Active (9.6 S) and Inactive (21 S) Oligomers of NHE3 in Microdomains of the Renal Brush Border*

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We have previously shown that Na\(^+\)-H\(^+\) exchanger isoform NHE3 exists as both 9.6 and 21 S (megalin-associated) oligomers in the renal brush border (1). To characterize the oligomeric forms of the renal brush border Na\(^+\)-H\(^+\) exchanger in more detail, we performed membrane fractionation studies. We found that similar amounts of NHE3 were present in microvilli and a non-microvillar membrane domain of high density (dense vesicles). Horseradish peroxidase-labeled endosomes were not prevalent in the dense membrane fraction. However, megalin, which localizes primarily to the intermicrovillar microdomain of the brush border, was enriched in the dense vesicles, implicating this microdomain as the likely source of these membranes. Immunolocalization of NHE3 confirmed that a major fraction of the transporter colocalized with megalin in the intermicrovillar region of the brush border. Immunoprecipitation studies demonstrated that in microvilli the majority of NHE3 was not bound to megalin, while in the dense vesicles most of the NHE3 coprecipitated with megalin. Moreover, sucrose velocity gradient centrifugation experiments revealed that most NHE3 in microvilli sedimented with an S value of 9.6, while the S value of NHE3 in dense vesicles was 21. Finally, we examined the functional state of NHE3 in both membrane fractions. As assayed by changes in acridine orange fluorescence, imposing an outwardly directed Na\(^+\) gradient caused generation of an inside acid pH gradient in the microvilli, indicating Na\(^+\)-H\(^+\) exchange activity, but not in the dense vesicles. Taken together, these data demonstrate that renal brush border NHE3 exists in two oligomeric states: a 9.6 S active form present in microvilli and a 21 S, megalin-associated, inactive form in the intermicrovillar microdomain of the apical plasma membrane. Thus, regulation of renal brush border Na\(^+\)-H\(^+\) exchange activity may be mediated by shifting the distribution between these forms of NHE3.

In the kidney, the activity of the Na\(^+\)-H\(^+\) exchanger isoform NHE3, located on the apical microvillar membrane of the proximal tubule, plays a major role in mediating transepithelial bicarbonate and NaCl reabsorption (2–4). Numerous physiological studies of brush border Na\(^+\)-H\(^+\) exchange have shown that this activity is regulated by such hormones as angiotensin II (5) and parathyroid hormone (6) as well as by systemic alterations in acid-base balance (2, 3). Several laboratories have presented evidence suggesting that NHE3 is regulated by posttranslational mechanisms that may include membrane trafficking between an intracellular compartment and the plasma membrane (7–9). Such models predict that NHE3 must be localized in a nonmicrovillar membrane compartment, which functions as a store of transporter, as well as on the microvillar membrane where NHE3 is active.

We have recently reported an association between NHE3 and the putative scavenger receptor megalin in renal brush border membranes (1). Moreover, we found that renal brush border NHE3 exists in two states with distinct sedimentation coefficients, a 9.6 S megalin-free form and a 21 S megalin-bound form (1). The purpose of the present study was to use membrane fractionation methods to determine whether these two oligomeric forms of NHE3 are expressed in distinct microdomains of the renal brush border and, if so, to compare the functional activity of NHE3 in these microdomains. Our findings are consistent with a model in which the 9.6 S form of NHE3 is present in the microvillar membrane and is active, whereas the 21 S megalin-associated form is concentrated in the intermicrovillar microdomain of the brush border and is inactive. Thus, regulation of renal brush border Na\(^+\)-H\(^+\) exchange activity may be mediated by shifting the distribution between these forms of NHE3.

