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Human Na^+/H^+ exchanger isoform 6 is found in recycling endosomes of cells, not in mitochondria

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Brett, Christopher L., Ying Wei, Mark Donowitz, and Rajini Rao. Human Na^+/H^+ exchanger isoform 6 is found in recycling endosomes of cells, not in mitochondria. *Am J Physiol Cell Physiol* 282: C1031–C1041, 2002. First published January 2, 2002; 10.1152/ajpcell.00420.2001.—Since the discovery of the first intracellular Na^+/H^+ exchanger in yeast, Nhx1, multiple homologs have been cloned and characterized in plants. Together, studies in these organisms demonstrate that Nhx1 is located in the prevacuolar/vacuolar compartment of cells where it sequesters Na^+ into the vacuole, regulates intravesicular pH, and contributes to vacuolar biogenesis. In contrast, the human homolog of Nhx1, Na^+/H^+ exchanger isoform 6 (NHE6), has been reported to localize to mitochondria when transiently expressed as a fusion with green fluorescent protein. This result warrants reevaluation because it conflicts with predictions from phylogenetic analyses. Here we demonstrate that when epitope-tagged NHE6 is transiently expressed in cultured mammalian cells, it does not colocalize with mitochondrial markers. It also does not colocalize with markers of the lysosome, late endosome, *trans*-Golgi network, or Golgi cisternae. Rather, NHE6 is distributed in recycling compartments and transiently appears on the plasma membrane. These results suggest that, like its homologs in yeast and plants, NHE6 is an endosomal Na^+/H^+ exchanger that may regulate intravesicular pH and volume and contribute to lysosomal biogenesis.

intraendosomal pH; Nhx1; intracellular NHE

THE FIRST INTRACELLULAR Na^+/H^+ exchanger (NHE) was identified in yeast by Nass et al. (22) in 1997. Named Nhx1, this transporter conferred salt-tolerant growth by Na^+ sequestration and was predicted to reside in an intracellular acidic organelle such as the vacuole. A chimera of Nhx1, alternatively named Nha2, with green fluorescent protein (GFP), colocalized with 4,6-diamidino-2-phenylindole staining of DNA and was therefore proposed to localize to mitochondria (26). However, a more detailed study with an array of compartmental markers used in conjunction with subcellular fractionation as well as confocal microscopy revealed that Nhx1 is found in the prevacuolar compartment of *Saccharomyces cerevisiae*, where cargo are sorted before delivery to the vacuole/lysosome (23). This unusual localization of a member of the well-known family of plasma membrane NHEs led us to speculate about a role for endosomal NHE in regulation of vesicle volume and pH and in vacuole biogenesis (23). Recently, Bowers et al. (3) confirmed the prevacuolar localization of Nhx1 and identified it as Vps44, a locus previously isolated from genetic screens for vacuolar protein-sorting mutants. Loss of function of Nhx1/Vps44 delays the delivery of proteins to the vacuole and results in an exaggerated and more acidic prevacuolar compartment.

Putative homologs of Nhx1 are readily identifiable in the genomes of other organisms, including plants, *Caenorhabditis elegans*, and humans. Of these, the gene for Nhx1 has since been cloned and characterized in several plant species, where localization supports a prevacuolar/vacuolar distribution (1, 6, 8, 11, 13, 32, 33, 35). Transgenic *Arabidopsis thaliana* overexpressing AtNhx1 are salt-tolerant and show increased Na^+/H^+ exchange activity in isolated vacuoles (1). Heterologous expression of AtNhx1 in yeast also results in prevacuolar/vacuolar localization and amiloride-sensitive, electroneutral Na^+/H^+ exchange (6, 11). In Japanese morning glory, a *Tpn4* insertion/loss-of-function mutation in the upstream region of the InNhx1 gene results in significant acidification of the vacuole, changing petal color from blue to purple (9, 35).

In humans, NHE6 (also known as KIAA0267) is the closest recognized homolog of Nhx1 on the basis of sequence identity and also was predicted to be intracellular by Nass et al. (22). Numata et al. (26) originally suggested that, like Nhx1/Nha2, human NHE6 might reside in mitochondria, on the basis of the experimental observation that transient expression of NHE6 and enhanced GFP (NHE6-eGFP) fusion protein in HeLa cells partially colocalized with the mitochondrial stain MitoTracker Red. This assignment was consistent with the relative abundance of NHE6 mRNA in highly metabolically active human tissues, including brain, skeletal muscle, and cardiac muscle. Also, they observed that the NH_2 terminus of NHE6 is unusually rich in positive charges that could serve as a mitochondrial targeting signal. However, in separate studies, a fusion of this putative mitochondrial signal sequence with GFP was targeted to the endoplasmic

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reticulum, and not mitochondria, when expressed in COS-7 cells (20). Furthermore, mitochondrial localization is inconsistent with phylogenetic analysis, which predicts that NHE6 should be present in an endosomal or lysosomal compartment, as has been shown for the yeast and plant homologs. It is noteworthy that Numata and Orlowski (25) have shown that NHE7, a recently discovered human NHE isoform that shares high sequence identity (86%) to NHE6, is found in the *trans*-Golgi network of mammalian cells, and not in mitochondria. Therefore a reevaluation of the localization of human NHE6 in mammalian cells is warranted. Here, we use a series of well-established compartmental markers in conjunction with immunofluorescent confocal microscopy, membrane fractionation, and surface biotinylation techniques to show that human NHE6, fused to GFP at the COOH terminus, is not localized to mitochondria but, rather, to endosomal compartments, consistent with other members of its NHE subgroup. These new data suggest that NHE6 may function in mammalian cells to regulate endosomal pH and volume and may play a role in lysosomal biogenesis.

METHODS

Media and cell culture. Chinese hamster ovary (CHO) cells were cultured in Ham's F-12 medium (Mediatech, Herndon, VA) containing 10% fetal bovine serum (Life Technologies, Gaithersburg, MD). Opossum kidney cells expressing NHE3 with a vesicular stomatitis virus glycoprotein (VSVG) epitope tag on the COOH terminus (OK-E3V cells, a generous gift from Dr. Josette Noel, Montreal, Canada) were cultured using protocols previously described (24).

DNA constructs. A partial human cDNA (KIAA0267) containing the NHE6 gene, with a 3-amino acid NH_2-terminal truncation, was generously provided by Dr. T. Nagase (Kazusa DNA Research Institute, Kisarazu, Japan). The NH_2-terminal Met-Ala-Arg tripeptide identified by Numata et al. (26) was inserted by using polymerase chain reaction (PCR) amplification of KIAA0267 with primers complementary to the 5′ (GGCGCGGGCCCGGATCCATGGCTCGGCGCGGCTGGCGGCGGGCA) and 3′ (GCAAGCGGGCCCGGCGGTGCTTGAAGAGC) ends. The primers were designed to create a product containing full-length human NHE6 flanked by 5′ *Apa*I and *Bam*HI and 3′ *Apa*I restriction sites. The *Apa*I sites were then used to place the full-length NHE6 in the pEGFP-N3 expression vector (Clontech Laboratories, Palo Alto, CA) to create an in-frame fusion of the COOH terminus of NHE6 and enhanced GFP (NHE6-eGFP; pEGFP-N3-NHE6). Epitope tagging of NHE1 and NHE3 in this location has been shown not to influence biosynthesis, targeting, or regulation.

