CHARACTERIZATION OF A SODIUM/ PROTON EXCHANGER INVOLVED IN THE SORTING OF PROTEINS IN YEAST

by

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A THESIS

Presented to the Department of Biology
and the Honors College of the University of Oregon
in partial fulfillment of the requirements
for the degree of
Bachelor of Arts

June 2000

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An Abstract of the Thesis of

Falguny I. Patel for the degree of Bachelor of Arts

in the Department of Biology to be taken June 2000

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Newly synthesized proteins are transported across the endoplasmic reticulum (ER) and delivered to the Golgi complex where transport pathways to the vacuole diverge. Proteins such as carboxypeptidase Y (CPY) go from the late Golgi through a prevacuolar compartment (PVC), to the vacuole. Nhx1p is a sodium/ proton exchanger that is localized to the PVC and predicted to have 10-12 transmembrane domains. Approximately 35% of newly synthesized CPY is missorted to the cell surface in *nhx1Δ* cells, as revealed by pulse-chase experiments. Studies have also shown that proteins following the CPY pathway are prevented from reaching the vacuole, resulting in an enlargement of the PVC. The results presented in this thesis show that Nhx1p is required for trafficking of proteins destined for the vacuole. In addition, residues have been identified that significantly affect the role of Nhx1p in vacuolar transport. Site-directed mutagenesis has yielded residues within transmembrane domains, presumed to affect ion-binding sites. Random mutagenesis using hydroxylamine, has yielded residues along the large C-terminal end that has been implicated in regulatory functions as well as transmembrane domains, strengthening the idea that they are important in the exchange

activity of Nhx1p. Characterization of Nhx1p through mutational analysis has shown its importance in protein trafficking and has provided insight towards its function. The transport of protons out of the PVC in exchange for sodium ions may be essential for the maintenance of the ionic environment inside the PVC necessary for the associations of other factors required for protein trafficking.

ACKNOWLEDGEMENTS

I wish to express my sincere thanks to Dr. Tom H. Stevens for giving me the opportunity to do research in his lab this past year. I would also like to thank Dr. Katherine E. Bowers whose mentorship has proven to be invaluable in the completion of this thesis. Through her patience and guidance, I have gained knowledge of basic molecular biology research as well as research techniques.

I also wish to thank members of the Stevens' lab: Dr. Laurie A. Graham, Dr. Elizabeth Conibear, Dr. Ben Powell, Dr. Young Kweon, and Andrew Jost, who have given me advice on numerous accounts and have been extremely helpful in answering my questions. And to everyone in the Stevens' lab as well as Dr. Dave Mitchell from the George Sprague lab, for all the goodies and the donuts!

Lastly, I would like to thank my family and friends. Especially for their belief in my abilities and constant encouragement and support. Finally, I thank God for inspiring me and giving me faith and insight.

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CHAPTER I

INTRODUCTION

Cells, and the structures that they are composed of, are too small to be seen or touched. Yet in spite of this handicap, cells are the subject of numerous publications each year. The study of molecular cell biology has arisen from human curiosity as well as human intelligence for devising the instruments and techniques by which discoveries can be made. At one end of the spectrum, astronomers are learning of objects that are out of the universe. At the other end, nuclear physicists focus their attention on subatomic particles that have equally inconceivable properties. It is clear that our universe consists of worlds within worlds.

Both cellular and molecular biology are inherently tied and based on the view that the knowledge of parts of a whole can explain the character and function of that whole. Thus, trying to explain things in terms of the machinery of the living system gets us closer to unraveling the mystery of life. New discoveries provide insight towards the complexity of the mechanisms that underlie cellular activity allowing us to get closer to drawing the "big picture." Modern research utilizes various biological models to answer questions that further our understanding of the cell. This current knowledge can then be directed and applied towards more tangible concepts such as medicine and agriculture.

The following chapters of this thesis focus on a novel protein involved in the sorting of proteins to their correct locations in the cell, using the yeast species *Saccharomyces cerevisiae* as a research model for biochemical and genetic studies.

A Research Model: Yeast

The yeast cell is a eukaryotic microorganism with a nucleus, which houses genetic information, and several other compartments. Cells that do not have a nucleus by contrast are classified as prokaryotes, for example bacteria. Eukaryotic cells are what make up you and me or more generally, multi-cellular organisms. Some of the properties that make yeast suitable for genetic studies are rapid growth rate, simple nutritional requirements, ease of isolating mutants, availability of entire genome sequence, and accessibility to genetic engineering techniques. In other words, yeast is a popular research model because it is a simple eukaryotic cell that can be manipulated easily.

A yeast cell is separated from its environment and defined by a border known as the plasma membrane, which is surrounded by a cell wall. Within this membrane is an aqueous or "wet" environment containing the nucleus and other membrane-bound compartments, which are known as organelles. These organelles are individually regulated and functionally distinct, each having their own inner environment. Organelles of the eukaryote include the nucleus, mitochondria, endoplasmic reticulum, Golgi complex, pre-vacuolar compartments (or endosomes), and vacuoles (or lysosomes). The nucleus contains all the information necessary for life in the form of DNA (deoxyribonucleic acid). DNA is decoded to make specific proteins needed for the cell to live and communicate with its surroundings.

Sorting of Proteins

The endoplasmic reticulum (ER) is a maze of parallel internal membranes that are continuous with the membrane of the nucleus. The ER has a number of functions but is most notably important in the synthesis of proteins. Once proteins are made they are either released into the cytosol or they enter the protein sorting pathway and are transported to the lumen of the ER. Vesicles, or tiny packages, containing the proteins bud off from the ER and are carried to the Golgi complex, which is a series of stacked membranes. The proteins are then chemically modified as they proceed through the stacks from *cis* (closest to ER) to *trans* (away from ER) Golgi. At the *trans* Golgi, or last Golgi subcompartment, the transport pathways diverge. The proteins are again packaged into vesicles and are directed into one of the several pathways (Bryant and Stevens, 1998). This sorting of proteins is necessary for proper function of the cell, to ensure that specific proteins are delivered to their specific destinations. If any of the pathways are disrupted these new proteins cannot get to where they are needed and the consequences of this can range from minor dysfunction to cell death.

Vacuolar Protein Sorting

My research specifically addresses proteins being sent to the vacuole. The vacuole in yeast, which is similar to the lysosome in mammals in terms of its functions, plays a key role in the physiology of this organism. Its functions include ion transport, pH homeostasis, osmoregulation, protein degradation, and storage of amino acids, small

ions, and polyphosphates (Bryant and Stevens, 1998; Jones et al., 1997). In order to perform these functions the vacuole requires specific proteins. These essential proteins are delivered to the vacuole by several known transport pathways - of which three are of relevance to this study (Figure 1).

Proteins such as the vacuolar enzyme carboxypeptidase Y (CPY) follow a route going from the *trans* Golgi via the pre-vacuolar compartment (PVC) to the vacuole in a pathway known as the CPY pathway (1). Other proteins such as alkaline phosphatase (ALP) take a direct route, known as the ALP pathway, from the *trans* Golgi to the vacuole, bypassing the PVC (2). The third route from the *trans* Golgi allows secretory proteins to be transported directly to the plasma membrane or cell surface (3). Proteins can then reach the vacuole following endocytosis of material from the plasma membrane. The regulation and detailed mechanism of each of these pathways is not completely understood. My project focuses on two of these, the CPY pathway and endocytosis. We know that proteins following these pathways must go through an intermediate compartment (the PVC) before reaching the vacuole.

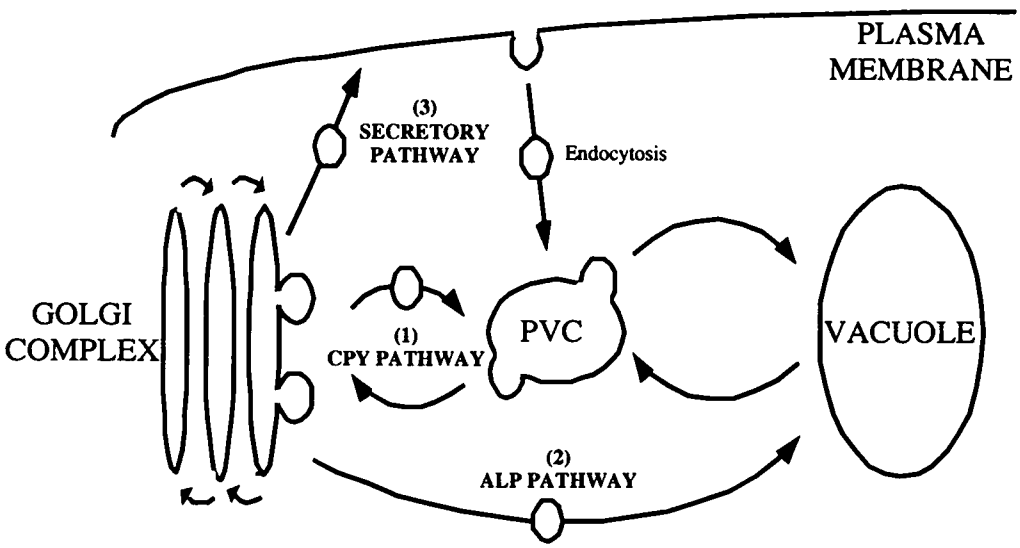


FIGURE 1. Protein trafficking in yeast. (1) Proteins transported via the carboxypeptidase Y (CPY) pathway go through the pre-vacuolar compartment (PVC) to reach the vacuole. (2) The alkaline phosphatase (ALP) pathway allows proteins to get to the vacuole directly, bypassing the PVC.
(3) A third route, the secretory pathway allows proteins transported to the plasma membrane to reach the vacuole following endocytosis.

Vacuolar Protein Sorting (VPS) Genes

Genetic screens in yeast have yielded many genes involved in the sorting of proteins to the vacuole (for review see Bryant and Stevens, 1998). These genes have been named Vacuole Protein Sorting genes or *VPS* genes. In addition, these genes can be classified into groups according to changes in size and shape of the vacuole due to a mutation or alteration of the gene. The *VPS* genes have been classified into six groups (A through F). Classification of *VPS* genes helps in the identification of genes that act at the same step of the pathway.

Class E VPS Genes

Class E *VPS* genes play a role at the pre-vacuolar compartment (PVC), and affect the exit of proteins to the vacuole or back to the Golgi complex. When these genes are mutated, the result is a much larger and exaggerated pre-vacuolar compartment (Raymond et al., 1992). This and further analysis has shown that protein products of the class E genes regulate transport through the PVC (Piper et al., 1995; Rieder et al., 1996). When their function is lost through mutation, the newly synthesized proteins being transported via the CPY pathway to the vacuole are held up in the PVC. Therefore, the enlarged PVC in class E mutants contains an accumulation of proteins such as CPY. Because the CPY sorting receptor also becomes trapped in the PVC, much of the newly synthesized CPY is secreted directly from the Golgi complex to the cell surface (Figure 2).

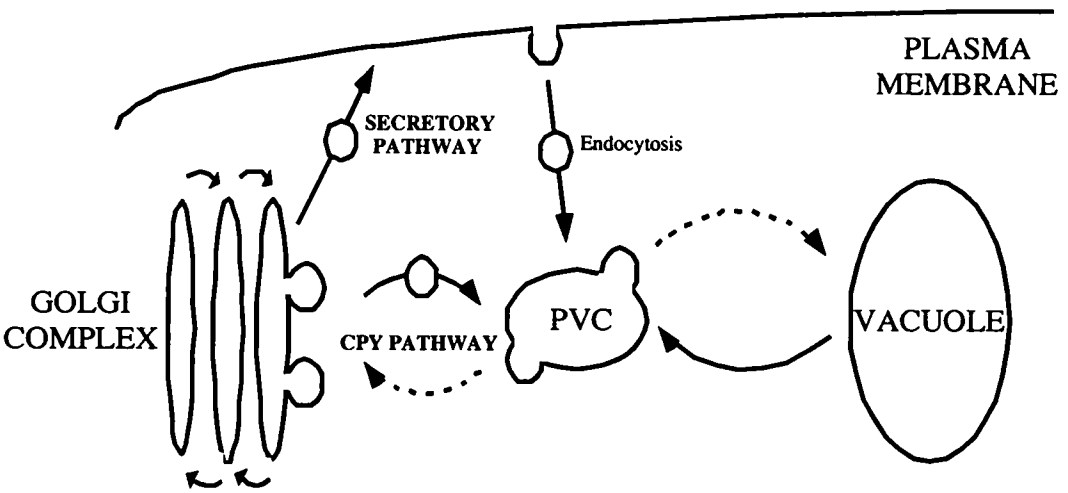


FIGURE 2. Trafficking to the vacuole is affected in *nhx1*Δ cells. In yeast cells in which the *NHX1* gene has been deleted (*nhx1*Δ cells), proteins following the CPY pathway are held up at the pre-vacuolar compartment (PVC). Transport to the vacuole is inhibited, represented by the dashed lines.

NHX1p: A Sodium/ Proton Exchanger

NHX1 (also called *VPS44*) plays a significant role in the transport of CPY to the vacuole. Yeast cells in which the *NHX1* gene has been deleted (*nhx1*Δ cells), secrete approximately 40% of newly synthesized CPY due to missorting of the protein to the cell surface instead of to the vacuole. These *nhx1*Δ cells exhibit the class E phenotype (physical characteristic) of large aberrant pre-vacuolar compartments. This suggests that *NHX1* is required for efficient trafficking of proteins out of the PVC (Bowers et al., 2000). Recent studies have shown that the protein product of the *NHX1* gene, Nhx1p, is localized to the pre-vacuolar compartment, which would further support its role at this particular step of the CPY pathway (Bowers et al., 2000; Nass and Rao, 1998).