MATERIALS AND METHODS

Antibodies to NHE3—In a previous paper, we described in detail the development and characterization of isoform-specific monoclonal antibodies (mAbs)\(^{1}\) to a restricted region of the carboxyl terminus of NHE3 (10). mAbs 2B9, 4F5, and 19F5 were raised to a fusion protein (fpNHE3–702-832) that reproduced the C-terminal 131 amino acids of rabbit NHE3 (11). mAb 2B9 was used throughout the present study. As shown in Fig. 1, this antibody immunoprecipitates and immunoblots the same protein that is precipitated and blotted by mAb 2B9, namely NHE3. Since this antibody is also specific for NHE3, it was used to immunoblot NHE3 in immune complexes precipitated with the mAbs (see Fig. 8).

A polyclonal antibody, raised in a goat to fpNHE3–702-832, was also used in this study. As shown in Fig. 1, this antibody immunoprecipitates and immunoblots the same protein that is precipitated and blotted by mAb 2B9, namely NHE3. Since this antibody is also specific for NHE3, it was used to immunoblot NHE3 in immune complexes precipitated with the mAbs (see Fig. 8).

A polyclonal antibody raised to a synthetic peptide representing amino acids 809–831 of rat NHE3 was a gift from Dr. Mark Knepper (National Institutes of Health, Bethesda, MD) (12). This antibody was used as affinity-purified IgG.

Antibodies to Megalin—In a previous paper (1) we described the development and characterization of mAb 10A3 to rabbit megalin. This antibody was used for immunoblotting and immunoprecipitation in

\(^{1}\) The abbreviations used are: mAb, monoclonal antibody; HRP, horseradish peroxidase; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl) ethynglycine; PLP, paraformaldehyde-lysine-periodate; TBS, Tris-buffered saline; MES, 4-morpholineethanesulfonic acid.
Homogenization was performed using a Potter-Elvehjem with a loose-fitting Teflon pestle. The homogenate was centrifuged for 15 min at 1900 × g using an SS-34 rotor. The supernatant was saved and again centrifuged for 20 min at 21,000 × g using the same rotor. The supernatant and the upper, light portion of the pellet were saved. These membranes, referred to as postmitochondrial microsomes, were collected by centrifugation for 30 min at 48,000 × g in the same rotor. The membrane pellets were resuspended in 5% OptiPrep™ (see below) at a concentration of 10–20 mg/ml.

The protease inhibitors (Sigma) pepstatin A (0.7 μg/ml), leupeptin (0.5 μg/ml), phenylmethylsulfonyl fluoride (40 μg/ml), and EDTA (1 mM) which were included in all membrane preparations, were special stock solutions using 20 mM Tricine (pH 7.8) and 8% sucrose according to the manufacturer’s protocols. Preformed OptiPrep™ gradients were made using a Gradient Master™ (Bio-comp Inc., New Brunswick, Canada). 1–5 mg of postmitochondrial microsomes (see above) in 5% OptiPrep™ were layered on the top of 15–25% OptiPrep™ gradients. Gradients were centrifuged to equilibrium (at least 2 h) at 100,000 × g for 21,000 × g using an SW 41 rotor in a Beckman ultracentrifuge. 1-ml fractions were manually collected from the top and stored at −70 °C. For analysis by immunoblotting and immunoprecipitation, equal volumes of each fraction were used.

Labeling Endosomes in the Proximal Tubule with HRP—Renal endosomes were labeled with HRP as follows. Harlan Sprague-Dawley rats were anesthetized with sodium pentobarbital intraperitoneal. Following an abdominal incision, 25 mg of HRP (Sigma) in 1 ml of phosphate-buffered saline was injected into the mesenteric vein. This vein was used because of its accessibility. 5–10 min after injecting the tracer, the kidneys were perfused in a retrograde manner via the abdominal aorta. When kidneys were used for membrane fractionation studies, they were perfused with homogenization buffer (see above). When the kidneys were used for morphological studies they were perfused with paraformaldehyde-lysine-periodate (PLP) fixative as described previously (20).

Tissue Preparation for Electron Microscopy and Immunocytochemistry—Rats were anesthetized with sodium pentobarbital injected intravenously, and the kidneys were perfusion-fixed with PLP fixative (21) as described previously (20).