A second construct was made to determine whether NHE6 is expressed on the plasma membrane and within the recycling compartments of cells (16). This construct involved engineering three copies of the hemagglutinin (HA) peptide in the coding region within the first predicted extracellular loop of human NHE6 between Glu^{55} and Glu^{56} (amino acids of NHE6 underlined: EGYPYDVPDYAGYPYDVPDYAGLVYPYDVPDYAGE) by using a modified inverse PCR mutagenesis protocol (10) to amplify pEGFP-N3-NHE6 with complementary primers to *1*) the 5′ region of the NHE6 gene after and including Glu^{56} with a single HA peptide epitope and an *Spe*I restriction site (*Spe*I restriction site is in bold type, HA peptide is underlined: G**ACTAGT**CTACCCCTACGACGTCCCCGACTACGCTGGTGAGATCGTGTCCGAGAAGCAA) and *2*) the 3′ region of the NHE6 gene before and including Glu^{55} with two copies of the HA peptide epitope and an *Spe*I restriction site (*Spe*I restriction site is in bold type, HA peptide is underlined: GG**ACTAGT**CCGGCGTAGTCGGGGACGTCGTAGGGGTAACCAGCGTAGTCGGGGACGTCGTAGGGGTAACCCTCGTCCATGGCTCTAGCCTC). The *Spe*I site was then used to circularize the resulting construct (pEGFP-HA_3-NHE6), which encodes a triple HA epitope-tagged NHE6-eGFP fusion protein (HA_3-NHE6-eGFP). All cDNA constructs were sequenced to confirm the presence of the mutations and to ensure that other random mutations were not introduced.

Antibodies and fluorescence microscopy. MitoTracker Red CMVRos, LysoTracker Red DND-99, AlexaFluor 568-conjugated human transferrin, anti-human cytochrome *c* oxidase IV (subunit I) mouse monoclonal antibody, and AlexaFluor 568-labeled goat anti-rabbit and anti-mouse IgG antibodies were purchased from Molecular Probes (Eugene, OR). The mouse monoclonal antibody against human LAMP-2 (H4B4) developed by Drs. J. Thomas August and James E. K. Hildreth was obtained from the Developmental Studies Hybridoma Bank (maintained by the University of Iowa, Iowa City, IA). The mouse monoclonal antibody against human influenza virus hemagglutinin protein (HA; clone 12CA5) was purchased from Roche Molecular Biochemicals (Indianapolis, IN). The rabbit polyclonal antibodies raised against TGN-38, α-mannosidase II, aquaporin-6, and rat mannose-6-phosphate receptor were generous gifts from Dr. Betty Eipper (University of Connecticut, CT), Dr. Kelley Moremen (University of Georgia, GA), Drs. Peter Agre and Masato Yasui (Johns Hopkins University, MD), and Dr. William J. Brown (Cornell University, NY), respectively. The mouse monoclonal antibodies raised against human Rab11 and EEA1 were purchased from BD Transduction Laboratories (Lexington, KY). The anti-human transferrin receptor monoclonal mouse antibody was purchased from Zymed Laboratories (San Francisco, CA).

CHO and OK-E3V cells were grown on eight-chamber glass slides and were transiently transfected with either pEGFP-HA_3-NHE6 or pEGFP-N3-NHE6 by using Lipofectamine 2000 and Lipofectace (Life Technologies), respectively, according to the manufacturer's instructions. Immediately before fixation or direct examination, CHO cells were grown to 70–80% confluency (or 2 days posttransfection) and OK-E3V cells were grown to 3–5 days postconfluency (or 5 to 7 days posttransfection). To compare the subcellular distribution of NHE6-eGFP and vital mitochondrial or lysosomal dyes or the cellular recycling compartments marked by transferrin, live CHO cells were incubated at 37°C for 30 min with the fluorescent probes diluted to the indicated concentrations in Ham's F-12 medium containing 10% fetal bovine serum: MitoTracker Red, 200 nM; LysoTracker Red, 50 nM; and AlexaFluor 568-conjugated human transferrin, 120 nM. Cells were then incubated in fluorescent probe-free medium and immediately imaged at room temperature.

To compare the subcellular distribution of NHE6-eGFP and antibodies against markers of cellular compartments, CHO or OK-E3V cells were fixed in 2% paraformaldehyde in PBS for 30 min, rinsed with PBS three times to remove residual fixative, and then permeabilized with 0.5% Triton X-100 in PBS for 15 min. Fixation, permeabilization, and all subsequent incubations were at room temperature. Cells were rinsed with PBS three times before incubation with 0.1% bovine serum albumin (BSA) in PBS for 1 h. Next, the permeabilized cells were incubated for 1 h with the indicated

primary antibody diluted in PBS containing 0.1% BSA at the following dilutions: LAMP-2, 1:200; EEA1, 1:100; Rab11, 1:100; mannose-6-phosphate receptor, 1:500; α-mannosidase II, 1:1,000; TGN-38, 1:500; and HA, 1:500. The cells were then washed with PBS three times over 30 min and then incubated for 1 h with either AlexaFluor 568-labeled goat anti-rabbit or anti-mouse antibodies diluted 1:500 in PBS containing 0.1% BSA. Finally, cells were again washed with PBS three times over 30 min and then mounted with Prolong antifade mounting medium (Molecular Probes).

To determine whether HA_3-NHE6-eGFP is expressed on the surface and within the recycling compartment of cells, CHO or OK-E3V cells were incubated either for 2 h at 4°C or for 1 h (also 0.5 and 2 h) at 37°C with anti-HA primary antibody diluted 1:500 in appropriate culture medium. Afterward, cells were fixed, permeabilized, and blocked with BSA as indicated above. Having already been exposed to primary antibody under specific conditions, cells were incubated for 1 h with AlexaFluor 568-labeled goat anti-mouse secondary antibody diluted 1:500 in PBS containing 0.1% BSA. Cells were then washed and mounted as described above.

Images were acquired by using a Zeiss LSM410 laser confocal microscope equipped with a Zeiss ×40 water-immersion lens (NA 1.2). Digitized images (8-bit) were recorded at eight-times frame averaging, processed with MetaMorph software (Universal Imaging, West Chester, PA), and assembled with Adobe Photoshop software (Adobe Systems, Mountain View, CA). Final images shown are representative of >90% cells observed expressing the indicated NHE6-GFP fusion protein. Each colocalization experiment was repeated at least three times.

Cell fractionation and Western analysis. Membrane fractionation experiments were performed by using transiently transfected CHO cells expressing HA_3-NHE6-eGFP protein grown to 70–80% confluency in 10-cm petri dishes. Subsequent manipulations were performed at 4°C. Cells were washed three times with wash buffer (PBS containing additions: 0.5 mM phenylmethylsulfonyl fluoride and 0.2% 2-mercaptoethanol) and washed once with potassium-containing buffer (KB; 150 mM KCl and 10 mM Tris, pH 7.3, also containing additions). The cells were then resuspended in KB, sonicated for 30 s (3 times), and centrifuged at 1,600 *g* for 10 min. The supernatant was collected and centrifuged at 10,000 *g* for 15 min, the resulting precipitate containing dense membranes (P10 sample) was saved, and the supernatant was further centrifuged at 100,000 *g* for 1 h. The resulting precipitate containing lighter membrane fractions (P100) was also saved, and both P10 and P100 samples were resuspended in equal volumes of KB containing 10% glycerol. Western analysis with mouse monoclonal anti-HA, anti-transferrin receptor, and anti-cytochrome *c* oxidase antibodies was performed following electrophoresis of samples from P10 and P100 membrane fractions run on the same 9% SDS-polyacrylamide gel. Bands were visualized by using ECL and exposed to preflashed X-ray film.

