Specific Association of Megalin and the Na⁺/H⁺ Exchanger Isoform NHE3 in the Proximal Tubule*

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Daniel Biemesderfer†, Tamas Nagy, Brenda DeGray, and Peter S. Aronson
From the Departments of Internal Medicine and of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut 06520-8029

We investigated whether the renal brush border Na⁺/H⁺ exchanger NHE3 exists in assemblies with other proteins in native kidney membranes. To this end we generated monoclonal antibodies (mAbs) against affinity purified NHE3 protein complexes. Hybridomas were selected based on ability to immunoprecipitate NHE3. One of the resulting mAbs (10A3) labeled a high molecular mass (>200 kDa) protein and stained primarily the coated pit region of the proximal tubule in a manner similar to that described for megalin (gp330). We then confirmed that both mAb 10A3 and a known anti-megalin mAb immunoprecipitated and immunoblotted the same protein, namely megalin. mAb 10A3 specifically co-precipitated NHE3 but not villin or NaPi-2 from solubilized renal membranes, indicating specificity of the NHE3-megalin interaction. When immunoprecipitations were performed using either 10A3 or anti-NHE3 mAb 2B9 after separation of solubilized renal proteins by sucrose velocity gradient centrifugation, we found that NHE3 exists in two states with distinct sedimentation coefficients, a 9.6 S megalin-free form and a 21 S megalin-bound form, and that when NHE3 assembles with megalin, epitopes within the carboxyl-terminal 131 amino acids of NHE3 are blocked. Taken together, these findings indicate that a significant pool of NHE3 exists as a multimeric complex with megalin in the brush border of the proximal tubule.

Na⁺/H⁺ exchangers (NHEs) mediate the electroneutral, amiloride-sensitive exchange of Na⁺ and H⁺ across plasma membranes in most eukaryotic cells. In the mammalian kidney this activity is particularly important as it contributes to the maintenance of acid-base balance and NaCl homeostasis. Although there are at least four NHE isoforms expressed in the kidney (1–8), recent studies from this laboratory (7, 9, 10) and from other (11) laboratories have shown that Na⁺/H⁺ exchanger isoform NHE3 is present on the apical (brush border) membrane of the proximal tubule and is the isoform responsible for most, if not all, of the described Na⁺/H⁺ exchange activity in this membrane domain (12).

Transporters often exist as components of multimeric protein complexes that stabilize their localization in specific membrane domains and/or ensure proximity to signaling pathways (13–15). Recent studies have shown that two homologous proteins known as NHE-RF (Na⁺/H⁺ exchanger regulatory factor) and E3KARP (NHE3 kinase A regulatory protein) play critical roles in the cAMP-mediated inhibition of NHE3 (16, 17). Both proteins have PDZ domains that may interact directly with NHE3 or may be adapters that link NHE3 to the cytoskeleton (18). Much of the data provided by these studies have been derived from experiments in cell culture or from yeast two-hybrid analysis.

We therefore investigated whether NHE3 exists in assemblies with other proteins in native kidney membranes. We report that the sedimentation coefficient for NHE3 solubilized from renal brush border membranes is greater than predicted for monomeric NHE3, indicating the presence of multimeric complexes. Moreover, by use of a strategy involving the generation of monoclonal antibodies to immunopurified NHE3 complexes, we find that a significant pool of NHE3 exists in association with the putative scavenger receptor megalin.

MATERIALS AND METHODS

Antibodies to NHE3—In a previous paper we described in detail the development and characterization of monoclonal antibodies to a restricted region of the carboxyl terminus of NHE3 (9). mAbs 2B9, 4F5, and 19F5 were raised to a fusion protein (fpNHE3–702–832) that reproduced the carboxyl-terminal 131 amino acids of the rabbit Na⁺/H⁺ exchanger NHE3 (1). By study of Na⁺/H⁺ exchanger-deficient LAP cells transfected with each of NHE1–4, we demonstrated that these three mAbs are specific for the 80-kDa NHE3 polypeptide (9). No cross-reactivity was observed to other NHE isoforms or any other proteins. By immunocytochemistry, all three mAbs stained the brush border of proximal tubule cells (9). These mAbs were used as purified IgG from hybridoma supernatants.