For the HRP tracer studies, 30-μm cryosections were prepared and incubated in diaminobenzidine. Sections were then prepared for electron microscopy as described previously (20). For light microscopic studies, Epon sections (0.5 μm) were cut with a glass knife, stained with toluidine blue, and examined using a Zeiss Axioshot microscope. For electron microscopy, ultrathin sections of Epon embedded tissue were cut and examined using a Zeiss 910 electron microscope.

Indirect immunofluorescence microscopy was performed using either serial cryosections of fixed tissue or paraffin sections of the same tissue that was further subjected to antigen retrieval. Cryosections were prepared and stained exactly as described previously (10). For antigen retrieval, fixed tissue was embedded in paraffin. 0.5–2.0-μm thick sections were cut using a glass knife mounted on the sectioning stage of a Reichert ultramicrotome. Sections were mounted on glass coverslips, deparaffinized using xylene, and rehydrated in graded ethanol and phosphate-buffered saline. Sections were next microwaved in buffer containing 10 mM citrate in Tris-buffered saline at 40% power for 20 min. After washing in TBS, the sections were further denatured using 1% SDS in TBS for 5 min. After washing in TBS the sections were immunolabeled as described previously (10).

Immunoprecipitation—Immunoprecipitation of soluble renal proteins was performed essentially as described previously (22). Renal membranes were solubilized at 4 °C in TBS (pH 7.4) containing 1% Triton X-100 and protease inhibitors phenylmethylsulfonyl fluoride, leupeptin, pepstatin, and EDTA as described above. The samples were cleared of insoluble material by centrifugation (15,000 × g for 10 min) using a table top centrifuge (Hermle™ model Z230M, National Labnet Co., Woodbridge, NJ). To the above supernatants were added primary antibody, 50 μg for mAbs or 10 μg of serum. When immunoprecipitating across sucrose gradients, the primary antibodies were added directly to the gradient fractions. Primary antibodies were allowed to incubate at 4 °C for 1 h. Immune complexes were collected using 5 μl/sample of Protein G-Sepharose 4B (Amersham Pharmacia Biotech). The beads were washed five times in solubilization buffer and then incubated in 50 μl of SDS-PAGE sample buffer for 1–3 min at 100 °C, followed by heat treatment for 5 min. The beads were then resuspended in sample buffer and analyzed by SDS-PAGE.
and the samples were prepared for SDS-PAGE and immunoblotting. Although we have found that heating NHE3 increases its tendency to form aggregates (see Fig. 7), heating is necessary in these experiments to completely reduce IgG to monomeric heavy and light chains.

Sucrose Velocity Gradient Centrifugation—Velocity gradient sedimentation was carried out according to Copeland et al. (23). Membranes were suspended in lysis buffer (pH 7.4) containing 20 mM MES, 30 mM Tris, 100 mM NaCl, and 1% Triton X-100. The samples were applied to the top of 5–25% continuous sucrose gradients. Sucrose solutions were prepared with lysis buffer containing 0.1% Triton X-100. After centrifugation for 12 h at 4 °C at 200,000 × g, the samples were fractioned by hand from the top. Sucrose concentrations of each fraction were calculated from the refractive index. Sedimentation coefficients were determined by comparison with standard proteins with known S values (aldolase, \( s_{20, w} = 7.3 \); catalase, \( s_{20, w} = 11.3 \); horse spleen apoferritin, \( s_{20, w} = 16.5 \); and bovine thymoglobulin, \( s_{20, w} = 19.3 \)) or by using the formula \( s_{20, w} = \frac{D}{t^2} \), where \( I \) is the time integral, \( \omega \) is rotor speed (radians/s), and \( t \) is time, as described by Griffith (24).

Buffers (Tris and MES), Triton X-100, and apoferritin were purchased from Sigma. Aldolase, catalase, and thymoglobulin were from Amersham Pharmacia Biotech.

