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Biochimica et Biophysica Acta 1712 (2005) 185 – 196
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
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Characterization of the ion transport activity of the budding yeast Na^+/H^+ antiporter, Nha1p, using isolated secretory vesicles

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Received 15 February 2005; received in revised form 25 March 2005; accepted 28 March 2005
Available online 14 April 2005

Abstract

The *Saccharomyces cerevisiae* Nha1p, a plasma membrane protein belonging to the monovalent cation/proton antiporter family, plays a key role in the salt tolerance and pH regulation of cells. We examined the molecular function of Nha1p by using secretory vesicles isolated from a temperature sensitive secretory mutant, *sec4-2*, in vitro. The isolated secretory vesicles contained newly synthesized Nha1p en route to the plasma membrane and showed antiporter activity exchanging H^+ for monovalent alkali metal cations. An amino acid substitution in Nha1p (D266N, Asp-266 to Asn) almost completely abolished the Na^+/H^+ but not K^+/H^+ antiport activity, confirming the validity of this assay system as well as the functional importance of Asp-266, especially for selectivity of substrate cations. Nha1p catalyzes transport of Na^+ and K^+ with similar affinity (12.7 mM and 12.4 mM), and with lower affinity for Rb^+ and Li^+. Nha1p activity is associated with a net charge movement across the membrane, transporting more protons per single sodium ion (i.e., electrogenic). This feature is similar to the bacterial Na^+/H^+ antiporters, whereas other known eukaryotic Na^+/H^+ antiporters are electroneutral. The ion selectivity and the stoichiometry suggest a unique physiological role of Nha1p which is distinct from that of other known Na^+/H^+ antiporters.

Keywords: Yeast Na^+/H^+ antiporter; Ion selectivity; Electrogenicity; Yeast secretory vesicle

1. Introduction

The intracellular ion environment is crucial for the survival of all organisms and is maintained by ion transporters both on the plasma membrane and on endomembranes. In particular, the cytoplasmic pH and the concentration of major metal cations such as Ca^{2+}, Mg^{2+}, Na^+ and K^+ are tightly regulated, since these are central to many cellular processes, such as proliferation, differentiation and osmoregulation.

Na^+/H^+ antiporters play a significant role in the regulation of pH and Na^+ concentrations in all types of cells from bacteria to higher eukaryotes. In an exchanging reaction with H^+, bacterial and yeast Na^+/H^+ antiporters extrude intracellular Na^+ to the out side, while mammalian transporters mediate influx of extracellular Na^+ to the inside of cells [1–3]. Several different types of bacterial Na^+/H^+ antiporters have been identified [4–8]. One of the most thoroughly characterized Na^+/H^+ antiporter protein, NhaA of *Escherichia coli* is an integral membrane protein that consists of 12 putative transmembrane domains and plays a central role in Na^+ extrusion from cells. By using the well-established methods to obtain membrane vesicles for ion transport assays, such as reconstitution of purified proteins into liposomes [9] and preparation of inverted plasma membrane vesicles from *E. coli* cells [10], the antiport activity of NhaA has been investigated in detail. The structure–function relationship of essential and important Asp residues in antiport activity as well as the packing of helices in the hydrophobic environment of the membrane are now becoming clear [11–16].

Abbreviations: NHA, Na^+/H^+ antiporter; NHE, Na^+/H^+ exchanger; SDS PAGE, SDS polyacrylamide gel electrophoresis; ORF, Open Reading Frame; ACMA, 9-Amino-6-Chloro-2-Methoxyacridine; EGFP, Enhanced Green Fluorescent Protein; pmf, proton motive force; CCCP, Carbonyl Cyanide *m*-Chlorophenylhydrazone

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doi:10.1016/j.bbamem.2005.03.011

In mammals, nine Na^+/H^+ exchanger isoforms (NHE1-9) have been identified and are suggested to play specific functions [17–22]. Mammalian NHEs transport extracellular Na^+ to the inside of the cell, while yeast and bacterial transporters transport intracellular Na^+ to the outside. The NHE isoforms consist of an integral membrane domain and a C-terminal hydrophilic domain which is highly divergent among the isoforms. Putative regulatory mechanisms associated with the C-terminal tail region have been extensively studied [23–25]. Mutations in the genes encoding NHEs have been known to cause severe diseases such as epilepsy [26,27] and hypertension [28], implying the importance of this protein in multicellular organisms. Despite their physiological importance, the molecular mechanism of ion transport of NHEs still remains unclear.

In the budding yeast, *Saccharomyces cerevisiae*, two different types of Na^+/H^+ antiporters have been identified. One of the antiporters is Nha1p, found at the plasma membrane, and the other is Nhx1p, which localizes to the endosomal prevacuolar compartment [29]. Nha1p was identified as a molecule showing similarity to *SOD2*, the Na^+/H^+ antiporter of *Schizosaccharomyces pombe* [30]. Deletion of the *NHA1* gene causes a loss of salt tolerance in yeast cells, and high sodium and potassium conditions increase the cytoplasmic pH in an Nha1p-dependent manner [31]. From these observations, Nha1p has been suggested to function as a Na^+, K^+/H^+ antiporter and to regulate the intracellular pH, and the Na^+ and K^+ concentrations [32,33]. Although the primary structure of Nha1p is very different from that of mammalian NHEs, the two domain structure found in NHEs is similar to the domain organization of Nha1p. The C-terminal hydrophilic domain of Nha1p is involved in the regulation of ion transport activity as in the NHEs [34–37]. Six independent regions in the C-terminal tail have been shown to be conserved among various yeast and fungal species. One of the conserved regions proximal to the integral membrane domain is involved in localization of Nha1p to cytoplasmic membranes and also plays an important role in antiporter activity [36]. Recently, we identified Cos3p, a novel membrane protein which interacts with the C-terminal hydrophilic tail of Nha1p and increases salt tolerance [37]. Analysis of the interaction between Nha1p and Cos3p may give some implications for the general understanding of the activation mechanism of all Na^+/H^+ antiporters including NHEs. Furthermore, a relationship between Nha1p and the cell cycle has been implied, as overexpression of Nha1p suppresses the G_1/S blockage of a conditional *sit4 hal3* mutant [38,39].

Although extensive efforts have been made to elucidate the structure–function relationship of Nha1p, the approaches taken in previous studies have been primarily based on molecular genetics. Biochemical analyses of the ion antiport activity of Nha1p are still at an early stage and there is a need to establish a direct measurement system for Na^+/H^+ antiporter activity. Although reconstitution of Nha1p activity in artificial liposomes using a crude plasma membrane fraction has been described, detailed analyses, in terms of substrate ion specificity and stoichiometry of H^+ and Na^+, have not been performed [31].