Nhx1p is a sodium/ proton exchanger, whose function is to transport protons out of the PVC in exchange for sodium ions (Nass and Rao, 1998). This activity of Nhx1p, in light of its effect on protein trafficking when mutated, may therefore be essential for the maintenance of the ionic environment inside the PVC necessary for the association of other cytosolic factors with the membrane (Bowers et al., 2000).

Nhx1p belongs to the NHE (sodium/ proton exchanger) family of proteins, which includes similar exchangers in other organisms. These NHE proteins are predicted to have related structure and are thought to exchange sodium ions for protons along ion gradients in the cell. The sodium/ proton exchangers of the NHE protein family found in mammalian systems are responsible for regulation of cellular volume, intracellular pH, and for reabsorption of NaCl (salt) across renal, intestinal and other epithelial membranes or linings (Orlowski and Grinstein, 1997). Loss of function or increased activity of

human NHE proteins has been associated with hypertension or high blood pressure (Garbers and Dubois, 1999). The mammalian sodium/ proton exchangers are typically found on the plasma membrane, whereas Nhx1p is found intracellularly on the membrane of the yeast PVC. Nhx1p has not been as extensively studied as the other exchangers. Nhx1p has been proposed to function so that cells can tolerate a high salt environment by exchanging sodium ions out of the cytosol into an intracellular compartment in exchange for protons, which are transported out of this compartment into the cytosol (Nass et al., 1997).

It is already known that Nhx1p is essential for protein trafficking to the yeast vacuole. It is also known that Nhx1p is localized at the pre-vacuolar compartment thereby affecting the CPY pathway of protein traffic. What is unknown is the specific role of the sodium/proton exchange activity of Nhx1p in yeast protein sorting. The goal of my study is to determine the role of the sodium/ proton exchange activity of Nhx1p, and also to characterize Nhx1p through a genetic study of specific residues (a chain of residues make up a protein, which is encoded by a gene) that are necessary for normal function. The determination of specific residues involved in the function of Nhx1p is also likely to give valuable information about residues that are important for the function of NHE proteins.

CHAPTER II

MATERIALS AND METHODS

Random Mutagenesis

Plasmid DNA is a ring of DNA separate from genomic DNA within the cell's nucleus. Genomic DNA is organized into chromosomes and dictates the manufacturing and function of proteins. Plasmid DNA, on the other hand is variable and can be removed from one cell and transferred (transformed) into another. The genetic products of both plasmid DNA and genomic DNA can function together within the same cell. A plasmid containing the *NHX1* gene was mutagenized to create random errors within the original DNA sequence. The chemical mutagen hydroxylamine was used which causes transitions in nucleotides (pairs of nucleotides make up a chain of DNA) converting cytosine (one type of nucleotide that constitutes the DNA sequence) to a compound that pairs with adenine (another type of nucleotide).

The pRS316 plasmid containing the *NHX1* gene with a triple MYC epitope tag inserted in-frame immediately before the stop codon of *NHX1* (pKEB60) was obtained from Katherine Bowers (T.H. Stevens lab). This plasmid was prepared from frozen *Escherichia coli* (*E. coli*) by the large-scale alkali lysis method (Maniatis et al., 1989). The pRS316 plasmid containing *NHX1*-MYC was mutagenized using 0.36M hydroxylamine in 0.05M potassium phosphate pH 6.0. Immediately after incubating 10µl of the plasmid prep with 90µl of the hydroxylamine solution at 70°C for 0 (no

hydroxylamine control), 30, 45, or 60 minutes, 10µl of 3M sodium acetate pH 4.8, 5µl single stranded DNA, and 300µl cold 100% ethanol was added. The samples were then spun for 5 min at 13,200 rpm, 4°C, in a benchtop centrifuge. The supernatant was removed and the pellet was washed with 300µl cold 70% ethanol. The sample was spun again for 2 min at 13,200 rpm, 4°C, supernatant removed, and the pellet was dried for 5 min at 37°C and then resuspended in 100µl H_2O. This ethanol precipitation procedure was repeated two more times after adding 10µl of 3M sodium acetate pH 4.8 and 300µl cold 100% ethanol. After the third precipitation, the DNA was resuspended in 20µl H_2O. Using 4µl of the mutagenized plasmid, *E. coli* were transformed by the $CaCl_2$ method (Maniatis et al., 1989) and plated out onto LB + 400µg/ml Ampicillin (Amp) agar plates.

Screening for CPY-Secreting *NHX1* Mutants

In order to screen for *NHX1* mutants that have only one or two point mutations, a timepoint must be selected that represents a low frequency of mutation. The number of colonies on each of the plates was counted to check for mutation of the Amp resistance gene - with increasing incubation time in hydroxylamine there should be fewer *E.coli* colonies. The 30 min timepoint mutagenized plasmids were chosen and were purified by the small-scale alkali lysis method for plasmid DNA preparation (Maniatis et al., 1989).

Following purification, the mutagenized plasmids were transformed into a strain lacking Nhx1p, a *nhx1*Δ yeast strain (KEBY10). The mutagenized library was plated on selective media (SD -Ura) at a density of 300 colonies per plate (total of 47 plates) and grown at 30°C (standard permissive temperature for growth of yeast). After observable

growth of colonies (two days), a CPY immunoblot overlay (see CPY Immunoblot Overlays) was done to check which colonies secreted CPY. Colonies that secreted CPY were selected and patched along with positive (cells that secrete CPY) and negative (cells that do not secrete CPY) controls: *nhx1*Δ cells and wildtype, respectively. These were incubated at 30°C overnight then assayed for CPY secretion by CPY immunoblot overlay. The patches that displayed CPY secretion were then streaked for single colonies. Three colonies were picked from each streak and patched along with positive and negative controls. This was then assayed for CPY secretion by overlay. From the three patches for each original colony, one was selected that clearly secreted CPY and its plasmid was isolated from yeast (Kaiser et al., 1994).

After purifying the plasmids, 3µl of each prep was transformed into *E. coli* by electroporation (Maniatis et al., 1989). A single colony was then selected from each transformation plate and purified by the small-scale alkali lysis method. To check for the presence of *NHX1* in the pRS316 plasmid a restriction digest was performed using Hpa I which cuts within the *NHX1* gene and yields fragment sizes of 6.7 and 1.2 kilobases (kb). The mutant plasmids containing *NHX1* were then transformed back into yeast (*nhx1*Δ cells). Each was then patched along with positive and negative controls and probed for CPY secretion by CPY overlay.

CPY Immunoblot Overlays

This technique allows the detection of CPY secretion. Yeast from a selective plate were replica plated, then overlayed with nitrocellulose filters and incubated at 30°C

for 12-16 hrs. As CPY is secreted by cells it sticks to the filter. The filter was then removed and the yeast were washed off. The filter was then transferred to a beaker containing blocking solution (5% milk in TTBS (Tris, Tween Buffered Saline). Proteins in the milk bind all of the non-specific sites on the filter, thus reducing the non-specific binding of antibodies to the filter. The filter was then incubated on a rotary shaker for 15-30 min. The blocking solution was poured off and more was added with a 1:50 dilution of CPY monoclonal antibody. This was incubated for 2 hrs. The primary antibody solution was poured out and the filter was then washed 3x in TTBS. More blocking solution was added along with a 1;10,000 dilution of anti-mouse secondary antibody conjugated to horseradish peroxidase (HRP) and incubated for 2hrs. The solution was poured off and the filter was then washed 3x in TTBS and 1x in PBS (Phosphate Buffered Saline). The filters were then incubated in chemiluminescent substrates for the HRP and exposed to film.

Site-Directed Mutagenesis

In order to create specific point mutations on the *NHX1* gene, the QuikChange mutagenesis kit (Stratagene) was employed following manufacturer's instructions. Two double mutants of *NHX1* were made by this method, D201N/E225Q and D201N/D230N. This was done by using the *NHX1* gene on a pRS316 plasmid with a triple HA epitope tag inserted in-frame immediately before the stop codon of *NHX1,* and a single mutation E225Q (pKEB45) or with a single mutation D230N (pKEB46) as templates, and the oligonucleotides (Keystone) 5' CATTATCTGCTACCAACCCTGTTACAATTC 3'

(D201N 5') and 5' GAATTGTAACAGGGTTGGTAGCAGATAATG 3' (D201N 3'). (This procedure replaces D201 of NHX1 with N to create a second mutation in each of the plasmids.) The sample reactions consisted of 5µl 10x reaction buffer, 25ng template, 125ng oligo D201N5', 125ng oligo D201N3', 1µl of a 10mM mix of dinucleotide triphosphates (dNTPs). The reactions were made up to 50µl total with distilled H_2O in a 0.6ml Eppendorf tube and then 2.5U Pfu DNA polymerase (Stratagene) was added. Control reactions were also set up without template DNA or without Pfu DNA polymerase. The samples were placed into a polymerase chain reaction (PCR) machine in which temperature cycling was programmed as follows: 95°C for 30 sec, 50°C for 1 min, and 68°C for 16 min (2 min/kb plasmid length) for 16 cycles.

The reactions were then run on a 1% agarose gel. Gels were run at 90 Volts (V) in TAE running buffer (40mM Tris-acetate, 1mM EDTA pH8.0). The gel revealed PCR products 8kb in size (5kb pRS316 plasmid plus 3kb *NHX1* including 500bp upstream and 450bp downstream of the open reading frame).

Following temperature cycling and gel electrophoresis, products were treated with 0.02U DpnI endonuclease and incubated for 1 hr at 37°C to digest methylated DNA (template) and to select for the DNA containing mutation. This DpnI- treated DNA was then used to transform *E. coli* by the $CaCl_2$ method. Plasmid DNA was then prepared for sequencing using the Wizard kit (Promega) according to the manufacturer's instructions.

Polymerase Chain Reaction

The *NHX1* mutants were amplified as a PCR product from yeast genomic DNA templates containing mutations on the *NHX1* gene using Pfu DNA polymerase (Stratagene) and the oligonucleotides 5' CCGATGAGTACGGTCGACATTAGC 3' and 5' GCTTATCGATAGCGGCGAGTTTCTC 3'. The sample reactions consisted of 5µl 10x reaction buffer, 25ng DNA template, 125ng oligo 5' *NHX1* 500, 125ng oligo 3' *NHX1* 450, 4µl of a 10mM mix of dNTPs. The reactions were made up to 50µl total with distilled H_2O and then 2.5U Pfu DNA polymerase was added. A control reaction was also set up without template DNA. The samples were placed into a PCR machine in which temperature cycling was programmed as follows: 95°C for 45 sec, 50°C for 45 sec, and 72°C for 8 min (2 min/ kb plasmid length) for 24 cycles.

The reactions were then run on a 1% agarose gel in TAE buffer at 90 V. The 3 kb PCR product included 500 bp upstream of the start codon and 450 bp downstream of the stop codon of *NHX1*. It was first cloned into PCR-Blunt (Invitrogen) and then subcloned into pRS316 using Sall (from 5' oligo) and Notl (from PCR-Blunt plasmid).

Protein Preparation, SDS-PAGE, and Western Blot Analysis

This is a technique for detecting specific proteins in a mixture. Proteins were extracted from yeast and run through a gel by electrophoresis. Electrophoresis is a process that uses an electric potential to separate the proteins principally based on size. Proteins have a net negative charge due to the negatively charged detergent (SDS) in the

buffer and therefore travel from the negatively charged cathode to the positively charged anode. The smaller a protein, the faster it will travel down the gel. The proteins are then transferred from the gel onto a filter from which the specific protein can be detected using an antibody specific to the protein or to the tag that is attached to it. It is possible to estimate the size of a protein by comparing the distance it has traveled with the distance traveled by marker proteins of known size. In this case, the filter was probed with an antibody specific to the epitope tag of Nhx1p-HA or Nhx1p-MYC.

Yeast strains were grown in selective media (SD -Ura) overnight at 30°C. 9ml of rich medium (YEPD) was added to the overnight cultures and cells were grown 3-4 hrs to mid-log phase (OD_{600} = 0.6-1.0). At this point, the cells were harvested, resuspended in 1ml H_2O, and transferred to an Eppendorf tube. The cells were re-pelleted at 6000rpm for 30 sec in a benchtop centrifuge. After decanting the supernatant, the pellet was resuspended in 200µl Thorner sample buffer (8M Urea, 5% SDS, 50mM Tris pH 6.8, 5% β-mercaptoethanol and bromophenol blue) and a small cap-full of glass beads. The samples were vortexed for 5 min at room temperature on a multivortexer to lyse the cells and then spun for 2 min at 13,200 rpm to pellet the beads and unbroken cells.

The supernatant of each sample, as well as appropriate molecular weight markers, was loaded onto a 10% polyacrylamide gel. The gel was run at 100 V and was stopped as the dye front reached the end of the gel. The proteins were then blotted onto nitrocellulose overnight at 200mA. The nitrocellulose was then stained with Ponceau dye and then blocked for 15 min in blocking solution (5% milk in TTBS). The filter was then incubated for two hours in anti-MYC or anti-HA primary antibody and blocking solution. The filter was washed three times in TTBS and incubated for 2 hrs in mouse-

anti-HA (1:200 dilution) or rabbit-anti-MYC (1:2,000 dilution) antibody conjugated to HRP (1:10,000 dilution) and blocking solution. The filter was washed again in TTBS and in PBS and then chemiluminescent substrates for HRP were added before exposing the filter to film.