Surface biotinylation. To estimate the relative amount of the HA_3-NHE6-eGFP on the cell surface, a surface biotinylation assay was performed by using transfected CHO cells expressing the HA_3-NHE6-eGFP protein. As previously described in detail (4), the cells were grown to 70–80% confluency in 10-cm petri dishes. All subsequent manipulations were performed at 4°C. Cells were then washed twice in PBS and once in borate buffer (in mM: 154 NaCl, 10 boric acid, 7.2 KCl, and 1.8 $CaCl_2$, pH 9.0). Surface plasma membrane proteins were then biotinylated by gently shaking the cells for two 20-min periods, each with 3 ml of borate buffer containing 1.5 mg of *N*-hydroxysulfosuccinimydyl-S,S-biotin. The cells were then washed five times with quenching buffer (in mM: 120 NaCl and 20 Tris, pH 7.4) and twice with PBS. Cells were scraped and solubilized with 1 ml of N+ buffer (in mM: 60 HEPES, pH 7.4, 150 NaCl, 3 KCl, 5 trisodium EDTA, 3 EGTA, and 1% Triton X-100), sonicated for 20 s, agitated on a rotating rocker for 30 min, and centrifuged at 12,000 *g* for 30 min to remove insoluble cellular debris. A portion of the resulting supernatant was retained as the total fraction, and two consecutive avidin precipitations were performed on the remainder. Afterward, the supernatant was retained as the intracellular fraction, the remaining avidin-agarose beads were washed five times in N+ buffer, and bound proteins were solubilized in equivalent volumes of sample buffer (110 mM Tris·HCl, 0.9% SDS, 0.8% EDTA, 5% glycerol, 1% 2-mercaptoethanol, and bromphenol blue), yielding the surface fraction. Western analysis with a mouse monoclonal anti-HA antibody was performed following electrophoresis of dilutions of the total and surface fractions run on the same 9% SDS-polyacrylamide gel. Bands were visualized with ECL, exposed to preflashed X-ray film, and quantified with a densitometer and ImageQuant software. The sample volumes for each surface fraction were compared against the volume of the total fraction needed to give the same densitometric value to estimate the surface percentage of total protein.

Phylogenetic analysis. More than 140 sequences of Na^+/H^+ antiporters have been identified in databases, and a representative set of NHE-like sequences was selected from diverse phyla and isoform types for phylogenetic analysis. Sequences were aligned by using ClustalW 1.5, and the tree was generated using PHYLIP 3.5c. Amino acid sequence source files are as follows (gene name, species, and accession no.): NHX1, *S. cerevisiae*, NP 010744; NHX1, *Schizosaccharomyces pombe*, T37706; human NHE6, *Homo sapiens*, NP 006350; human NHE7, *H. sapiens*, AAK54508; worm NHX1, *C. elegans*, T22848; NHX1, *A. thaliana*, AAG51408; Atriplex NHX1, *Atriplex gmelini*, BAB11940; Japanese morning glory NHX1, *Ipomoea nil*, BAB16380; rice NHX1, *Oryza sativa*, BAB83337; crab NHE, *Carcinus maenas*, AAC26968.1; worm NHE1, *C. elegans*, CAC42390; rat NHE1, *Rattus norvegicus*, A40204; mouse NHE1, *Mus musculus*, Q61165; human NHE1, *H. sapiens*, A31311; rat NHE2, *R. norvegicus*, P48763; rabbit NHE2, *Oryctolagus cuniculus*, P50482; rat NHE4, *R. norvegicus*, P26434; human NHE3, *H. sapiens*, NP 004165; rat NHE3, *R. norvegicus*, NP 036786; rabbit NHE3, *O. cuniculus*, P26432; rat NHE5, *R. norvegicus*, Q9Z0X2; and human NHE5, *H. sapiens*, NP 004585.

RESULTS

NHE6 is not found in the mitochondria of mammalian cell. Previously, Numata et al. (26) created a construct of NHE6, tagged at the COOH terminus with eGFP, which was transiently expressed behind the strong cytomegalovirus promoter in cultured HeLa cells. In these cells, a partial overlap of the fluorescent signal from NHE6-eGFP with the mitochondrial marker MitoTracker Red was demonstrated after fixing and permeabilization (26). Using a similar NHE6-eGFP construct expressed in a variety of cell lines, we have not been able to see mitochondrial localization. In Fig. 1, *A–F*, live CHO cells labeled with MitoTracker Red show mitochondrial staining in a characteristic elongated "snakelike" appearance. Virtually no overlap of fluorescence signal is seen with NHE6-eGFP in multiple fields of cells. Furthermore, there is no colocalization of indirect immunofluorescence signal from anti-