For biochemical analysis of the Nha1p activity, we developed a direct assay system of the antiporter activity of yeast Nha1p. For this purpose, we used yeast secretory vesicles isolated from a yeast *sec4-2* mutant defective in secretion. The secretory vesicle is a sealed membrane vesicle and should carry a set of plasma membrane ion transporters including Nha1p. Another advantage of using secretory vesicles in an assay system for ion transporters is that the membrane proteins are integrated into the vesicle uniformly with the correct topology. Secretory vesicles have been used to assay the yeast plasma membrane H^+-ATPase Pma1p [40] and other heterologously expressed mammalian transporters as well [41–46]. Proteins on the secretory vesicles have acquired post-translational modifications at the ER and Golgi-apparatus. Thus, we expected that the secretory vesicles contain mature Nha1p which has undergone all the post-translational modifications required for functional expression on the plasma membrane. Using this assay system, we demonstrate here that Nha1p catalyzes low affinity transport of Na^+ and K^+ in an electrogenic manner. These findings suggest that the budding yeast Nha1p is a novel type of monovalent cation/H^+ antiporter protein in eukaryotic cells.

2. Materials and methods

2.1. Strains and media

The yeast ROY21 strain (*ena1*Δ::*HIS3*::*ena4*Δ *nha1*Δ::*LEU2 sec4-2*) was derived from MTsec4 (YPH499 derivative: *MAT*a *ura3-52 lys2-80 ade2-10 trp1-*Δ*63 his3-*Δ*200 leu2-*Δ*1 sec4-2*), kindly provided by Dr. Yoshinori Ohsumi (National Institute of Basic Biology, Okazaki, Japan). The chromosomal *NHA1* locus of MTsec4 was substituted by homologous recombination of the 3.9 kb *Xba*I–*Nde*I *NHA1* fragment containing insertion of the *LEU2* gene [34]. After confirming the disruption by genomic PCR, the resulting *sec4-2 nha1*Δ cell was mated with SK5 (*ena1*Δ::*HIS3*::*ena4*Δ *nha1*Δ::*LEU2*) [34], and the resulting diploid was sporulated in sporulation medium (1% potassium acetate and 0.025% glucose). His^+ Leu^+ (i.e., *ena1*Δ::*HIS3*::*ena4*Δ *nha1*Δ::*LEU2*) haploid clones were selected by culturing on synthetic dextrose (SD) medium lacking histidine and leucine. Temperature sensitivity (*sec* phenotype) was verified by impaired growth on plates and invertase accumulation at 37 °C.

Yeast cells were grown in YPD medium containing 1% yeast extract, 2% peptone and 2% glucose, or SD medium containing 0.67% yeast nitrogen base and 2% glucose, supplemented with appropriate nutrients. The salt sensitivity of yeast cells was examined by culturing in medium containing 0.4M NaCl or 1 M KCl (pH 5.5). SD medium

containing 0.2% glucose was used for induction of invertase. Yeast cells were grown at 30 °C (*SEC4*) or at 25 °C (*sec4-2*) for propagation, but at 37 °C for vesicle accumulation.

2.2. Plasmids

To construct the multi-copy expression vector pKT10-P_{NHA1} containing the yeast *NHA1* promoter, the *NHA1* promoter region (0.7 kbp) was amplified by PCR with oligonucleotide primers #1 and #2 (see below), from the genomic *NHA1* locus [34]. The PCR product was digested with *Bam*HI–*Kpn*I, and substituted for the GAP promoter (*Bam*HI–*Kpn*I) of the pKT10 vector [47]. To obtain pNHA1, the *NHA1* ORF (*Kpn*I–*Sal*I, 3.0 kbp) from pKT10-NHA1 [34] was introduced downstream of the *NHA1* promoter region in pKT10-P_{NHA1}. The amino acid substitution of Asp-266 to Asn in Nha1p was performed by PCR using primers #3 and #4 to generate pNHA-D266N. The oligonucleotide primers used were 5′-AATTAACCCTCACTAAAGGG-3′ (#1), 5′-AGCCATGGTACCTAGCTAAGTTCAGGAT-3′ (#2), 5′-GGGTGTGAATGACCTATTGGTATC-3′ (#3), 5′-GATACCAATAGGTCATTCACACCC-3′ (#4).

pNHA1-6xHis and pNHA1-D266N-6xHis were derived from pP_{GAP}NHA1-EGFP and pP_{GAP}NHA1-D266N-EGFP, respectively [36]. The EGFP fragment (*Sph*I–*Sal*I) of each plasmid was substituted using the *Sph*I–*Sal*I 6xHis adaptor, then the NHA1-6xHis or NHA1-D266N-6xHis fragment (*Kpn*I–*Sal*I) was introduced into pKT10-P_{NHA1}. The structure of the pNHA1-EGFP has been described previously [36]. The gene encoding the HA-tagged Pma1p was introduced into pRS314 to generate pRS314-HA-PMA1 [48].

2.3. Preparation of secretory vesicles

Secretory vesicles were isolated by differential centrifugation and a subsequent gel filtration chromatography step, as described by Walworth and Novick [49], with some minor modifications. Yeast cells carrying either pKT10-P_{NHA1}, pNHA1-6xHis or pNHA1-D266N-6xHis were grown to mid-exponential phase (OD_{600}=0.6–1.0) at 25 °C in 2.5 l of SD medium. Cells were collected, washed with distilled water, and resuspended in prewarmed SD medium with 0.2% glucose, followed by a 2-h incubation at restrictive temperature (37 °C) to induce vesicle accumulation and the expression of a secretory vesicle marker, invertase. Ten minutes before harvest, 10 mM NaN_3 was added to the culture. Cells were collected by centrifugation at 3000 × *g* for 5 min, washed with ice-cold distilled water containing 10 mM NaN_3, resuspended at a density of 50 OD_{600} units/ml in spheroplast buffer (1.2 M sorbitol, 50 mM K_2HPO_4 [pH 7.5]), 10 mM NaN_3, 40 mM β-mercaptoethanol, and 125 units/ml Zymolyase 100 T (Nihon Seikagaku, Tokyo, Japan), and incubated at 37 °C for 45 min. The resulting spheroplasts were collected by a brief centrifugation, resuspended in 25 ml of sorbitol buffer (0.8 M sorbitol, 10 mM triethanolamine, 1 mM EDTA/acetic acid [pH 7.2]), and lysed on ice with 20 strokes of tight pestle in a Dounce homogenizer (Wheaton, Millville, NJ). The lysate was centrifuged at 10,000 × *g* for 10 min to remove unbroken cells, cell debris, nuclei, and mitochondria (P10). The supernatant (S10) was further centrifuged at 100,000 × *g* for 1 h to yield a pellet containing secretory vesicles (P100), and the supernatant (S100). The P100 fraction was resuspended in 3 ml of sorbitol buffer and loaded onto a 1.5-cm × 90 cm Sephacryl S-1000 (Amersham Pharmacia, Piscataway, NJ) gel filtration column at 4 °C. The material was eluted in sorbitol buffer at a flow rate of 15.6 ml/h; 4 ml fractions were collected. Protease inhibitors were included in the sorbitol buffer at the following concentrations: Phenylmethanesulfonyl fluoride, 1 mM; leupeptin, pepstatin, aprotinin, 1 μg/ml each.

2.4. Protein measurement and enzyme assays

Protein was analyzed by BCA (Bicinchonic Acid) protein assay using bovine serum albumin as a standard (Pierce, Rockford, IL). Invertase activity was measured by the method of Goldstein and Lampen [50], a colorimetric method to quantify glucose production with glucose oxidase, peroxidase, and *o*-dianisidine. The assay was modified by the addition of 0.1% Triton X-100 to lyse vesicles. Vacuolar α-mannosidase activity was assayed as described previously [37].

