Four isoforms of the Na+/H+ exchanger (NHE6–NHE9) are distributed to intracellular compartments in human cells. They are localized to Golgi and post-Golgi endocytic compartments as follows: mid- to trans-Golgi, NHE8; trans-Golgi network, NHE7; early recycling endosomes, NHE6; and late recycling endosomes, NHE9. No significant localization of these NHEs was observed in lysosomes. The distribution of these NHEs is not discrete in the cells, and there is partial overlap with other isoforms, suggesting that the intracellular localization of the NHEs is established by the balance of transport in and out of the post-Golgi compartments as the dynamic membrane trafficking. The overexpression of NHE isoforms increased the luminal pH of the compartments in which the protein resided from the mildly acidic pH to the cytosolic pH, suggesting that their in vivo function is to regulate the pH and monovalent cation concentration in these organelles. We propose that the specific NHE isoforms contribute to the maintenance of the unique acidic pH values of the Golgi and post-Golgi compartments in the cell.

The luminal ionic composition of intracellular compartments differs from the cytoplasm, and each compartment is characterized by a unique, organelle-specific ion concentration. This specific ionic composition is thought to be an important determinant for organelle function and is maintained by the concerted action of ion transporters on the membrane (1, 2). Organelles of the secretory and endocytic pathways exhibit differential weak acidity in their lumen with a gradient of pH values decreasing toward the trafficking destination, from ER (pH ~7.1) to Golgi (pH ~6.2–7.0), trans-Golgi network (TGN) (pH ~6.0), and secretory granules (pH ~5.0) and from early and late endosomes (pH ~6.5) to lysosomes (pH ~4.5) (1, 3, 4). This progressive acidification is essential for compartmentalizing cellular events, such as post-translational modifications, sorting of newly synthesized proteins into the secretory pathway, and the degradation or recycling of internalized ligand-receptor complexes and fluid-phase solutes in the endocytic pathway (3, 5). Even pH differences of less than 0.5 between organelles can be essential for the compartmentalizing cellular events (6).

The differential ionic milieu of the organelles is maintained by a suite of ion carriers on the membrane, including pumps, channels, and transporters. Luminal acidity is primarily generated by the vacuolar-type H+-translocating ATPase (V-ATPase) (4, 5). Because the V-ATPase is electrogenic, the pumping activity could be affected by membrane potential and availability of permeant counterions such as chloride and potassium. In vitro studies using isolated endosomes and Golgi suggested a critical role for Cl− in shunting the inside-positive membrane potential generated by H+ pumping (7–9). However, although Cl− and K+ serve as counterions for H+ pumping, their conductances on the Golgi complex and the TGN were large compared with the rate of H+ pumping, arguing against modulation of Cl− and K+ conductances as the mechanism for pH regulation and suggesting that the electrical potential across the membrane is not a determinant of steady-state pH in the Golgi and TGN (10–14). Instead, the steady-state pH is thought to be controlled by the balance between the rate of H+ pumping by V-ATPase and the magnitude of H+ leak from the organelle lumen. The H+ leak mechanism is postulated based on the rapid dissipation of the proton gradient across the membrane after inhibiting the V-ATPase with bafilomycin A1, however the molecular mechanism is still unknown (10, 11, 13–15). The decreasing pH values of organelles along the secretory pathway is established by gradually increasing the density of active H+-pumps from the ER to the Golgi while concomitantly decreasing the H+ permeability from ER to Golgi to secretory granules (14). Thus, the H+ leak acts as a key determinant of organellar pH. These data emphasize the importance of identifying the molecular components of this system, which could conceivably involve proton channels, proton-coupled cotransporters, or proton-exchanging transporters.

Na+/H+ exchanger (NHE) proteins are integral membrane proteins that mediate electroneutral exchange of H+ for Na+ and K+ across the membrane, down their concentration gradients (16). NHEs are composed of an N-terminal 10–12 membrane-spanning domain mediating the ion exchange and a large hydrophilic C-terminal domain that serves a regulatory function in ion transport (17, 18). To date, eight NHE isoforms have been identified in mammals (19). NHE1–NHE5 are localized to the plasma membrane in various cell types (20–28), and NHE6 and NHE7 reside on organellar membranes (29, 30). NHE8 is the most recently identified isoform, but its cellular localization remains unknown (31). Plasma membrane NHEs are known to participate in the maintenance of intracellular pH and cell volume and are also crucial for absorption of Na+ across epithelia (19, 32). Recently, it was reported that NHE7...
on the trans-Golgi network and the plant vacuolar homologue AtNHX1 catalyze low affinity transport of Na⁺ and K⁺ in exchange for H⁺ (29, 33). However, the physiological function of the organellar membrane NHEx is still unknown.

In this study, we characterized organellar alkali-cation/proton exchanger proteins and found that four NHE isoforms are distributed to Golgi and post-Golgi compartments. From these observations we suggest a mechanism regulating the luminal pH and cation composition of the intracellular compartments by NHExs.