FM4-64 Staining

FM4-64 (N-(3-triethylammoniumpropyl)-4-(p-diethyl-hexatrienyl)) is a fluorescent dye that is taken up by yeast cells through the endocytic pathway. This endocytic marker can be visualized by fluorescence microscopy. Overnight cultures of yeast were grown in selective media (SD -Ura) at 30°C. The following day, rich medium (YEPD) was added and the cells were allowed to grow to OD_{600} = 0.8-1.6. Staining was done by a 15 min incubation of the cells in 40μM FM4-64 at 30°C followed by a 30 min chase in fresh medium at 30°C. Cells were viewed using a 100x oil immersion lens on a fluorescence microscope and images captured with a digital camera.

Pulse-Chase Immunoprecipitations

This technique is a useful way to measure the amounts of CPY, as well as to track the forms of CPY that lies inside and outside the cells at various time points. First a "pulse" of radioactive amino acids (cysteine and methionine) is added to the culture medium for a brief period during which time it is incorporated into proteins (including CPY). The fate of the newly synthesized, radioactive molecules is examined during the

subsequent period when the radioactive label is removed from the cell medium, an excess of unlabeled methionine and cysteine is added (the "chase"), and CPY is immunoprecipitated.

Yeast cultures were grown overnight at 30°C in minimal media (SD -Ura, -Met) to 1 OD_{600}/ml. For each timepoint to be immunoprecipitated, 0.5 OD_{600} was taken, pelleted and resuspended at OD_{600} = 1.0/ml in fresh minimal media (without methionine) with 20mM KPO_4, pH 5.7 and 2mg/ml BSA (bovine serum albumin), and incubated for 15 min at 30°C. Cells were then pulse-labeled with 200μCi Express [^{35}S] labeling mix (New England Nuclear) per OD_{600} for 10 min at 30°C.

The chase was initiated by the addition of 100μg/ml each of unlabeled cysteine and methionine, and after incubation for the appropriate time (0 min and 40 min) at 30°C, 500μl aliquots were removed and the chase was terminated by addition of 50mM sodium azide and incubation on ice. The cells were pelleted by centrifugation and the supernatant (extracellular fraction) was reserved. After the pellets were spheroplasted using oxalyticase, 0.5% SDS was added to lyse the cells and the samples were incubated at 100°C for 5 min. Then 0.1% SDS, 0.1% Triton X-100, 2mM EDTA and 90mM Tris pH 8.0 (1x IP buffer) was added to the intracellular fraction. 100μl 10x IP buffer was added to each extracellular fraction and the samples incubated at 100°C for 5 min before adding 350μl H_2O. Both intracellular and extracellular fractions were precleared with 50μl of a 10% slurry of fixed *Staphylococcus aureus* cells (IgGSorb) for 15 min on ice. 1μl anti-CPY serum was added and the samples were incubated for 1 hr on ice, followed by a second incubation for 1 hr on ice after addition of 50μl 10% IgGSorb. IgGSorb with bound immune complexes was pelleted and washed three times in 1x IP buffer.

Immunoprecipitated proteins were resuspended in 20μl SDS sample buffer with 10% β-mercaptoethanol and then separated on 7% SDS polyacrylamide gels.

Immunofluorescence Microscopy

This technique uses fluorescent dyes that are coupled to a purified antibody to detect a desired protein. When the dye-antibody complex is added to a cell it will bind to the specific antigen and the protein of interest will become labeled with the dye, thereby allowing one to visualize the desired protein through a microscope.

Cells were grown in YEPD at 30°C to $1OD_{600}$/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, 50mM KPO_4 pH 6.5. Cells were converted to spheroplasts using zymolyase and permeabilized by treatment with 5% SDS for 5 min for visualization of Vph1p or Pep12p. Cells were allowed to adhere to poly-L-lysine multiwell coated slides. Non-specific binding was blocked by incubation of the cells in PBS with 5mg/ml BSA and 1% normal goat serum. All antibodies were diluted in PBS with 5mg/ml BSA, which was also used for washes. Antibodies against Vph1p and Pep12p were preabsorbed to yeast proteins to remove non-specific binding by incubation with *vph1Δ* and *pep12Δ* cells, respectively. Antibody incubations were performed at room temperature in a humid chamber: 2 h for primary antibodies and 1 h for secondary and tertiary antibodies. Polyclonal serum against Vph1p was used at a 1/100 dilution, and monoclonal serum against Pep12p (Molecular Probes) was used at a 1/1000 dilution. The dye-antibody complexes biotin-conjugated goat anti-rabbit and streptavidin-conjugated fluorescein

isothiocyanate (FITC) antibodies were obtained from Jackson Immunoresearch. Images were generated using a microscope.

CHAPTER III

RESULTS

Endosomal Trafficking is Affected in *nhx1Δ* cells

Proteins may reach the vacuole by one of several pathways. One route allows proteins to be transported to the cell surface from the Golgi complex and then to the vacuole following endocytosis. The endocytic pathway in yeast can be traced using a fluorescent dye known as FM4-64. A chemical is said to be fluorescent if it absorbs light at one wavelength and emits light at a specific and longer wavelength within the visible spectrum. Using a fluorescence microscope, fluorescent light emitted by the sample is then used to form an image, or in this case, to find where it becomes localized within a yeast cell. FM4-64 is lipophilic ("loves" lipids) and incorporates into the plasma membrane. From here, this dye is endocytosed and transported to the vacuole by the endocytic pathway.

Yeast cells were incubated for 15 min with FM4-64 at 30°C allowing sufficient time for the dye to incorporate into the membrane, and then for 30 min in fresh medium (no dye) at 30°C so that the dye can reach its target within the cell following endocytosis. Under a fluorescence microscope, the dye was visualized at the vacuolar membranes of wild type cells (figure 3). However, in *vps27Δ* cells (cells lacking the *VPS27* gene which is an established class E gene), there was an accumulation of FM4-64 in the pre-vacuolar compartments (PVC) just outside of the vacuoles, in addition to slight staining of the

vacuolar membranes (figure 3). Cells that lacked the *NHX1* gene (*nhx1Δ*) appeared similar to the *vps27Δ* cells with the dye internalized within the PVC and traces encircling the vacuoles.

I also studied the accumulation of FM4-64 in combination with the trafficking of the protein Ste3p. There are two yeast mating types: a and α. For mating to occur, one type of cell secretes a ligand that is recognized and binds to a specific receptor on the plasma membrane of the other type of cell. For example, a cells secrete a-factor which binds an a-factor receptor on an α cell. This a-factor receptor is also known as Ste3p. Ste3p is a transmembrane protein that is endocytosed from the plasma membrane, both constitutively and after binding its ligand (a factor) and then sent to the vacuole where it is degraded (Davis et al., 1993).

A green fluorescent protein (GFP) attached to the C-terminal end of Ste3p (allow visualization of Ste3p in living cells) revealed that Ste3p accumulated within the vacuole in wild type cells (figure 4) (Gerrard et al., 2000; Piper et al., 1995). On the other hand, FM4-64 (as shown above), which is a lipophilic dye, made a distinct "ring" around the vacuole in wild type cells (figure 4). In *nhx1Δ* and *vps27Δ* cells, however, Ste3GFP accumulated within the PVC (figure 4). In addition, with pictures of FM4-64 and Ste3GFP merged together, it was apparent that both colocalize to the PVC in these class E mutant cells (figure 4).

These experiments have indicated that endosomal trafficking is defective in *nhx1Δ* cells. Both Ste3p and FM4-64 accumulate within the PVC and are prevented from reaching the vacuole in *nhx1Δ* cells. Because *nhx1Δ* cells are phenotypically similar to *vps27Δ* cells, *NHX1* can be classified as a class E gene.

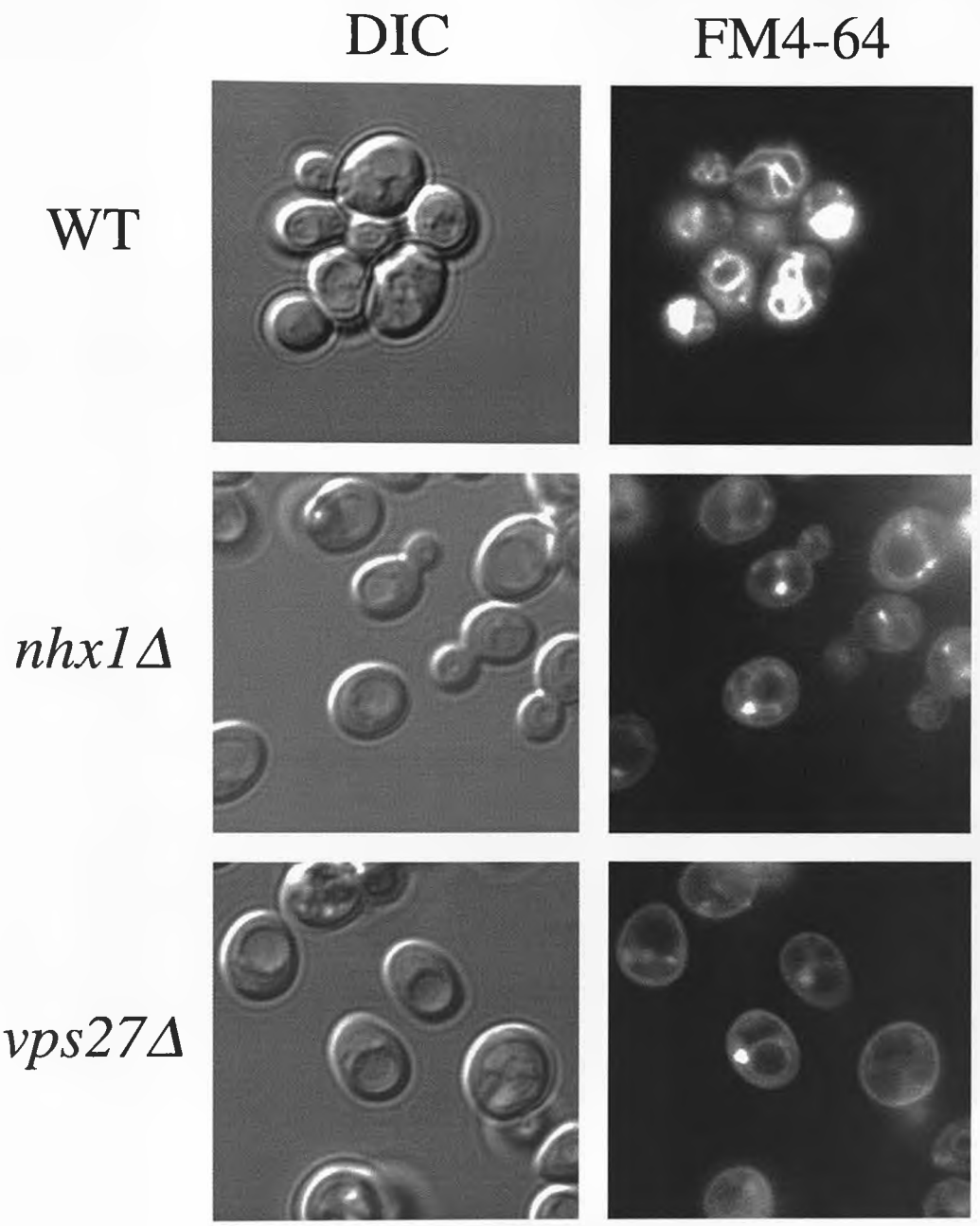


FIGURE 3. *nhx1*Δ cells display a class E morphological phenotype similar to *vps 27*Δ cells. Wild-type, *nhx1*Δ and *vps27*Δ *cells* were labeled with FM4-64 for 15 min at 30°C and then incubated in fresh medium for 30 min at 30°C. Photographs were taken of FM4-64 under a red fluorescence channel. In addition, differential interference contrast (DIC) photographs were taken simultaneously of the same cells. In wild-type cells, FM4-64 is exclusively seen in the vacuole whereas in both *nhx1*Δ and *vps27*Δ cells, FM4-64 is held up within a compartment adjacent to the vacuole