2.5. Alkali metal cation/H^+ antiport assay

Alkali metal cation/H^+ antiport activities on the secretory vesicles were measured by monitoring the change in fluorescence of the ΔpH probe, ACMA (9-amino-6-chloro-2-methoxyacridine). Secretory vesicles at the peak fractions of invertase activity were pooled, pelleted at 100,000 × *g* for 1 h and resuspended in sample buffer (0.6 M sorbitol, 10 mM HEPES-Tris [pH 7.5]). Secretory vesicles (25 μg protein) were added to 2 ml of assay buffer containing 0.6 M sorbitol, 10 mM HEPES–Tris (pH 7.5), 100 mM choline-chloride, and 1 μM ACMA at 30 °C. After stabilization of fluorescence, alkali metal cation chlorides were added to initiate the antiport reaction. Fluorescence (ex. 410 nm and em. 480 nm) was recorded using a spectrofluorometer (JASCO FP-750, Jasco Corp., Tokyo, Japan). Mg-ATP, CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), valinomycin and nigericin were added at the indicated concentrations when required.

2.6. Fluorescence microscopy

Yeast cells harboring pNHA1-EGFP were grown to the early logarithmic phase in SD medium, washed, and transferred to SD medium with 0.2% glucose. The cells were incubated for an additional 2 h at either 25 °C or 37 °C, and then observed with a fluorescence microscope (BX-

51, Olympus, Tokyo, Japan) equipped with a 100× oil-immersion objective and a GFP filter. Images were recorded using an ORCA-ER1394 digital camera (Hamamatsu Photonics, Hamamatsu City, Japan).

2.7. Immunoblotting

Total cell lysate (20 μg protein) was subjected to 7.5% SDS-PAGE and transferred to a GVHP membrane filter (Millipore, Billerica, MA). The membranes were incubated with anti-GFP serum (Molecular Probes, Eugene, OR), anti-Nha1p polyclonal antibody [37] or an anti-penta-His antibody, and then HRP-conjugated secondary antibodies. To characterize the vesicle purification, 10 μl of each fraction was used. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Bioscience, Piscataway, NJ).

3. Results

3.1. Preparation of yeast secretory vesicles from a sec4-2 mutant

To examine ion transport by Nha1p in vitro using secretory vesicles, we constructed a yeast strain carrying a temperature sensitive mutation, *sec4-2*, and also lacking *NHA1* and *ENA* genes. The *SEC4* gene encodes a Rab GTPase essential for the docking and fusion of secretory vesicles to the plasma membrane [51]. The *sec4-2* mutation has been shown to confer rapid and reversible temperature sensitive accumulation of secretory vesicles in yeast cells [52]. This suggests that the secretory vesicles accumulated in this mutant are functionally active and sealed membrane vesicles. *ENA* genes (*ENA1-ENA4*) encoding plasma membrane Na^+-translocating ATPases [53] were disrupted to reduce overall Na^+ transport activity on the vesicle membrane.

The salt sensitivity of *sec4-2* cells expressing Nha1p derivatives was tested on agar plates to examine the expression and function of Nha1p derivatives (Fig. 1A). Cells expressing wild type Nha1p as well as versions tagged at the C-terminus (Nha1p-6xHis and Nha1p-EGFP) showed the same level of resistance to high concentrations of Na^+ and K^+. The growth of cells carrying an empty vector as the control was greatly inhibited by Na^+ and also suppressed by K^+. All Nha1p derivatives were expressed at the same level, as detected by an anti-Nha1p antibody (Fig. 1B). These results indicate that Nha1p-6xHis and Nha1p-EGFP were functionally expressed at the same level as the wild type Nha1p in *sec4-2* cells.

We examined whether or not newly synthesized Nha1p is delivered to the plasma membrane through the *SEC4*-

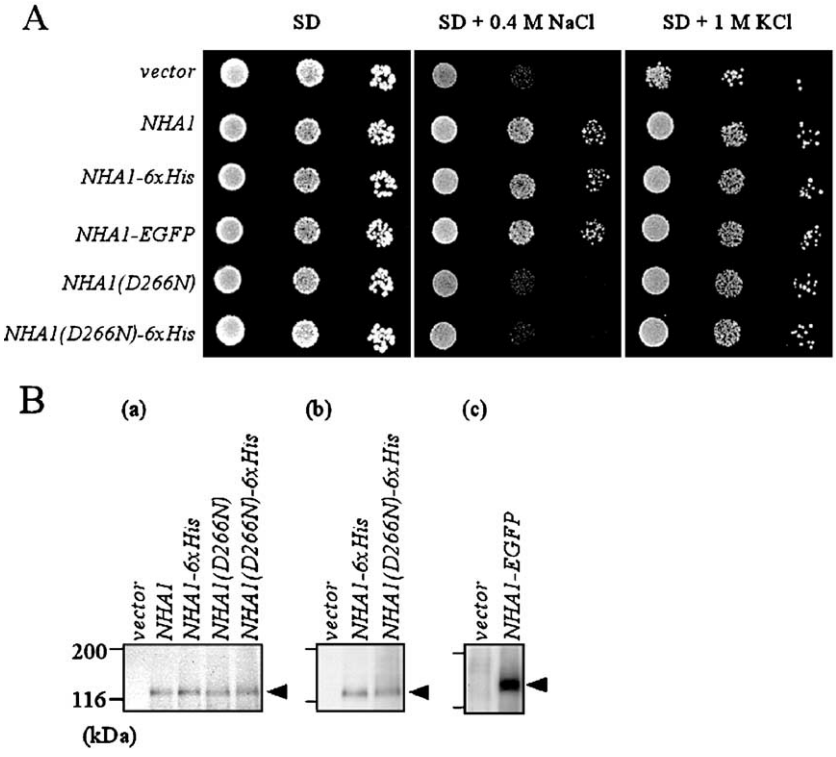


Fig. 1. Salinity resistant cell growth by functional expression of Nha1p tagged versions and a D266N mutant. (A) ROY21 (*nha1*Δ *ena1-4*Δ *sec4-2*) cells carrying either pKT10-P_{NHA1} (vector), plasmids pNHA1 (*NHA1*), pNHA1-6xHis (*NHA1-6xHis*), pNHA1-EGFP (*NHA1-EGFP*), pNHA1-D266N (*NHA1(D266N)*) or pNHA1-D266N-6xHis (*NHA1(D266N)-6xHis*) were grown until the early logarithmic phase and 10-fold serial dilutions were spotted onto SD plates at pH 5.5 containing 0.4 M NaCl or 1 M KCl, as indicated. The cells were incubated at 25 °C for 4 days. (B) Whole cell extracts were subjected to SDS-PAGE and immunoblotted with (a) an anti-Nha1p antibody, (b) an anti-penta-His antibody, or (c) an anti-GFP antibody.