SDS-PAGE and Immunoblotting—Protein samples were solubilized in SDS-PAGE sample buffer and separated by SDS-PAGE using 7.5% polyacrylamide gels according to Laemmli (25). For immunoblotting, proteins were transferred to polyvinylidene difluoride (Millipore Immobilon-P) at 500 mA for 6–10 h at 4 °C with a TransphorTM transfer electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA) and the samples were prepared for SDS-PAGE and immunoblotting. Immunoblotting was performed as follows. Sheets of polyvinylidene difluoride containing transferred protein from entire gels were incubated first in Blotto (5% nonfat dry milk in phosphate-buffered saline, pH 7.4) for 1–3 h to block nonspecific binding of antibody, followed by overnight incubation in primary antibody. Primary antibodies, diluted in Blotto, were used at dilutions ranging from 1:1000 to 1:5000. The sheets were then washed in Blotto and incubated for 1 h with an appropriate HRP-conjugated secondary antibody diluted 1:2000 in Blotto. After washing three times in Blotto, once in phosphate-buffered saline (pH 7.4), and once in distilled water, bound antibody was detected with the ECLTM chemiluminescence system (Amersham Pharmacia Biotech) according to the manufacturer’s protocols. In some experiments, polyvinylidene difluoride blots were reprobed with additional primary antibodies (see Fig. 2) after stripping away the first antibody. This was accomplished by incubating the polyvinylidene difluoride sheets in 2% SDS, 100 mM β-mercaptoethanol, 50 mM Tris (pH 6.9) for 60 min at 70 °C.

Measuring Na⁺/H⁺ Exchange in Membrane Vesicles—Na⁺/H⁺ exchange was measured by monitoring the fluorescence change of the weak base dye acridine orange (26). These experiments were designed so that the H⁺ modifier site (27) would be equally activated regardless of the topological orientation of the membrane vesicles. The vesicles were loaded with either 100 mM Na₂SO₄ or 100 mM (NH₄)₂SO₄, buffered with 10 mM Hepes, pH 6.5, by preincubination in these solutions for 2 h at 37 °C. In all experiments, the protein concentrations of the samples were matched. The final protein concentration was ~10 mg/ml. After loading, samples (10 μl) were added to cuvettes containing 9.5 μM acridine orange, 100 mM K₂SO₄, and 10 mM Hepes, pH 6.5. Thus, an outward Na⁺/H⁺ gradient was imposed in the absence of an initial pH gradient. Generation of an inside acid pH gradient was monitored by measuring fluorescence quenching at room temperature by use of a PerkinElmer Life Sciences spectrofluorometer (excitation, 493 nm; emission, 535 nm).

RESULTS

Separation of NHE3-containing Dense Membranes—To examine the subcellular location of NHE3 in the proximal tubule, we evaluated the distribution of NHE3 in rat renal cortical microsomes following their separation by density gradient centrifugation. In the experiment illustrated in Fig. 2, fractions from 15–25% OptiPrep™ density gradients were analyzed by immunoblotting using antibodies to proteins known to be markers for specific subcellular domains within cells of the proximal tubule. As shown in Fig. 2, NHE3 was detected in membranes with two distinct densities. A relatively light fraction was found at the top of the gradient and had the same density as that of microvillar markers such as villin and the Na-Pi cotransporter, NaPi-2. A second peak of NHE3 was found in very dense membranes, which lacked a distinct peak of other microvillar markers but which overlapped peaks of megalin and clathrin. This experiment implied that in the proximal tubule a major fraction of total NHE3 is located in a nonmicrovillar membrane compartment represented by the dense membranes.