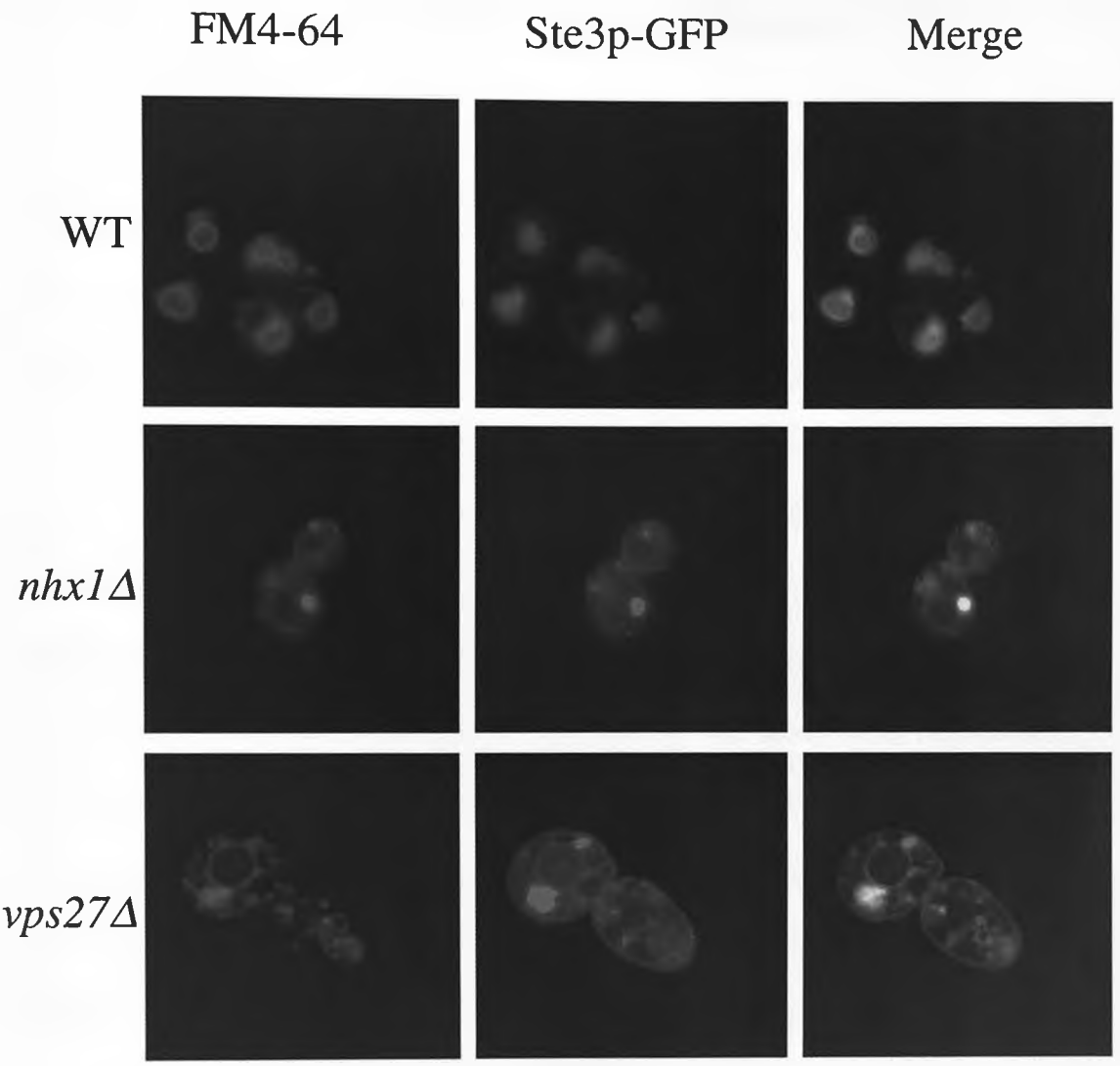


FIGURE 4. FM4-64 and Ste3pGFP colocalize to the PVC in *nhx1*Δ cells. Wild-type, *nhx1*Δ, and *vps27*Δ cells transformed with Ste3GFP encoded on a plasmid, were labeled with FM4-64 for 15 min at 30°C and then in fresh medium for 30 min at 30°C. Photographs of FM4-64 were taken under a red fluorescence channel and Ste3GFP under a green fluorescence channel. The two were overlapped to create the merged image and to see colocalization of FM4-64 and Ste3pGFP where the color is yellow.

CPY Pathway is Affected in *nhx1Δ cells*

In the CPY pathway, proteins reach the vacuole via the intermediary PVC. Because Nhx1p is thought to be localized at the PVC, we examined the localization of proteins resident to the PVC and vacuole in *nhx1Δ* cells. Using these as organelle markers we were able to localize specific proteins and organelles within cells by immunofluorescence microscopy. This technique employs fluorescent dyes coupled to purified antibodies specific to the desired protein. When this fluorescent dye-antibody complex is added to a cell it will bind to the chosen antigens that will light up when illuminated.

Pep12p was used as a marker for the PVC (Bowers et al., 2000; Gerrard et al., 2000). This protein is found in punctate structures throughout the cytoplasm in wild type cells (figure 5). Another marker used in this study was Vph1p. This protein makes up part of the vacuolar ATPase and is localized to the vacuole membrane where it is transported via the CPY pathway following synthesis in wild type cells (figure 5).

In *vps27Δ* cells, Vph1p and Pep12p both colocalized within the PVC (figure 5). Similarly, cells lacking Nhx1p revealed the class E morphological phenotype as shown in figure 5. Cells lacking Nhx1p or Vps27p accumulated Vph1p and Pep12p within an enlarged PVC that was located immediately beside the vacuole. This experiment shows that deletion of the *NHX1* gene resulted in a defective CPY pathway. Proteins that normally passed through the PVC and reached the vacuole, were instead no longer able to reach the vacuole and internalized within the PVC. This experiment demonstrates that Nhx1p, like Vps27p, is required for trafficking of proteins out of the PVC.

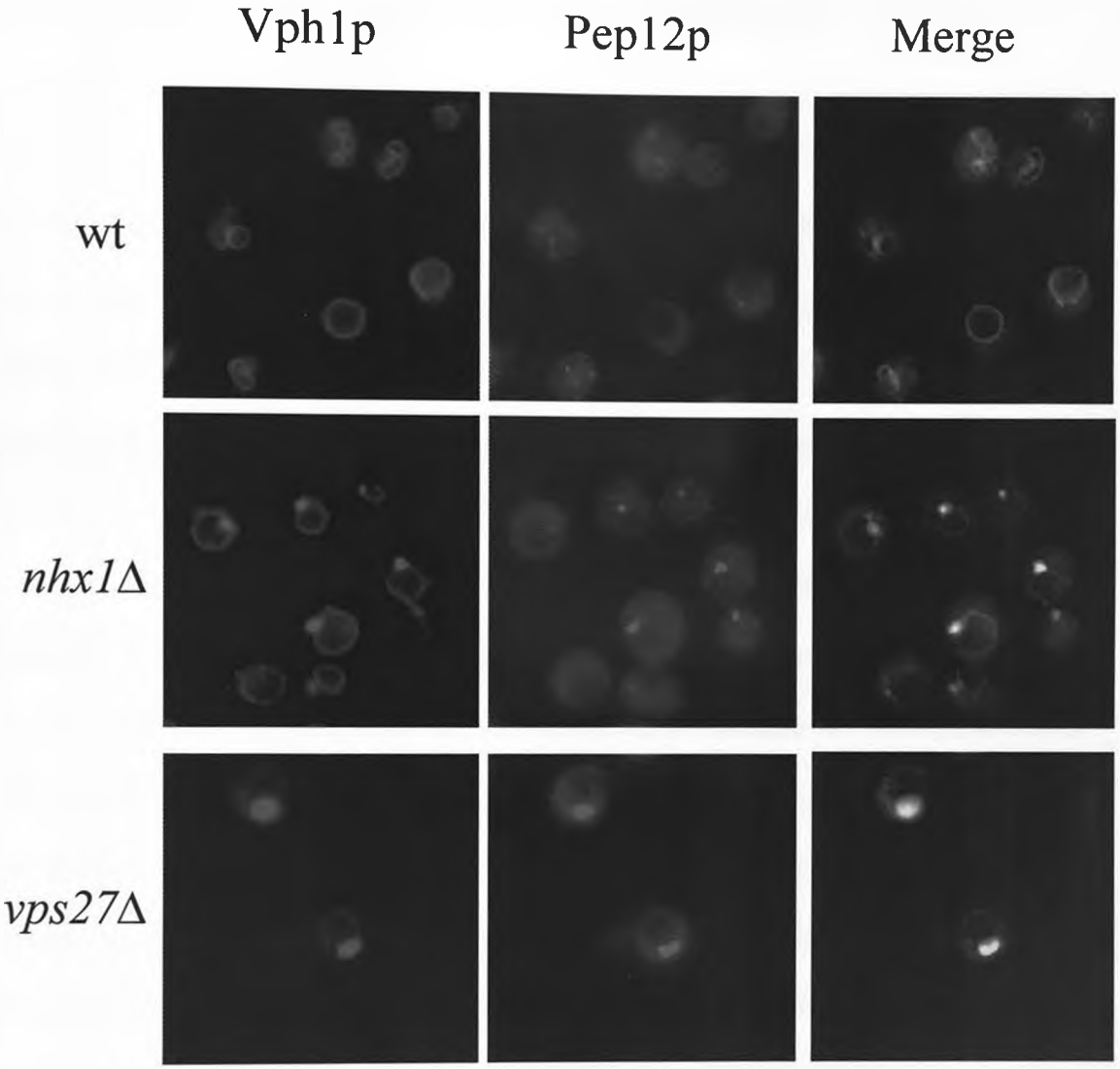


FIGURE 5. Pep12p and Vph1p colocalize to the PVC in *nhx1*Δ cells. Immunofluorescence was performed (see materials and methods) in wild-type, *nhx1*Δ, and *vps27*Δ cells. Pep12p was visualized by using a monoclonal antibody followed by an anti-mouse biotin and streptavidin-FITC. The same cells were incubated in a polyclonal antibody against Vph1p followed by an Alexa-anti-rabbit antibody in order to visualize Vph1p. Photographs of Pep12p were taken under the green fluorescent channel. The same cells were photographed for Vph1p under the red fluorescent channel. These two were overlapped to create the merged image revealing colocalization of Pep12p and Vph1p in *nhx1*Δ and *vps27*Δ cells.

Characterization of Original *VPS44* Mutants

An earlier screen for vacuolar protein sorting mutants (*vps* mutants) resulted in the identification of more than 50 genes that affect CPY sorting (Raymond et al., 1992). Four of the mutants generated by this study were characterized by complementation analysis and classified as *VPS44* (*NHX1*) mutants. The DNA of these four mutants was amplified by PCR (see materials and methods), subcloned into the plasmid pRS316, and put back into a yeast strain lacking the *NHX1* gene (*nhx1Δ*) for further analysis.

In order to determine what the mutations were and how these could affect the protein Nhx1p, the DNA was sequenced. The identification of residues that are needed for proper function may help elucidate how this protein functions and perhaps its role in CPY sorting. DNA is composed of four different nucleotides (A, T, C, and G) and is read three nucleotides at a time. Three sequential nucleotides make up a codon that encodes the specific amino acid to be inserted as the protein product is being synthesized. The sequences of each of the four mutants' DNA was compared to that of wild type *NHX1* to identify and map any errors in nucleotide sequence.

Figure 6a illustrates various regions of the Nhx1 protein sequence in alignment with its closest homologues and correlates the specific mutations found in the mutant sequences to particular regions of the protein. Mutant 1 was found to have a single mutation in the loop region between the putative transmembrane domains 10 and 11 (figure 6a). A nucleotide replacement resulted in a change in the protein sequence. The original codon representing a tryptophan (W; an amino acid) was subsequently changed to a stop codon. A stop codon directs protein synthesis to stop at its location in the

sequence. The result of this is a truncated protein without transmembrane domains 11 and 12, as well as a long C-terminal region. This C-terminal region has been implicated in having a regulatory function in mammalian NHE homologues (Orlowski and Grinstein, 1997).

Similarly, mutant 2 was found to have a change in the nucleotide sequence that resulted in a truncation in the loop region just before transmembrane domain 11. The deletion of a nucleotide resulted in a frameshift of the protein sequence, changing all subsequent codons (figure 6a). Shortly after the point of nucleotide deletion, a stop codon was found which signals a stop in the formation of the mutant protein as depicted in figure 6a. Without a full length Nhx1 protein, including the last transmembrane domains and C-terminal region, both mutants 1 and 2 failed to complement the CPY secreting phenotype when expressed in yeast (data not shown).

Mutant 3, unlike mutants 1 and 2, was found to have a mutation that changed a conserved residue, rather than one resulting in truncation. Figure 6a shows that the serine (S; a polar, uncharged amino acid) at position 306 (C-terminus) in the protein sequence is conserved or present in four NHE homologues of Nhx1p. As a result of the mutation, the polar, uncharged serine was changed to a nonpolar amino acid, phenylalanine (F). Because this mutant fails to complement the CPY secretion phenotype when expressed in *nhx1Δ* cells, this alteration suggests that this conserved serine in the C-terminal region is necessary for wild type function of Nhx1p, perhaps for having a role in protein regulation.

