The Epithelial Sodium-Hydrogen Antiporter Na\(^+\)/H\(^+\) Exchanger 3 Accumulates and Is Functional in Recycling Endosomes*

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Na\(^+\)/H\(^+\) exchangers (NHEs) mediate electroneutral exchange of Na\(^+\) for H\(^+\) and thereby play a central role in pH regulation and Na\(^+\) homeostasis. NHE3, the predominant epithelial isoform, is found in apical membranes of renal and intestinal epithelial cells, where it contributes to NaCl (re)absorption. NHE activity has been detected in endosome vesicles of epithelial cells, but the precise compartment involved and its functional role have not been defined. Many aspects of the targeting machinery that defines the compartmentation and polarity of epithelia are also functional in nonepithelial cells. We therefore compared the targeting of NHE1, the basolateral isoform, with that of NHE3 in Chinese hamster ovary cells. To circumvent the confounding effects of endogenous exchangers, epitope-tagged constructs of NHE1 and NHE3 were stably expressed in antiport-deficient (AP-1) cells. While NHE1 was found almost exclusively in the surface membrane, NHE3 was also found intracellularly, accumulating in a juxtanuclear compartment. Confocal microscopy showed this compartment to be distinct from the Golgi, trans-Golgi network, and lysosomes. Instead, NHE3 colocalized with transferrin receptors and with cellubrevin, markers of recycling endosomes. The activity of NHE3 in endomembranes was assessed by targeting pH-sensitive probes to the recycling endosomes using a chimeric cellubrevin construct with an accessible extracellular epitope. Fluorescence ratio imaging indicated that cellubrevin resides intracellularly in an acidic compartment. In AP-1 cells, endosomal acidification was unaffected by omission of Na\(^+\) but was dissipated entirely by concanamycin, a blocker of H\(^+\)-ATPases. In contrast, the cellubrevin compartment was more acidic in NHE3 transfectants, and the acidification was only partially reduced by concanamycin. Moreover, removal of extracellular Na\(^+\) resulted in a significant alkalization of the endocytic compartment. These results indicate that NHE3 is present and active in recycling endosomes. By recruiting NHE3 to the plasma membrane, modulation of vesicular traffic could contribute to the regulation of Na\(^+\) reabsorption across epithelia.

To maintain the intracellular pH near neutrality, most mammalian cells actively extrude H\(^+\) in exchange for extracellular Na\(^+\), a process mediated by the Na\(^+\)/H\(^+\) exchanger (NHE) or antiporter. Exchange of Na\(^+\) for H\(^+\) across the plasma membrane is a tightly coupled, electroneutral process that is generally sensitive to inhibition by amiloride and benzoyl guanidinium compounds. NHEs are integral membrane phospho(glyco)proteins with 10–12 predicted transmembrane domains and a sizable carboxy-terminal hydrophilic domain Ca\(^2+\) believed to face the cytoplasm. Six members of the NHE family have been identified to date. NHE1, thought to be the “housekeeping” isoform, is found on the plasma membranes of virtually all animal cells, including the basolateral membrane of epithelial cells, where it is believed to control cytosolic pH (pH\(_c\)) to play a role in cell volume regulation (3). NHE2, -3, -4, and -5 display a more restricted tissue distribution, probably reflecting specialized functions, whereas NHE6 is expressed in all tissues examined to date. NHE3 is the best characterized of these isoforms; it is predominantly expressed in kidney and gut, where it is found on the apical membranes of polarized epithelial cells. This isoform is thought to play a central role in transepithelial reabsorption of Na\(^+\).

NHE activity has been described in endosomes of the renal cortex, where it was postulated to play a potential role in the maintenance of endosomal pH (4, 5). While the early studies of renal subcellular fractions did not identify the isoform detected functionally, they demonstrated that the endosomal NHE was poorly sensitive to amiloride (4, 5). Independent pharmacological studies have revealed that, of the isoforms known to date, NHE3 is the least sensitive to pharmacological antagonists (1, 3, 6), suggesting that this is the antiporter present in renal endosomes. This assumption was recently substantiated by immunolocalization studies using antibodies to NHE3, where this isoform was detected not only on the brush border membrane of renal cells, but also in intracellular vesicles (7).

