolytically active. Thus, by both biochemical and morphological criteria *NHX1* is a class E *vps* gene. However, the trafficking phenotype of *nhx1*Δ cells is not as severe as that seen in *vps27*Δ cells.

The Function of Nhx1p in Protein Trafficking Is Distinct from that of the V-ATPase and the Chloride Channel Gef1p

Nhx1p, an endosomal sodium/proton exchanger, is proposed to sequester sodium ions inside the PVC in exchange for protons that are released into the cytosol (Nass *et al.*, 1997; Nass and Rao, 1998, 1999). It has been proposed that the proton gradient created by the V-ATPase across the PVC membrane is used by Nhx1p to transport sodium ions into this compartment (Nass and Rao, 1998; Gaxiola *et al.*, 1999; Quintero *et al.*, 2000). It is reasonable to assume therefore that Nhx1p function depends on the proton pumping activity of the V-ATPase. In addition, the chloride channel Gef1p (which has been shown to partially colocalize with Nhx1p; Gaxiola *et al.*, 1999) is proposed to transport chloride ions into the PVC and late Golgi (Figure 7A). Transport of chloride anions would serve to neutralize the membrane potential across the PVC membrane generated by the V-ATPase, allowing further acidification of the compartment (Gaxiola *et al.*, 1998, 1999; Schwappach *et al.*, 1998). We have studied the protein trafficking phenotype caused by loss of the V-ATPase or Gef1p, compared with that seen after loss of Nhx1p.

The V-ATPase is a protein complex consisting of 13 subunits, with a peripheral membrane V_1 subcomplex required for the hydrolysis of ATP, and an integral membrane V_0 subcomplex involved in proton translocation (reviewed in Graham and Stevens, 1999; Forgac, 2000). Deletion of any subunit of the complex (except Vph1p, which has a second isoform in yeast, Stv1p; Manolson *et al.*, 1994) results in loss of proton translocation. Gef1p is a putative voltage-gated chloride channel of the CLC family, and possibly functions as a homodimer (reviewed in Jentsch *et al.*, 1999). We have constructed yeast strains lacking Vma2p (a V_1 subunit of the V-ATPase), Vma3p (a V_0 subunit), or Gef1p, for phenotypic comparison with *nhx1*Δ cells.

As shown in Figure 7B, wild-type and *gef1*Δ cells secreted 4 ± 2% and 10 ± 0.5% of their newly synthesized CPY, respectively. This is consistent with a previous study, which demonstrated that *gef1* mutant cells do not secrete significant amounts of CPY (Li *et al.*, 1999). In contrast to *gef1*Δ, *vma2*Δ cells secreted 22 ± 2% of newly synthesized CPY, which is more than wild-type, but less than the 34 ± 7% secreted by *nhx1*Δ cells (Figure 7B). Figure 7B also shows that although ~20% of CPY was secreted in the *vma2*Δ strain, the majority of the intracellular CPY was mature after 40 min. Cells lacking the V_0 subunit Vma3p also secreted 22 ± 3% of newly synthesized CPY. Therefore, although cells lacking the V-ATPase show some secretion of CPY, they secrete significantly less CPY than cells lacking Nhx1p (Figure 7B).

We have also investigated the proteolytic cleavage of Vps10p in *vma2*Δ, *vma3*Δ, *gef1*Δ, and *nhx1*Δ. As shown in Figures 6 and 7C, Vps10p was more rapidly cleaved to a smaller product in *nhx1*Δ than in wild-type cells. However, in *vma2*Δ, *vma3*Δ, and *gef1*Δ cells, the kinetics of Vps10p proteolytic cleavage was indistinguishable from wild-type (Figure 7C). These data suggest that although *vma2*Δ and *vma3*Δ strains secrete some CPY, they have a trafficking phenotype distinct from that observed for *nhx1*Δ. This was