2 In the course of preparing this manuscript, the human NHE8 cDNA containing the entire ORF was identified at the Kazusa DNA Research Institute (clone hh04825s1). The nucleotide sequence is available at GenBank (accession number AL050307) provided by Chil-
Purification of NHE8 Expressed in Yeast—Yeast TTY3 cells expressing NHE8–6His or NHE8/E220Q,D225N–6His were cultured in synthetic medium containing 2% glucose, 0.5% casamino acids, and appropriate supplements. The cells (8000 A600 units) were lysed in 70 ml of lysis buffer (50 mM potassium phosphate (pH 7.5), 500 mM NaCl, 5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin) by two passages through a French press, followed by centrifugation at 500,000 g for 5 min. The supernatant (S30) was spun at 100,000 × g for 1 h, and the resulting pellet fraction (P100) was resuspended in lysis buffer (5 mg of protein/ml). The solution was mixed with 4 volumes of lysis buffer containing 0.5% n-dodecyl-β-D-maltoside (DDM), 20% glycerol, and 10 mM imidazole and was incubated for 30 min at 4 °C with gentle shaking. Insoluble material was centrifuged at 30,000 × g for 30 min. The supernatant (S30) was applied to a 1-ml column of Hi-Trap Chelating HP charged with Ni2+. The column was washed with lysis buffer containing 0.15% DDM, 20% glycerol, and 10 mM imidazole, followed by elution buffer (50 mM potassium phosphate (pH 7.4), 500 mM NaCl, 0.675% DDM) containing 90 mM imidazole and was incubated for 30 min at 4 °C and then dialysed against 2.5-mL spin column filled with Sephadex G-50 (Amersham Biosciences) and a Beckman LS6500 liquid scintillation counter (Beckman Coulter, Inc., Fullerton, CA).

Fluorescence Microscopy—Cells were fixed with 2% formaldehyde and then permeabilized in phosphate-buffered saline containing 1% bovine serum albumin, 0.04% saponin, and 2% normal goat serum (34). The cells were incubated with primary antibodies against NHE proteins, the epitope tags, or organellar marker proteins and then stained with fluorescently labeled secondary antibodies. After washing with phosphate-buffered saline, samples were fixed with 10% glycerol and then observed under an Olympus IX51 microscope equipped with differential interference contrast optics (Olympus, Tokyo, Japan). COS7 cells were transfected with pCMV-NHE6-HA or pCDNA3.1-NHE9-mycHis and incubated for 2 days. Cells were serum-starved for 30 min at 37 °C to deplete them of transferrin, chilled on ice, and allowed to bind Alexa 594-Tfn (60 μg/ml in phosphate-buffered saline) for 45 min on ice. Cells were washed briefly in phosphate-buffered saline and placed in Dulbecco’s modified Eagle’s medium at 37 °C. After incubation for the indicated times, cells were fixed, permeabilized, and stained with anti-HA or anti-Myc antibodies.

Measurement of Organelle pH in Vivo—Between 2 and 4 days after transfection, cells were imaged at 26 °C with an inverted fluorescence microscope (IX70, Olympus, Tokyo, Japan) equipped with a cooled charge-coupled device camera (ORCA-ER, Hamamatsu Photonics, Hamamatsu, Japan). Cells were incubated initially for 5 min with IncuCyte LiveCell medium containing 0.5% DMSO to allow for the equilibration of NHE activity. The IncuCyte LiveCell medium was then exchanged with buffer containing 440-nm (ECFP) and 505-nm (EYFP) excitation wavelengths. The medium was then switched to a series of high KCl calibration solutions containing 125 mM KCl, 20 mM NaCl, 0.5 mM CaCl2, 0.5 mM MgCl2, and 25 mM of one of the following buffers: HEPES (5.0 to 7.0, Sigma), MES (pH 6.5 to 5.5, Roche), or acetate (5.0 to 4.0). The microscopy was performed using a Night Vision 10x/0.30 objective. Images were acquired beginning 3 min after pH adjustment with a 7.5-min acquisition period. Organelle pH images were captured at 10-min intervals, and pixel intensities were spatially averaged after background subtraction.