Sequencing data failed to reveal any apparent mutations in the sequence of mutant 4. The above findings have, however, indicated that without the final transmembrane

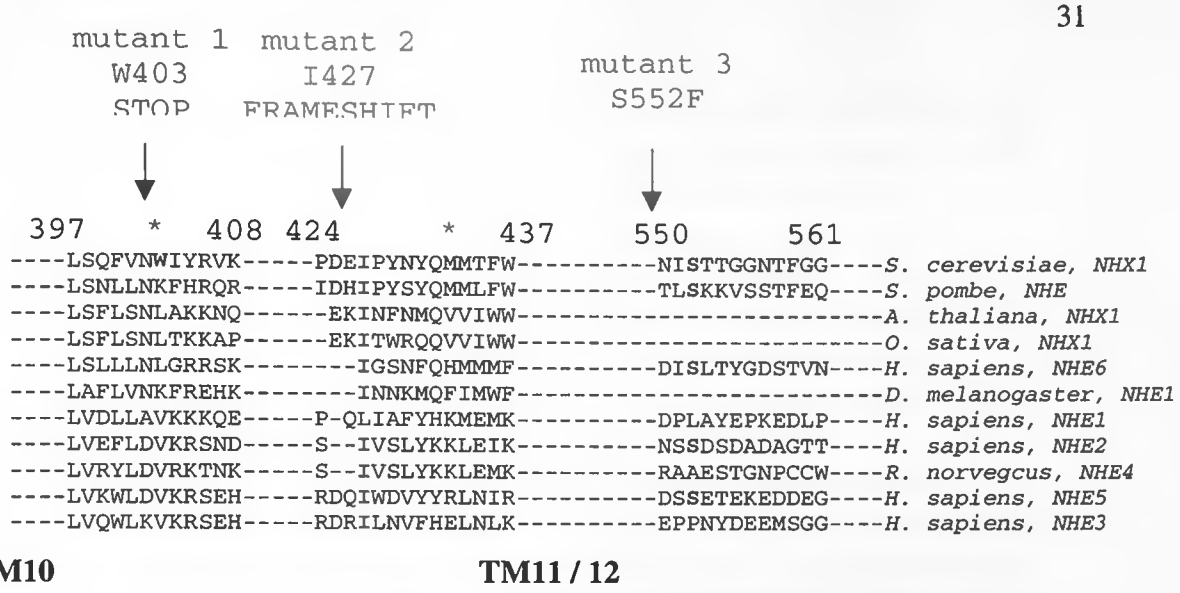
domains and C-terminal end, Nhx1p cannot function normally, resulting in secretion of CPY. In addition, the mutation found on mutant 3 reveals a critical amino acid in the C-terminal region that is necessary for proper function.

Further experiments were performed to determine the amount of CPY secreted by these mutants in comparison to wild type, *nhx1Δ*, and *nhx1Δ* with the complementing plasmid containing *NHX1*. Yeast cells were assayed for secretion of CPY by pulse-chase immunoprecipitation. By this method, newly synthesized CPY is labeled with radioactive cysteine and methionine, allowing it to be tracked outside of the cell as well as from compartment to compartment within the cell. Figure 6b shows that after a 10 min pulse of radiolabel, three intracellular forms were apparent in wild type cells. These three forms have distinct sizes and are separated by polyacrylamide gel electrophoresis. They include the newly synthesized form that is released from the ER (P1), the larger form that is modified by the Golgi Complex.(P2), and the smaller mature form that is proteolytically cleaved within the vacuole (M) (Bryant and Stevens, 1998). The different sizes of each of these forms distinguish its extent of travel through the CPY pathway. After a 40 min chase with unlabeled cysteine and methionine, newly synthesized CPY was largely intracellular as the mature form (M) in wild type cells. Thus, in wild type cells, CPY is correctly sorted to the vacuole. In comparison, *nhx1Δ* cells secreted 44% of newly synthesized CPY that was of the Golgi-modified form (P2) and part of the extracellular fraction (figure 6b). In cells that lack Nhx1p, CPY is not sorted properly to the vacuole and is instead secreted from the Golgi complex. On the other hand, *NHX1*-HA on the pRS316 plasmid was able to complement the CPY secretion phenotype when transformed into *nhx1Δ* cells. The amount of CPY (P2 form) secreted by these cells

containing a complementing plasmid (5%) was found to be similar to wild type secretion level (5%). Therefore, the presence of a plasmid or of the HA epitope tag did not affect Nhx1p function in the sorting of CPY.

Each of the VPS44 mutants were subcloned into the pRS316 plasmid and then transformed into *nhx1Δ* cells. Therefore, the only Nhx1p being expressed in the cell was the mutant version. After a 40 min chase, in these cells carrying mutant plasmids 29%, 46%, 40%, and 39% (mutants 1, 2, 3, and 4, respectively) of newly synthesized CPY was secreted into the extracellular medium in the P2 form (figure 6b). The truncated proteins as well as the mutant carrying the S552F mutation were unable to complement the CPY secretion of CPY cells. The mutant proteins did not secrete as much CPY as cells lacking Nhx1p but significantly more than wild type cells (figure 6b). These findings indicate the inability of these mutants to recover the *nhx1Δ* phenotype due to their specific mutations as revealed by DNA sequencing. The Golgi-modified form (P2) that is secreted does not reach the PVC or vacuole – compartments which are proteolytically active and are responsible for the cleaved mature form (M) of CPY. Secretion of the P2 form of CPY indicates that the transport of CPY from the Golgi complex to the PVC and then the vacuole is defective in these mutants.

a)


mutant 1
W403
STOP
mutant 2
I427
FRAMESHIFT
mutant 3
S552F
397 * 408 424 * 437 550 561
----LSQFVNWIYRVK-----PDEIPYNYQMMTFW----------NISTTGGNTFGG----S. cerevisiae, NHX1
----LSNLLNKFHRQR-----IDHIPYSYQMMLFW----------TLSKKVSSTFEQ----S. pombe, NHE
----LSFLSNLAKKNQ------EKINFNMQVVIWW----------A. thaliana, NHX1
----LSFLSNLTKKAP------EKITWRQQVVIWW----------O. sativa, NHX1
----LSLLLNLGRRSK--------IGSNFQHMMMF----------DISLTYGDSTVN----H. sapiens, NHE6
----LAFLVNKFREHK--------INNKMQFIMWF----------D. melanogaster, NHE1
----LVDLLAVKKKQE-----P-QLIAFYHKMEMK----------DPLAYEPKEDLP----H. sapiens, NHE1
----LVEFLDVKRSND-----S--IVSLYKKLEIK----------NSSDSDADAGTT----H. sapiens, NHE2
----LVRYLDVRKTNK-----S--IVSLYKKLEMK----------RAAESTGNPCCW----R. norvegcus, NHE4
----LVKWLDVKRSEH-----RDQIWDVYYRLNIR----------DSSETEKEDDEG----H. sapiens, NHE5
----LVQWLKVKRSEH-----RDRILNVFHELNLK----------EPPNYDEEMSGG----H. sapiens, NHE3
TM10
TM11 / 12

b)

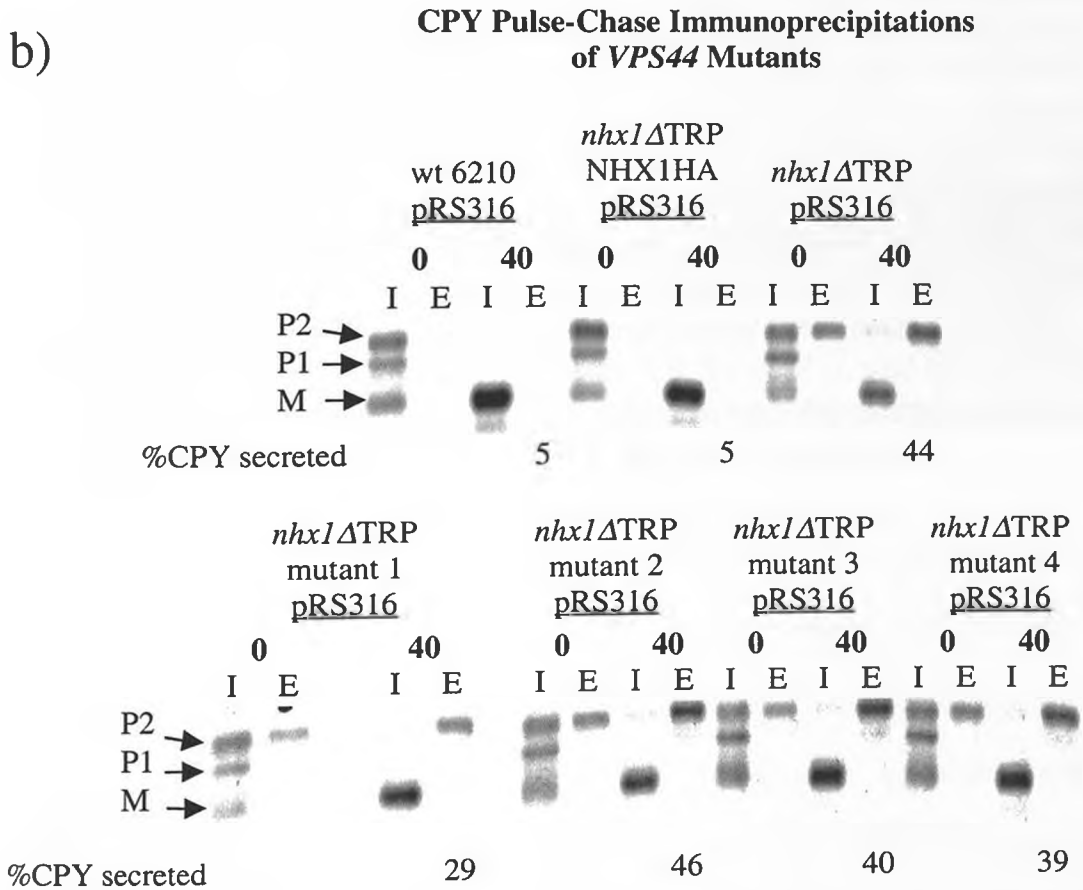

CPY Pulse-Chase Immunoprecipitations
of VPS44 Mutants
wt 6210
pRS316
nhx1ΔTRP
NHX1HA
pRS316
nhx1ΔTRP
pRS316
0 40 0 40 0 40
I E I E I E I E I E I E
P2
P1
M
%CPY secreted
5
5
44
nhx1ΔTRP
mutant 1
pRS316
nhx1ΔTRP
mutant 2
pRS316
nhx1ΔTRP
mutant 3
pRS316
nhx1ΔTRP
mutant 4
pRS316
0 40 0 40 0 40 0 40
I E I E I E I E I E I E I E I E
P2
P1
M
%CPY secreted
29
46
40
39

FIGURE 6. Mutations found in original *VPS44* mutants. a) Alignment of partial NHE protein sequences. Nhx1p has several homologues found in a wide range of organisms. Those that have the highest degree of similarity to baker's yeast (*S. cerevisiae*) *NHX1* include: fission yeast (*S. pombe*) *NHE*, corn (*A. thaliana*) *NHX1*, rice (*O. sativa*) *NHX1*, fruit fly (*D. melanogaster*) *NHE1*, rat (*R. norvegicus*) *NHE4*, and human (*H. sapiens*) *NHE* genes 1, 2, 3, 5, and 6. The protein sequences were aligned and segments that bear the specific mutations (in boldface type) are depicted here. Two segments within the loop region between predicted transmembrane domains 10 and 11 show the location of mutations in mutants 1 and 2, indicated by the arrows. The positions of the truncations that result from the mutations are indicated by asterisks. The third segment in the C-terminus, after the putative transmembrane domain 12, shows the serine to phenylalanine mutation found in mutant 3 at position 552 in the Nhx1p protein sequence. **b) CPY secretion in *nhx1*Δ cells expressing original *VPS44* mutants.** The strains used in this experiment include wild-type, *nhx1*Δ with *NHX1*-HA on pRS316, *nhx1*Δ with pRS316 (empty plasmid), and *nhx1*Δ with mutants 1, 2, 3, and 4 on pRS316 plasmids. The CPY immunoprecipitation procedure was followed (see materials and methods). Following a 10 min "pulse" in [^{35}S] cysteine and methionine and a 0 or 40 min "chase" in non-radioactive cysteine and methionine, CPY was immunoprecipitated from intracellular (I) and extracellular (E) fractions for each time point. The different forms of newly synthesized CPY are indicated: Golgi-modified form (P2), ER form (P1), and mature vacuolar form (M). The percentage of CPY secreted into the extracellular medium after 40 min was calculated by phosphoimager analysis.

Highly Conserved Acidic Residues Required for Exchange Activity of Nhx1p

Previous experiments, as well as fluorescence studies and CPY immunoprecipitations documented in this thesis, have shown that Nhx1p is required for the exit of proteins out of the PVC. There are two possibilities for the role of Nhx1p in protein trafficking: (1) the presence of Nhx1p in the PVC is required as an anchor protein, perhaps allowing binding of other class E proteins in the PVC; or (2) the sodium / proton exchange activity is necessary for proper trafficking of proteins. Changing specific residues or amino acids of Nhx1p, which affect only its exchange activity, not its localization or expression, will help distinguish between these two models. Studies of other exchangers have shown that specific acidic residues within transmembrane domains (parts of the protein that span across the membrane) are important for activity (Dibrov et al., 1998). When the Nhx1p sequence is lined up with its NHE protein homologues from a wide range of organisms, there are four acidic residues that are highly conserved – each protein sequence has the same acidic residue at the same relative position four times in the sequence (see figure 7a).

In order to determine whether these residues play a role in the activity of Nhx1p, they were genetically modified (Bowers et al., 2000). The four acidic (positively charged) amino acid residues were changed to uncharged residues using a technique called site-directed mutagenesis (see materials and methods). The aspartic acid (D) at position 201 was changed to asparagine (N) (D201N); glutamic acid (E) at position 225 to glutamine (Q) (E225Q); aspartic acid (D) at position 230 to asparagine (N) (D230N);

and glutamic acid (E) at position 355 to glutamine (Q) (E355Q) as depicted in figure 7a. These mutations were made in the pRS316 plasmid carrying the *NHX1*-HA gene.