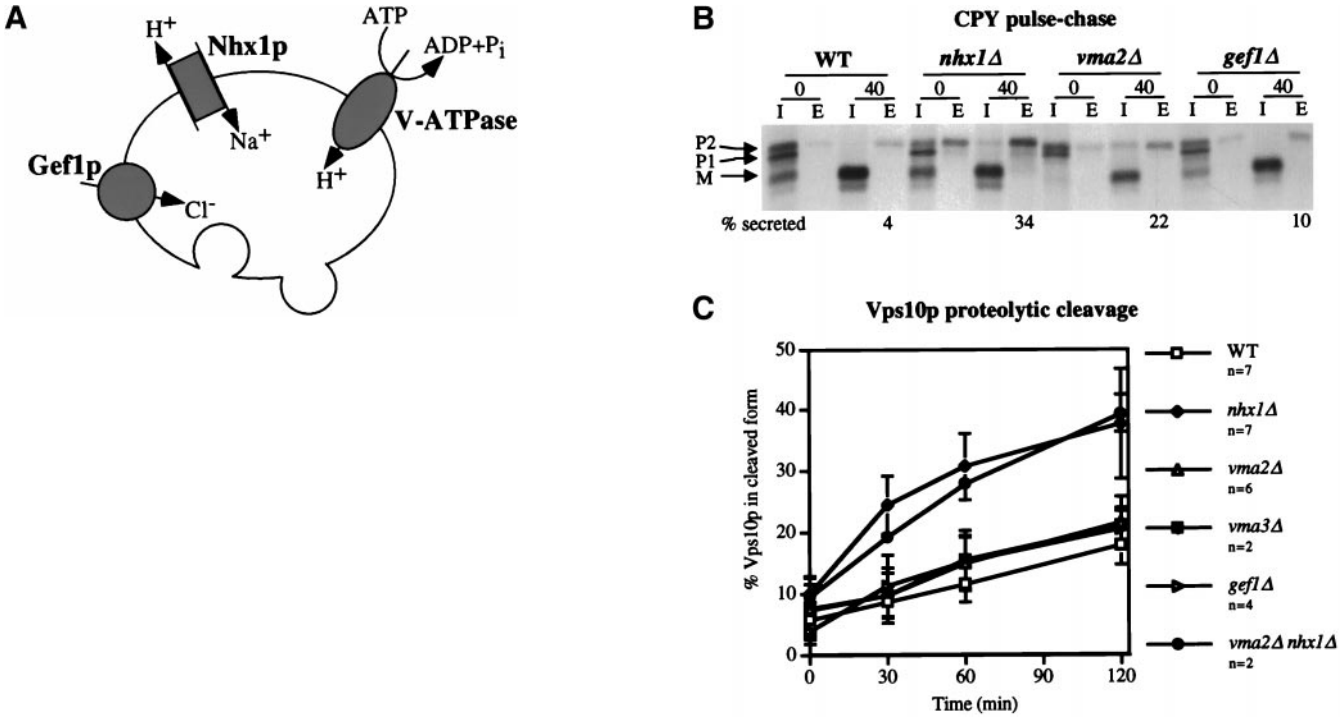


Figure 7. Analysis of the trafficking defect of *vma*Δ and *gef1*Δ cells. (A) Proposed ion transporters of the PVC. The V-ATPase actively pumps protons into the PVC, while Nhx1p passively exchanges sodium ions for protons driven by ion gradients across the membrane. Gef1p is a putative voltage-gated ion channel thought to transport chloride ions into the PVC. (B) CPY processing and secretion. Wild-type (RPY10), *nhx1*Δ (KEBY13), *vma2*Δ (KEBY27), and *gef1*Δ cells (KEBY32) were labeled with [^{35}S]methionine and cysteine for 10 min and then chased for 0 or 40 min at 30°C. CPY was immunoprecipitated as described in Figure 1, and in the MATERIALS AND METHODS. The amount of CPY secreted after a 40-min chase was quantified by using phosphoimager analysis, and is shown underneath the gel. The % CPY secreted for each strain is the average value obtained over several independent experiments (7 for WT and *nhx1*Δ, 3 for *vma2*Δ, and 2 for *gef1*Δ). (C) Proteolytic cleavage of Vps10p. Wild-type (RPY10), *nhx1*Δ (KEBY13), *vma2*Δ (KEBY27), *vma3*Δ (KEBY29 with pTS18), *gef1*Δ cells (KEBY32), and *nhx1*Δ *vma2*Δ cells (KEBY35) were metabolically labeled as described above and chased for 0, 30, 60, or 120 min. Vps10p immunoprecipitates were separated on a SDS polyacrylamide gel, which was exposed to a phosphoimager screen and the band intensities quantified. The amount of the lower, cleaved form of Vps10p (*) was calculated as a percentage of total Vps10p at each time point. Each point on the graph represents the mean of several independent experiments (n = number of experiments), and the error bars represent SDs from the mean.

supported by immunofluorescence experiments that localized Pep12p and Vph1p in *vma2*Δ, *vma3*Δ, or *gef1*Δ cells. As described above, *nhx1*Δ cells exhibit an aberrant PVC compartment next to the vacuole where Pep12p accumulates, distinct from its punctate localization in wild-type cells (Figures 3, 4, and 8). Pep12p did not accumulate in a class E-like compartment in *vma2*Δ, *vma3*Δ, or *gef1*Δ cells and instead exhibited a more wild-type, dispersed PVC staining (Figure 8; Bowers and Stevens, unpublished results). Vph1p also accumulated in the class E-like compartment of *nhx1*Δ cells (Figures 3, 4, and 8). However, no similar compartment was seen in *vma2*Δ and *gef1*Δ cells, and Vph1p antibodies stained the vacuole membrane (Figure 8; Bowers and Stevens, unpublished results). In *vma3*Δ cells, Vph1p is localized to the ER because the V_0 V-ATPase subcomplex is unable to assemble correctly (Jackson and Stevens, 1997).

The data presented here demonstrate that inactivation of the V-ATPase by the loss of a subunit, or loss of the chloride channel Gef1p does not have the same effect on protein trafficking in the CPY pathway as loss of the sodium/proton exchanger Nhx1p. *gef1*Δ cells showed no detectable trafficking defects of CPY, Vps10p, Vph1p, or Pep12p in our experiments, whereas *vma*Δ strains showed some secretion of CPY, and no defect in Vps10p cleavage, or Pep12p and Vph1p localization. Thus, loss of Nhx1p creates a much more severe effect on protein trafficking to the vacuole than loss of the V-ATPase. There are at least two interpretations of these results. One idea is that Nhx1p requires a proton gradient for function, and can still function in the absence of the V-ATPase (suggesting that the PVC is acidified in these cells by an alternative mechanism). The second hypothesis is that Nhx1p is only required if the V-ATPase is present, to reduce the proton gradient or raise the pH within the PVC (Figure 7A). To attempt to distinguish between these hypotheses, we constructed a double mutant *nhx1*Δ *vma2*Δ yeast strain. If Nhx1p is needed in the presence of the V-ATPase for correct protein trafficking, then we might expect the double mutant to phenocopy the *vma2*Δ single delete cells.