RESULTS

Novel Organellar Na+/H+ Exchangers, NHE8 and NHE9—Previously, seven isoforms of the NHE protein have been identified using molecular biological approaches (19). Recent progress on the human genome sequencing project provided a powerful tool to identify novel molecules showing characteristic primary structures, and this enabled us to identify all genes encoding organellar NHE members. We searched the human genome sequence, and found that six regions (chromosomes 3q24, 10q11.21, 12q23.1, 22q11.21, and Xq21.2) contain none of the exon-intron boundaries predicted from the genomic structures of the NHE family and carry both nonsense and frameshift mutations. We searched the EST databases for cDNA clones corresponding to the regions at chromosomes 20q13.3 and 3q24, and found KIAA0939 (named NHE8) and BI600394 (named NHE9) for chromosomes 3q24, 10q11.21, 12q23.1, 20q13.3, 22q11.21, and Xq21.2 contain sequences showing similarity to previously known NHEs, in addition to the seven loci for previously known NHEs and one NHE3 pseudogene (45). Sequence analysis revealed that four of the six chromosome regions (chromosomes 10q11.21, 12q23.1, 22q11.21, and Xq21.2) contain none of the exon-intron boundaries predicted from the genomic structures of the NHE family and carry both nonsense and frameshift mutations. We did not find the corresponding cDNAs in the mammalian EST data bases. Thus, these regions are thought to be pseudogenes. We searched the EST databases for cDNA clones corresponding to the regions at chromosomes 20q13.3 and 3q24, and found KIAA0939 (named NHE8) and BI600394 (named NHE9) for chromosomes 3q24, 10q11.21, 12q23.1, 20q13.3, 22q11.21, and Xq21.2 contain sequences showing similarity to previously known NHEs, in addition to the seven loci for previously known NHEs and one NHE3 pseudogene (45). Sequence analysis revealed that four of the six chromosome regions (chromosomes 10q11.21, 12q23.1, 22q11.21, and Xq21.2) contain none of the exon-intron boundaries predicted from the genomic structures of the NHE family and carry both nonsense and frameshift mutations. We did not find the corresponding cDNAs in the mammalian EST data bases. Thus, these regions are thought to be pseudogenes.
FIG. 1. A, alignment of amino acid sequence of NHE isoforms. The alignment was generated using the ClustalV algorithm (73). Amino acid residues conserved over three or more isoforms, and transmembrane regions predicted by TMHMM2.0 (43) are inverted and shaded, respectively. The amino acid sequences used are as follows: NHE1 (human, P19634); NHE6 (human, Q92581); NHE7 (human, AAK54508); NHE8 (mouse, AB089793); and NHE9 (human, AB089794).

B, hydropathy plots of amino acid sequences of mouse NHE8 (upper panel) and human NHE9 (lower panel). Average hydropathy indexes were calculated by the Kyte-Doolittle method using a window of 9. Positive and negative values represent hydrophilicity and hydrophobicity, respectively. Twelve predicted membrane-spanning domains are indicated by the bars below each plot.

C, phylogenetic tree of mammalian NHE isoforms. The phylogenetic relationships among the NHE isoforms were calculated using the ClustalV algorithm. Amino acid sequence used are as follows: NHE2 (human, AF073299); NHE3 (human, U28043); NHE4 (rat, AAA41703); NHE5 (human, XM_007868).
this clone contained a nonsense (C→A, 26 nt from initiation ATG) and a frameshift (deletion of C at 272 nt from initiation ATG) mutation in the possible coding region when compared with the human genomic sequence. We cloned NHE9 cDNAs from several human cell lines and determined the nucleotide sequence.

Amino acid sequence analysis of NHE8 and NHE9 revealed that both proteins are predicted to have 12 membrane-spanning domains and a large C-terminal domain, characteristic of eukaryotic NHEs (Fig. 1, A and B). The overall similarity of NHE8 to other isoforms is relatively low (23–27% identities to other NHE isoforms), whereas the membrane-spanning regions are more conserved (~50% identity). NHE9 showed high similarity to NHE6 (55% identity) and NHE7 (57% identity) throughout the entire length. We note that the sequences in four regions, the N terminus (aa 1–29 in NHE9), the second loop (aa 70–127), and two C-terminal hydrophilic domains (aa 495–517 and aa 570–645), are significantly divergent in NHE6, NHE7, and NHE9 isoforms. The charged residues in the membrane-spanning domains (Aasp-184, Aasp-196, and Aasp-225; Glu-220, Glu-293, Glu-309, and Glu-354; and Arg-421 in NHE8) are conserved in NHE8 and NHE9 (Fig. 1A).

From these examinations, we concluded that the human NHE family is composed of nine members. Phylogenetic analysis of the amino acid sequences revealed that the nine NHE isoforms are classified into two groups of NHE1–NHE5 and NHE6–NHE9 (Fig. 1C). NHE8 and NHE9 are grouped with NHE6 and NHE7, which are thought to reside on recycling endosomes and the trans-Golgi network (29, 30). These results suggest that NHEs can be classified into two groups, plasma membrane NHEs (NHE1–NHE5) and organelle-specific NHEs (NHE6–NHE9) that include the novel NHE members NHE8 and NHE9.

Tissue and Cellular Distribution of NHE6–NHE9 Isoforms—We examined the distribution of NHE8 and NHE9 transcripts in human tissues by Northern blotting analysis. Previous studies have revealed that plasma membrane NHEs show unique and isoform-specific tissue expression, but NHE6 and NHE7 are ubiquitous (21, 26, 28, 29, 46). Poly(A)⁺ RNA prepared from various human tissues was hybridized with ³²P-labeled DNA probes corresponding to the open reading frames of human NHE8 and NHE9 and then autoradiographed. NHE8 (6.4 and 4.6 kb) and NHE9 (3.7 kb) transcripts were detected in all human tissues tested (Fig. 2A), suggesting that these isoforms are ubiquitously expressed. The highest amounts of NHE8 transcript were found in skeletal muscle and kidney. The NHE9 transcript was detected at similar levels in all the human tissues. We further examined the expression of NHE8 and NHE9 in several human cultured cell lines, HEK293 (embryonic kidney), HeLa (cervix), HeLa (cervix), and Caco-2 (colon), by RT-PCR. The broad and ubiquitous expression of NHE8 and NHE9 shown by Northern blotting (Fig. 2A) was confirmed at the cellular level. The transcripts of the NHE6 and NHE7 were also detected in all the cell lines by the RT-PCR analysis, suggesting that NHE6–NHE9 isoforms each play a role in basic cellular functioning.