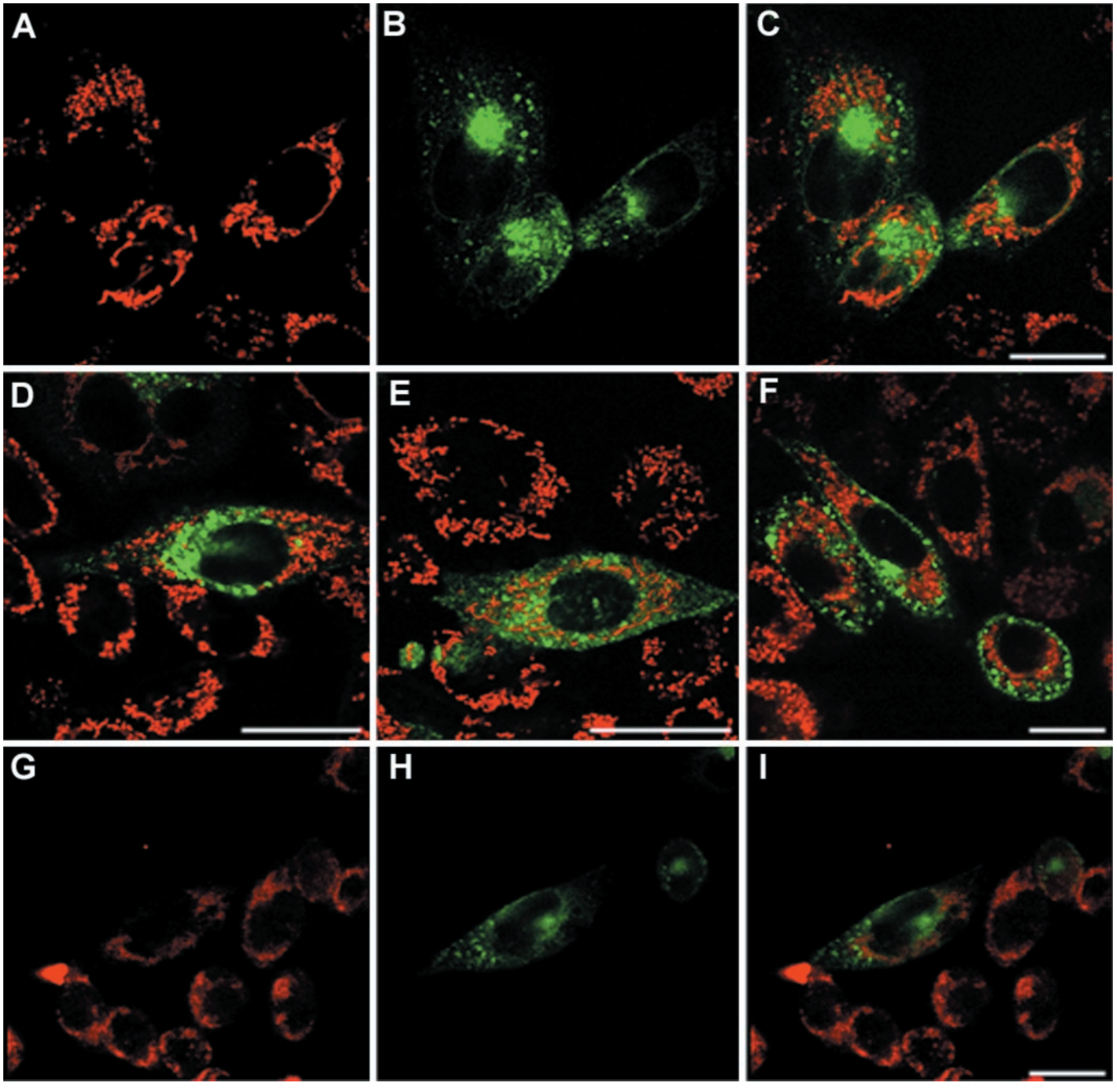

Fig. 1. Na^+/H^+ exchanger isoform 6-enhanced green fluorescent protein (NHE6-eGFP) protein is not found in mitochondria of Chinese hamster ovary (CHO) cells. Live CHO cells transiently expressing NHE6-eGFP (*B*; green) were stained with MitoTracker Red (*A*; red). Overlay of signals from NHE6-eGFP and MitoTracker Red in this (*C*) and 3 other fields of cells (*D–F*) are shown. In *G–I*, CHO cells transiently expressing NHE6-eGFP were fixed, permeabilized, and treated with antibody against cytochrome *c* oxidase. Signals from NHE6-eGFP (*H*; green), cytochrome *c* oxidase (*G*; red), and the overlay (*I*) are shown. Scale bars represent 30 μm.

cytochrome *c* oxidase antibody and NHE6-eGFP (Fig. 1, *G–I*). Rather, NHE6-eGFP shows a scattered vesicular distribution with a distinct juxtanuclear concentration. A similar distribution of NHE6-eGFP is seen in the polarized proximal tubule line from opossum kidney (e.g., see Figs. 4 and 6). We inserted a triple HA epitope in the extracellular loop of NHE6 between the first and second predicted transmembrane spans. HA_3-NHE6 also localized to a juxtanuclear concentration of vesicles that was distinct from mitochondria (not shown), confirming that localization was independent of the nature and position of the epitope tag.

NHE6 is present in the recycling compartments of cells including early and recycling endosomes, but not in lysosomes. Next, we examined whether NHE6 was found in the lysosomes of mammalian cells, which are equivalent to the vacuoles of plants and fungi. Neither the vital lysosomal dye LysoTracker Red nor antibod-

ies to LAMP-2, a lysosomal marker, colocalized with NHE6-eGFP in CHO cells (Fig. 2, *A–F*). Instead, NHE6-eGFP was found in the recycling endosomal compartments labeled by AlexaFluor 568-conjugated transferrin (Fig. 2, *G–I*) and transferrin receptor (not shown). This was further exemplified by fractionation of cells expressing epitope-tagged NHE6 (Fig. 3). Western analysis shows that HA_3-NHE6-eGFP was distributed in both P10 and P100 fractions, much like transferrin receptor. In contrast, the mitochondrial marker cytochrome *c* oxidase was found almost exclusively in the P10 fraction. Further confirmation of the endosomal distribution of NHE6 was obtained in the OK-E3V cell line, where specific colocalization was demon-