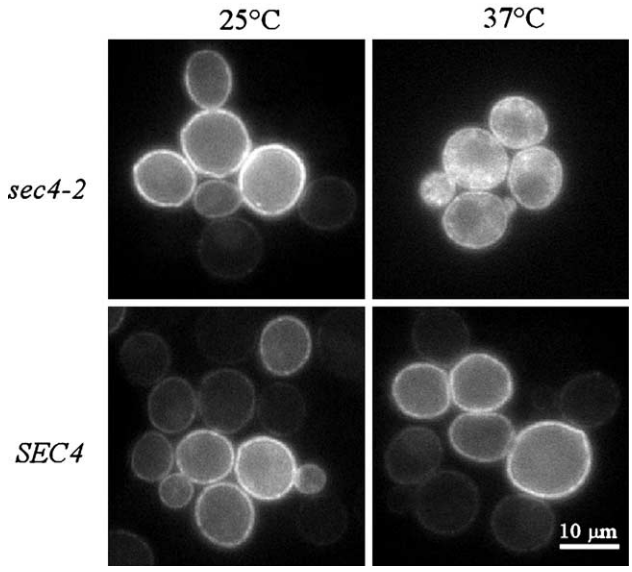


Fig. 2. Accumulation of secretory vesicles carrying Nha1p in ROY21 cells. ROY21 (*nha1*Δ *ena1-4*Δ *sec4-2*) and SK5 (*nha1*Δ *ena1-4*Δ *SEC4*) cells expressing Nha1p-EGFP were cultured until the early logarithmic phase at 25 °C in SD medium, transferred to SD medium with 0.2% glucose, and further incubated for 2 h at either 25 °C or 37 °C. Then, the cells were observed by fluorescence microscopy.

dependent secretory pathway, i.e., whether or not *sec4-2* cells accumulate secretory vesicles containing Nha1p at the restrictive temperature. Yeast cells expressing Nha1p-EGFP cultured at the permissive temperature of 25 °C were shifted to the restrictive 37 °C, incubated for 2 h, and observed by fluorescence microscopy (Fig. 2). In the wild type cells, even after incubation at 37 °C, the EGFP signal was exclusively observed on the cell surface. However, in *sec4-2* cells incubated at the restrictive temperature, a large portion of the signal was observed as punctate structures accumulating throughout the cytoplasm. This suggests that secretory vesicles containing Nha1p accumulate in *sec4-2* cells at the restrictive temperature.

We isolated secretory vesicles from the *sec4-2* mutant cells using the method of Walworth and Novick [49]. Yeast membrane fractions (P100) were prepared by differential centrifugation and further subjected to a size-exclusion gel chromatography. Two peaks of protein were detected in the gel filtration fractions (Fig. 3). The first protein peak, which was not observed in preparations from cultures grown at the permissive temperature (data not shown), overlapped with invertase activity, the secretory vesicle marker enzyme, Pma1p and Nha1p (Fig. 3, bottom panel), as detected by

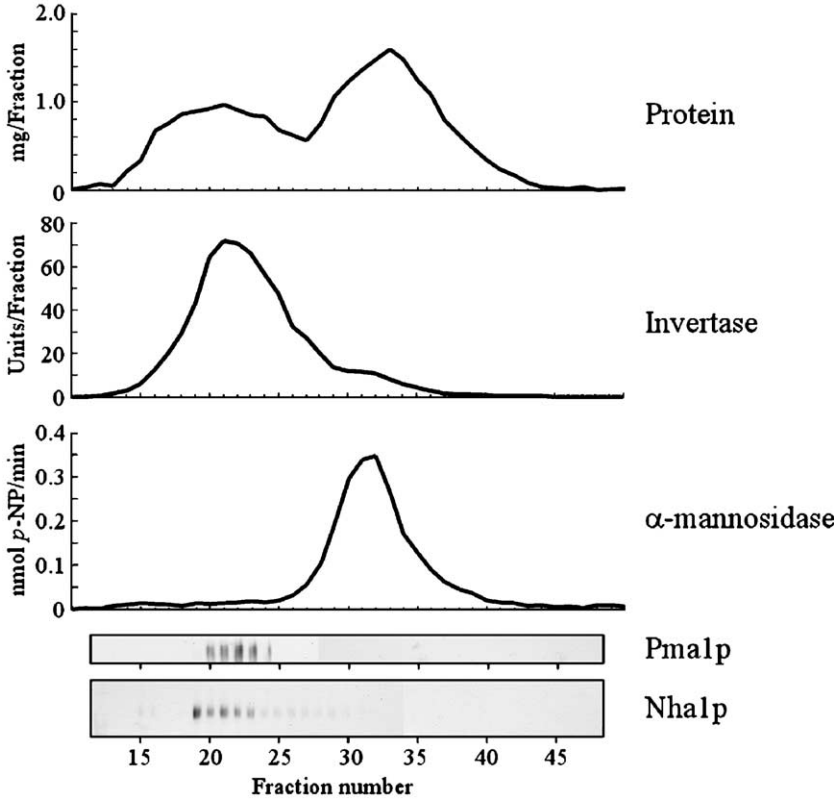


Fig. 3. Elution profile of vesicle preparations from *sec4-2* cells. The P100 fraction of ROY21 cells expressing Nha1p-6xHis was subjected to Sephacryl S-1000 gel filtration and 4 ml fractions of the eluates were collected. An aliquot of each fraction was assayed for protein and invertase and α-mannosidase activities, as described in Materials and methods. Distribution of HA-Pma1p (Pma1p) and Nha1p-6xHis (Nha1p) were analyzed by immunoblotting with anti-HA and anti-Nha1p antibodies, respectively.

Table 1
Purification table of secretory vesicle preparation

Fraction	Protein		Invertase		Specific activity	Fold purification[a]	Distribution of Nha1p[b]
	mg	%TL	U	%TL	U/mg		%TL
Total	346	100	1466	100	4.24	1	100
P10	113	33	358	24	3.17		61
S100	237	68	165	11	0.70		<1
P100	17.2	5.0	625	43	36.3	8.59	20
Pool	2.73	0.79	209	14	76.6	18.1	<8

[a] Fold purification was calculated with respect to the specific activity of Total fraction.
[b] Distribution of Nha1p-6xHis was calculated by quantifying the intensity of signals in immunoblotting detection.

Western blotting of the fractions. This result confirms that Nha1p is delivered to the plasma membrane via the *SEC4*-dependent secretory pathway. Vacuolar α-mannosidase activity eluted in the second protein peak. Thus, secretory vesicles carrying Nha1p eluted in fractions of the first protein peak and were clearly separated from the vacuolar membrane. The plasma membrane and other organelles such as mitochondria and ER were shown to be eliminated by the differential centrifugation and gel chromatography [49]. The overall distribution of organelle markers was the same in cells with either pKT10-P_{NHA1} or pNHA1-NHA1-D266N-His (data not shown).

Purification table of a typical vesicle preparation is summarized in Table 1. The invertase was enriched in the P100 fraction 8.6-fold compared to the total cell lysate (Total). Following the gel filtration chromatography, the invertase activity in the pooled fractions increased 18-fold over the activity in the cell lysate (Total) fraction. Eight percent of the total Nha1p was estimated to be present in the pooled fractions by immunoblot analysis.