Distinct apical and basolateral early endosomes have been described in epithelial cells, but there is mounting controversy as to whether specialized apical and basolateral recycling en-

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*The abbreviations used are: NHE, Na\(^+\)/H\(^+\) exchanger; Chv, cellubrevin; CHO, Chinese hamster ovary; HA, hemagglutinin; pHe, endosomal pH; pHe, cytosolic pH; Tf, transferrin; Tf-R, transferrin receptor; FITC, fluorescein isothiocyanate; Ab, antibody; PBS, phosphate-buffered saline; TGN, trans-Golgi network; BCECF, 5,6-bis(carboxyethyl)carboxyfluorescein; TRITC, tetramethylrhodamine isothiocyanate.
dosomes exist or whether they are interconnected in a larger endosomal network (8–11). This controversy stems, at least in part, from the lack of unique markers for apical endosomes, which have been poorly characterized. This contrasts with the extensive biochemical and morphological knowledge of endosomes in nonpolarized cells, where sorting, recycling, and late endosomes have been studied in detail (for reviews, see Refs. 12–14). It has become apparent that, like epithelial cells, nonpolarized cells also possess the targeting machinery to segregate apical and basolateral proteins to distinct microdomains within the cell (15, 16). When endotubulin, a protein found in apical endosomes of intestinal cells, was expressed heterologously in fibroblasts, it was found to distribute to a subpopulation of early endosomes, which are likely analogous to apical endosomes (11). This suggests that nonpolarized cells can be used as a simplified model system to study the targeting of epithelial proteins suspected of entering apical endosomes.

The purpose of the present experiments was to define the properties and subcellular targeting of NHE3 using heterologous expression in nonpolarized cells. For comparison, cells were also transfected with NHE1, which is predominantly targeted to the basolateral membranes of epithelial cells (1). To study the activity of the heterologously transfected exchangers in isolation, we used cells that were deficient in endogenous NHE activity (for details, see Refs. 17 and 18). We found that, unlike NHE1, which is largely plasmaemal, NHE3 concentrates in an endomembrane compartment. The nature of this compartment and the functionality of NHE3 therein were assessed using confocal microscopy and fluorescence ratio imaging.

MATERIALS AND METHODS

Reagents—The acetylomethyl ester of BCECF-AM, FITC-, or TRITC-labeled transferrin (Tfn) and FITC-labeled dextran were purchased from Molecular Probes, Inc. (Eugene, OR). Horseradish peroxidase-conjugated goat anti-mouse Ab, Cy3-, and FITC-labeled donkey anti-rabbit and anti-mouse Ab were obtained from Jackson Laboratories, Inc. (West Grove, PA). FITC-labeled anti-T-cell antigen (CD25) Ab was from Serotec. FITC-labeled goat anti-human P(ab) fragments and mouse monoclonal anti-HA Ab were bought from Cappel (Durham, NC) and BabCo (Berkeley, CA), respectively. Rabbit polyclonal Ab to mouse monoclonal anti-HA Ab were obtained from Cappel (Durham, NC). FITC-labeled goat anti-human F(ab) fragments and rabbit and anti-mouse Ab were obtained from Jackson Laboratories, Inc. (West Grove, PA). Appropriate dilutions: HA, 1:1000; mannosidase II and calnexin, 1:200. Next, the permeabilized cells were then incubated in PBS containing 0.1% bovine serum albumin for 15 min. The cells were washed, and mounted. To label lysosomes, AP-1NHE3HA cells were co-transfected with pCDH-hNAT-Moesin and pMCIhph, which contains a gene that confers resistance to hygromycin B. Cells were plated on coverglasses and coverglasses were washed with 0.25% sodium hypochlorite, washed, and mounted. To examine the subcellular localization of HA-tagged proteins, cells were fixed in 2% paraformaldehyde in PBS at 30 min, placed in 100 mM glycine in PBS for 15 min to scavenge any residual fixative, and then permeabilized with 0.1% Triton X-100 in PBS containing 0.1% bovine serum albumin for 15 min. The cells were then incubated in PBS with 5% donkey serum for 20 min. Fixation, permeabilization, and subsequent steps were performed at 4 °C. After permeabilization, cells were then incubated for 1 h with the indicated primary Ab diluted in PBS with 5% donkey serum at the following dilutions: HA, 1:1000; mannosidase II, 1:500; calnexin, 1:200. Next, the cells were washed with PBS three times over 15 min and incubated for 1 h with either Cy3-labeled anti-mouse Ab, FITC-labeled anti-rabbit Ab, or both. Finally, cells were mounted with DAKO mounting medium.