Cation/H⁺ Exchange Activity of NHE8—We examined the alkali-cation/H⁺ exchange activity of the NHE8 gene product. NHE8 protein tagged with His₆ was expressed in yeast cells, purified using a nickel-nitrilotriacetic acid column, and subjected to ion-transport assays using artificial liposomes. The NHE8 expressed in yeast cells appeared as a 55-kDa protein on SDS-PAGE (Fig. 3A), which is slightly smaller (~10 kDa) than the calculated mass, as has been noted for other eukaryotic Na⁺/H⁺ exchanger proteins (47–50). The protein was localized to intracellular compartments, primarily the ER (Fig. 3B).

Microsomal membranes were isolated from the yeast cells expressing NHE8–6xHis, and the proteins were solubilized using n-dodecyl-β-D-maltoside. The protein (Fig. 3C) was reconstituted in soybean phospholipid vesicles containing the pH indicator pyranine in the presence of (NH₄)₂SO₄. Dilution of the proteoliposome solution in reconstitution buffer without (NH₄)₂SO₄ resulted in an immediate fluorescence diminution of vesicle-trapped pyranine, reflective of the internal acidification of the vesicles (Fig. 4A, time 30 s). When proteoliposomes were diluted under equilibrium conditions (equal concentrations of ammonium inside and outside the vesicles), the lumen of the liposome was not acidified (data not shown). The internal pH, estimated using standard pH buffers, was 5.73. When vesicles were diluted in the presence of (NH₄)₂SO₄, the internal pH as indicated from pyranine fluorescence was 7.5. The variation of pyranine fluorescence (pKᵥ, 7.2) was approximately linear between pH 5.7 and 7.5 (data not shown) and therefore is directly related to intravesicular pH changes. The addition of NaCl (300 mM) increased the pyranine fluorescence, which indicates the alkalization of the liposome lumen to pH 6.4 (Fig. 4A). No significant recovery of pyranine fluorescence was observed upon the addition of organic cation, choline chloride, or addition of NaCl to control liposomes that did not contain the NHE8 protein (Fig. 4A). The addition of (NH₄)₂SO₄ fully collapsed the pH gradient in both liposomes and proteoliposomes. These observations are consistent with NHE8-mediated Na⁺/H⁺ exchange activity.
dependent proton efflux from the liposomes. The ion exchange reaction showed saturable kinetics with increasing cation concentrations (Fig. 4B). The affinity of NHE8 for Na⁺ and K⁺ was relatively low, with apparent \( K_m \) values of 130 and 75 mM, respectively. The affinities for Li⁺, Cs⁺, and Rb⁺ were much lower, and the \( K_m \) value was difficult to determine because it is relatively high (>200 mM) (data not shown). The maximum initial rate of vesicle alkalinization (\( V_{max} \)) obtained from fitting the data was 3.6 and 3.8 \( \text{nmol min}^{-1} \). We examined Na⁺ uptake into the proteoliposomes. If the increase in pH in the liposomes is because of proton efflux coupled with Na⁺ influx through Na⁺/H⁺ antiport, then the proteoliposomes should sequester Na⁺. As expected, \( ^{22}\text{Na}^+ \) uptake was observed with these proteoliposomes (Fig. 4B), whereas \( ^{22}\text{Na}^+ \) uptake was negligible in liposomes without NHE8 and in proteoliposomes carrying NHE8 mutated at the glutamate 220 and asparagine 225 residues (Fig. 4B), which are important for the exchange activity in NHE1 (51, 52). These results indicate that NHE8 is capable of performing alkali-cation/H⁺ exchange.

**Intracellular Distribution of NHE6–NHE9 Isoforms**—The similarity of primary structures among NHE6 to NHE9 isoforms suggested that these isoforms act on the intracellular compartments. We examined the subcellular localization of NHE8 and NHE9 in COS7 cells, transfected with cDNAs encoding either NHE8, NHE9, or epitope tags fused to the C terminus of the NHE proteins. Fluorescence microscopy revealed that NHE8 proteins were found primarily in curvy tubular structures at the cell periphery and the peri-nuclear region (Fig. 5A). A portion of the signal was detected in punctate structures dispersed throughout the cell. Double staining of NHE8 with organelle-marker proteins suggested that the localization of NHE8 is quite similar to that of GM130, a marker for cis-Golgi compartments (Fig. 5A). However, the signals from NHE8 and GM130 are not overlapping but rather closely adjacent to one another at higher magnifications (Fig. 5A). The same result was obtained by staining NHE8-FLAG transiently expressed in COS7 cells (data not shown). We did not find colocalization with protein markers for other compartments, including ER (calnexin), early endosomes (EEA1), late endosomes and lysosomes (Lamp-2), TGN and late endosomes (cation-independent mannose 6-phosphate receptor) (data not shown). From these observations, we concluded that NHE8 is mainly localized to the mid- to trans-Golgi compartments.