3.2. Na^+/H^+ antiport activity in secretory vesicles

The secretory vesicles in the invertase peak fractions (fraction 20–22) were assayed for Na^+/H^+ antiport activity. As shown in Fig. 3, the isolated secretory vesicles contained a plasma membrane H^+-ATPase, Pma1p. We tested for Pma1p-dependent acidification of vesicles by adding Mg-ATP to the solution, in order to use the proton motive force generated by Pma1p as the driving force for Nha1p. A fluorescent ΔpH probe, ACMA, was used to monitor the pH inside secretory vesicles. The dye accumulates in membrane vesicles according to the strength of the pH gradient across the membrane, which leads to a decrease in the fluorescence intensity. Surprisingly, the fluorescence intensity decreased (quenching) significantly even when Mg-ATP was omitted from the solution. The magnitude of quenching was almost the same as that in the presence of Mg-ATP (Fig. 4A). This quenching was reversed by adding NH_4Cl, suggesting the presence of pH gradient across the membrane. Since vanadate and bafilomycin did not affect the quenching

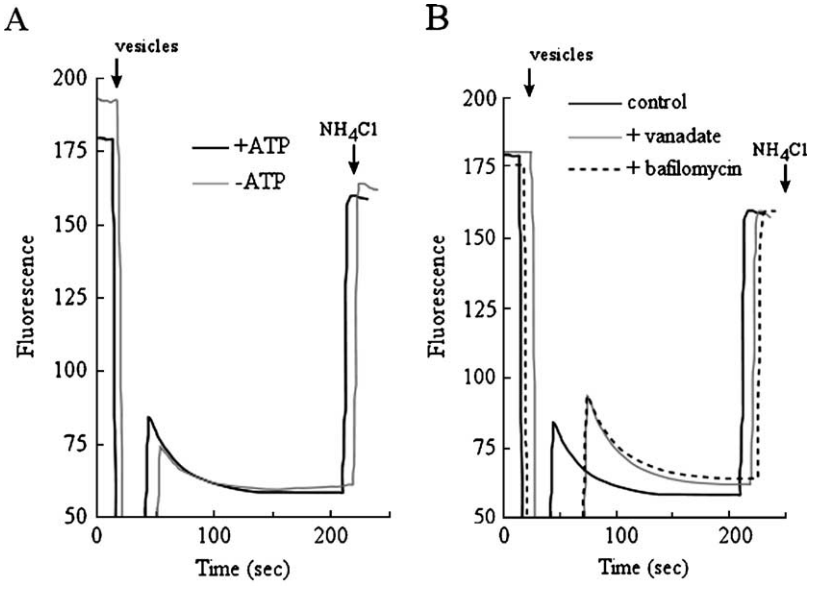


Fig. 4. Preformed pH gradient across the secretory vesicle. (A) ACMA fluorescence was monitored in the secretory vesicles prepared from ROY21 cells carrying pNHA1-6xHis in the presence and absence of ATP. The secretory vesicles (25 μg protein) were suspended into 2 ml assay solution with or without 1 mM Mg-ATP. NH_4Cl (5 mM) were added at the times indicated. ACMA fluorescence was monitored using an excitation wavelength of 410 nm and emission at 480 nm. (B) Secretory vesicle was preincubated with either 100 μM vanadate or 100 nM bafilomycin, and then added to the assay solution lacking Mg-ATP. As a control, secretory vesicle was added without incubation of inhibitors.

(Fig. 4B), the pH gradient neither depends on Pma1p driven by residual ATP in the vesicle fraction or on contamination by vacuolar H^+-ATPase. Addition of Mg-ATP after the quenching (at time 200 s after vesicle addition) did not provoke further quenching (data not shown), suggesting that this pH gradient is saturated under these conditions.

From these observations, we concluded that the secretory vesicles possess an acidic pH gradient whose generation is independent of Pma1p. We measured Nha1p-dependent ion transport activity with this preformed pH gradient in the absence of Mg-ATP. Na^+/H^+ antiport causes H^+-efflux from the vesicles, and results in a shift in vesicular pH to alkalinity. The Na^+/H^+ antiport reaction was started by the adding alkali metal cations to the mixture. Nha1p activity was estimated by measuring the recovery of fluorescence caused by the addition of Na^+ which reflects the shift in vesicular pH to alkalinity by H^+-efflux. Low fluorescence recovery after addition of NaCl was observed in vesicles carrying Nha1p, but this was indistinguishable from that of control vesicles lacking Nha1p (Fig. 5A). In the presence of Mg-ATP, the reverse in fluorescence intensity after the addition of NaCl was significantly increased in the vesicles containing Nha1p, but not in control vesicles lacking Nha1p. In the latter, the recovery in fluorescence was weak even in the presence of ATP and was approximately the same as that observed for Nha1p-carrying vesicles without ATP. As shown in Fig. 5B, the extent of the increase in fluorescence intensity was proportional to the concentration of Na^+. There was some recovery in fluorescence after the addition of NaCl even in the control vesicles isolated from cells lacking Nha1p expression. However, in vesicles carrying Nha1p, the increase in fluorescence intensity was significantly greater than that observed in vesicles lacking Nha1p. Therefore, we conclude that the difference in the