*nhx1*Δ *vma2*Δ cells had the growth phenotypes associated with cells lacking the V-ATPase (they failed to grow at neutral pH, or when 100 mM $CaCl_2$ was added to the growth medium). In addition, they secreted 37% of newly synthesized CPY and were thus indistinguishable from the

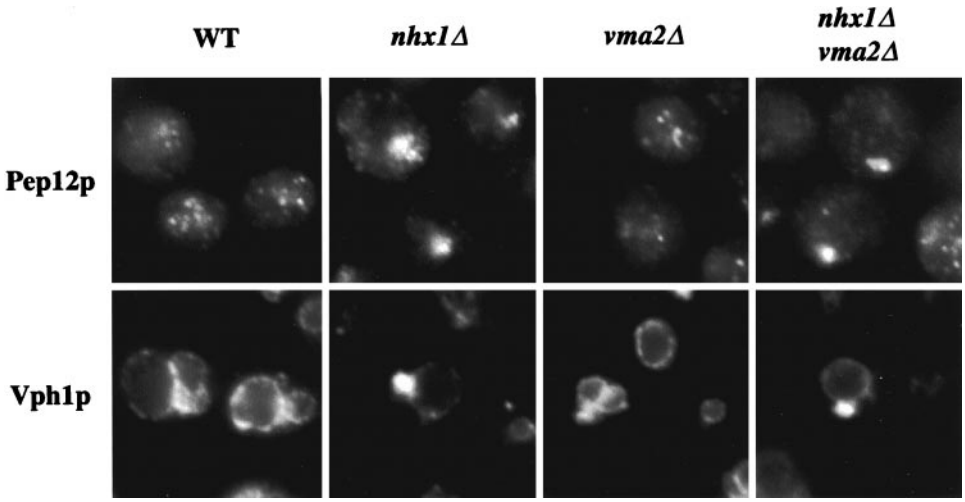


Figure 8. Unlike *nhx1*Δ or *nhx1*Δ *vma2*Δ cells, *vma2*Δ cells do not show a class E Vps$^-$ morphological phenotype. Immunofluorescence was performed as described in Figure 4, with wild-type (WT: SF8389Dα), *nhx1*Δ (KEBY15), *vma2*Δ (KEBY26), and *nhx1*Δ *vma2*Δ (KEBY34) cells and anti-Pep12p and anti-Vph1p antibodies. Images were captured by using a fluorescence microscope fitted with a digital camera.

*nhx1*Δ cells with respect to CPY sorting. Interestingly, *nhx1*Δ *vma2*Δ cells showed the class E phenotype of *nhx1*Δ cells, as measured by increased kinetics of Vps10p proteolytic cleavage (Figure 7C), and by immunofluorescence of Vph1p or Pep12p (Figure 8).

Three Acidic Residues Are Required for the Function of Nhx1p in Protein Trafficking

Our results show that Nhx1p is essential for correct protein trafficking in the yeast CPY pathway. However, the trafficking phenotype of yeast strains with an inactive V-ATPase is not as severe as the class E phenotype of *nhx1*Δ cells. These results were surprising because Nhx1p has been proposed to use the proton gradient generated by the V-ATPase to sequester sodium ions inside the PVC, and would thus rely on this proton gradient for its exchange function. We have investigated whether Nhx1p exchange activity might be important for vacuolar protein transport, or whether the presence of the protein is important, perhaps as a scaffold for other factors.

To study the effect of Nhx1p activity on protein trafficking, we sought to make mutant cells that correctly expressed and localized a form of Nhx1p predicted to lack ion exchange activity. Glutamic acid 262 of NHE1 has previously been shown to be essential for the sodium/proton exchange activity of the mammalian protein (Fafournoux *et al.*, 1994). In addition, studies of sodium/proton exchangers unrelated to the NHE family have shown that specific acidic residues within transmembrane domains are important for exchange activity (Inoue *et al.*, 1995; Dibrov *et al.*, 1998). Interestingly, multiple sequence alignment of NHE protein sequences from a wide range of organisms reveals that there are four highly conserved acidic residues within putative transmembrane domains, including E262 of NHE1, which aligns with E225 of Nhx1p (Figure 9A). We have substituted these four conserved acidic amino acids for uncharged, polar residues in Nhx1p by site-directed mutagenesis: aspartic acid (D) 201 was changed to asparagine (N), glutamic acid (E) 225 to glutamine (Q), D230 to N, and E355 to Q. Each of these mutations was made in *NHX1*-HA (see above) carried on a CEN-based plasmid. These plasmids were then transformed into *nhx1*Δ yeast to test for protein expression. Western blotting showed that all four of these mutant Nhx1p-HA proteins were full length and expressed at levels indistinguishable from wild-type Nhx1p-HA (Figure 9B), indicating that these mutations do not affect Nhx1p stability.

As shown in Figure 9C, Nhx1p-HA in wild-type cells is localized to a small punctate structure typical of the PVC (Pep12p staining is shown for comparison). To produce these images, cells containing one copy of *NHX1*-HA integrated into the genome were transformed with *NHX1*-HA on a CEN plasmid, thus producing 2–3 copies of *NHX1*-HA per cell. This level of expression allowed us to see the PVC localization of the protein. Overexpression of Nhx1p in our strains (from a 2 μ plasmid with the endogenous *NHX1* promoter) led to retention of Nhx1p-HA in the ER (Bowers and Stevens, unpublished results). Also shown in Figure 9C is the localization of Nhx1p-HA to the class E compartment of *vps27*Δ cells, where it colocalizes with Pep12p. The *vps27*Δ mutant background allows concentration of Nhx1p in the aberrant PVC of these cells, where it can be easily visualized by immunofluorescence. We thus transformed *vps27*Δ cells with wild-type or mutant forms of *NHX1*-HA. As shown in Figure 9D, the D201N, E225Q, D230N, and E355Q point mutant forms of Nhx1p-HA all correctly localized to the class E compartment.