To compare the distribution of NHE3HA and TGN38, AP-1NHE3HA/TGN38 cells were fixed, permeabilized, and labeled with the anti-HA Ab followed by Cy3-conjugated anti-mouse secondary Ab, as described above. On the other hand, the cells were transfected with FITC-conjugated anti-CD25 Ab, washed, and mounted. To label lysosomes, AP-1NHE3HA cells were incubated with 30 µg/ml fixable FITC-conjugated dextran for 12 h at 37 °C. The dextran was then chased over a 4-h period. Cells were then fixed with 2% paraformaldehyde at 4 °C for 20 h and then permeabilized and stained for HA as described above. For Tfn-R localization experiments, cells were washed three times with serum-free medium and then incubated at 37 °C with the same medium containing 20 µg/ml of either FITC- or TRITC-labeled Tfn for 30 min. The cells were then washed, chased for 15–60 min, fixed, and permeabilized as above. To determine the time course of transferrin internalization, cells were washed three times with serum-free medium and incubated at 37 °C with the same medium containing 100 µg/ml FITC-conjugated goat anti-human IgG for 30 min. Cells were then washed and incubated for 1 h in serum-free medium at 37 °C and then fixed and permeabilized as above. The distribution of Cbv was also examined by first fixing and permeabilizing the cells and then labeling with the FITC-conjugated Ab.
Comparative results were obtained with both methods.

Images were acquired using a Leica laser-confocal microscope with a ×100 plan-apochromat lens (1.4 N.A.). Digitized images were processed with Adobe Photoshop (Adobe Systems, Inc., Mountain View, CA) and assembled and labeled in PowerPoint (Microsoft, Seattle, WA).

Fluorescence Ratio Imaging pH Measurements—To determine endosomal pH (pHe), cells plated on a 25-mm glass coverslip (Fisher 48 h prior to use were incubated with the FITC-conjugated F(ab) fragment of goat anti-human Ab or with FITC-labeled Tf as described above. The coverslip was then placed in a Leiden CoverSlip Dish (Medical Systems Corp., Greenvale, NY) in a temperature-regulated perfusion chamber (Open Perfusion Micro-Incubator; Medical Systems Corp.) on the stage of an inverted microscope (Axiovert 135; Zeiss Oberkochen, Germany) equipped with a ×63 oil immersion objective and epifluorescence optics. An electronically controlled shutter and filter-wheel (Sutter Instruments Co., Novato, CA) was used to alternately position two excitation filters (490BP10 and 440BP10 nm) in front of a xenon lamp. The fluorescent signals were processed through a 535BP25 nm band-pass filter. The digitized images were captured on a cooled CCD camera (Princeton Instruments Inc., Princeton, NJ). Data were recorded every 60–120 s by irradiating the cells for 1 s at each wavelength. At the end of each calibration, control was obtained by equilibrating the cells in isosmotic KCl of each experiment, calibration was obtained by equilibrating the cells

pHc was determined as described previously. Briefly, cells plated on coverslips were loaded with a 2 μM concentration of the acetoxy-methyl ester precursor of BCECF at 37 °C for 10 min, washed, and placed in Leiden CoverSlip holders as above. pHc was determined in a fashion identical to that described for endosomes with the exception that a much shorter excitation period (75 ms) and neutral density filters were used to minimize photolysis of the dye and the attendant cytotoxicity. NHE activity was assessed as the rate of Na+/H+ -induced recovery of pHc following an acid load imposed by preloading with NH4Cl (for details, see Ref. 22).