NHE3-rich Dense Membranes Are Not Derived from Endosomes—Studies from other laboratories have suggested that regulation of NHE3 might involve trafficking to the plasma membrane (microvilli) from an endosomal compartment (7, 28, 29). To determine if the NHE3-rich dense membranes are derived from endosomes, we labeled the endosomal compartment of the proximal tubule with HRP (Fig. 3, A and B) and examined the density of labeled endosomes in the OptiPrep™ gradient. As seen in Fig. 3 (A and B), when the tracer was localized ultrastructurally it was found only in endosomes. It is important to note that the tracer was never found in tubular lumina, where it might be artificially trapped during homogenization (see Fig. 3A). Importantly, as seen in Fig. 3C, the HRP-labeled endosomes were predominantly found at the top of the gradient and did not have the same density as the...
NHE3-rich dense membranes. These data indicate that the NHE3-rich, dense membranes are not derived from endosomes.

Identification of an Intermicrovillar Pool of NHE3 by Immunocytochemistry—The most direct approach to identify NHE3-containing membranes is to localize the transporter using immunocytochemistry. Indeed, we previously demonstrated by immunoelectron microscopy with anti-NHE3 monoclonal antibodies that NHE3 is almost exclusively expressed on the microvilli of the renal brush border surface (10). However, we subsequently found that the epitopes to which these antibodies bind on NHE3 are not available when NHE3 was complexed with megalin (1). Hence, under the usual conditions for immunocytochemistry, these antibodies may have failed to detect sites of NHE3 expression where NHE3 is complexed with megalin.

Accordingly, we sought to utilize antigen retrieval as a method to expose the megalin-associated NHE3 in the proximal tubule. Figs. 4 and 5 compare staining of rat kidney with the same anti-NHE3 mAb (2B9) using cryosections (Fig. 4), as described previously (10), or on paraffin sections following denaturing by antigen retrieval (Fig. 5). In this experiment, double labeling was performed with anti-NHE3 mAb (2B9) and polyclonal antibodies to megalin or the microvillar enzyme γ-glutamyltranspeptidase. As shown previously, in the proximal tubule, staining for NHE3 on cryosections is restricted to the microvilli and colocalizes with staining for γ-glutamyltranspeptidase (Fig. 4A). In contrast, double labeling with an anti-megalin antibody shows little if any overlap (Fig. 4B).

However, following antigen retrieval, intense staining for NHE3 was seen at the base of the microvilli (Fig. 5, A and B). In double labeling experiments on these sections, the staining for NHE3 more closely overlapped that of megalin (Fig. 5B) than that of γ-glutamyltranspeptidase (Fig. 5A). These data strongly suggest that the dense, NHE3-rich membranes identified by density centrifugation are derived from the intermicrovillar region of the brush border.

To verify the existence of a pool of NHE3 in the intermicrovillar region of the brush border in the absence of antigen retrieval, we also performed immunocytochemical studies utilizing a rabbit anti-NHE3 polyclonal antibody developed by Knepper and co-workers (12). As shown in the double labeling experiment in Fig. 6, this anti-NHE3 antibody (seen in green) is distinct from the anti-NHE3 mAb 2B9 (seen in red) and stains a pool of NHE3 that was at the base of the microvilli. This staining pattern is consistent with that previously described by Kwon et al. using the same antibody (31). This observation indicates that the findings in Fig. 4 were not artifactual due to the antigen retrieval procedure and confirms the identification of an intermicrovillar plasma membrane pool of NHE3.

Biochemical Comparison of NHE3-rich Dense Membranes and Microvilli—The preceding membrane fractionation and immunocytochemistry experiments indicated that there are two pools of NHE3 in the brush border of the rat proximal tubule. One population is located in the microvillar membrane, while the other is located in the intermicrovillar membrane, the latter corresponding to the dense membranes found with...
density fractionation. We next evaluated biochemical properties of these two membrane fractions.

It should be noted that we have observed proteolytic activity in the rat but not the rabbit kidney that degrades NHE3 when solubilized in nonionic detergents (data not shown). Therefore, these and subsequent studies were performed using membrane preparations isolated from rabbit kidney.