As shown in Figure 10A, wild-type cells secreted ~5% of newly synthesized CPY into the extracellular medium, as did *nhx1*Δ cells with the complementing *NHX1*-HA plasmid. *nhx1*Δ cells expressing the E355Q mutant of Nhx1p also secreted around 5% CPY, consistent with this mutation having no effect on the function of Nhx1p in vacuolar trafficking. In contrast, the D201N, E225Q, and D230N mutant proteins were unable to complement the CPY secretion of *nhx1*Δ cells. *nhx1*Δ cells expressing these mutant proteins did not secrete as much newly synthesized CPY as *nhx1*Δ cells with empty plasmid, but secreted significantly more than wild-type cells (Figure 10A). These results suggest that Nhx1p with either a D201N, E225Q, or D230N mutation is unable to function normally in protein trafficking of CPY to the yeast vacuole. To see whether the effect of the point mutations was addi-

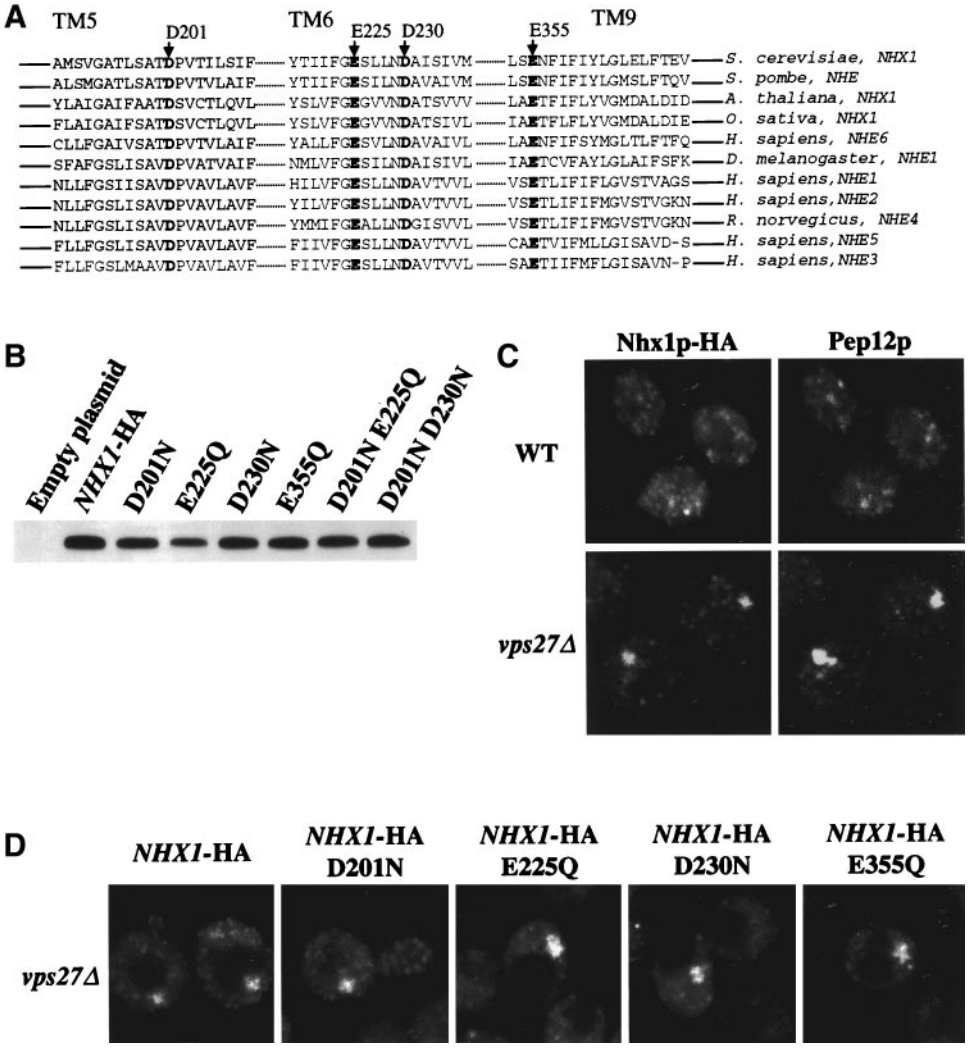


Figure 9. Mutation of conserved acidic residues in Nhx1p. (A) Partial sequence alignment of NHE protein sequences. NHE proteins with homology to Nhx1p have been found in many organisms. Those shown here are baker's yeast (*S. cerevisiae*) Nhx1p; fission yeast (*S. pombe*) NHE; thale cress (*A. thaliana*) NHX1; rice (*Oryza sativa*) NHX1; fruit fly (*Drosophila melanogaster*) NHE1; human (*Homo sapiens*) NHE proteins 1, 2, 3, 5, and 6; and rat (*Rattus norvegicus*) NHE4. The alignment was generated by using complete amino acid sequences, though only predicted transmembrane domains 5, 6, and 9 are shown. Acidic residues conserved throughout the NHE family (D201, E225, and D230 in Nhx1p) are in bold type. (B) *NHX1*-HA point mutant constructs make full-length proteins, expressed to wild-type levels. Whole-cell lysates were prepared from *nhx1*Δ cells (KEBY10) transformed with pRS316 (empty plasmid), or pRS316 with wild-type or mutant forms of *NHX1*-HA (pKEB38, 44, 45, 46, 47, pFP1, or pFP2). Fifteen micrograms of total protein was loaded per lane. Although E225Q appears to express slightly less Nhx1p-HA than the other strains, this was not significant over several experiments. In addition, this strain has the same point mutation as the D201N E225Q mutant, which shows wild-type expression levels in this experiment. (C) Localization of Nhx1p-HA. Rabbit polyclonal antibodies against the HA epitope were used to immunolocalize Nhx1p-HA in wild-type cells (WT; KEBY11 with pKEB38), or *vps27*Δ cells (*vps27*Δ; KEBY12 with pKEB38). Cells were costained by using a monoclonal antibody to Pep12p. (D) Localization of Nhx1p-HA point mutants. *vps27*Δ cells were transformed with pRS316 containing wild-type or mutant forms of *NHX1*-HA (pKEB38, 44, 45, 46, or 47). Nhx1p-HA proteins were immunolocalized with rabbit polyclonal antibodies against the HA epitope. The images in C and D are confocal micrographs.

tive, we constructed double mutants of D201N and E225Q, and D201N and D230N. These double mutants were expressed to wild-type levels in *nhx1*Δ cells (Figure 9B), and had CPY secretion levels that were not significantly different from those seen for the D201N, E225Q, or D230N single mutants (Figure 10A).