In order to test the models proposed above, it was important to check that the mutants correctly expressed and localized Nhx1p and that the mutations presumably affected ion exchange across the PVC membrane. All four of these mutant genes were introduced into a yeast strain in which the *NHX1* gene had been deleted (*nhx1*Δ). Because the gene for wild-type (normal) Nhx1p has been deleted, and a plasmid encoding mutant Nhx1p has been inserted, the cell expresses only Nhx1p containing the specific mutation. To test for protein expression a western blot was performed. The *NHX1* gene has a tag (HA), which allows detection of its protein product. In a western blot, proteins are extracted from a yeast culture and run through a gel using an electric potential to separate the proteins based on size. The smaller a protein, the faster it will travel down the gel. The proteins are transferred from the gel onto a filter from which Nhx1p can be detected using an antibody specific to the tag (HA) that is attached to Nhx1p. The *nhx1*Δ yeast strain was able to produce these four mutant Nhx1 proteins at the same level as wild-type Nhx1p as shown in figure 7b, indicating that the mutations do not affect the stability of Nhx1p but rather its activity. A CPY immunoblot overlay assay revealed that only D201N, E225Q, and D230N had detectable secretion of CPY, whereas E355Q complemented the CPY secretion phenotype of *nhx1*Δ (data not shown). It is clear that these three mutants display the *vps* mutant phenotype because they secrete CPY and therefore, these three no longer function normally in sorting CPY to the vacuole. According to my hypothesis, this link between CPY secretion and sodium / proton exchange activity is a good indication of residues important for function of Nhx1p.

Initial experiments suggested that D201N secreted slightly more CPY than the other two mutants, E225Q and D230N (Bowers et al., 2000). Therefore, double mutants were constructed in which D201N was combined with either E225Q or D230N to see if the effect of the single point mutations is additive.

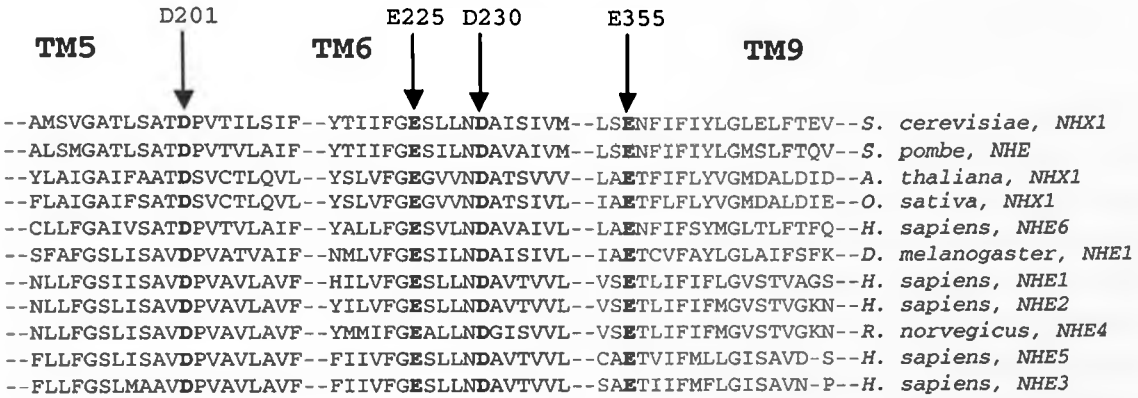
The mutants were created by site-directed mutagenesis, and then the genes containing the mutations were sequenced to determine whether the correct specific mutations were made (D201N/E225Q and D201N/D230N). The mutant plasmids were introduced into a *nhx1*Δ strain and the level of Nhx1p produced by these double mutants was then compared to that of wild-type Nhx1p by western blot, to make sure that the mutation did not affect expression. Figure 7c shows that the levels of 70kD Nhx1p produced by both the mutants and wild-type are indistinguishable, pointing out that the additional mutations did not affect stability of the protein. In addition, a CPY immunoblot overlay assay showed that the double mutants failed to complement the CPY secretion phenotype of *nhx1*Δ (data not shown).

To further test and compare the amount of CPY secreted by each of the single mutants and the double mutants, CPY immunoprecipitations were performed (Bowers et al., 2000). As shown in figure 8a, in a single experiment, wild-type cells secreted about 7% of newly synthesized CPY (P2) into the extracellular medium. Similarly, *nhx1*Δ cells with the complementing *NHX1*-HA plasmid secreted 5%, indicating that the presence of plasmid or the HA epitope tag did not affect the function of Nhx1p. *nhx1*Δ cells with an empty (non-complementing) plasmid, however, secreted about 26% of the P2 form of newly synthesized CPY, consistent with previous data (Bowers et al., 2000). Figure 8a also reveals that *nhx1*Δ cells that express the E355Q Nhx1p mutant secreted 5% CPY,

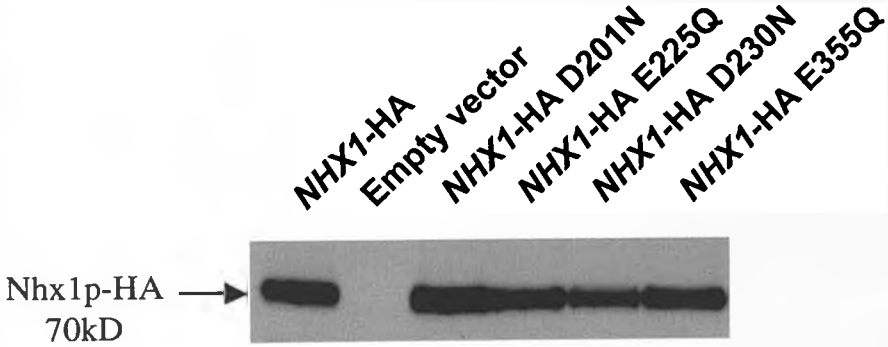
similar to the levels secreted by wild-type cells and *nhx1*Δ cells with the complementing plasmid. On the other hand, D201N, E225Q, and D230N mutations resulted in the secretion of 18%, 19%, and 22% CPY, respectively (figure 8a). This amount is more than that secreted by wild-type cells, however, it is also slightly less than *nhx1*Δ cells with empty plasmid. In comparison, when the double mutants were expressed in *nhx1*Δ cells, D201N/E225Q secreted more than any of the single mutants (30%), whereas the amount of CPY secreted by the D201N/D230N mutant (20%) was not significantly different than the amount secreted by any of the single mutants (figure 8a). A compilation of several CPY immunoprecipitation experiments revealed that the secretion levels of both of the double mutants expressed in *nhx1*Δ cells were not significantly different from the amount of CPY secreted by the three single mutants (figure 8b). These results show that either a D201N, E225Q, or D230N mutation on Nhx1p results in a protein that is unable to function normally. The combination of two of these residues required for function did not appear to have an additive effect, but rather maintained the CPY secretion phenotype.

Earlier fluorescence studies with the single mutant D201N revealed the correct localization of the mutant Nhx1p to the PVC, in addition, *nhx1*Δ cells expressing Nhx1p with the D201N mutation appeared to have the vacuolar morphological phenotype of class E *vps* mutants (Bowers et al., 2000). The above findings indicate that the point mutations created do not affect stability or localization, but rather fail to allow the correct sorting of CPY to the vacuole. Therefore, residues D201, E225 and D230 are very likely to be required for the exchange activity of Nhx1p, which is necessary for trafficking of proteins to the vacuole via the CPY pathway.

a)


D201
E225
D230
E355
TM5
TM6
TM9
--AMSVGATLSATDPVTILSIF--YTIIFGESLLNDAISIVM--LSENFIFIYLGLELFTEV--S. cerevisiae, NHX1
--ALSMGATLSATDPVTVLAIF--YTIIFGESILNDAVAIVM--LSENFIFIYLGMSLFTQV--S. pombe, NHE
--YLAIGAIFAATDSVCTLQVL--YSLVFGEGVVNDATSVVV--LAETFIFLYVGMDALDID--A. thaliana, NHX1
--FLAIGAIFSATDSVCTLQVL--YSLVFGEGVVNDATSIVL--IAETFLFLYVGMDALDIE--O. sativa, NHX1
--CLLFGAIVSATDPVTVLAIF--YALLFGESVLNDAVAIVL--LAENFIFSYMGLTLFTFQ--H. sapiens, NHE6
--SFAFGSLISAVDPVATVAIF--NMLVFGESILNDAISIVL--IAETCVFAYLGLAIFSFK--D. melanogaster, NHE1
--NLLFGSIISAVDPVAVLAVF--HILVFGESLLNDAVTVVL--VSETLIFIFLGVSTVAGS--H. sapiens, NHE1
--NLLFGSLISAVDPVAVLAVF--YILVFGESLLNDAVTVVL--VSETLIFIFMGVSTVGKN--H. sapiens, NHE2
--NLLFGSLISAVDPVAVLAVF--YMMIFGEALLNDGISVVL--VSETLIFIFMGVSTVGKN--R. norvegicus, NHE4
--FLLFGSLISAVDPVAVLAVF--FIIVFGESLLNDAVTVVL--CAETVIFMLLGISAVD-S--H. sapiens, NHE5
--FLLFGSLMAAVDPVAVLAVF--FIIVFGESLLNDAVTVVL--SAETIIFMFLGISAVN-P--H. sapiens, NHE3

b)


NHX1-HA
Empty vector
NHX1-HA D201N
NHX1-HA E225Q
NHX1-HA D230N
NHX1-HA E355Q
Nhx1p-HA
70kD

c)

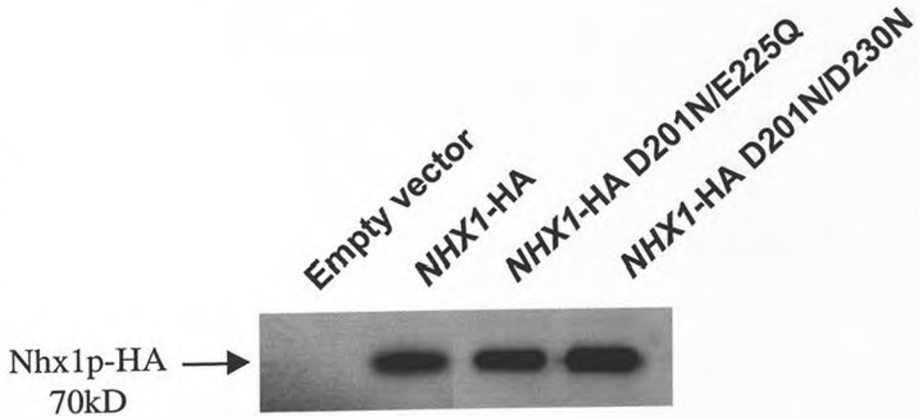
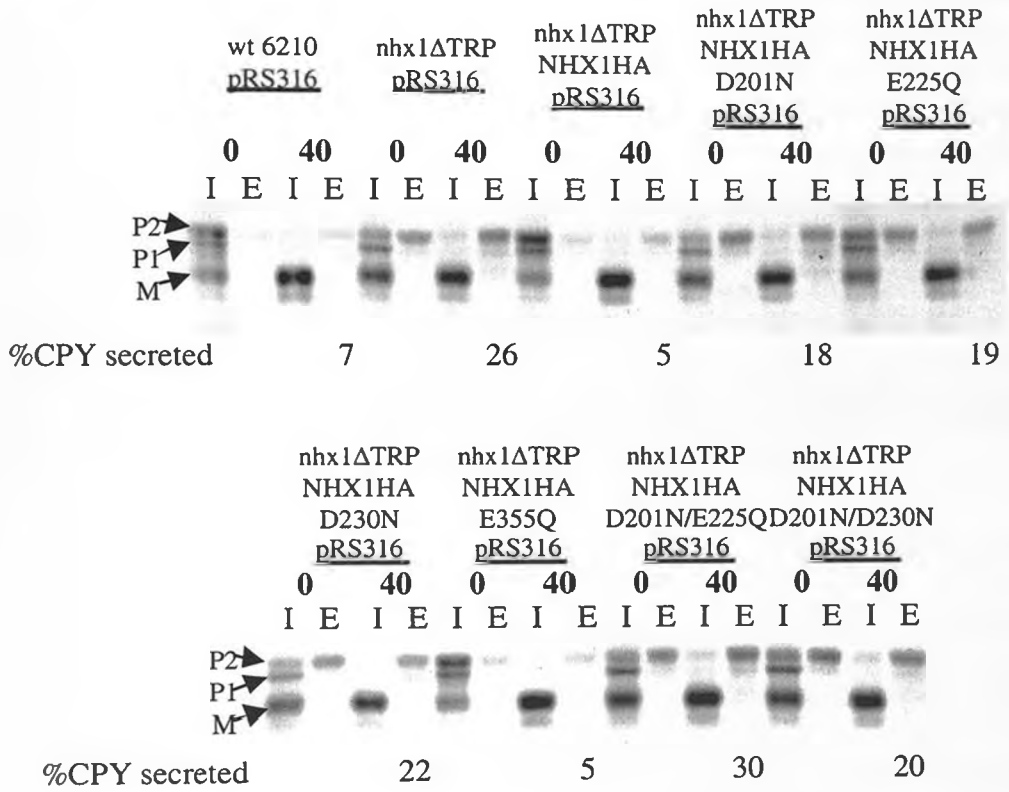