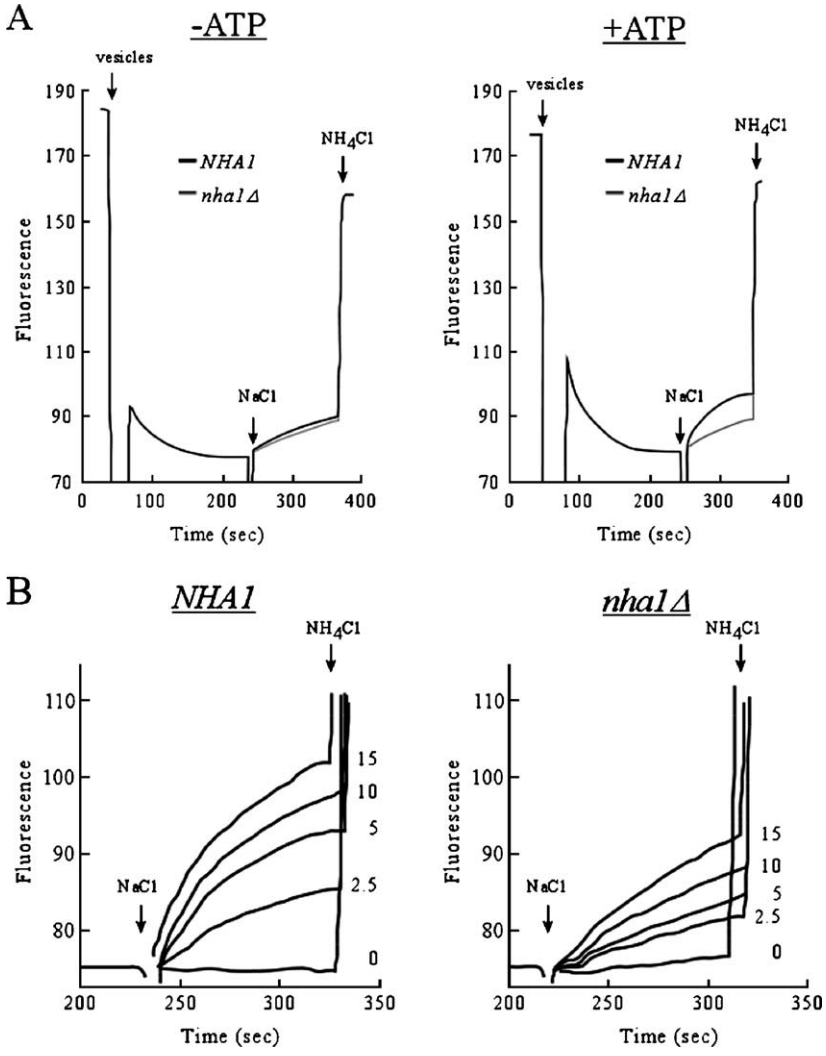


Fig. 5. Na^+/H^+ antiport activities on secretory vesicles in the presence or absence of Nha1p. (A) Secretory vesicles were prepared from ROY21 cells carrying pNHA1-6xHis (*NHA1*, black line) and empty vector (*nha1*Δ, gray line). ACMA fluorescence after the addition of NaCl was compared in the absence (−*ATP*, left) and presence (+*ATP*, right) of 1 mM Mg-ATP. The Na^+/H^+ antiport was initiated by the addition of 5 mM NaCl. (B) Various concentrations of NaCl (0–15 mM) were added to secretory vesicles carrying pNHA1-6xHis (*NHA1*, left) or to vesicles from cells transformed with empty vector (*nha1*Δ, right).

fluorescence recovery between vesicles with and without Nha1p in the solution containing Mg-ATP represents Na^+/H^+ antiport activity catalyzed by Nha1p.

3.3. Electrogenic antiporter activity of Nha1p in secretory vesicles

To address why the preformed ΔpH does not drive Nha1p, and why apparent Nha1p activity is increased by ATP, we further characterized the isolated secretory vesicles. Addition of a proton ionophore CCCP did not reduce the preformed ΔpH at all (Fig. 6A). This might be due to the electric back force of the membrane potential (ΔΨ), because CCCP-dependent H^+-efflux is an efflux of positive charge. In agreement with this interpretation, following addition of the K^+-ionophore valinomycin together with KCl assisted CCCP to completely dissipate the preformed ΔpH by compensating the ΔΨ with the positive charge of K^+ (Fig. 6A). This result implies that the vesicles did not sustain an inside positive membrane potential, and could not allow CCCP-mediated H^+-efflux. Furthermore, this indicates that movements of other ions across the membrane (counter anions to the outside or cations to the inside) did not compensate electrically for the efflux of the positive charge of H^+ under the conditions tested. Essentially, the same result was obtained when nigericin, a K^+/H^+ antiporter ionophore, was added with KCl (data not shown). These findings and the apparent ATP-dependence of Nha1p activity shown in Fig. 5 suggest that Nha1p mediates electrogenic transport, i.e., the transport of more protons per single sodium ion, leading to a net efflux of positive charge. In the absence of ATP, Nha1p could not continuously transport H^+ as observed with CCCP, an electrogenic protonophore (Fig. 6A). In the presence of ATP, Pma1p transports the positive charge of H^+ into the vesicles, which consequently allows the antiport of Nha1p counteract the back force of membrane potential. In agreement with this, the recovery in fluorescence intensity was greatly reduced when the vesicles were preincubated with vanadate to inhibit Pma1p activity (Fig. 6B).

We examined electrogenicity by using valinomycin and low concentrations of KCl (0.25 mM) instead of Mg-ATP. In the presence of valinomycin and KCl, the outside positive ΔΨ created by Nha1p, should be compensated by valinomycin mediated K^+-influx. As shown in Fig. 6C, apparent Nha1p activity was enhanced by valinomycin and KCl. Valinomycin or KCl alone did not increase a recovery in ACMA fluorescence. This result supported the notion that Nha1p mediates electrogenic Na^+/H^+ antiport.

3.4. Ion selectivity of Nha1p and effect of Asp-266 mutation

To investigate the ion selectivity of Nha1p, we examined the transport of five alkali metal cations in the antiport with H^+ (Fig. 7). Nha1p showed low affinity Na^+/H^+, K^+/H^+, Rb^+/H^+ and Li^+/H^+ antiport. The Li^+/H^+ antiport was so weak that we could not estimate the Michaelis constant (*K*m) for Li^+. Cs^+/H^+ antiport activity was not detected

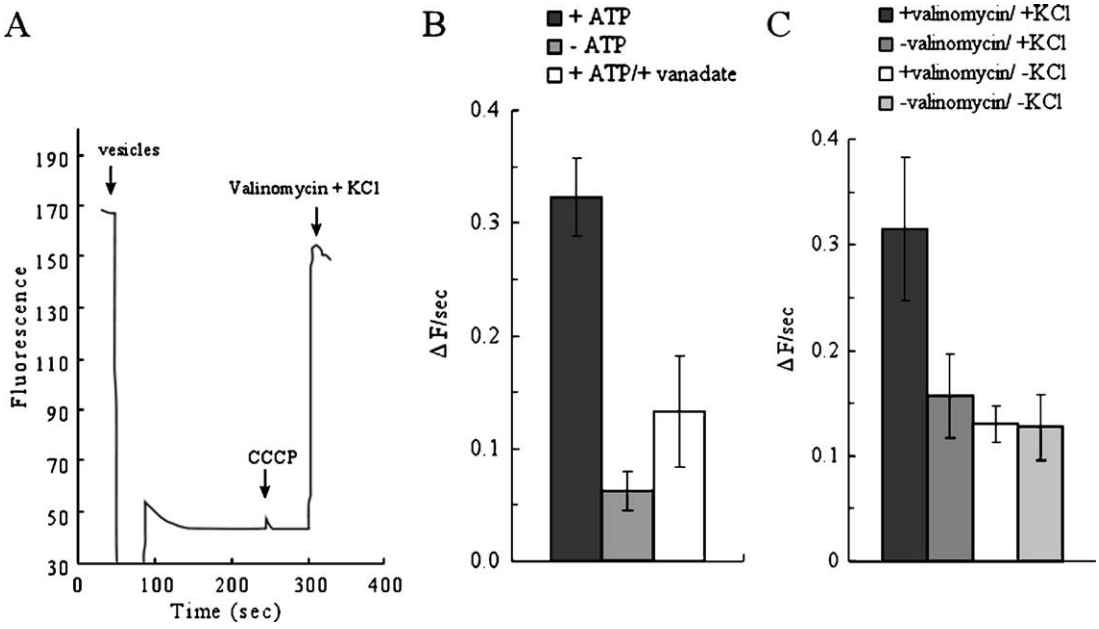


Fig. 6. Electrogenic ion transport by Nha1p. (A) Secretory vesicles (25 μg protein) were added to 2 ml of assay buffer without 1 mM ATP. CCCP (10 μM), valinomycin (5 μM), and KCl (5 mM) were added as indicated. (B) Secretary vesicles carrying Nha1p-6xHis was assayed for Na^+/H^+ antiport activity after incubation with vanadate. The reverse in the fluorescence (Δ*F*/s) after the addition of 5 mM NaCl was plotted. *+ATP*: 1 mM Mg-ATP was added to the solution, *−ATP*: Mg-ATP was omitted from the solution, *+ATP/+vanadate*: vesicle was incubated with 100 μM vanadate and then added to solution containing 1 mM Mg-ATP. (C) Effect of valinomycin and KCl on the Nha1p activity. 0.25 mM KCl and 0.25 μM valinomycin were added to the solution instead of Mg-ATP, and assayed for Na^+/H^+ antiport activity. The reverse in the fluorescence (Δ*F*/s) after the addition of 5 mM NaCl was plotted.