As a further test of the function of Nhx1p point mutants in membrane trafficking, we investigated the morphological phenotype of these cells. *nhx1*Δ cells expressing the point mutant forms of Nhx1p-HA were analyzed by fluorescence microscopy using the dye FM4-64. As described in Figure 5, FM4-64 accumulates in the class E-like compartment of

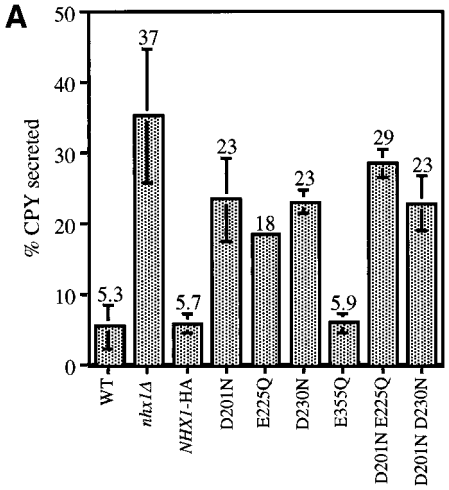
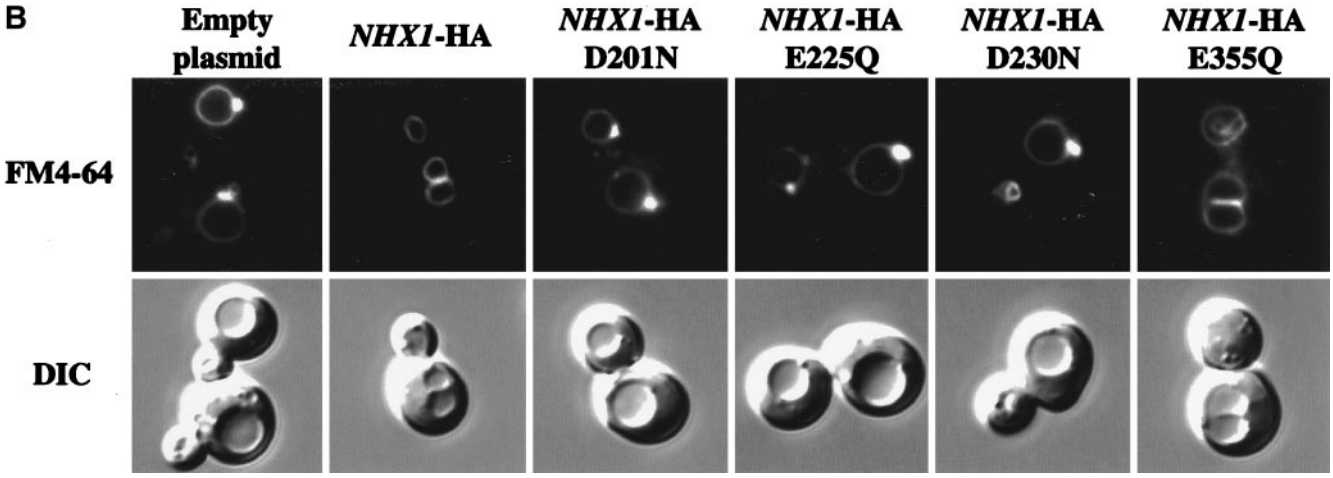


Figure 10. Phenotype of *nhx1*Δ cells expressing Nhx1p-HA point mutants. The strains used in these experiments were wild-type (WT; SEY6210 with empty plasmid), *nhx1*Δ (KEBY10 with empty plasmid), *nhx1*Δ with *NHX1-HA* on a CEN plasmid (NHX1-HA; KEBY10 with pKEB38), and *nhx1*Δ with *NHX1-HA* point mutants on CEN plasmids (D201N, E225Q, D230N, E355Q, D201N E225Q, and D201N D230N; KEBY10 with pKEB44, pKEB45, pKEB46, pKEB47, pFP1, and pFP2, respectively). (A) CPY secretion. Cells were labeled in [^{35}S]cysteine and methionine for 10 min and incubated in excess nonradioactive cysteine and methionine for 40 min. CPY was immunoprecipitated as described in Figure 2 and the MATERIALS AND METHODS. The gel was exposed to a phosphoimager screen, and the amount of CPY secreted into the extracellular medium was quantified as the percentage of total CPY in the sample. Each bar on the graph represents the mean percentage of CPY secreted over five separate experiments for WT, *nhx1*Δ, and *NHX1*-HA; three separate experiments for D201N, E225Q, D230N, and E355Q; and two separate experiments for D201N E225Q and D201N D230N. The numbers above each bar are the mean averages of CPY secreted over all experiments, and the error bars represent SDs from this mean. (B) FM4-64 staining. Cells were stained with FM4-64 as described in Figure 5. Fluorescence images are shown, as well as differential interference contrast (DIC) images for the same cells.