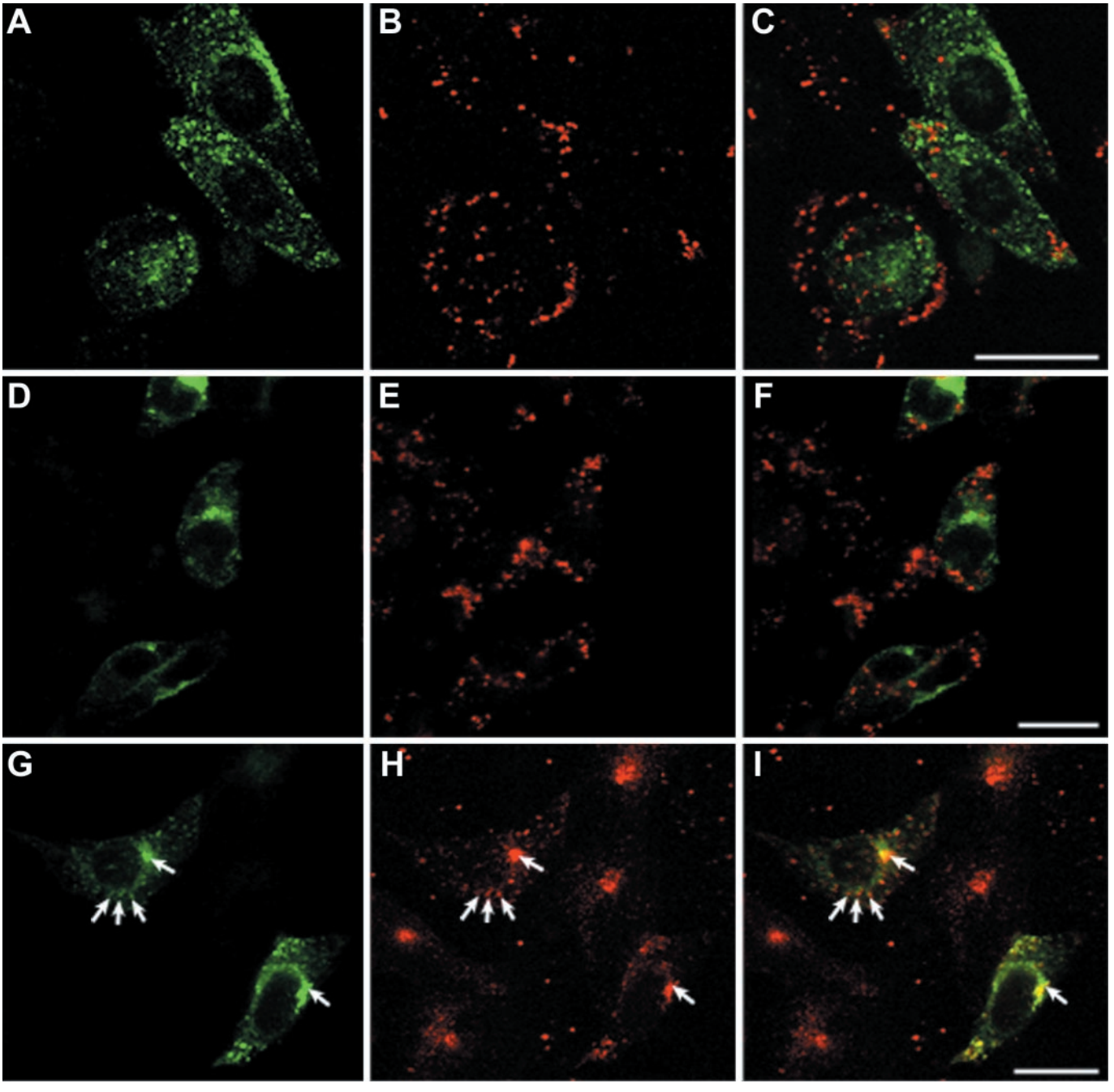

Fig. 2. Lysosomal markers do not colocalize, whereas labeled transferrin colocalizes, with NHE6-eGFP in CHO cells. Live CHO cells transiently expressing NHE6-eGFP (*A*; green) were stained with LysoTracker Red (*B*; red) for 30 min at 37°C before imaging. Overlay of the signals is shown in *C*. CHO cells transiently expressing NHE6-eGFP were fixed, permeabilized, and treated with a monoclonal antibody against human LAMP-2. Signals from NHE6-eGFP (*D*; green), LAMP-2 (*E*; red), and the overlay (*F*) are shown. The subcellular pattern of transiently expressed NHE6-eGFP (*G*; green) was similar to that of AlexaFluor 568-conjugated transferrin (*H*; red) in live CHO cells, as shown by the overlay of the images (*I*; colocalization shown as yellow, indicated by arrows). Scale bars represent 30 μm.

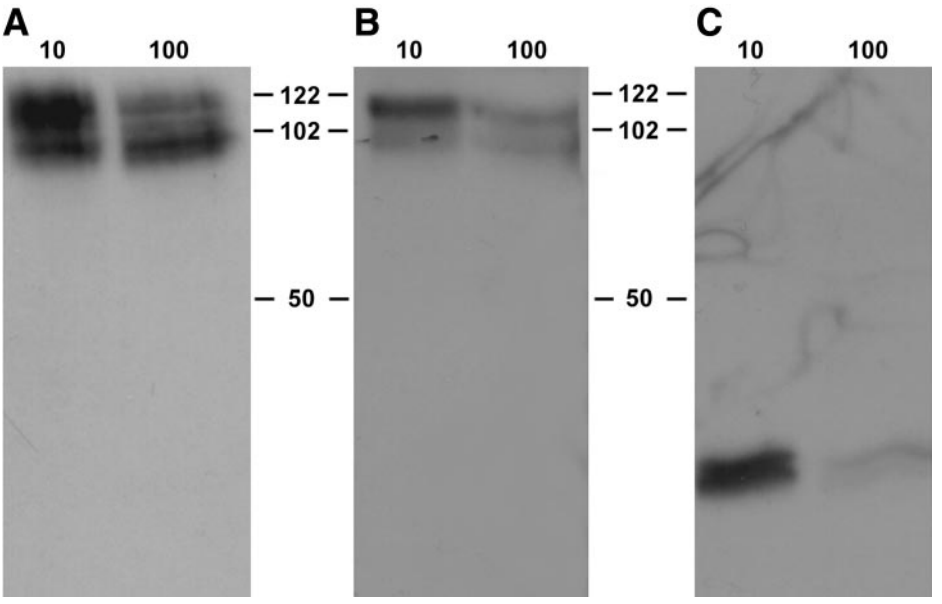

Fig. 3. NHE6 distribution in membrane fractions is similar to that of transferrin receptor. CHO cells transiently expressing HA_3-NHE6-eGFP were lysed and fractionated into 10,000 *g* (10) and 100,000 *g* (100) pellets by centrifugation as described in METHODS. Equal volumes were separated by SDS-PAGE and subjected to Western analysis using antibody against HA (NHE6) (*A*), transferrin receptor (*B*), and cytochrome *c* oxidase (*C*). HA, hemagglutinin.

strated with EEA1, the early endosomal marker, and with Rab11, a marker of recycling endosomes (Fig. 4, *A–F*). Like transferrin receptors, NHE6-eGFP was found to be excluded from the late endosomes, as judged by lack of colocalization with mannose-6-phosphate receptor (Fig. 4, *G–I*).

Golgi and TGN markers do not colocalize with NHE6. Human NHE7, an isoform highly similar to NHE6, was recently cloned and localized to the *trans*-Golgi network (TGN) by Numata and Orlowski (25). It was therefore of interest to determine whether the juxtanuclear concentration of NHE6 coincided with markers for the Golgi or TGN. Here, we show that the pattern of distribution of NHE6-eGFP is different from that of α-mannosidase II and TGN-38, despite some spatial overlap (Fig. 5, *A–C* and D–F, respectively). It is therefore unlikely that NHE6 is a resident protein of the Golgi or the TGN.

NHE6 transiently appears on the plasma membrane. Because our data indicate that NHE6 is found in the recycling compartments of cells, we addressed the possibility of transient trafficking to the plasma membrane. The triple HA tag was introduced into the predicted extracellular loop of NHE6 between the first and second transmembrane helices of the COOH terminus-tagged NHE6-eGFP, as originally described for NHE3 (16). OK-E3V cells expressing this construct (pEGFP-HA_3-NHE6) were examined by confocal fluorescence microscopy to demonstrate that both HA and eGFP tags labeled the same pool of NHE6 in fixed and permeabilized cells (Fig. 6, *A–C*). This procedure was then modified to stain only the surface pool of HA-NHE6-GFP by the addition of anti-HA antibody to live cells at a temperature nonpermissive to endocytosis (4°C). Figure 6, *D–F*, shows very low levels of surface HA-labeling compared with the total cellular pool of NHE6 indicated by the eGFP signal. However, when anti-HA antibody was added to live cells under conditions permissive for recycling (37°C), a high degree of coincidence of the two signals was observed (Fig. 6, *G–I*), indicative of extensive labeling of the recycling compartments due to either antibody binding to transiently presented external epitope at the plasma membrane or delivery of the antibody to the recycling compartments by endocytosis.

The presence of NHE6 on the plasma membrane was confirmed with a surface biotinylation assay on intact cells. Figure 7 demonstrates that a small fraction (~5%) of the total pool of NHE6 appears on the plasma membrane in CHO cells. Taken together, our data indicate that the NHE6 isoform localizes to early and recycling endosomal compartments, with low, possibly transient, expression on the cell surface.

DISCUSSION

In contrast to a previous report (26), our results demonstrate that human NHE6, when transiently expressed in cultured cells, is not present in mitochondria but, rather, is present in the recycling compartment. This is similar to previous findings in yeast where a mitochondrial localization of the NHE6 homolog, Nhx1, was also considered and shown not to occur (23). While this article was being prepared, the signal peptide of NHE6 was shown to target to the endoplasmic reticulum, consistent with localization to a compartment of the secretory pathway (20). In light of these data, the molecular identity of the physiologically characterized mitochondrial Na^+-selective antiporter remains unknown (see Ref. 2). Another uncharacterized NHE isoform may localize to mitochondria; one such candidate is KIAA0939, a cDNA isolated from brain with relatively low sequence identity to other known human NHE isoforms (21). Alternatively, the antiporter may belong to a separate gene family. It is interesting to note the structural and mechanistic diversity of systems capable of conducting Na^+/H^+ exchange that are emerging from studies in bacteria.