Empty vector
NHX1-HA
NHX1-HA D201N/E225Q
NHX1-HA D201N/D230N
Nhx1p-HA
70kD

FIGURE 7. Mutations of highly conserved residues of the NHE protein family do not affect the stability of Nhx1p. a) Alignment of partial NHE protein sequences showing conserved acidic residues. These residues are common in 10 NHE protein homologues from various organisms (see figure 6a). Segments of the aligned sequences including putative transmembrane domains 5, 6, and 9 are shown here depicting the residues that are shared by the NHE protein family in boldface type.
b) Nhx1p bearing single point mutations are produced at the same level as wild-type Nhx1p. A western blot (see materials and methods) was performed following protein extraction from *nhx1*Δ with the complementing *NHX1*-HA plasmid, *nhx1*Δ with the empty vector, and *nhx1*Δ with each of the single point mutants. Nhx1p-HA (70kD) was detected using an antibody specific to the HA tag. Nhx1p-HA was expressed in all lanes except that containing *nhx1*Δ cells with the empty plasmid. **c) Double point mutations on *NHX1* yield similar levels of Nhx1p as wild-type *NHX1*.** Proteins were extracted from *nhx1*Δ cells with the complementing plasmid, *nhx1*Δ with the empty vector, and *nhx1*Δ with each of the double point mutants. In this experiment, extracts were equally loaded (30μg) following a protein assay. Both of the double mutants were found to express mutant Nhx1p at the same level as wild-type Nhx1p.

a)

CPY Pulse-Chase Immunoprecipitations of Site Specific Mutants



b)

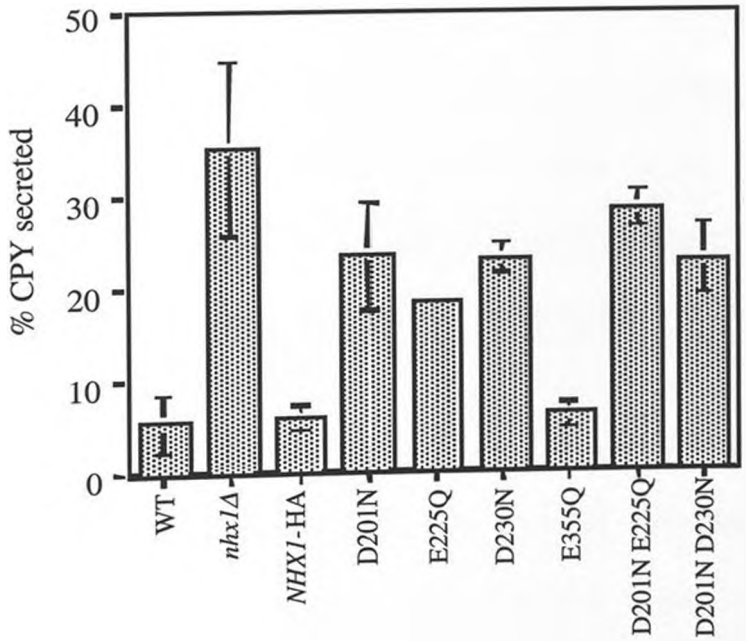


FIGURE 8. Site specific mutations of highly conserved residues results in CPY secretion. a) CPY secretion in *nhx1*Δ cells expressing single and double point mutants. The strains used in this experiment include wild-type, *nhx1*Δ with an empty plasmid, *nhx1*Δ with *NHX1*-HA on pRS316, *nhx1*Δ with single point mutations on pRS316, and *nhx1*Δ with double point mutations on pRS316. The time points (0, 40) and fractions (I, E) for this CPY immunoprecipitation are indicated as described in figure 6b, as well as the different CPY forms (P2, P1, M). The % CPY secreted by each strain was analyzed using a phosphoimager and then calculated from the % total CPY in the sample. **b) Comparison of CPY secretion levels among point mutants.** A series of CPY immunoprecipitation experiments were previously conducted (Bowers et al., 2000). Each bar represents the average of % CPY secreted by each strain mentioned above from several separate experiments, including the one shown above (a).

Identification of Residues Essential for Nhx1p Function

In order to determine residues important for the function of Nhx1p, the *NHX1* gene was randomly mutagenized and screened using CPY secretion as a functional assay. A tagged version of *NHX1* on a pRS316 plasmid was mutagenized (see materials and methods) using a chemical mutagen called hydroxylamine. The mutagenized plasmid was incorporated into a *nhx1*Δ yeast strain so that the only version of Nhx1p being expressed is that encoded by the plasmid. The cells were then screened to identify those that secrete CPY.

When vacuolar protein sorting is blocked, CPY is diverted from the pathway and is instead secreted at the cell surface. Therefore, mutants (in which sorting is defective) are identified by an assay for CPY secretion. Approximately 14,000 colonies (47 plates at 300 colonies/ plate) were screened by CPY immunoblot overlays. The assay revealed 63 colonies (0.45%) that appeared to secrete CPY. These colonies were tested again for CPY secretion along with positive (*nhx1*Δ) and negative (wild-type) controls. 35 of the picked colonies appeared to secrete CPY similar to that of the positive control and were streaked for single colonies to ensure that only the one colony that secretes CPY was isolated. After retesting the isolated colonies, 32 were found to definitely secrete CPY.

To determine that the mutation causing CPY secretion was located on the plasmid and not in the yeast genomic DNA, it was necessary to rescue the plasmids from the yeast cells. After the plasmids were isolated, they were transformed into *E. coli* to be amplified. The plasmid ws then enzymatically digested using the enzyme Hpa1 to check that the *NHX1*-MYC gene was indeed present within the plasmid pRS316, yielding

products of size 3kb and 5kb. 30 of the 32 recaptured plasmids were found to have the correct plasmid and were transformed back into *nhx1*Δ cells. This screen was aimed to find mutants that affected only the exchange activity of Nhx1p, not its expression or localization. Therefore, a western blot was performed to find mutants that are fully expressed in a *nhx1*Δ yeast strain. Of the 30 mutants rescued with correct plasmids, 7 were found to produce full-length Nhx1p (see figure 9). The 70kD protein was detected by using an antibody specific to the MYC epitope tag attached to the C-terminus.

The mutagen created random errors in the sequence, some of which may be insignificant, and others which are responsible for the mutant phenotype. To narrow down the mutations that result in CPY secretion when expressed in *nhx1*Δ, the mutant *NHX1*-MYC DNA was subcloned (see figure 10). Essentially, each mutant gene was cut in half using the enzyme Spe1. Each half was also cut out of the plasmid using Xho1 at the N-terminus and Not1 at the C-terminus. In addition, a wild-type copy of *NHX1*-MYC on pRS316 was digested with the same enzymes, but the larger fragments were isolated and purified. This larger fragment consists of the plasmid vector as well as one half of wild-type *NHX1* (figure 10). The mutant half containing the N-terminus was ligated into the plasmid with wild-type half containing the C-terminus. Similarly, the mutant half containing the C-terminus was ligated into the plasmid with the wild-type half containing the N-terminus. After subcloning each half of the mutant *NHX1* DNA, each half in pRS316 was transformed into *E. coli*, amplified and isolated. They were then incorporated back into a *nhx1*Δ strain to test which half contains the mutations responsible for the CPY secretion phenotype of Nhx1p. This was done by CPY immunoblot overlay. The overlay revealed that the mutant halves of mutants 40 and 54

containing the N-terminus, failed to complement the *nhx1*Δ phenotype. The mutant halves of 3, 8, and 61 containing the C-terminus, also secreted CPY. Neither mutant halves of 7 and 59 showed CPY secretion, indicating that perhaps mutations in both halves are necessary to create the mutant phenotype. The N-terminus halves of mutants 40 and 54, as well as the C-terminus halves of mutants 3, 8, and 61 were then sequenced to identify where the specific mutations fell, and subsequently how these mutations affected the protein sequence of Nhx1. The entire *NHX1* genes of mutants 7 and 59 were also sequenced to determine the mutations in both halves.

Mutations have been mapped for mutants 3, 40, and 54. The other mutants 7, 8, 59, and 61 await further analysis of the sequencing data. Mutant 3 was found to have mutations at positions 441 and 442 of the Nhx1 protein sequence. The arginine at position 441 was changed to a histidine (R441G). Both of these are polar, charged amino acids, however, histidine, bears a larger side chain with an imidazole ring. The substitution of a larger side chain may affect the ion exchange activity of Nhx1p, resulting in the mutant phenotype. Mutant 3 was found to have a second mutation changing the glycine (lacks a side chain) at position 442 to a serine (G442S), which has a hydrophilic, polar, uncharged side chain. This substitution may also be responsible for the secretion of CPY by mutant 3. Both R441 and G442 are highly conserved residues in predicted transmembrane domain 11. Because this mutant expresses a stable full-length protein it is possible that either of these mutations or the combination of the two are essential for the exchange activity of Nhx1p.

Two mutations were also mapped onto the protein sequence of mutant 40. At position 138 of the protein sequence, the glycine was substituted by arginine (G138R).

This change from an amino acid that has no side chain to one that has a large hydrophilic side chain in putative transmembrane domain 3 is likely to have a significant effect on the function of Nhx1p. G138 is also a conserved residue throughout the NHE protein family, implying that its presence may be required for the proper sorting of CPY to the vacuole. A second mutation found at position 414 (the loop between putative transmembrane domains 10 and 11) resulted in the replacement of serine by asparagine (S414N). This replacement, however, does appear to be significant because both residues have similar characteristics (polar, uncharged). In addition, S414 is not conserved among similar exchangers. Further testing by site-directed mutagenesis would reveal which of these residues are in fact essential for normal function of Nhx1p.

Mutant 54 was also found to have two nucleotide changes. The first was found to cause the substitution of valine at position 193 of the fifth predicted membrane-spanning domain by glycine (V193G). This valine is not conserved among other members of the NHE protein family, however, the change from a nonpolar, hydrophobic residue to glycine in a transmembrane domain may have some effect on the protein's sodium/ proton exchange activity. On the other hand, at position 228 of the Nhx1p sequence, a highly conserved lysine was substituted by a valine (L228V) in the putative transmembrane domain 6. It is unclear whether this mutation would have a significant effect of the protein's function because both residues are similarly nonpolar, hydrophobic residues.

All of the above mapped mutations must be further analyzed by constructing point mutants at each of the resides. Mutations that involve a drastic change in residues and that are also conserved in other exchangers across various other organisms may be more

likely to have some importance in the function of Nhx1p and its role in the CPY pathway. Each of these mutants express full-length protein, as mentioned before in figure 9, indicating stability. testing each of the mutations, therefore, will elucidate the residues that are essential for Nhx1p function, without affecting its stability or localization.

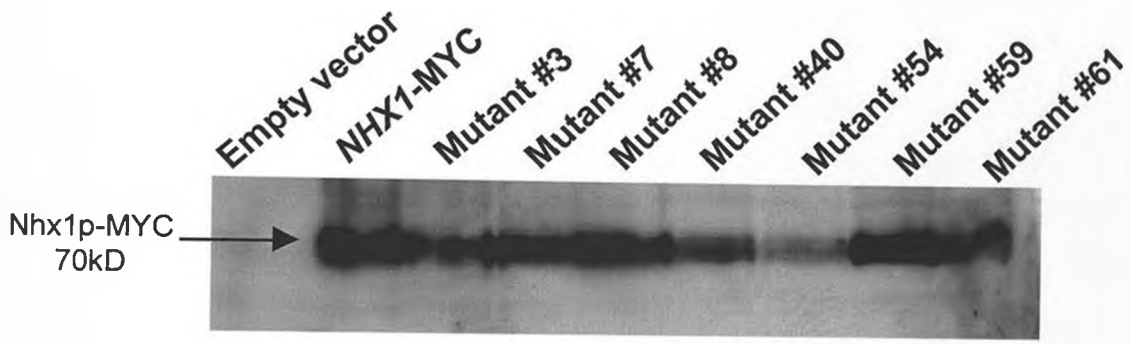


FIGURE 9. *nhx1*Δ cells expressing seven of the random Nhx1p mutants. Proteins were extracted from the following strains: *nhx1*Δ with an empty vector (pRS316), *nhx1*Δ with complementing *NHX1*-MYC on pRS316, and *nhx1*Δ cells expressing each of the seven random mutants (specified by their colony number). The proteins (70kD) were detected by using an antibody specific to the MYC epitope tag attached to the end of Nhx1p. This western blot reveals the expression of full-length Nhx1p-MYC in all lanes except that of the delete strain having an empty plasmid.