We next investigated the intracellular localization of NHE9 by immunofluorescence microscopy. This protein is also localized to intracellular compartments. The signals were found as punctate structures, highly concentrated around the nucleus and dispersed throughout the cell periphery (Fig. 5B). NHE9 showed a partial colocalization with NHE6 in punctate structures between the cell periphery and the peri-nuclear region (Fig. 5C). Since the distribution of NHE9-positive compartments is similar to that of endosomes, we performed double staining with EEA1, Lamp-2, and cation-independent mannose 6-phosphate receptors. No significant overlap of NHE9 with Lamp-2 or cation-independent mannose 6-phosphate receptor was observed, and only a small portion of NHE9 was colocalized with EEA1 (Fig. 5D). Signals from NHE9 were found in compartments labeled by rhodamine-conjugated transferrin (Fig. 5E), suggesting the localization of NHE9 to the recycling endosomes rather than EEA1-residing early endosomes. To differentiate among endosomal compartments in the recycling pathway, rhodamine-labeled transferrin was internalized and chased in the presence of nonlabeled transferrin for up to 30 min. After fixation, NHE6 and NHE9 were detected by immunostaining. Early after internalization (2–10 min), the major population of transferrin-labeled structures was found at the cell periphery and labeled with NHE6 (Fig. 6), suggesting that NHE6 is localized to an early stage of recycling pathway. Consistently, most of the NHE6-positive compartments contained EEA1, a marker for early endosomes (data not shown). The colocalization with NHE6 was transient and decreased after longer chase periods. The signals from labeled transferrin were translocated to the juxtanuclear region, and the number of transferrin-labeled endosomes containing NHE9 increased slowly from 10 to 15 to 30 min of chase (Fig. 6). These observations, together with the partial colocalization of NHE6 and NHE9 (see Fig. 5C), suggest that the proteins reside on compartments of the...
early and late stages of recycling pathway, respectively, with partial overlap, which are possibly early endosome and recycling endosome, respectively.

Overexpression of Na\(^+\)/H\(^+\) Exchangers Causes Alkalinization of the Compartment—The isoform-specific distribution of NHE6–NHE9 prompted us to investigate their function in regulation of organellar pH and cation concentration. Some GFP mutants are reported to exhibit pH-sensitive fluorescence (53, 54). We examined the pH sensitivity of EGFP (F64L and S65T), EYFP (S65G, V68L, S72A, and T203Y), and ECFP (F64L, S65T, V66W, N146I, M153T, and V163A) expressed in E. coli cells. EGFP and EYFP showed an acidification-dependent decrease in the fluorescence emission at 510 and 528 nm, respectively (Fig. 7A). The apparent pK\(_a\) values of EGFP and EYFP were 5.9 and 5.4 with Hill coefficients (n) of 2.1 and 2.3, respectively. The pH-dependent change in fluorescence of ECFP was smaller than that of EGFP or EYFP (pK\(_a\) 6.2). The fluorescence change was reversible in the pH range 4–8 for all three proteins (data not shown).

Because these pH-dependent changes span the pH range of most subcellular compartments, we reasoned that these GFP mutants could be used as physiological pH indicators for Golgi and endosomes. EYFP was fused with integral membrane protein markers for the mid- to trans-Golgi, GaIT (55), or the recycling endosome VAMP3 (56). The N-terminal 82-aa residues of GaIT fused to GFP has been shown to localize to the mid- and trans-Golgi with the GFP moiety residing in the Golgi lumen (54). COS7 cells expressing GaIT-GFP and either NHE8-
FLAG or NHE8-ECFP were observed under the fluorescence microscope. NHE8-FLAG and NHE8-ECFP were colocalized with GalT-GFP and GalT-EYFP, respectively (Fig. 8). This confirms the mid- and trans-Golgi localization of NHE8 protein. Steady-state Golgi pH in COS7 cells was 6.50 ± 0.05, which is consistent with values measured in HeLa and Vero cells (Fig. 9A) (15, 54). Overexpression of NHE8 dissipated the acidic pH of the Golgi complex and increased the pH by about 0.78 pH unit to pH 7.28 ± 0.15 (Fig. 9A), indicating that NHE8 mediates proton efflux from the Golgi lumen.

We examined the pH in recycling endosomes using VAMP3-GFP. VAMP3 is a type II integral membrane protein on the recycling endosome (56) and the chimera of VAMP3-GFP localizes to recycling endosomes with GFP residing in the endosome lumen (57). The VAMP3-EYPF was localized to punctate structures in the peri-nuclear region and cell periphery of COS7 cells (Fig. 8). Most interestingly, NHE9-ECFP was partially colocalized with VAMP3-EYFP in the peri-nuclear region (Fig. 8), but NHE6-ECFP was not significantly colocalized with VAMP3-EYFP (data not shown). The resting pH in recycling endosomes of COS7 cells was 6.73 ± 0.03, slightly higher than that reported previously (pH 6.2; Refs. 58 and 59). When NHE9-ECFP was overexpressed in cells, the pH in the recycling endosomes increased 0.41 pH unit to 7.14 ± 0.07 (Fig. 9B). Most interestingly, overexpression of NHE6 did not cause the alkalinization of VAMP3 compartments (pH 6.73 ± 0.01, Fig. 9B), consistent with the differential localization of NHE9 and NHE6 in cells.