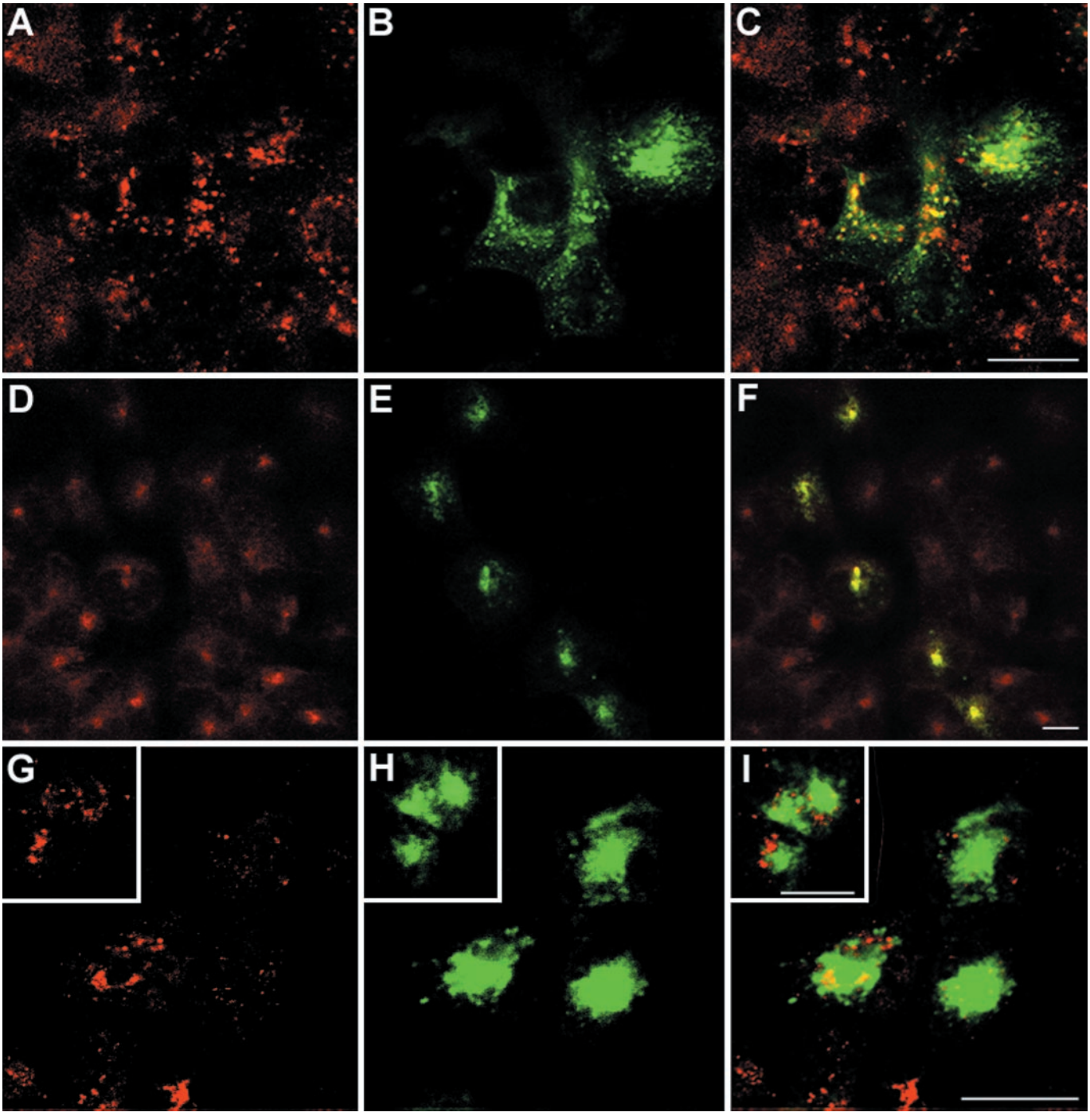

Fig. 4. NHE6-eGFP is found in early and recycling, but not late, endosomes in opossum kidney (OK)-E3V cells. OK-E3V cells transiently expressing NHE6-eGFP (*B*, *E*, and *H*; green) were stained with antibodies to EEA1 (*A*; red), Rab11 (*D*; red), or mannose-6-phosphate receptor (*G*; red). Overlays of signals from NHE6-eGFP and the respective vesicular markers are shown in *C*, *F*, and *I*. Boxed insets represent an additional field of cells. Scale bars represent 30 μm.

Recent work by Wei et al. (34) demonstrates that Na^+/H^+ exchange in *Bacillus subtilis* can be coupled to malate-lactate exchange by YqkI, a homolog of the bacterial Na^+/H^+ antiporter NhaC. The nuo-L, -M, and -N subunits of bacterial complex I (or NADH:ubiquinone oxidoreductase) of the electron transport chain share sequence homology with bacterial Na^+/H^+ antiporters and have been proposed to confer to the redox pump a dual role with Na^+ antiporter-like function (29). Similar mechanisms may conceivably exist in the inner membranes of eukaryotic mitochondria.

Evidence for intracellular electroneutral Na^+/H^+ exchange has been documented in a variety of mammalian tissues, and in each case this activity colocalized