*nhx1*Δ cells. Consistent with the CPY secretion data (Figure 10A), *nhx1*Δ cells expressing the D201N, E225Q, and D230N mutant forms of Nhx1p-HA accumulated FM4-64 in a large, aberrant structure next to the vacuole (Figure 10B). In cells expressing either wild-type Nhx1p-HA or the E355Q mutant FM4-64 was seen exclusively on the vacuole membrane. As expected from our previous data (Figures 3, 4, and 5), Pep12p and Vph1p also accumulated in the class E-like compartment of cells expressing the D201N, E225Q, or D230N mutant forms of Nhx1p-HA, but showed wild-type staining patterns in cells expressing wild-type Nhx1p-HA or the E355Q mutant (Bowers and Stevens, unpublished results). Thus, we have created point mutant forms of Nhx1p that are expressed and localized normally but fail to function in the trafficking of proteins to the vacuole.

DISCUSSION

We report the identification of the yeast vacuolar protein sorting gene *VPS44* as *NHX1*. The *NHX1* gene encodes a sodium/proton exchanger, Nhx1p, of the NHE family. Nhx1p is localized to the PVC, and our data indicate that Nhx1p is required for protein trafficking out of this compartment.

Three Acidic Resides Are Essential for the Function of Nhx1p in Protein Trafficking

Of all the NHE proteins identified so far, Nhx1p is most closely related (50% identical) to a putative protein from the yeast *Schizosaccharomyces pombe*. NHE proteins are also found in plants, insects, amphibians, crustaceans, and mammals. The yeast Nhx1p sequence is 21–24% identical to mammalian NHE proteins 1–5, and 31% identical (60% similar) to human NHE6. The highest sequence identities are found within the putative transmembrane domains, suggesting an important role for these regions in the function of NHE proteins.

We have identified four acidic residues within putative transmembrane domains of NHE proteins that are con-

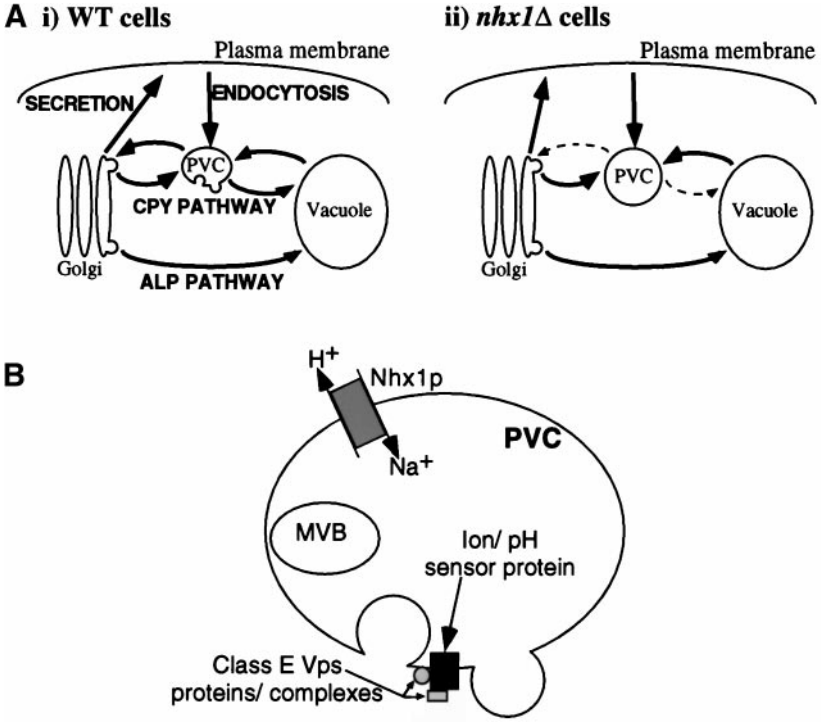


Figure 11. Function of Nhx1p in yeast protein trafficking through the PVC. (Ai) Protein trafficking pathways from the late Golgi to the vacuole in wild-type cells. Proteins can reach the vacuole via several different routes. Proteins such as CPY follow a pathway to the vacuole that includes transport through an endosomal PVC. The ALP pathway allows some proteins to reach the vacuole via an alternate route, bypassing the PVC. Proteins can also reach the vacuole following endocytosis from the plasma membrane. The endocytic and CPY pathways converge at or before the PVC. (Aii) Nhx1p functions to mediate exit from the PVC. In *nhx1*Δ cells, exit from the PVC to the Golgi and to the vacuole is inhibited (represented by dashed arrows). (B) Model for the function of Nhx1p in protein trafficking. A specific environment inside the PVC is essential for protein trafficking. This environment is dependent upon the activity of Nhx1p. The lumenal ion concentration and pH are monitored by a transmembrane protein or sensor, which transmits this information to the cytosolic face of the membrane. The binding of cytosolic proteins to the PVC membrane is dependent on the correct intralumenal environment as determined by the sensor. Candidates for these cytosolic factors include the class E proteins/complexes that control exit from the PVC (vesicular budding into the cytosol) and multivesicular body (MVB) formation (vesicular budding into the PVC lumen).

served across all species (Figure 9A). Mutation of these residues in Nhx1p to uncharged, polar residues by site-directed mutagenesis results in proteins that are expressed to wild-type levels and localized correctly to the PVC (Figure 9). However, three of these mutant proteins are unable to function in protein transport out of the PVC, as measured by the secretion of CPY and the accumulation of the endocytic dye FM4-64 in an aberrant prevacuole structure (Figure 10). Although we have not measured sodium/proton exchange activity directly, there is some evidence that the acidic resides we mutated in Nhx1p may be important for ion exchange. By sequence alignment with other NHE proteins, one of these residues (E225) is equivalent to E262 of human NHE1. Interestingly, when E262 of NHE1 is mutagenized to I, the protein is expressed correctly and able to dimerize, but shows no detectable sodium/proton exchange activity (Fafournoux *et al*., 1994). Our results are also consistent with the idea that acidic residues in transmembrane domains 5 and 6 of the NHE proteins constitute ion binding sites of these exchangers (Counillon and Pouyssegur, 2000). Our results suggest that Nhx1p does not act as a scaffold for other proteins at the PVC, but rather that the ion exchange activity of Nhx1p may be essential for correct vacuolar trafficking in yeast. However, further experiments are needed to link the protein trafficking phenotypes of the *nhx1*Δ cells to the sodium/proton exchange activity of Nhx1p.