For the experiment illustrated in Fig. 7, renal cortical microvilli were isolated by divalent cation aggregation (17), and dense membranes were isolated by centrifugation on an Opti-Prep™ gradient as above (fractions 5–7 in Fig. 2). Then equal quantities of protein from each preparation were separated by SDS-PAGE and subjected to immunoblotting using antibodies to the microvillar protein villin, to the predominantly intermicrovillar protein megalin, and to NHE3. As shown in Fig. 7, although villin was enriched in the microvillar fraction and megalin in the dense membrane fraction, staining of NHE3 was approximately equal in both membrane preparations. This experiment shows that although the microvillar and intermicrovillar microdomains of the proximal tubule brush border are biochemically distinct, as first demonstrated by Rodman et al. (32), the level of expression of NHE3 appears to be nearly equal between these two domains.

We previously demonstrated that renal brush border NHE3 exists in two states with distinct sedimentation coefficients, a 9.6 S megalin-free form, and a 21 S megalin-bound form (1). We next sought to determine the relative abundance of these oligomeric forms of NHE3 in the microvillar and intermicrovillar microdomains of the brush border.

To determine if the two oligomeric forms of NHE3 are differentially expressed in microvilli and dense (intermicrovillar) membranes, we performed immunoprecipitation experiments using equal quantities of both membrane preparations. Megalin-associated NHE3 (21 S form) was identified by coprecipitation with the anti-megalin mAb 10A3 (1). NHE3 not associated with megalin (9.6 S form) was identified by precipitation with the anti-NHE3 mAb 2B9, which binds to an epitope that is not available when NHE3 is complexed with megalin (1). As shown in Fig. 8, the anti-NHE3 mAb precipitated more NHE3 from the microvilli than from the dense membranes. In contrast, the anti-megalin mAb coprecipitated more NHE3 from the dense membranes than from microvilli. Indeed, Fig. 8 demonstrates that in the dense membranes more NHE3 could be precipitated by the anti-megalin mAb than by the anti-NHE3 mAb, indicating that the majority of NHE3 in this membrane domain is complexed with the receptor.

Since we had previously shown that the sedimentation coefficient for megalin-free NHE3 is 9.6 S, whereas for megalin-associated NHE3 is 21 S (1), we performed density velocity centrifugation to confirm the differential localization of these forms in microvilli and dense membranes. In the experiment illustrated in Fig. 9, equal quantities of solubilized microvilli and dense membranes were subjected to density velocity centrifugation, and then each fraction was analyzed by immunoblotting for the presence of NHE3. Shown in Fig. 9, the predominant form of NHE3 in microvilli had an S value of 9.6, while the majority of NHE3 in the dense membranes had an S value of 21. Taken together with the preceding immunoprecipitation data, these findings indicate that distinct oligomeric forms of NHE3 are differentially expressed in the microdomains of the renal brush border: the megalin-free 9.6 S form in microvilli and the 21 S megalin-associated form in dense (intermicrovillar) membranes.

Physiologic Comparison of NHE3-rich Dense Membranes and Microvilli—The fact that there are two distinct oligomeric forms of NHE3 that are differentially located within microdomains of the renal brush border led us to postulate that these forms may have different functional characteristics. Therefore, we next sought to compare the transport activity of the 9.6 S microvillar form of NHE3 with the 21 S intermicrovillar oligomer of the transporter. Again, rabbit microvilli were prepared using the divalent cation precipitation method, and rabbit dense vesicles were collected from appropriate fractions of

**Fig. 6.** Indirect immunofluorescence microscopy localizing NHE3 in the proximal tubule using antibodies to two epitopes within the C terminus of NHE3. PLP-fixed rat kidney was prepared for cryosectioning. Sections were double-labeled for NHE3 using mAb 2B9 (the epitope lies between amino acids 702 and 756) and rabbit anti-NHE3 synthetic peptide (the epitope lies between amino acids 809 and 831). Staining with mAb 2B9 is seen in red (arrowheads), while the staining with the polyclonal antibody is seen in green (small arrows). Note that there is little overlap in the staining patterns of the two anti-NHE3 antibodies.