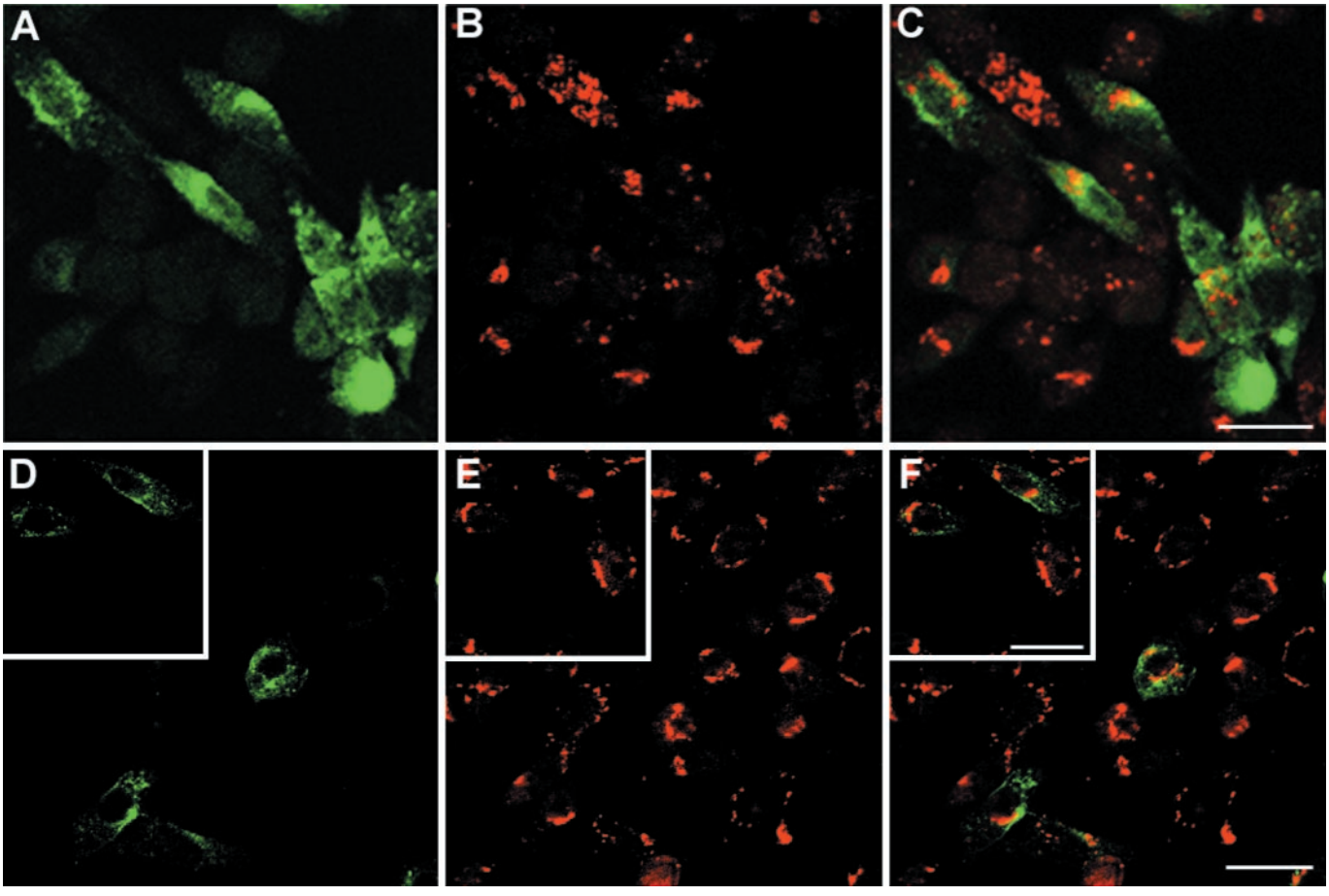

Fig. 5. Golgi and *trans*-Golgi network (TGN) markers do not have subcellular distributions similar to that of NHE6-eGFP in CHO cells. CHO cells transiently expressing NHE6-eGFP (*A* and *D*; green) were stained with rabbit polyclonal antibodies to either α-mannosidase II, a Golgi marker (*B*; red), or TGN-38, a TGN marker (*E*; red). Overlays of the signals are shown in *C* and *F*. Boxed insets indicate an additional field of cells. Scale bars represent 30 μm.

with vacuolar (V-type) H^+-ATPase (V-ATPase) activity, suggesting endosome-type residence for Na^+/H^+ exchange (15, 31). The basic properties of these exchange activities are remarkably similar to one another but significantly distinct in several respects from the cloned NHE isoforms found predominantly in the plasma membrane (e.g., Ref. 18). We suggest that these endosomal Na^+/H^+ exchange activities likely represent a novel and physiologically important subset of Na^+/H^+ exchangers, which includes NHE6.

Certainly other NHE isoforms could contribute to endosomal Na^+/H^+ exchange as well. For example, the predominantly surface-expressed NHE3 has been shown to be present in subapical endosomes where it contributes to pH regulation (7, 12). Despite these similarities, we propose that the intracellular function and distribution of NHE3 and NHE6 are distinct, based on two observations. First, the tissue distribution of NHE6 mRNA is different from that of NHE3 mRNA (5, 26, 27). Second, NHE3 functions to acidify the intracompartmental space (7, 12), whereas the NHE6-like isoforms, NHE7 and Nhx1 from yeast and plants, have both been shown to alkalinize the inside of compartments. Thus NHE7 and AtNhx1 have been shown to transport both Na^+ and K^+ into intracellular compartments in exchange for H^+ (25, 32). The intracellular NHE isoforms may well have ion selectivities distinct from those on the plasma membrane, allowing them to utilize K^+ gradients to move H^+ out of the endosomes.

A phylogenetic analysis of a representative subset of NHE isoforms is shown in Fig. 8. NHE6, NHE7, and Nhx1 constitute a distinct cluster, separate from NHE1–NHE5. This grouping of sequences potentially represents a separation of physiological function and subcellular location. We suggest that the plasma membrane-type NHEs, represented by NHE1–NHE5, show Na^+ gradient-driven H^+ transport, whereas the intracellular NHEs, represented by NHE6, NHE7, and Nhx1, function as H^+ gradient-driven Na^+ transporters. Importantly, this subset of NHE excludes NHE3, consistent with the proposed function of this isoform (5, 7, 12).

Therefore, on the basis of phylogenetic classification and functional data from experiments performed on Nhx1 and NHE7, we propose that NHE6 functions to export H^+ from the early and recycling endosomes of cells in exchange for the import of Na^+, thus alkalin-