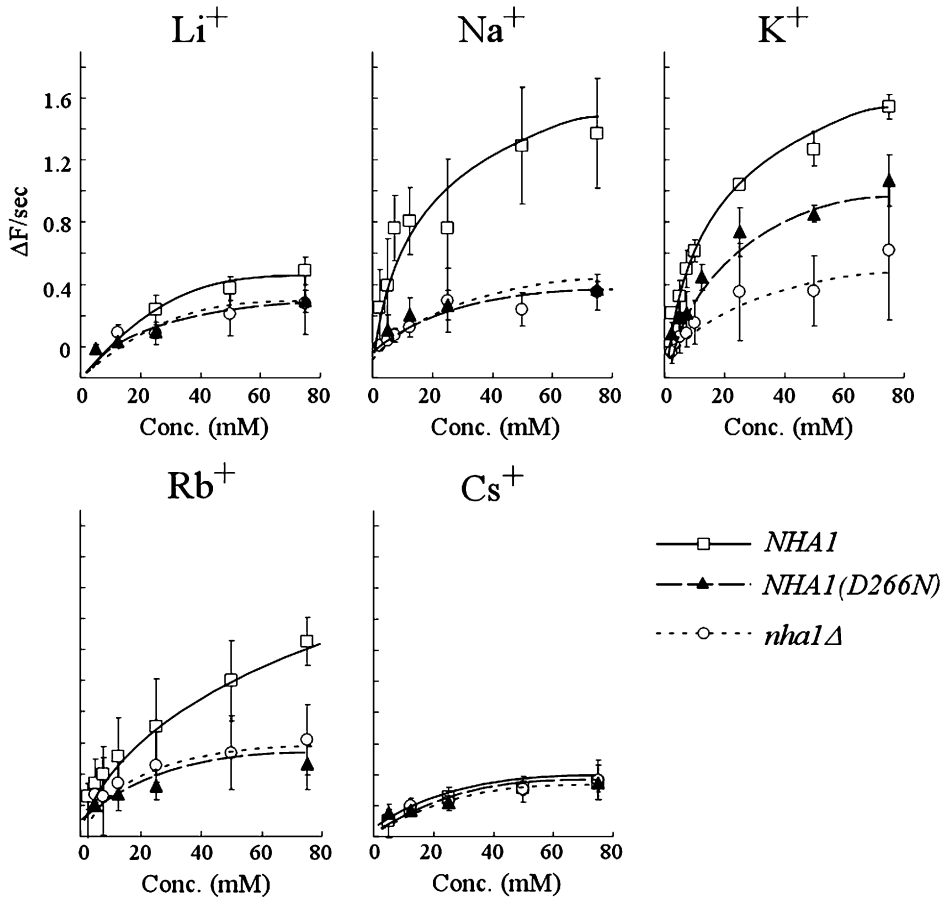


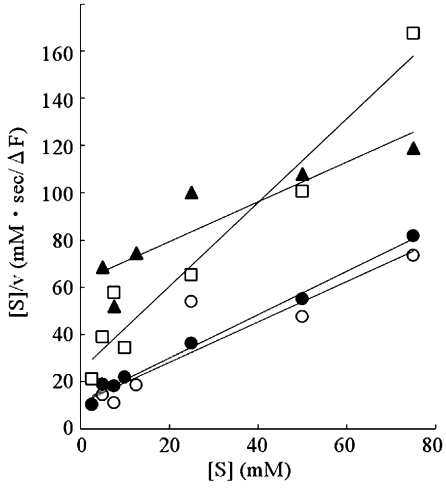
Fig. 7. Substrate specificity of Nha1p and Nha1p(D266N). Various concentrations of Li^+, Na^+, K^+, Rb^+ and Cs^+ were tested, each at a concentration of 0–75 mM. The reverse in ACMA fluorescence after the addition of the cations (ΔF/s) is plotted against concentration of cation added. Secretory vesicles were prepared from ROY21 cells carrying pNHA1-6xHis (*NHA1*), pNHA1-D266N-6xHis (*NHA1(D266N)*), or empty vector (*nha1*Δ).

under the conditions tested. To estimate *K*m and *V*max values, the background activity on the secretory vesicles from cells carrying empty vector (*nha1*Δ in Fig. 7) was subtracted from the total activity (*NHA1* in Fig. 7) and the Nha1p dependent portions of Na^+-, K^+- and Rb^+/H^+ antiport activity were analyzed by Hanes–Woolf plot. The estimated *K*m values of Nha1p for Na^+ and K^+ (12.7 and 12.4 mM, respectively) were rather smaller than that for Rb^+ (42.4 mM). The *V*max values were also similar for Na^+ and K^+ (1.17 and 1.08 [ΔF/s], respectively), but slightly larger than for Rb^+ (0.89 [ΔF/s]) (Fig. 8).

The mutant carrying an amino acid replacement of Asp-266 to Asn, Nha1p(D266N), did not confer Na^+ resistance in yeast, but conferred K^+ tolerance to the same extent as the wild type Nha1p (Fig. 1 and Ref. [33]). We examined the ion transport activity of Nha1p(D266N), and found that the mutant Nha1p catalyzes K^+/H^+ antiport but not Na^+/H^+ and Rb^+/H^+ antiport (Fig. 7). The *K*m value of Nha1p(D266N) for K^+ was slightly greater than that of the wild type, but the *V*max value was decreased by about 50% (Fig. 8). This result demonstrates directly the functional importance of Asp266 in the Na^+/H^+ antiport, especially in recognition of substrate ions.

4. Discussion

In *E. coli*, inverted plasma membrane vesicles have contributed to the characterization of the Na^+/H^+ antiporter. Attempts to prepare inside-out vesicles from yeast plasma membranes have not been successful. However, isolated secretory vesicles are one reasonable candidate for an assay system of the Na^+/H^+ antiporter in yeast. Here, we successfully detected Na^+/H^+ antiport activity, which depends on the expression of Nha1p, in isolated *sec4-2* secretory vesicles. Analysis using this system revealed the electrogenicity and the ion selectivity of Nha1p and suggested that Nha1p represents a novel type of Na^+/H^+ exchanger. Nha1p catalyzed low affinity Li^+/H^+, K^+/H^+ and Rb^+/H^+ antiport, as well as Na^+/H^+ antiport. The estimated *K*m values of Nha1p for these cations were 12.7 mM, 12.4 mM and 42.4 mM, for Na^+, K^+, and Rb^+, respectively. Li^+/H^+ antiport activity was too low to estimate the *K*m, and Cs^+/H^+ antiport was not detected. These results indicate that Nha1p activity is dependent on the substrate ion size and suggest that Na^+ and K^+ are major substrates of Nha1p under physiological conditions. Among the members of the plasma membrane type Na^+/H^+ exchanger family, from



		Km (mM)	Vmax (ΔF/sec)
Nha1p	Na^+	12.7	1.17
	K^+	12.4	1.08
	Rb^+	42.4	0.89
Nha1p(D266N)	Na^+	-	-
	K^+	13.9	0.56
	Rb^+	-	-

Fig. 8. Kinetic parameters of ion transport of Nha1p on secretory vesicles. Nha1p dependent portions of Na^+-, K^+- and Rb^+/H^+ antiport activity and Nha1p(D266N) dependent K^+/H^+ antiport activity on the secretory vesicles shown in Fig. 7 were analyzed by Hanes–Woolf plot. Nha1p for Na^+ (open circles), K^+ (filled circles) and Rb^+ (filled triangles); Nha1p(D266N) for K^+ (open squares).

bacteria to higher eukaryotes, only *S. cerevisiae* Nha1p, *C. albicans* Cnh1p and *P. sorbitophila* Nha1p mediate K^+/H^+ antiport, implying a specialized function for these yeast antiporters, that is, regulation of K^+ concentration and pH [54,55].