**Fig. 7.** Immunoblots comparing the protein composition of dense membranes with microvilli. 50 µg of either microvillar membrane vesicles (M) or dense membranes (DV) (fractions 5–7 in Fig. 2) were separated by SDS-PAGE and prepared for immunoblotting. Lanes were probed using mAbs to villin, megalin, and NHE3.
OptiPrep™ density gradients. To avoid variability between animals, microvilli were prepared from one kidney and the dense vesicles from the other kidney of the same animals. Na\(^+\)-H\(^+\) exchange transport activity was assayed as the rate of generation of an inside acid pH gradient in response to an outward Na\(^+\) gradient. Generation of a pH gradient was monitored by measurements of fluorescence of the weak base dye probe acridine orange (26). Because the topology of the dense (intermicrovillar) membranes is not known, membrane vesicles were initially equilibrated at pH 6.5 to ensure that the cytoplasmic H\(^+\) modifier site (27) was equally activated whether the membranes were right-side-out, as is known to be the case for microvilli (17), or inside-out.

As shown in the right panel of Fig. 10, when Na\(^+\)-loaded dense membranes were added to the cuvette, the outward Na\(^+\) gradient failed to generate a significant inside acid pH gradient. Importantly, rapid generation of an inside acid pH gradient was observed when NH\(_4\)\(^+\)-loaded dense membranes were added to the cuvette, resulting from efflux of NH\(_3\) by nonionic diffusion. This pH gradient was collapsed by the addition of NH\(_4\)\(^+\) to the external medium after 200 s. One trivial explanation for the inability of the outward Na\(^+\) gradient to generate a pH gradient was that the dense membranes were leaky or not in the form of sealed vesicles. The fact that an outward NH\(_4\)\(^+\) gradient was capable of generating an inside acid pH gradient indicates that sealed membrane vesicles were indeed present. Thus, the inability of an outward Na\(^+\) gradient to generate an inside acid pH gradient in the dense membranes indicates that Na\(^+\)-H\(^+\) exchange activity is low or absent in this preparation. In contrast, as shown in the left panel of Fig. 10, an outward Na\(^+\) gradient generated a large pH gradient in microvillar membranes, indicating brisk Na\(^+\)-H\(^+\) exchange activity as previously observed by use of similar assays (26). Thus, although the level of expression of NHE3 is similar in microvilli and dense membranes (as shown in Fig. 7), Na\(^+\)-H\(^+\) exchange activity was only detected in the microvillar membranes. These findings imply that the 9.6 S megalin-free form of NHE3 that predominates in microvilli is active, whereas the 21 S megalin-bound form of NHE3 that predominates in the dense (intermicrovillar) membranes is inactive.

**DISCUSSION**

This study describes the localization of two oligomeric forms of NHE3 to distinct microdomains of the renal brush border. Using density centrifugation combined with specific brush border antibodies, we have shown that a 9.6 S oligomer of NHE3 is predominately expressed on microvilli, while a 21 S, megalin-associated form is concentrated in the intermicrovillar region of the brush border. As shown by Rodman et al. (32), this is a

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**Fig. 8. Immunoprecipitation of NHE3 and the NHE3-megalin complex from microvilli and dense membranes.** 100 µg of microvillar membrane vesicles (Microvilli) or NHE3-rich, dense membranes (Dense Vesicles) made from rabbits were solubilized in 1% Triton X-100 in TBS and prepared for immunoblotting. Immunoprecipitations were performed with antibodies to NHE3 (mAb 2B9) or to megalin (mAb 10A3). Immune complexes were prepared for immunoblotting, and the blot was probed with goat anti-NHE3. Molecular weights, expressed as 10\(^-3\) \(M_\text{r}\), are presented on the left. NHE3 is seen as either a monomer (arrowhead) or an aggregate (small arrow). Note that in the dense membranes more NHE3 coprecipitates with the anti-megalin antibody (10A3) than can be precipitated with the anti-NHE3 antibody (2B9).