**DISCUSSION**

In this study, we showed that four types of Na+/H+ exchanger proteins, including the two novel isoforms NHE8 and NHE9, were distributed to intracellular compartments, Golgi complex, and post-Golgi endosomal compartments in human cells. The distribution of these NHEs was isoform-specific, but there was partial overlap among the distributions. This suggests that different NHEs function in particular organelles and regulate the specific compositions of monovalent alkali cations and protons in the Golgi and post-Golgi compartments. Four Organellar NHE Isoforms in the Human NHE Family—By searching the human genome sequence, we found that the NHE family is composed of nine members that are classified into two groups, NHE1–NHE5 and NHE6–NHE9. The sequence analysis of the entire human genome and EST data base enabled us to identify all members of this protein family; indeed, all NHE isoforms reported previously were found in the data base. Although the overall similarity of amino acid sequences among the NHE isoforms is relatively low (17–67% identity), all the NHE isoforms possess a characteristic secondary structure composed of multiple transmembrane domains at the N terminus and a large hydrophilic domain at the C terminus. Charged amino acid residues in the predicted trans-
membrane segments are conserved in all members of this family. Although little is known about the specific amino acids involved in the exchanging transport and about the mechanism of operation in mammalian NHEs, mutations in the conserved residues, glutamine 262, glutamine 391, and asparagine-267 (in NHE1), greatly reduced the exchange activity (Fig. 4) (51, 52). These data suggest the importance of the charged residues in ion transport across the membrane and essentially the same molecular mechanism of ion transport mediated by the NHE isoforms. We showed that NHE8 mediates the exchange of H⁺/H⁺ for Na⁺/H⁺ using the recombinant protein reconstituted in liposomes. Most interestingly, the luminal acidity of the liposome was not fully collapsed by the exchange reaction but appeared to reach an equilibrium at the mildly acidic pH of 6.4 (Fig. 4). These results indicate that NHE8 acts as an ion exchanger on the membrane and also suggest that this protein modulates the acidic pH of the lumen. Further investigations of the pH sensitivity and ion selectivity are now in progress using the in vitro assay and will provide insight into the molecular mechanism of transport and the in vivo function of this protein.

In contrast to the NHE1–NHE5 isoforms, which are known to act on the plasma membrane and which exhibit distinctive patterns of tissue and cell expression in order to fulfill their tissue- and cell-specific functions, NHE6–NHE9 isoforms are ubiquitously and concurrently expressed in single cells. In addition, the yeast endosomal NHE homologue, Nhx1p (60) falls into the NHE6–NHE9 group by phylogenetic analysis (data not available).

![Fig. 6. Distribution of NHE6 and NHE9 in recycling endosomal compartments.](image) COS7 cells expressing NHE6 and NHE9 were serum-starved for 45 min, incubated with rhodamine-conjugated transferrin on ice, and chased at 37 °C in the presence of nonlabeled transferrin for up to 30 min. The cells were fixed, immunostained with NHE6-HA (left) and NHE9-myc (right), and observed under a fluorescence microscope. Bars indicate 20 μm.
Organellar Na\(^{+}/H^{+}\) Exchanger Proteins in Human

Isoform-specific Localization of NHEs—Microscopic observations revealed that NHE6–NHE9 isoforms were localized to distinct intracellular compartments. NHE6 was found in early recycling endosomes, NHE7 in the trans-Golgi network, NHE8 in the mid- and trans-Golgi, and NHE9 localized to late recycling endosomes. Most interestingly, the distributions of these isoforms are not discrete in these compartments but partially overlap one another. Post-Golgi compartments are connected by dynamic membrane flow of exocytic and endocytic membrane trafficking (61, 62). The overlapping distribution of the NHE isoforms suggests that they are circulating in the post-Golgi membrane traffic, and the distribution of the isoforms would be maintained by the balance of export from and retention in each compartment. Most interestingly, the amino acid sequence of four regions, the N terminus (aa 1–29 in NHE9), second loop (aa 70–127), and two segments in the C-terminal hydrophilic domain (aa 495–517 and 570–645), are diverged in the NHE6, NHE7, and NHE9 isoforms. Recent topological analyses of plant vacuolar and yeast endosomal Na\(^{+}/H^{+}\) exchangers suggested that the N termini and second loop are exposed to the cytoplasm and that the C-terminal domain resides in the organelle lumen (63, 64). This topological feature implies that the N terminus and the second loop are involved in the localization of the NHEs through recognition by cytoplasmic machinery for vesicular membrane trafficking and that the C-terminal region may act as a regulatory domain for ion transport by sensing the intra-compartment ionic concentration. Further examination will uncover how these isoforms are distinguished and localized to specific compartments.