Where specified, the cells were incubated at 37 °C in serum-free medium supplemented with colchicine (25 μM, 60 min), wortmannin (10 μM, 45 min), or forskolin (10 μM, 10 min) prior to loading with BCECF or before being fixed and permeabilized to examine the effects of these drugs on the subcellular distribution of NHE3.

RESULTS

Functional Expression of Epitope-tagged NHE1 and NHE3—To facilitate the detection of NHE3 in heterologous expression systems, an immunogenic epitope derived from the influenza virus HA was attached to the carboxyl terminus of the protein, yielding NHE3-HA. The site of attachment was chosen because truncation analyses showed earlier that this region of the antiporter is not essential for NHE3 function (22). To increase the availability and immunogenicity of the protein, a construct was also made expressing three tandem HA repeats (NHE3–3HA). For comparison, the ubiquitous NHE1 isofrom was tagged with a single HA epitope (NHE1-HA). Schematic representations of these constructs are shown in Fig. 1A. Such constructs were stably transfected into an antiport-deficient CHO cell line, termed AP-1, and the expression and intactness of the protein were assessed by immunoblotting with anti-HA Ab. Typical results are shown in Fig. 1B. Immunoreactive bands of the predicted molecular mass (~100 kDa for NHE1-HA; ~86 and 88 kDa for NHE3-HA and NHE3–3HA, respectively) were readily detectable in whole-cell extracts. Cells transfected with untagged NHE1 or NHE3 failed to react with the Ab, implying that the immunoreactivity is specific to the HA epitope.

The functional activity of the transfected epitope-tagged constructs was assessed fluorimetrically in BCECF-loaded cells (Fig. 2). Antiport activity was induced by the addition of Na+ to the media that had been previously acid-loaded by an ammonium prepulse (22). As shown in Fig. 2A, NHE1-HA cells responded to the addition of Na+ with a vigorous alkalization that restored normal pHc within 1–2 min. Like wild-type NHE1, the epitope-tagged protein was exquisitely sensitive to 1 μM HOE 693, which reduced the rate of H+/K+ extrusion to levels comparable with that observed in the absence of Na+ (cf. open circles in Fig. 2, A and B). The epitope-tagged NHE3-HA was similarly functional, inducing a conspicuous alkalization upon the addition of Na+. The rate of H+/K+ extrusion was noticeably lower in the NHE3-HA transfectants than in the NHE1-HA counterparts, despite the presence of comparable amounts of protein (e.g., Fig. 1). This may reflect differences in the intrinsic activity of the exchangers, but it may also be a consequence of differential subcellular localization (see below). Unlike NHE1, NHE3 is virtually insensitive to micromolar doses of HOE 693 (1, 6) but maintains strict Na+ dependence. This pharmacological profile was preserved in the epitope-tagged constructs (Fig. 2C). Other physiological properties were also maintained in NHE3-HA. Specifically, the inhibitory effect of cAMP, induced by the addition of forskolin, was clearly apparent in the HA-tagged antiporter (Fig. 2D). Similar results were obtained with the triple HA-tagged NHE3, while no inhibition was observed in forskolin-treated NHE1-HA transfectants (not shown).

![Fig. 1.](image-url)
Jointly, these observations indicate that when expressed in a heterologous system, the epitope-tagged forms of NHE1 and NHE3 retain their functional and pharmacological properties.