Nhx1p Is Required for Trafficking out of the PVC

Our results show that yeast cells lacking the sodium/proton exchanger, Nhx1p, secrete ~35% of newly synthesized CPY. *nhx1*Δ cells form an aberrant PVC structure next to the vacuole in which late Golgi, PVC, and vacuolar proteins accumulate. In addition, we have shown that proteins trapped in this aberrant PVC are subject to abnormal proteolytic cleavage, consistent with the PVC containing active proteases (Raymond *et al*., 1992; Piper *et al*., 1995). Compared with *vps27*Δ cells, *nhx1*Δ cells have a similar class E compartment next to the vacuole, but less of each marker protein appears trapped in this compartment. For example, although Vph1p accumulates in the aberrant PVC of *nhx1*Δ cells, some is also seen on the vacuole membrane (Figure 3). The morphological phenotypes are consistent with the lower levels of CPY secretion, and slower kinetics of Vps10p and A-ALP cleavage in *nhx1*Δ cells compared with *vps27*Δ cells (Figures 2 and 6). Thus, our results show that the phenotype of *nhx1*Δ cells is similar, though somewhat weaker, to that seen for *vps27*Δ cells. *VPS27* belongs to the class E subset of *VPS* genes (Raymond *et al*., 1992; Piper *et al*., 1995). Thus, Nhx1p is likely to act at the same step in trafficking to the vacuole as the other class E Vps proteins (Figure 11A). Class E Vps proteins are thought to control protein trafficking out of the PVC (to the late Golgi, and to the vacuole; Piper *et al*., 1995; Rieder *et al*., 1996; Finken-Eigen *et al*., 1997). In addition, recent reports suggest that class E Vps proteins are essential for the formation of multivesicular bodies, a process that is also dependent on the phosphatidylinositol 3-phosphate 5-kinase Fab1p (Babst *et al*., 1997, 1998).

Thirteen other class E *VPS* genes have been identified to date: *REN1 (VPS2)*, *VPS4 (END13)*, *VPS20*, *VPS22*, *VPS23 (STP22)*, *VPS24*, *VPS25*, *VPS27*, *VPS28*, *VPS31*, *SNF7 (VPS32)*, *VPS36 (VAC3)*, and *VPS37* (Raymond *et al*., 1992; Davis *et al*., 1993; Tu *et al*., 1993; Piper *et al*., 1995; Nothwehr *et al*., 1996; Rieder *et al*., 1996; Babst *et al*., 1997, 1998, 2000; Finken-Eigen *et al*., 1997; Luo and Chang, 1997; Li *et al*.,

1999). Although several of the class E *VPS* genes have yet to be cloned, those that have been identified encode cytosolic or peripheral membrane proteins. This has led to the hypothesis that the class E proteins associate transiently with the PVC membrane and function (perhaps as complexes) in trafficking out of the PVC and in multivesicular body formation (Babst *et al.*, 1998). To support this idea, recent studies have shown that both Vps24p and Snf7p transiently associate with the PVC membrane. Vps24p and Snf7p require the activity of an AAA-type ATPase, Vps4p, for dissociation from the membrane (Babst *et al.*, 1998). Vps23p and Vps28p have also been demonstrated to be part of a cytosolic protein complex (Babst *et al.*, 2000).

We hypothesize that the sodium/proton exchange activity of Nhx1p is important for its role in protein trafficking. There is evidence from studies of mammalian cells that the internal environment of endosomes is critical for the binding of cytosolic factors to the membrane. The binding of a subset of COP1 coat proteins to early endosome membranes is dependent on the pH within the organelle (Aniento *et al.*, 1996; Gu *et al.*, 1997). This has led to the hypothesis that an unknown transmembrane protein acts as a pH sensor, transmitting information about the internal environment across the membrane to the cytosolic face (Gu and Gruenberg, 1999).

Given that *nhx1*Δ cells show a class E Vps$^-$ phenotype, we suggest that sodium/proton exchange regulates the environment inside the PVC, and that this internal environment determines the binding of other, cytosolic proteins to the PVC membrane (Figure 11B). These cytosolic proteins may control vesicle budding from the PVC, multivesicular body formation (budding of membrane into the PVC), and/or the sorting of membrane proteins at this compartment. Some amount of membrane association of these cytosolic factors to the PVC membrane, even in the absence of Nhx1p may account for the less severe class E phenotype of *nhx1*Δ cells compared with deletion of other class E genes. Possible candidates for association with the PVC in a Nhx1p-dependent manner include proteins that appear to act at the same transport step such as the class E Vps proteins (or complexes of proteins).

We show that Nhx1p plays a role in endosomal protein transport in yeast. Is this role conserved in higher organisms? Of the six NHE proteins identified in mammalian cells, NHE6 shows higher sequence homology than the others to Nhx1p. NHE6 is also the only mammalian NHE to fall into the subset of intracellular NHE proteins identified by phylogenetic analysis (Fukuda *et al.*, 1999). However, NHE6 has been localized to the mitochondrial inner membrane, suggesting that it is unlikely to be the Nhx1p orthologue (Numata *et al.*, 1998). The identity of a mammalian Nhx1p orthologue localized to endosomes therefore remains unclear, but it is possible that the plasma membrane NHE proteins undergo endocytosis and remain active in endosomal compartments (D'Souza *et al.*, 1998). Several other species, such as fruit fly, rice, and thale cress have intracellular NHE proteins with homology to Nhx1p (Figure 9). It will be interesting to see whether these NHE proteins also prove to be involved in intracellular protein trafficking. Of the other yeast class E Vps proteins, Vps23p, Vps27p, and Vps4p are known to have mammalian orthologues (TSG101, Hrs, and SKD1, respectively), suggesting that their functions are conserved (Komada *et al.*, 1997; Li *et al.*, 1999; Scheuring *et al.*, 1999). This is supported by experimental evidence that suggests Tsg101, Hrs, and SKD1 play roles in transport through the endosomal system (Komada *et al.*, 1997; Komada and Soriano, 1999; Babst *et al.*, 2000; Bishop and Woodman, 2000; Yoshimori *et al.*, 2000). Thus, it seems that the functions of the yeast class E Vps proteins are conserved in higher organisms.