Role of NHE6–NHE9 Isoforms in Maintaining Organelle Ion Homeostasis and Their Unique Acidity—Previous studies revealed that intracellular compartments are mildly acidified to specific pH values and exhibit a gradient of decreasing pH from ER (pH ~7.1) to Golgi (pH ~6.2–7.0), trans-Golgi network (pH ~6.0), and secretory granules (pH ~5.0) and from early and late endosomes (pH ~6.5) to lysosomes (pH ~4.5) (1, 3, 4). The low pH environment is crucial for a number of well defined processes, such as the activation of endoproteases (65), the maturation and modification of secretory proteins (66), and the dissociation of receptor-ligand complexes (67, 68). Steady-state pH values of ER, Golgi, and secretory vesicles appeared to be controlled by rates of H\(^{+}\) pumping and by the gradual decrease in organelle H\(^{+}\) permeability from ER to Golgi to secretory vesicles in the secretory pathway, whereas the membrane potential in Golgi and secretory vesicles is small and not perturbed by large changes in Cl\(^{−}\) and K\(^{+}\) conductances, indicating that membrane potential is not a determinant of steady-state pH values (10, 11, 13–15).

Based on these findings, we hypothesized the role of NHEs in controlling luminal pH through the cation/H\(^{+}\) exchange activity, and examined the organellar pH using pH-dependent GFP mutants. Overexpression of NHE8 and NHE9 caused luminal alkalinization to near cytosolic pH of the compartments in which they reside. This observation suggests that these NHEs effuse luminal H\(^{+}\) in exchange for cytosolic cations. Electro-neutral one for one ion exchange driven by the concentration gradients across the membrane equalizes the ratios of inside and outside ion concentrations, i.e., \([A^{+}]_{i}/[A^{−}]_{o} = [B^{+}]_{o}/[B^{−}]_{i}\). The Golgi luminal [K\(^{+}\)] is ~107 mM, which is only slightly lower than the cytosolic concentration (12). Because of the much higher concentration of K\(^{+}\) than H\(^{+}\), the electroneutral exchange by overexpressed NHEs would dissipate the trans-membrane \(\Delta\)pH of ~0.7 without changing both Golgi and cytosolic K\(^{+}\) concentrations and neutralize the compartment to near the cytosolic pH. This assumption is validated by incubating cells in the presence of nigericin, a K\(^{+}\)/H\(^{+}\) ionophore, which caused rapid neutralization of the Golgi pH (data not shown). The protein levels of NHE8 and NHE9 proteins in overexpressing cells were increased by 51 ± 33-fold (55 overexpressing cells, total 150 cells) and 99 ± 83-fold (67 overexpressing cells, total 138 cells), respectively, compared with the endogenous levels under the fluorescence microscope. Further examination such as knock-down experiments would be required to confirm that endogenous NHE8 and NHE9 are involved in the organel-

**FIG. 8. Localization of GFP mutants fused with GalT and VAMP3.** Cells expressing the GFP markers and NHE isoforms were observed under the fluorescence microscope. Upper panels, fixed COS7 cells expressing GalT-EGFP (left) and NHE8-FLAG (right). Lower panels, living cells expressing GalT-YFP (left) and NHE8-ECFP (right). The GFP markers and NHE isoforms were observed with the appropriate filter sets as described under “Experimental Procedures.” Bars indicate 20 μm. N, nucleus.
lar pH regulation. A detailed analysis of the relation between protein levels and luminal pH is currently in progress to provide further insight into the action of NHEs to control the organellar pH.

Recently, a plant vacuolar Na\(^+/\)H\(^+\) exchanger has been suggested to play a role in the regulation of vacuolar pH (69). Vacular pH is known to be a main determinant for flower coloration, and a shift from reddish purple buds to blue open flowers correlates with an increase in the vacuolar pH. The vacuolar Na\(^+/\)H\(^+\) exchanger proteins utilize the proton electro-chemical gradient generated by the V-ATPase and H\(^+\) translocating pyrophosphatase to couple the movement of H\(^+\) down its concentration gradient in exchange for cytosolic alkali cation (70). Mutations in the gene encoding the vacuolar Na\(^+/\)H\(^+\) exchanger protein NHX1 causes excessive acidification of vacuolar lumen and, consequently, emuppling of flowers of the Japanese morning glory. The fact that NHX1 confers salt tolerance to plants by sequestering excess Na\(^+\) in the vacuole (71) is consistent with the idea that the vacuolar Na\(^+/\)H\(^+\) exchanger transports luminal H\(^+\) out of the vacuole, exchanging it for cytosolic Na\(^+\). These observations support our idea that K\(^+\)/H\(^+\) exchange mediates the H\(^+\) leak that has been proposed to regulate the luminal pH of intracellular compartments and that specific NHE isoforms present on Golgi and post-Golgi compartments are involved in controlling the unique acidic pH of these compartments. We found no apparent localization of NHE isoforms to lysosomes, which is consistent with the previous observation that rat liver lysosomes have no detectable Na\(^+/\)H\(^+\) exchange activity (72). Lysosomes are the most acidic compartment of the cell, pH ≈ 4.5, and it may be that the H\(^+\) leak mechanism is omitted to accomplish such high acidification, or an alternative mechanism may exist in the lysosomal system.