Subcellular Distribution of NHE1-HA and NHE3-HA—Immunofluorescence confocal microscopy was used to define the subcellular distribution of NHE1 and NHE3 in AP-1 cells. As described earlier for the unmodified form of NHE1, the epitope-tagged protein was found predominantly at the surface membrane (Fig. 3A). Transverse (x versus z) reconstructions confirmed the superficial staining and additionally showed some accumulation at or near lamellipodia, consistent with an earlier report (23) and with the notion that epithelial basolateral proteins expressed in nonepithelial cells concentrate in lamellipodia (24). Only a small fraction of NHE1-HA appeared to be intracellular, possibly reflecting immature protein that can occasionally be detected by SDS-polyacrylamide gel electrophoresis as a lower molecular weight, nonglycosylated species.

By contrast, NHE3-HA appeared to be largely intracellular, with only a fraction present at or near the surface membrane (Fig. 3, C and D). The distribution of NHE3-HA was punctate, with noticeable accumulation in a juxtanuclear location. Identical results were obtained using the triple-tagged form of NHE3, NHE3–3HA (not illustrated). The differential localization of the two isoforms can be attributed neither to the epitope tag nor to varying levels of expression, which are similar for NHE1 and NHE3 (see Fig. 1).

To define the site(s) where NHE3-HA accumulates in AP-1 cells, we undertook double-labeling studies using recognized
organellar markers. Overexpression of proteins driven by constitutive promoters can lead to defective processing and trapping in the endoplasmic reticulum. However, staining of the reticulum with calnexin showed a diffuse pattern that is distinctly different from that of NHE3-HA (not shown). Instead, the juxtanuclear accumulation observed resembles that reported for the Golgi complex, perinuclear lysosomes, and, in some cells, recycling endosomes. These possibilities were examined, and representative data are presented in Figs. 4 and 5. The site of accumulation of NHE3-HA was very near the location of α-mannosidase II, a marker of medial and trans-Golgi cisternae (25) (Fig. 4, A versus D). However, the location and morphology of the Golgi cisternae are distinct from that of NHE3-HA, implying that the antiporter resides in a separate compartment. Similarly, the trans-Golgi network marker TGN38 (26) was found adjacent to NHE3-HA, in a neighboring but separate compartment (Fig. 4, B versus E). The site of NHE3-HA accumulation was also readily discernible from lysosomes, which in CHO cells distribute randomly throughout the cell (Fig. 4, C and F).

The location of recycling endosomes was probed using two markers: the SNARE protein Cbv and the Tfn-R complex. The latter was detected using fluorescent Tfn, while Cbv was identified immunologically. Cells were transfected with a chimeric construct encoding the cytosolic domain of Cbv, which is responsible for intracellular targeting, fused to human IgG heavy chain (IgGH). Anti-human IgG H antibodies were used for detection. Typical results are shown in Fig. 5. As expected, both Tfn-R (Fig. 5, A and C) and Cbv (Fig. 5E) showed a punctate distribution with accumulation in a compact pericentriolar complex. The distribution of NHE3-HA overlapped extensively with those of Tfn-R and Cbv (Fig. 5), suggesting that the antiporter localizes to recycling endosomes.

Effect of Colchicine on NHE3-HA Distribution and Activity—Recycling endosomes are thought to accumulate in the vicinity of the nucleus by centripetal movement along microtubules (27). To confirm that NHE3-HA is located in juxtanuclear endosomes, cells were treated with colchicine, an agent known to disassemble microtubules. As illustrated in Fig. 6A, colchicine induced an extensive dispersal of NHE3-HA, yielding a fine punctate distribution with disappearance of the juxtanuclear accumulation. The effect of colchicine on NHE3-HA function, a measure of the number of plasmalemmal transporters, was assessed in acid-loaded cells, as above. Fig. 6B shows that, despite the noticeable rearrangement of intracellular NHE3-HA, transport activity at the surface membrane was unaffected. These findings are in good agreement with earlier observations that, while microtubule disruption results in a dispersion of the recycling endosomal compartment, it has no effect on the recycling of Tfn-R (27).
endosomal localization of 
Na+/H+ exchangers