**Fig. 9. Sucrose velocity gradient centrifugation to assess size of NHE3 oligomers in microvilli and dense membranes.** 500 µg of microvillar membrane vesicles (Microvilli) or NHE3-rich, dense membranes (Dense Vesicles) were solubilized in 1% Triton X-100 in Tris/MES buffer (see "Materials and Methods"). After centrifugation, the supernatant was applied to the top of 5–25% sucrose gradients, and the gradients were centrifuged for 12 h at 200,000 \( \times \) g using an SW 41 rotor. 0.75-ml fractions were collected across the gradients, and 50 µl of each were prepared for SDS-PAGE and immunoblotting. Blots were probed for NHE3 using mAb 2B9.

**Fig. 10. Na\(^+\)-H\(^+\) exchange activity measured by acridine orange fluorescence in microvilli and dense membranes.** Microvillar membrane vesicles (A) and NHE3-rich, dense membranes (B) were prepared from rabbits, and the samples were matched for total protein. In both panels fluorescence units (abscissa) are expressed as a function of time (ordinate). Squares, recordings from membranes that were loaded with Na\(_2\)SO\(_4\); circles, recordings from membranes that were loaded with (NH\(_4\))\(_2\)SO\(_4\). Note that in A an outwardly directed Na\(^+\) gradient acidifies the intravesicular space of the microvilli and results in quenching of the dye. There is no such activity in B when a similar gradient is imposed using the NHE3-rich, dense membranes.
distinct microdomain of the apical plasma membrane of the proximal tubule. In addition, transport studies indicate that NHE3 is active in microvilli but inactive in intermicrovillar membranes.

The mAb to NHE3 (2B9) that was used in this study is specific to a region within the C terminus of the transporter that lies between amino acids 702 and 756 (10). This region of NHE3 is blocked from antibody binding when the transporter is complexed with megalin (1). The fact that epitopes within the C-terminal region of NHE3 are blocked when NHE3 is complexed with megalin raises questions regarding the interpretation of previous immunocytochemical studies using antibodies made to the C terminus of NHE3. Most (10, 33, 34) but not all (12) of these studies localized NHE3 largely to the microvillar membrane. In fact, when used for immunoelectron microscopy, our mAbs failed to label the intermicrovillar microdomain of the renal brush border (10). We hypothesized that the apparent discrepancy between previous immunocytochemical studies and the present membrane fractionation study, showing large amounts of NHE3 in intermicrovillar membranes, may have resulted from the masking of C-terminal epitopes on NHE3 when the transporter is complexed with megalin. Our present immunocytochemical studies using antigen retrieval show this to be the case. When kidney sections were denatured during the antigen retrieval procedure, we visualized a large pool of NHE3 that colocalized with megalin in the intermicrovillar region of the brush border, consistent with our membrane fractionation data showing a major pool of NHE3 in megalin-rich, nonmicrovillar (and nonendosomal) dense membranes. Immunocytochemical localization of this pool of NHE3 was not an artifact of the antigen retrieval procedure, since similar staining was found without antigen retrieval by use of a polyclonal anti-NHE3 antibody raised to a peptide encompassing the C-terminal 22 amino acids of NHE3 (12).

Previous studies have localized Na\(^+\)/H\(^+\) exchange activity to association with megalin and sequestration in the coated pits as a prelude to endocytosis in proximal tubule cells. In conclusion, we have demonstrated that renal brush border NHE3 exists in two oligomeric states: a 9.6 S active form present in microvilli and a 21 S, megalin-associated, inactive form in the intermicrovillar microdomain. Future studies will be needed to examine whether regulation of renal brush border Na\(^+\)/H\(^+\) exchange activity may be mediated by shifting the distribution between these forms of NHE3.
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J. Biol. Chem. 2001, 276:10161-10167.
doi: 10.1074/jbc.M008098200 originally published online December 18, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M008098200

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