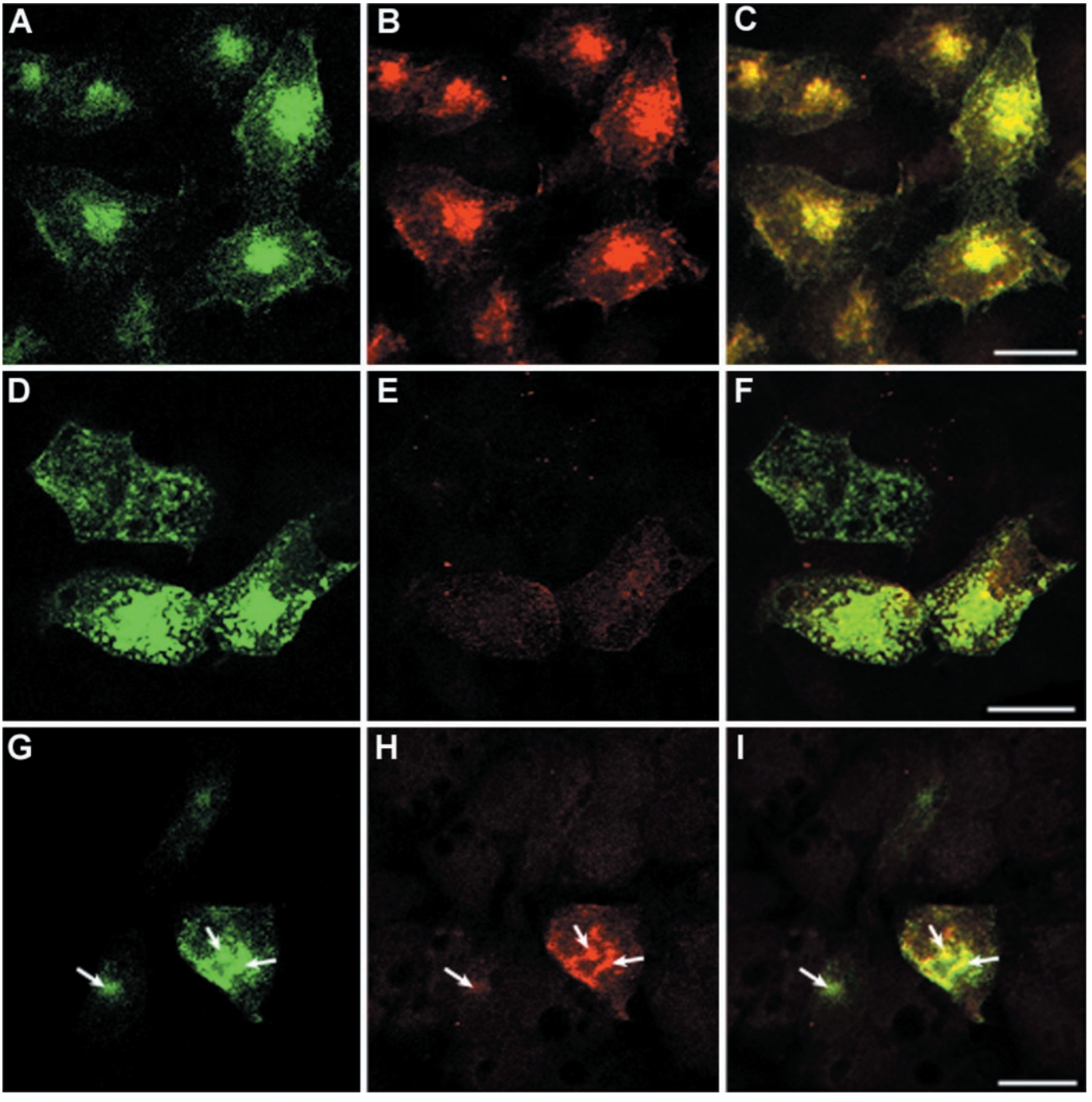

Fig. 6. HA_3-NHE6-eGFP is found in the recycling compartments of OK-E3V cells. OK-E3V cells transiently expressing NHE6-eGFP (*A*, *D*, and *G*; green) with an extracellular/luminal triple HA epitope were stained with anti-HA antibody (*B*, *E*, and *H*; red) under 3 independent experimental conditions. As a control (*A–C*), cells were fixed and permeabilized before being stained with the anti-HA antibody to show complete overlap of eGFP and HA signals (*C*; yellow). *D–F*: cells stained with the anti-HA antibody when live at 4°C. *G–I*: cells stained with the anti-HA antibody when live at 37°C before being fixed. Overlay of the eGFP and HA signals is shown in *F* and *I* (arrows indicate areas of colocalization). Scale bars represent 30 μm.

izing the endosomal lumen. The vectorial Na^+ transport by NHE6 would be driven by the H^+ electrochemical gradient established by the V-ATPase. There is emerging evidence for distinct isoforms of V-ATPase subunits localizing to discrete subcellular compartments in yeast and mammals, where they may confer compartment-specific function (19, 30). Under certain conditions of cell stress (e.g., protracted myocardial ischemia), NHE6 may function to sequester sodium into the endosomes, which could either be stored within the lysosome (analogous to yeast and plants) or excreted into the extracellular space via recycling endosomes (17).

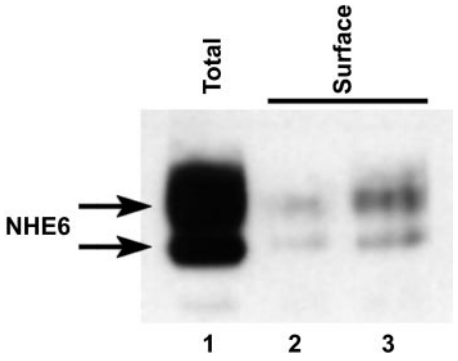

Fig. 7. HA_3-NHE6-eGFP is expressed on the plasma membrane of CHO cells. Surface biotinylation was performed on CHO cells transiently expressing HA_3-NHE6-eGFP, as described in METHODS. SDS-PAGE of the total cellular fraction (10-μl sample; *lane 1*) and surface fractions (10- and 20-μl samples; *lanes 2* and *3*, respectively) from the same preparation was subjected to Western analysis using anti-HA antibody.

NHE6-dependent Na^+ entry into the compartmental lumen could also be important for endosomal volume control when coupled to Cl^- and water entry. Preliminary data generated in our laboratory showing that aquaporin-6 (an intracellular water channel known to facilitate Cl^- movement) and NHE6 colocalize in OK cells support this idea (data not shown). The proposed role of NHE6 as a volume regulator may be important for vesicle fusion and, thus, endosome/lysosome biogenesis (as already demonstrated in yeast; Ref. 3). NHE6 function may therefore be relevant to a number of lysosomal biogenesis and lysosomal storage diseases (37). Control of lysosomal/endosomal pH is an important determinant in the survival mechanisms of pathogenic bacteria, such as *Salmonella typhimurium*. These microbes establish themselves within alkaline endosome-like compartments of host cells and escape killing by halting lysosomal biogenesis (14, 28). Finally, as a pH regulator of the early and recycling endosomes, NHE6 may function to regulate the rate of surface receptor recycling (12, 36).

Thus our finding that NHE6, a member of a newly recognized class of endosomal NHE, is present within the recycling compartments of mammalian cells, and not mitochondria, supports the importance of endosomal Na^+/H^+ exchange in numerous physiological and pathophysiological events.

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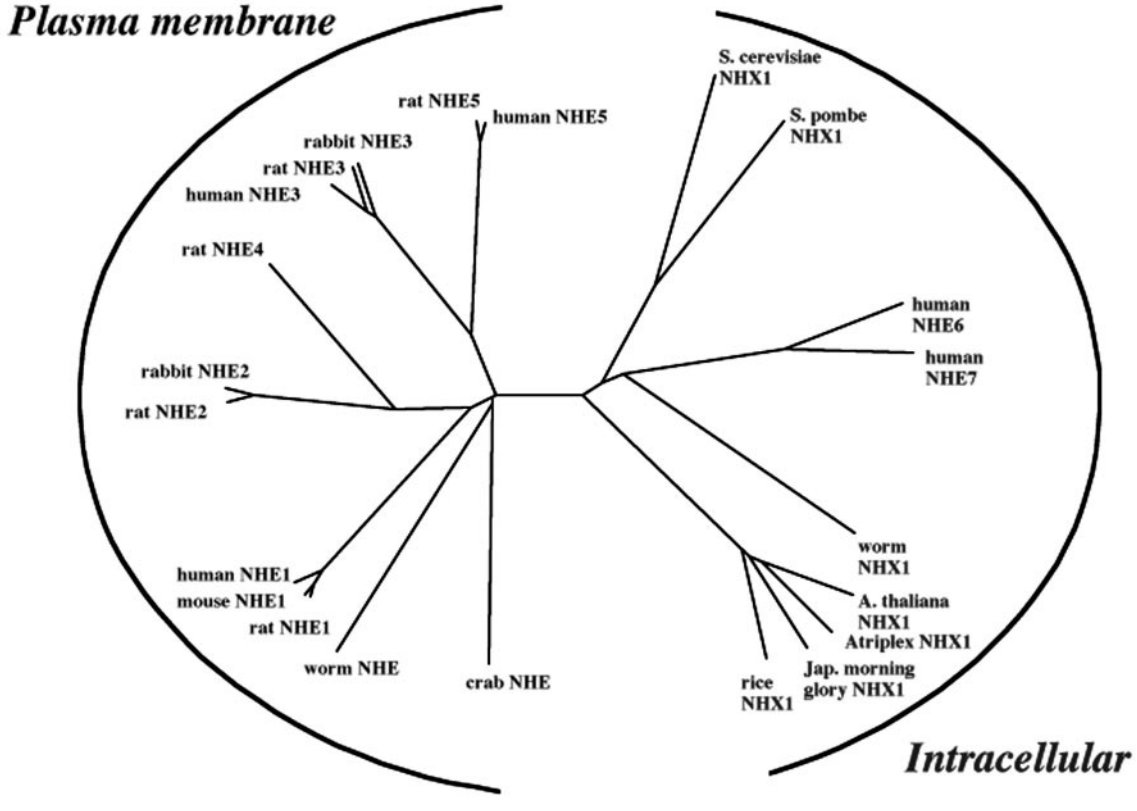

Fig. 8. Phylogenetic analysis of the NHE family. Selected NHE amino acid sequences were aligned with ClustalW 1.5 and analyzed with PHYLIP 3.5c. Two distinct phylogenetic clusters are apparent. Human NHE7 and multiple plant and yeast homologs of Nhx1 are a subset of the NHE family that functions predominantly within intracellular compartments. This cluster also contains human NHE6. NHE1–NHE5 form a separate cluster that functions predominantly on the plasma membrane. Amino acid sequence source files are listed in METHODS.

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