Tfn-R is accumulated, ostensibly due to a compaction of the recycling endosomes and the trans-Golgi network (28, 29). To further characterize the compartment where NHE3-HA is localized, we treated cells with brefeldin A (5 μg/ml for 1 h) and examined the subcellular distribution and transport activity of the antipporter. Brefeldin promoted compaction and tubulation of the NHE3-HA compartment, as described earlier for endosomes (Fig. 7A). It also increased the staining at or near the surface membrane. As shown in Fig. 7B, this was accompanied by an increase in the rate of Na+-induced recovery from an acid load (initial rate = 0.24 ± 0.05 pH units/min after brefeldin A versus 0.12 ± 0.01 pH units/s in control; means ± S.E. of four determinations). Although a change in the intrinsic activity of the transporters cannot be ruled out, the data are more readily explained by an increased number of plasmalemmal exchangers, consistent with the enhanced superficial staining. It is noteworthy that while brefeldin reportedly decreases the number of Tfn-R in the membrane, it increases the amount of other recycling proteins at the surface (30–32).

Phosphatidylinositol 3-kinase is implicated in various stages of endosomal traffic (33). This has been concluded primarily from experiments using wortmannin, a toxin that is a potent inhibitor of the kinase. In CHO cells, wortmannin was recently shown to decrease the number of surface Tfn-R receptors by increasing the rate of endocytosis while decreasing exocytosis (34). In addition, wortmannin induces a marked tubulation and enlargement of endosomes containing Tfn-R and fluid phase markers (33). To evaluate the role of phosphatidylinositol 3-kinase in NHE3-HA distribution and function, cells were pre-treated with 10 nM wortmannin for 30–45 min at 37 °C. This accentuated the accumulation of NHE3-HA in the juxtanuclear region and promoted coalescence of vesicles into larger tubulo-vesicular structures. Concomitantly, the peripheral staining diminished appreciably (not illustrated). The sum of the above studies provides strong evidence that NHE3-HA behaves in a manner similar to that described for the Tfn-R and is therefore probably localized in the recycling endosomal compartment.

NHE3-HA Is Functional in Endosomes—To determine if NHE3-HA in the endomembrane compartments is functional, we took advantage of its colocalization with Cbv. Although it is accumulated primarily in endosomes, Cbv has the propensity to cycle to the plasma membrane (35). This constitutive translocation process was harnessed to deliver a pH-sensitive fluorophore specifically into the recycling compartment (see Fig. 8A for a schematic representation of this strategy). Cells expressing NHE3-HA and the Cbv-IgGH chimera described above were incubated with FITC-conjugated F(ab) fragments of antibodies to human IgGH. After binding to the extracellular domain of the chimera, the labeled antibodies were internalized and ferried retrogradely to the recycling endosomes, where they accumulate. The fluorescein moiety attached to the antibody then serves as a spectroscopic reporter of the luminal pH of the endosomal compartment, which was monitored by dual excitation ratio imaging, as detailed under “Materials and Methods.” Typical results are presented in Fig. 8, B and C. The steady-state pH of the endocytic Cbv-containing compartment (pHE) averaged 6.77 ± 0.06 (mean ± S.E., n = 4) in antiport-deficient AP-1 cells, in the range reported earlier for recycling endosomes using other approaches (12, 36). As expected, the moderate endosomal acidification was fully dissipated by the addition of concanamycin, a selective inhibitor of vacuolar H+ pumps. Similar results were obtained using fluoresceinated transferrin in TrVb1 cells (Table I).

The pH in cells transfected with NHE3-HA was significantly lower than that in AP-1 or NHE-1-expressing cells (TrVb1).
which over time accumulated in the RE by virtue of the constitutive cycling of the chimera. To attach a pH-sensitive fluorophore to Cbv, a chimeric form of this protein expressing extracellular human IgG (see top of diagram) was transfected into either AP-1 or AP-1NHE3HA cells. The extracellular IgG domain was used to attach a fluoresceinated anti-human IgG F(ab) fragment (top left), which over time accumulated in the RE by virtue of the constitutive cycling of the chimera. B and C, determination of pH_E by fluorescence imaging. Cells expressing the Cbv-human IgG chimera were incubated with FITC-conjugated anti-human IgG F(ab) for 30 min and then washed, and the label was chased for 60 min. pH was then measured by dual excitation ratio fluorescence imaging as described under “Materials and Methods.”