In this study, we used *sec* vesicles which have been extensively employed to analyze Pma1p activity. Unexpectedly, there are a few differences between our results and those of the previous reports [40]. First, we detected an ATP-independent decrease of ACMA fluorescence in the secretory vesicles, which has not been previously reported. The ATP-independent quenching of fluorescence certainly reflects the preformed pH gradient because addition of NH_4Cl into the solution recovered it to the original level (Fig. 4). Addition of both CCCP and valinomycin (Fig. 6A) or nigericin alone (data not shown) also reversed the quenching, indicating that the quenching and the reversal in fluorescence are caused by proton movement across the membrane. We did not detect acidification of the secretory vesicles by ATP-driven Pma1p, which was reported previously [40]. This difference would be due to following reasons. First, we used low glucose medium during vesicle accumulation to induce the expression of a marker enzyme of the secretory vesicle, invertase. The activity of Pma1p in secretory vesicles is tightly regulated by glucose and about 11-fold lower when cells were grown under low glucose conditions [40]. Second, we performed all measurements at pH 7.5 to obtain maximum Nha1p activity. Pma1p activity at pH 7.5 is about 20% of that at the optimal pH, pH 6.25. The activity of Pma1p in low glucose condition would be insufficient to acidify the secretory vesicles.

Our results here are the first to demonstrate that Nha1p is an electrogenic transporter. Eukaryotic transporters such as mammalian NHEs and Sod2 of the fission yeast *S. pombe* have been suggested to mediate electroneutral transport [1,56]. In contrast, the H^+ and Na^+ stoichiometry of a bacterial transporter, the NhaA of *E. coli*, is 2 H^+ per 1 Na^+ [57] and NhaB antiports 3 H^+ per 2 Na^+ [58]. Although, we examined the $\Delta\Psi$ altered by Nha1p using oxonol V, a fluorescent probe of the membrane potential, no significant change in fluorescence was detected because of the low fluorescence intensity of the dye (<1/50 compared to ACMA). Turbidity of the vesicles seriously interfered with the detection of oxonol V fluorescence (ex. 610 nm, em. 639 nm) when the vesicles and the dye concentration were increased. In addition, low Pma1p activity was insufficient to generate the $\Delta\Psi$ to be detected with oxonol V. From these reasons, we examined the electrogenicity of Nha1p using valinomycin and K^+. The stoichiometry and molecular mechanism of Nha1p may be more similar to bacterial antiporters rather than other eukaryotic antiporters. One possible explanation for the electrogenic antiporter activity of Nha1p is related to the working pH range of Nha1p under physiological conditions, which is presumed to be a relatively large pH range because it is a component of a unicellular organism. The cytoplasmic pH of the yeast cell is reported to range from 6.5 to 7.5. Under conditions where the extracellular pH increases, the ΔpH gradually decreases and the contribution of the $\Delta\Psi$ to the total *pmf* increases. Even at an extracellular of pH 7.0, it is estimated that about 90% of the *pmf* arises from the $\Delta\Psi$ [59,60]. If Nha1p mediates an electroneutral process, this antiporter will not sufficiently function even at the neutral extracellular conditions. On the contrary, by mediating an electrogenic transport, i.e., if Nha1p utilizes both the $\Delta\Psi$ and the ΔpH as the driving force, Nha1p can successfully functions near the neutral and alkaline pH of the external environment.

We detected Na^+/H^+ antiporter activity in control vesicles which did not contain Nha1p. This Nha1p-independent activity is lower than that of Nha1p but not negligible (Fig. 5). The activity was observed even in the mutants lacking Nhx1p, an endosomal Na^+/H^+ antiporter and Kha1p, a K^+/H^+ antiporter on the plasma membrane [61] (data not shown), demonstrating that the antiporter activity is not related to these transporters. As the vacuoles were well separated from the secretory vesicles in our preparations, the antiport activity is not due to the previously reported antiport system on the vacuolar membrane [62]. This suggests the presence of another Na^+/H^+ antiport system in the secretory vesicles.

It has been suggested that the important role of acidic residues like Asp and Glu in transmembrane domains (TM) is a general feature of Na^+/H^+ antiporter activity [63]. For

example, in the NhaA of *E. coli*, two neighboring aspartic acid residues, Asp-163 and Asp-164, in TM5 were identified as essential residues for antiport activity. The importance of another aspartic acid, Asp-133, in TM4 has also been demonstrated. In human NHE1, two important residues, Glu-262 and Asp-267, in TM7 have been reported [64]. The negative charge of these acidic residues is thought to be involved in binding of the positive charge of substrate ions. Two aspartic acid residues in Nha1p, Asp-266 and Asp-267 in TM8, are well conserved among fungal Nha1p [50,52], and mutations in these residues result in a large decrease of Na^+ resistance, but not in K^+ tolerance (Yasui et al., in preparation). This suggests that these two aspartic acids are essential for Na^+ transport, but do not participate in K^+ transport. We examined the activity of Nha1p(D266N) to clarify this at the molecular level and to confirm the validity of this assay system. Nha1p(D266N) showed a complete loss of Na^+/H^+ antiport activity, indicating that this residue plays an important role in the antiport process. Nha1p(D266N) retained K^+/H^+ activity without affecting the affinity for K^+, although the *V*max decreased to about 50% of wild type Nha1p. These results indicate the functional importance of Asp-266 in the Na^+/H^+ antiport, as well as its role in the ion selectivity. Asp-266 contributes to the K^+/H^+ as well as Na^+/H^+ antiport, and Na^+ and K^+ are probably recognized by partially overlapping sites.

This study is the first to directly measure the *S. cerevisiae* Nha1p activity in biological membrane vesicles. We expect that this assay system will be useful for further detailed analysis to reveal the structure–function relationships in the antiport activity of Nha1p. Furthermore, this system will be applicable to studies revealing the physiological function of Nha1p such as the functional analysis of the C-terminal putative regulatory domain, and investigations of the interaction between Nha1p and Cos3p.

Acknowledgements

This study was supported by a Grant-in-Aid of Scientific Research by the Ministry of Science, Education, Sports, Culture and Technology of Japan. The authors thank Prof. Yoshinori Ohsumi and Akihiko Nakano for providing us yeast *sec* mutant strains.

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