Further analyses of the organellar NHE isoforms by examining the ion transporting activity, including the ion selectivity and pH dependence in vitro and by exchanging isoforms residing on the compartments in vivo, will uncover the role of NHEs in organellar ion homeostasis and the mechanism of organelle pH regulation in the cell.
Organellar Na\(^+\)/H\(^+\) Exchanger Proteins in Human

33. Venema, K., Quintero, F. J., Parde, J. M., and Donaire, J. P. (2002) J. Biol. Chem. 277, 2413–2418
34. Nakamura, N., Yamamoto, A., Wada, Y., and Futai, M. (2000) J. Biol. Chem. 275, 6523–6529
35. Nakamura, N., Hirata, A., Ohsumi, Y., and Wada, Y. (1997) J. Biol. Chem. 272, 11344–11349
36. Kamauchi, S., Mitsui, K., Ujike, S., Haga, M., Nakamura, N., Inoue, H., Sakajo, S., Ueda, M., Tanaka, A., and Kanazawa, H. (2002) J. Biochem. (Tokyo) 131, 821–831
37. Banuelos, M. A., Klein, R. D., Alexander-Bowman, S. J., and Rodriguez-Higares, A. (1990) J. Biol. Chem. 265, 6523–6529
38. Banuelos, M. A., Klein, R. D., Alexander-Bowman, S. J., and Rodriguez-Higares, A. (1990) J. Biol. Chem. 265, 6523–6529
39. Rose, M. D., Winston, F. D., and Hieter, P. (1990) Methods in Yeast Genetics: A Laboratory Course Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
40. Sambrook, J., Frisch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
41. Tanaka, K., Nakafuku, M., Tamanoi, F., Kaziro, Y., Matsumoto, K., and Toh-e, A. (1999) Methods in Yeast Genetics: A Laboratory Course Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
42. Buch-Pedersen, M. J., Venema, K., Serrano, R., and Palmgren, M. G. (2000) J. Biol. Chem. 275, 39167–39173
43. Sonnhammer, E. L. L., von Heijne, G., and Krogh, A. (1998) in Methods in Yeast Genetics: A Laboratory Course Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
44. Nakamura, T., Hsu, C., and Rao, R. (1998) J. Biol. Chem. 273, 21054–21060
45. Kokke, F. T., Elsawy, T., Bengtsson, U., Wasmuth, J. J., Wang, J., Jabs, E., Tse, C. M., Donowitz, M., and Brant, S. R. (1996) J. Biol. Chem. 271, 678–683
46. Numata, M., Petrecca, K., Lake, N., and Orlowski, J. (1998) J. Biol. Chem. 273, 6951–6956
47. Biemesderfer, D., Pizzonia, J., Abu-Alfa, A., Reilly, R., Igarashi, P., and Aronson, P. S. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8504–8508
48. Hoogerwerf, W. A., Tsao, S., Devuyst, O., Levine, S. A., Yun, C. H., Yip, W. H., Gabel, C. A., and Maxfield, F. R. (1990) J. Biol. Chem. 265, 8504–8508
49. Counillon, L., Pouyssegur, J., and Reithmeier, R. A. (1994) Biochemistry 33, 10483–10490
50. Fafournoux, P., Noel, J., and Pouyssegur, J. (1994) J. Biol. Chem. 269, 2589–2596
51. Murtazina, R., Booth, B. J., Bullis, B. L., Singh, D. N., and Pfeiffer, L. (2001) Eur. J. Biochem. 268, 4764–4775
52. Patterson, G. H., Knobel, S. M., Sharif, W. D., Kain, S. R., and Pistor, D. W. (1997) Biophys. J. 73, 2783–2790
53. Llopis, J., McCaffery, J. M., Miyawaki, A., Farquhar, M. G., and Tsien, R. Y. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 6603–6608
54. Llopis, J., McCaffery, J. M., Miyawaki, A., Farquhar, M. G., and Tsien, R. Y. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 6603–6608
55. Roth, J., and Berger, E. G. (1982) J. Cell Biol. 93, 22875–22881
56. Barrett, A. J., and Heath, M. F. (1977) in Lysosomes: A Laboratory Handbook (Dingle, J. T., ed) pp. 19–145, North-Holland Publishing Company, Amsterdam
57. Davis, C. G., Goldstein, J. L., Sudhof, T. C., Anderson, R. G., Russell, D. W., and Brown, M. S. (1987) Nature 326, 760–765
58. Borden, L. A., Einstein, R., Gabel, C. A., and Maxfield, F. R. (1990) J. Biol. Chem. 265, 8497–8504
59. Apse, M. P., Aharon, G. S., Snedden, W. A., and Blumwald, E. (1999) Plant Cell Physiol. 40, 451–461
60. Blumwald, E., Aharon, G. S., and Apse, M. P. (1999) Biochim. Biophys. Acta 1465, 140–151
61. Apse, M. P., Aharon, G. S., Snedden, W. A., and Blumwald, E. (1999) Science 285, 1256–1258
62. Van Dyke, R. W. (1995) Annu. Rev. Plant Physiol. 46, 223–239
63. Higgins, D. G., Bleasby, A. J., and Fuchs, R. (1992) Comput. Appl. Biosci. 8, 189–191