After acquisition of base-line values, the cells were treated with 100 nM concanamycin (arrows). Because this additional component was observed only in cells expressing NHE3-HA, we hypothesized that removal of extracellular Na^+ as a result of Na^+/H^+ exchange. In support of this notion, we found that removal of extracellular Na^+ rendered the pH_E of NHE3-transfected cells indistinguishable from that of untransfected AP-1 cells (Table I). Omission of Na^+ from the medium had no effect on pH_E in the NHE-deficient AP-1 cells. These findings indicate that the NHE3 resident in endosomes is functional and contributes measurably to the acidification of pH_E. The relative insensitivity of NHE3 to amiloride and its analogs precluded pharmacological confirmation of this conclusion.

### TABLE I

| Cellubrevin-IgG                   | Wild-type cells | NHE3-transfected cells |
|-----------------------------------|-----------------|------------------------|
| 140 mM Na^+                       | 6.77 ± 0.06 (n = 7) | 6.22 ± 0.13 (n = 7) |
| 0 mM Na^+                         | 6.70 ± 0.15 (n = 3) | 6.80 ± 0.06 (n = 3) |

| Transferrin | Wild-type cells | NHE3-transfected cells |
|-------------|-----------------|------------------------|
|             | 6.73 ± 0.02 (n = 4) | 6.14 ± 0.05 (n = 4) |

* ND, not determined.

**DISCUSSION**

Measurements of Na^+ flux or pH in subcellular fractions of epithelial cells had earlier demonstrated NHE activity in endomembrane vesicles (4, 5, 37). However, neither the type of exchanger involved nor the precise intracellular compartment is known. This is likely due to the coexistence of multiple isoforms of NHE and the complex subcellular architecture of epithelial cells. In the present study, we tried to circumvent these difficulties by transfecting individual isoforms of the exchangers into AP-1 cells. These cells are antiport-deficient, eliminating the confounding effects of multiple NHE isoforms. Moreover, AP-1 cells, which were derived from CHO cells, are nonpolar and relatively flat, facilitating visualization of intracellular compartments.

We found by immunofluorescence that NHE1 was largely present on the surface membrane of AP-1 cells (Fig. 2). This observation is consistent with results of biochemical experiments, where the predominant glycosylated form of NHE1 was quantitatively degraded by extracellular chymotrypsin2 and

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2 L. Shrode, J. Orlowski, and S. Grinstein, unpublished observations.
was readily accessible to impermeant biotin derivatives (38). By contrast, NHE3 was largely inaccessible to impermeant probes (not shown). While this may reflect a paucity of extracellularly oriented side chains, it could instead indicate that this isoform is localized predominantly in intracellular compartments. The latter interpretation is supported by the microscopic visualization of epitope-tagged antiporters, which revealed that NHE3 is located in endomembrane structures, some of which accumulate in a juxtanuclear location. Like the Golgi and TGN, the latter are maintained in a pericentriolar pattern by centripetal transport along microtubules. However, dual labeling experiments showed that the NHE3-containing vesicles are morphologically distinct from the TGN and Golgi complex (Fig. 4. A versus C and B versus E). Furthermore, treatment with brefeldin A to disperse the Golgi stacks by retrograde fusion with the endoplasmic reticulum failed to scatter the NHE3-containing vesicles.