Nhx1p Can Function in the Absence of the V-ATPase or the Chloride Channel Gef1p

Previous studies have proposed that Nhx1p sequesters sodium ions into the PVC in exchange for protons, by using the proton gradient generated by the V-ATPase (Nass *et al.*, 1997; Nass and Rao, 1998, 1999; Gaxiola *et al.*, 1999; Quintero *et al.*, 2000; Figure 7A). In addition, a putative voltage-gated chloride channel of the CLC family (Gef1p) is proposed to transport chloride ions into the PVC (Gaxiola *et al.*, 1999; Figure 7A). Gef1p is thought to neutralize the membrane potential generated by ion transporters such as the V-ATPase and Nhx1p, to allow further acidification of the PVC (Gaxiola *et al.*, 1998, 1999; Schwappach *et al.*, 1998). Thus, activity of Nhx1p (which presumably requires a proton gradient for function) may depend on the activities of the V-ATPase and Gef1p.

Our data indicate that the chloride channel Gef1p is not required for protein transport to the yeast vacuole because cells lacking Gef1p do not secrete significant amounts of CPY, or show abnormal processing of Vps10p. Furthermore, cells lacking Gef1p showed no characteristics of the *vps* mutant classes by immunofluorescent localization of Pep12p, Vph1p, Vma2p, or Vps10p, or by uptake of the endocytic tracer FM4-64 (Levi, Bowers, and Stevens, unpublished results). Cells lacking Gef1p therefore do not have the same phenotype as cells lacking Nhx1p.

We find that loss of V-ATPase activity by the deletion of a gene encoding either a V_1 subunit (Vma2p), or a V_0 subunit (Vma3p), results in less secretion of CPY than loss of Nhx1p. In addition, cells lacking the V-ATPase (unlike cells lacking Nhx1p) do not show abnormal cleavage of Vps10p, and do not accumulate the aberrant PVC structure typical of class E *vps* mutants (Figures 7C and 8). We have also constructed a yeast strain lacking both the V-ATPase and Nhx1p (*vma2*Δ *nhx1*Δ). This double deletion strain shows a CPY secretion phenotype, Vps10p proteolytic cleavage, and morphological phenotype identical to that seen for *nhx1*Δ cells (Figures 7C and 8; Bowers and Stevens, unpublished results). Our results establish that loss of V-ATPase function produces a less severe vacuolar trafficking phenotype than the loss of Nhx1p. Because loss of Nhx1p either in the presence (*nhx1*Δ cells) or absence (*nhx1*Δ *vma2*Δ cells) of the V-ATPase produces a similar protein trafficking phenotype, this suggests that Nhx1p can function even in the absence of the V-ATPase. The *nhx1*Δ *vma2*Δ results also argue against the lack of Vps10p cleavage in the *vma2*Δ cells being due to lower protease activity in the PVC of cells lacking an active V-ATPase.

Experiments in mammalian cells have shown that the acidification of endosomes is mediated by the V-ATPase, and is required for protein trafficking in the endocytic system (Johnson *et al.*, 1993; Clague *et al.*, 1994; van Weert *et al.*, 1995; Aniento *et al.*, 1996). Studies on yeast protein traffick-

ing in the absence of V-ATPase function, however, have led to conflicting results (Nelson and Nelson, 1990; Yamashiro *et al*., 1990; Klionsky *et al*., 1992a,b; Yaver *et al*., 1993; Morano and Klionsky, 1994). Our results, comparing cells lacking Nhx1p to cells lacking the V-ATPase, indicate that a sodium/proton exchanger can function in the absence of the V-ATPase. This leads us to hypothesize that yeast are able to acidify the PVC even in cells lacking the V-ATPase. What then functions to acidify the PVC of these yeast cells?

Cells devoid of a functional V-ATPase are unable to grow in medium buffered to pH 7.5. However, they are able to survive at pH 5.0, suggesting that in low pH medium the essential function of the V-ATPase is bypassed. The cellular basis for this phenotype is unclear, although some have suggested that acidification of an intracellular compartment is essential for yeast cell viability (Nelson *et al*., 2000). It has been suggested that fluid phase endocytosis might deliver protons from the external medium to endosomes, allowing cell survival (Nelson and Nelson, 1990; Munn and Riezman, 1994). However, a recent study by Plant *et al*. (1999) argues against this idea because blocking endocytosis in *vma* mutants does not prevent acidification of the vacuole in low pH medium. It is also possible that mislocalization of the plasma membrane P-type ATPase, Pma1p, in cells lacking the V-ATPase may allow growth at low pH (Nelson *et al*., 2000). It has also been suggested that transport of ammonium ions may allow equilibration of the pH across the plasma membrane and intracellular membranes under acidic growth conditions (Plant *et al*., 1999). Our results are consistent with the idea that under low pH growth conditions, the PVC of yeast cells lacking the V-ATPase still maintains a proton gradient with respect to the cytosol. This proton gradient, established even in the absence of the V-ATPase, would allow Nhx1p to exchange sodium ions for protons across the PVC membrane, a function critical for normal vacuolar protein sorting.

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