A polyclonal antibody, raised in guinea pigs to fpNHE3–702–832, was also used in this study. This antibody is also specific for NHE3 (19) and was used to immunoblot NHE3 in immune complexes precipitated with the mAbs.

Additional Primary Antibodies—A mAb raised to NHE1 (4E9) has been described previously (19). This antibody was raised to a fusion protein representing the carboxyl terminus (amino acids 514–818) of perine NHE1 (4). A mAb (mouse IgG) to villin was purchased from AmAC (Westbrook, ME). This mAb was raised to the carboxyl-terminal headpiece region of purified pig villin (20). A polyclonal antibody raised to the renal brush border Na-P cotransporter, NaPi-2, was provided by Drs. Heini Murer and Jurg Biber, Institute of Physiology, University of Zurich-Irchel, Zurich, Switzerland (21). A mAb raised to megalin was provided by Drs. Dotsenko Kerjaschki and Markus Eknner, University of Vienna, Austria (35).

Antibody Conjugates—Fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (heavy and light chain), used at 10 μg/ml, was purchased from Zymed Laboratories Inc. (San Francisco, CA). Horseradish peroxidase-conjugated rabbit anti-guinea pig IgG (heavy and light chain-specific), goat anti-mouse (γ chain-specific), or goat anti-rabbit (heavy and light chain-specific) were purchased from Zymed Laboratories Inc. (San Francisco, CA).
ries Inc. (San Francisco CA) and used at 0.5 μg/ml.

Preparation of Rabbit Renal Membrane Fractions—Adult male New Zealand White rabbits (sacrificed by intravenous injection of sodium pentobarbital (Butler Co., Columbus, OH)). Brush border membrane vesicles (BBMV) or microsomes were isolated from renal cortex as described previously (7). Protease inhibitors (Sigma) pepstatin A (0.7 μg/ml), leupeptin (0.5 μg/ml), phenylmethylsulfonyl fluoride (40 μg/ml), and EDTA (1 mM) each were included in the preparation. Protein concentrations were determined by the method of Lowry et al. (22).

Sucrose Velocity Gradient Centrifugation—Velocity gradient sedi- mentation was carried out according to Copeland et al. (23). Rabbit renal microsomes were solubilized in lysis buffer (pH 7.4) containing 20 mM MES, 30 mM Tris, 100 mM NaCl, and 1% Triton X-100. Insoluble material was removed by centrifugation at 200,000 g in a Beckman ultracentrifuge for 1 h at 4 °C. The supernatants were applied to the top of a 5–25% continuous sucrose gradients. Sucrose solutions were prepared with lysis buffer containing 0.1% Triton X-100. After centrifuga- tion for 12 h at 4 °C, 40,000 rpm in an SW 41 rotor, the gradients were fractionated by hand from the top. Sucrose concentrations of each fraction were calculated from the refractive index. Sedimentation coeffi- cients were determined by comparison to 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 thyroglobulin, $s_{20, w} = 19.3$), or by using the equation $s = 0.167$ (Coomassie blue dye intensity/molecular mass). In the top of the gradient, $s$ is the top of the gradient, $t$ is the time, as described by Griffith (24). Buffers (Tris and MES), Triton X-100, and apoferritin were purchased from Sigma. Aldolase, catalase, and thyroglobulin were from Amer- sham Pharmacia Biotech.

Immunofluorescence Purification of Native NHE3—An anti-NHE3 affin- ity matrix was prepared by cross-linking affinity purified mAb 2B9 to protein A-Sepharose CL-4B beads (Amersham Pharmacia Biotech) with dimethylpimelimidate dihydrochloride (Sigma) according to the “two layer” method described by Schneider et al. (25). Rabbit anti-mouse IgG (Zymed Laboratories Inc.) was used as a bridging antibody.