Several lines of evidence suggest that the organelles containing NHE3 are endosomes. First, HA-tagged NHE3 was found to colocalize with Cbw and with Tfn-R, the best available endosomal markers. Secondly, like endosomes, the NHE3-containing vesicles showed juxtanuclear coalescence and tubulation upon treatment with brefeldin. Similarly, the enhancement of endosomes reported in wortmannin-treated cells occurred also in the case of NHE3 vesicles. Treatment with this drug reportedly reduces the surface exposure of Tfn-R by increasing the rate of internalization while simultaneously inhibiting exocytosis (33, 34). The inhibition of transport noted in Fig. 5B is consistent with a parallel reduction in the number of surface NHE3 molecules. Jointly, these observations indicate that NHE3 is abundant in endosomes, accumulating in a pericentriolar subpopulation, which is likely to be the recycling compartment. In this regard, it is noteworthy that the cytosolic tail of NHE3 contains a consensus endocytic sequence (YXXL), which is not present in NHE1. Endosomal accumulation is unlikely to result from overexpression of NHE3 because NHE1, which was expressed at comparable levels (Fig. 1B), was restricted to the plasma membrane. Moreover, overexpression of proteins with endocytic sequences promotes their accumulation in the surface membrane and not in endosomes (39).

There is precedent for the colocalization of Cbw with transporters believed to reside in apical endosomes. Aquaporin-2, an epithelial isoform of the water channel, was reported to be present in vesicles also expressing Cbw (40). The colocalization of NHE3 with Tfn-R is, at first glance, unexpected. In epithelial cells, the bulk of the Tfn-R is found in basolateral membranes and endosomes. In these cells, separate subpopulations of basolateral and apical endosomes have been postulated to exist (11, 13). However, this notion has been recently challenged in studies that reported mixing of the two types of endosomes (8, 10). Finally, the apical endosomal protein endotubulin was also found to co-segregate with Tfn-R in pericentriolar endosomes (11). Thus, the colocalization of NHE3 with Tfn-R in heterologous expression systems possibly reflects the endosomal localization of this exchanger in native systems.

Measurements of pH$_{e}$ using two different probes indicated that NHE3 is active in endosomes, transporting luminal Na$^{+}$ in exchange for cytosolic H$^{+}$. Sustained endosomal acidification via NHE requires a continuous supply of luminal Na$^{+}$. Luminal Na$^{+}$ could be provided by the Na$^{+}$/K$^{+}$-ATPase, which has been reported to be active in the endosomal membrane (41, 42). However, this is not a universal observation (4), and a recent report using Cbw constructs could not detect Na$^{+}$ pump activity in recycling endosomes (43). Alternatively, Na$^{+}$ could be continuously provided by pinocytosis of Na$^{+}$-rich extracellular fluid. The latter possibility is consistent with the rapid distribution of the bafilomycin-insensitive component of endosomal acidification upon removal of the extracellular Na$^{+}$ (Table I). Whether NHE3 contributes to endosomal acidification in epithelial cells awaits direct confirmation; however, an amiloride-insensitive NHE has been shown to modulate the acidification of rat liver endocytic vesicles immediately after their formation (37).

The observation that NHE3 can reside in recycling endosomes raises the possibility that regulation of its activity may be mediated by recruitment of transporters to and from the plasma membrane. A similar mechanism has been proposed for the regulation of epithelial water and H$^{+}$ transport by aquaporin-2 and H$^{+}$/K$^{+}$-ATPases, respectively (44, 45). In accordance with this notion, Mircheff and colleagues (46–48) have reported a shift in the density of the vesicular compartment expressing NHE3 following treatment of renal cells with parathyroid hormone or after the induction of hypertension. These observations are consistent with an intracellular redistribution of the exchangers following treatment with agents that reduce the rate of transport. Moreover, stimulation of NHE3 by epidermal growth factor was found to be sensitive to wortmannin (49), suggesting that vesicular traffic may be involved. This conclusion should be tempered by earlier findings that phospatidylinositol 3-kinase also participates in the growth factor activation of NHE1, an isoform present almost exclusively at the surface membrane (50). Nevertheless, the possibility that NHE3 may be regulated by modulation of vesicular traffic is attractive.
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