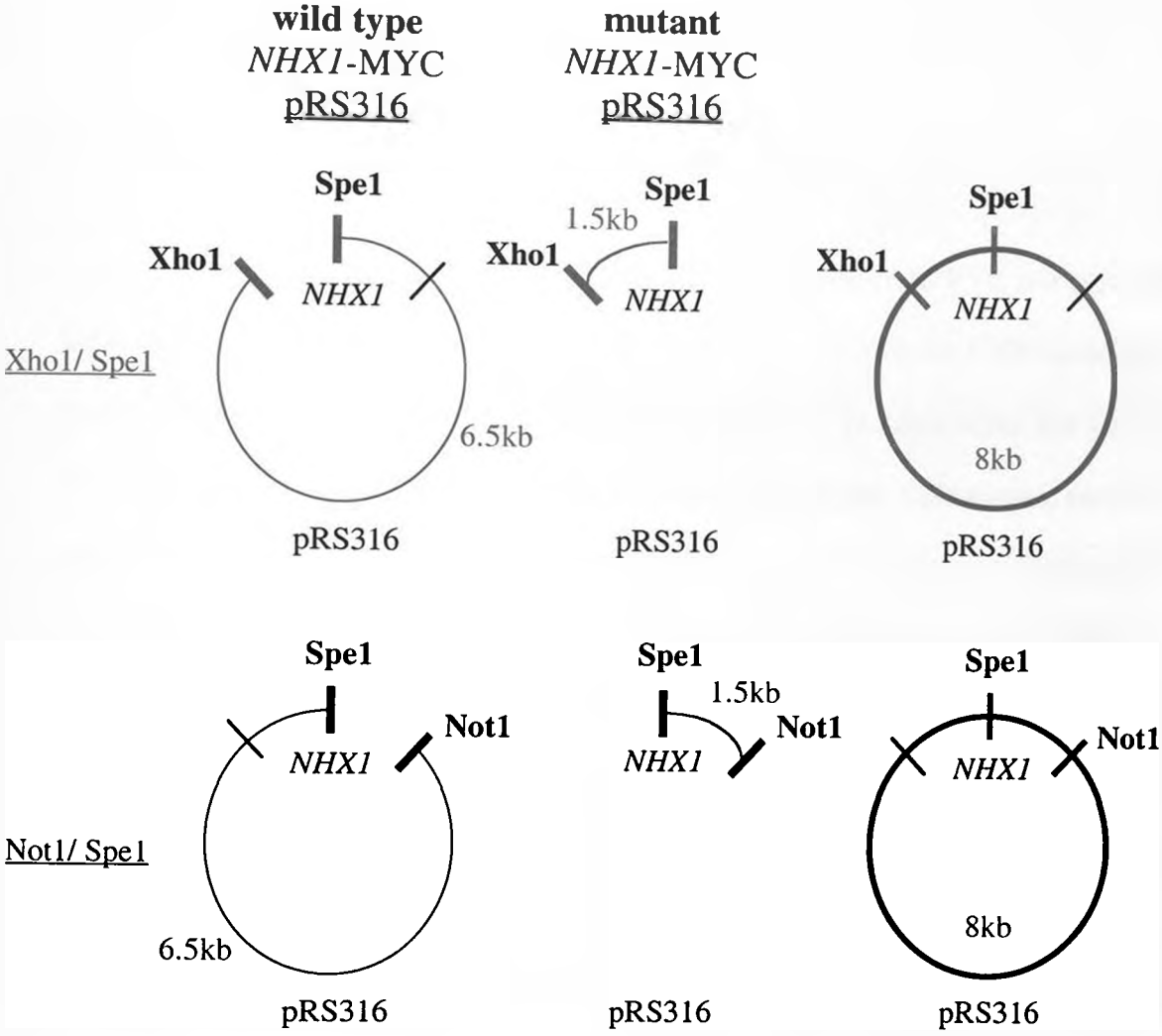


FIGURE 10. Method of subcloning a mutant-half into a vector containing the other wild type-half of *NHX1* to distinguish which half bears the mutation responsible for the CPY secretion phenotype. Wild-type *NHX1*-MYC on pRS316 was enzymatically digested with a combination of either Xho1 and Spe1, or Not1 and Spe1. The larger fragment (6.5kb) was isolated by gel electrophoresis and purified. Each of the seven mutant *NHX1*-MYC genes on pRS316 was also digested with both combinations, Xho1/Spe1 and Not1/Spe1. The smaller fragment (1.5kb) containing half of the mutated *NHX1*-MYC was isolated and purified. Thus, each mutant was cut in half, one half including the N-terminal region and the other half including the C-terminal region. Each of these halves containing mutations were then cloned (ligated) into pRS316 including the other half of *NHX1* bearing no mutations (wild-type).

CHAPTER IV

DISCUSSION OF RESULTS AND FUTURE DIRECTIONS

Previous experiments indicate that Nhx1p is localized to the PVC and required for the exit of proteins out of this compartment. Yeast cells in which the *NHX1* gene has been deleted result in an aberrant PVC next to the vacuole. Proteins of the late Golgi, PVC and vacuole accumulate here within this aberrant structure. In addition, earlier studies have shown that *nhx1*Δ cells secrete approximately 35% of newly synthesized CPY. This phenotype of *nhx1*Δ cells resembles that of class E *vps* mutants. These findings suggest that Nhx1p acts at the same step in trafficking to the vacuole as other class E Vps proteins, at the PVC.

It is possible that the sodium/ proton exchange activity of Nhx1p is essential for its role in the sorting of proteins to the vacuole. The exchange of sodium ions and protons may regulate the environment within the PVC and thereby determine the binding of other class E Vps proteins. Studies in mammalian cells have revealed that the environment within endosomes regulates the binding of cytosolic factors to the membrane. Perhaps this mechanism is true in other systems.

Sequence alignment reveals that Nhx1p is most closely related to a protein found in the yeast *Schizosaccharomyces pombe*. Other homologues of the NHE protein family are found in thale cress *A. thaliana*, rice *O. sativa*, fruit fly *D. melanogaster*, human *H. sapiens*, and rat *R. norvegicus*. The highest sequence identities are found in the predicted

transmembrane domains suggesting their importance in the role and function of NHE proteins.

We have identified four acidic residues within predicted transmembrane domains that are conserved across all species (figure 7a). When these residues are mutagenized to polar, uncharged residues in Nhx1p, the protein product is expressed to wild-type levels and also localizes to the PVC. The function of three of these mutant proteins, however, is compromised. The secretion of CPY, as well as the accumulation of late Golgi, PVC, and vacuolar proteins in an aberrant prevacuolar compartment indicate that these mutant proteins cannot function normally in protein trafficking out of the PVC. Mutation of similar residues in NHE proteins, similarly, affect vacuolar trafficking, suggesting that the sodium/ proton exchange activity is necessary for protein trafficking. It is likely that these residues D201, E225, and D230 affect exchange activity, maintaining the idea that acidic residues in transmembrane domains 5 and 6 of NHE proteins act as ion-binding sites (Counillon and Pouyssegur, 2000).

Double mutants were created to see if secretion became additive after combining D201N with E225Q and D201N with D230N. Both single and double mutants were expressed at the same level as wild-type Nhx1p, indicating that the mutations did not affect the stability of the protein. Pulse-chase experiments show that a combination of mutations do not have an additive effect, while maintaining the significance of mutations at D201N, E225Q, and D230N.

In addition to analyzing specific point mutations it was necessary to create random mutations as well, in order to characterize Nhx1p. Hydroxylamine mutagenesis followed by a screen using CPY secretion as a functional assay for Nhx1p function

yielded 30 mutant plasmids (out of 14,000 screened colonies). These 30 plasmids were then transformed into an *nhx1*Δ strain and it was found that 7 of the mutants were able to produce a full length protein.

The open reading frame of the *NHX1* gene is 2000 base pairs and the use of hydroxylamine implies that mutations may have been made all across the gene, making it difficult to determine which mutaitons are actually responsible for the CPY secretion phenotype of these mutants. Therefore, the gene was split in half and subcloned or inserted back into a plasmid containing the other half that was never exposed to the mutagen. After testing each half for CPY secretion, the halves that did secrete were sequenced so that extraneous mutations could be ruled out. After preliminary screening and sequencing, mutants 3, 40 and 54 have been found to have significance in regards to the function of Nhx1.

In mutant 3, two consecutive residues at positions 441 and 442 in transmembrane domain 11 were substituted. Coincidentally, both of these residues are highly conserved in the NHE protein family. Arginine at 441 was changed to histidine, both of which are polar charged residues. However, histidine has a larger side chain and imidazole ring which definitely may have some effect on the function of Nhx1p.

In mutant 40, glycine 138 is highly conserved and it has been changed to arginine, which has a much larger side chain and is also hydrophilic. This mutations like the other occurs in a transmembrane domain, indicating perhaps that residues that make up the transmembrane domains may be more sensitive to mutations and have some function in the ion exchange activity of Nhx1.

Mutant 54 was found to have mutations in domains 5 and 6, V193G and L228V. The valine to glycine change at 193 involves going from a residue with a nonpolar side chain to the absence of a side chain. This may indeed affect the function of the protein. The second mutation seems less likely to be responsible for the mutant phenotype because both residues involved are similarly nonpolar hydrophobic residues. These mutations all await further testing to determine which ones actually result in CPY secretion. In addition, the sequences of other mutants need to be further analyzed in order to find mutations that occur within the open reading frame of *NHX1*.

As mentioned earlier, a screen was performed that resulted in the derivation of several *VPS* genes. This screed also yielded a few mutants of *VPS44*, otherwise known as *NHX1*. Each of the four mutants from this screen were found to result in the secretion of CPY. Pulse-chase experiments confirmed that the mutants secreted significantly more than wild-type secretion levels of 5%, but not as much as *nhx1*Δ cells. Sequencing data revealed truncations that left out transmembrane domains 11 and 12, as well as the large C terminal domain that has been implicated in regulatory functions. One of the mutants was found to have a mutations here in the C-terminal end, leaving intact all transmembrane domains, thus strengthening the idea that the C-terminal end has some role in the function of Nhx1p.

Fluorescence studies have indicated the importance of Nhx1 in the trafficking of proteins to the vacuole. Through the analysis of mutants, several residues have been found to be potentially essential for the ion exchange activity of Nhx1p. These residues await further analysis including sequencing, as well as testing by site-directed mutagenesis. Most of the mutations have indicated that the transmembrane domains may

in fact be important in the exchange activity of Nhx1p. This is also represented by the high sequence similarity of these regions in other homologues, including NHE proteins. In addition, further experimentation regarding the C-terminal end should help to explain its possible regulatory functions and whether it actually has phosphorylation and ATP-binding sites. It may also be interesting to go back to the random mutants that did not express full-length proteins to see what mutations they have that result in the secretion phenotype after tagging the N-terminal end. Another use for an N-terminal epitope tag is on the *VPS44* mutants, which would allow us to check whether a full-length protein can be expressed. Further analysis of residues will ultimately allow us to characterize Nhx1p in terms of its role and function in vacuolar protein sorting. The results in this thesis indicate that the sodium/ proton exchange activity may be needed for the maintenance of the ionic environment inside the PVC that determines the associations of other factors required for protein trafficking.

GLOSSARY

alkaline phosphatase (ALP): vacuolar membrane protease (enzyme); transported to vacuole via ALP pathway.

alkaline phosphatase (ALP) pathway: direct route carrying ALP from *trans* Golgi to vacuole.

antibody: protein compound produced by plasma cells in response to specific antigens and having the capacity to fight against the antigens.

C-terminus (C-terminal region): in proteins, region of amino acids that ends in a carboxy (-COOH) group. The carboxy terminus varies in number and type of amino acids for individual proteins.

carboxypeptidase Y (CPY): soluble vacuolar hydrolase (enzyme); transported to vacuole via CPY pathway.

carboxypeptidase Y (CPY) pathway: carries CPY from *trans* Golgi to the pre-vacuolar compartment to the vacuole.

DNA (deoxyribonucleic acid): describes the structure of molecules that comprise genes, units of genetic information, that are passed from generation to generation.

eukaryote: organism whose cells possess nuclei and other membrane-bounded organelles.

hydroxylamine: chemical mutagen that causes nucleotide transitions.

immunoblot overlay assay: technique for detecting proteins secreted by cells which sticks onto a piece of nitrocellulose filter which is overlaid over the cells. Labeled antibodies then detect the specific protein.

mutagenesis: process or act of introducing a mutation or DNA base change.

mutation: any change in DNA; may include a change in the nucleotide base pairs of a gene, a rearrangement of genes within the chromosomes so that their interactions produce different effects, or a change in the chromosomes themselves.

NHE: family of sodium (N) / proton (H) exchanger (E) proteins.

NHX1: gene which encodes the sodium/proton exchanger Nhx1p.

Nhx1p: protein product encoded by the gene *NHX1*.

nucleotide: basic building block of DNA; base pairs with another nucleotide and forms a chain.

nucleus: an organelle in eukaryotes that contains the DNA and serves as the control center of the cell.

organelle: one of the specialized structures within the cell, such as the nucleus, mitochondria, Golgi complex, ribosome, or vacuole.

pH the negative algorithm of the hydrogen ion concentration of a solution.

phenotype: the physical or chemical expression of an organism's genes.

plasma membrane: the selectively permeable surface membrane that encloses the cell contents and through which all materials entering or leaving the cell must pass.

plasmid: small circular DNA molecule that carries genes separate from the main bacterial DNA.

polymerase: enzyme that catalyzes the formation of a polymer, and molecule built from repeating subunits of the same general type, such as amino acids in proteins, nucleotides in nucleic acid, or sugar units in polysaccharides.

pre-vacuolar compartment: the compartment preceding the vacuole through which materials pass before reaching the vacuole (ie. CPY)

prokaryote: cell that lacks a nucleus and other membrane-bounded organelles; include the bacteria, members of the kingdom Prokaryotae.

proteolysis: cleavage of a protein.

pulse-chase immunoprecipitations: a type of experiment in which a radioactive small molecule is added to a cell for a brief period (the pulse) during which it is incorporated into macromolecules. The fate of the newly-synthesized, radioactive, molecules is examined during the subsequent period when the radioactive small molecule is removed from the cell medium and an excess of the same small molecule, but unlabeled, is added (the chase). This can be done at different time points to track the amount of molecule/ protein internally and externally to the cells.

VPS: vacuolar protein sorting gene.

western blot: technique for detecting specific proteins in a mixture. After separation of the proteins by gel electrophoresis, proteins are blotted to a filter paper and specific ones detected by labeled antibodies.

wild-type: phenotypically normal (naturally occurring) form of a gene or organism.

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