For purification of native NHE3 complexes, brush border membrane vesicles were solubilized at 4 °C in 0.1% octyl glucoside (Roche Molecular Biochemicals) in Tris-buffered saline (pH 7.4). Samples were cleared by centrifugation at 15,000 g for 10 min. The supernatants were applied to the affinity matrix and allowed to incubated overnight in the cold (4 °C). The column was washed extensively with solubilization buffer and monitored for protein by measuring the absorbance at 280 nm using a Gilford Spectrophotometer 260 (Gilford Instruments, Inc., Oberlin, OH). When the wash buffer was determined to be free of unbound protein, bound NHE3 complexes were eluted using glycine in PBS/Triton X-100 and once in PBS/BSA/Triton X-100. Protein samples were solubilized in SDS-PAGE sample buffer and separated by SDS-PAGE using 7.5% polyacrylamide gels according to Laemmli (28). For immunoblotting, proteins were transferred to PVDF (Millipore Immobilon-P) at 500 mA for 6–10 h at 4 °C with a Transphor™ transfer electrophoresis unit (Hoefer Scientific Instruments, San Francisco) and stained with Pon- yene (Sigma) for 1 h. Membranes were washed 3× in solubilization buffer and once in PBS (pH 7.4) at 20 °C.

Tissue Preparation for Immunocytochemistry—Rabbits were anes- thetized with sodium pentobarbital injected intravenously, and the kidneys were perfusion-fixed with paraformaldehyde/lysine/periodate fixative (29) as described previously (30). Fixed tissue was processed and stained using the immunoperoxidase method exactly as described previously (30). 0.5% screening hybridomas, the culture supernatant or hybridoma media, which served as controls, were used undiluted for labeling. Thin sections of Epon-embedded tissue were examined using a Zeiss 910 electron microscope.

RESULTS

Solubility Properties of Renal Na⁺/H⁺ Exchangers—Our ini- tial studies were designed to establish the optimal conditions for solubilizing the apical Na⁺/H⁺ exchanger NHE3 from rabbit renal membranes. In these experiments, rabbit renal mi- crosomes were solubilized with several nonionic (Triton X-100, octyl glucoside, and C₁₂E₄) or zwitterionic (CHAPS) detergents and then subjected to differential centrifugation. The resulting fractions were analyzed by immunoblotting. For comparison, we assessed the solubility of both NHE3 and the basolateral Na⁺/H⁺ exchanger, NHE1, which is also expressed at high levels in rabbit renal cortex (8).

We assessed solubility based on the presence of protein in the supernatant following centrifugation. In this study, low speed centrifugation is defined as 15,000 × g for 10 min, and high speed centrifugation is carried out at 200,000 × g for 1 h. Therefore, the presence of a protein in the high speed super- natant indicates complete solubilization. In Fig. 1, lane 1 rep- resents proteins present in the low speed pellet, lane 2 the high
Fractions were collected, and 100 μl of supernatant was applied to the top of a 5–25% sucrose gradient, and the gradient was centrifuged in a Ti41 rotor at 40,000 g for 12 h at 4 °C. 0.75-ml samples (7 mg) were solubilized in 10 ml of MES buffer containing 1% Triton X-100. After centrifugation at 200,000 g for 10 min, the resulting low speed pellet is seen in panels A and B, lane 1. The resulting supernatant was centrifuged for 1 h at 200,000 × g. The high speed pellet is seen in lane 2, and 1/10 of the high speed supernatant is seen in lane 3. The blots were then stripped of bound antibody, and the blot was reprobed with a polyclonal antibody to NHE3 (panel B).

Fig. 1: Western blots showing the solubility of renal Na/H exchangers. Rabbit renal cortical microsomes (200 μg) were added to buffer (TBS, pH 7.4) containing either no detergent, 1% Triton X-100, 4% CHAPS, 2% octyl glucoside, or 0.1% C12E8. Incubation was performed at 4 °C for 30 min. Samples were centrifuged at 15,000 × g for 10 min, and the resulting low speed pellet is seen in panels A and B, lane 1. The resulting supernatant was centrifuged for 1 h at 200,000 × g. The high speed pellet is seen in lane 2, and 1/10 of the high speed supernatant is seen in lane 3. The blots were then stripped of bound antibody, and the blot was reprobed with a polyclonal antibody to NHE3 (panel B).

Speed pellet, and lane 3 the high speed supernatant.

As indicated in Fig. 1, the solubility properties of NHE1 and NHE3 are different. Although NHE1 can be detected in the high speed pellets following CHAPS and octyl glucoside solubilization, most of this isoform is soluble (present in the high speed supernatant) in Triton X-100 and C12E8 (Fig. 1, panel A). In contrast, NHE3 was completely insoluble (present in the pellets) when we used CHAPS or octyl glucoside and was only partially soluble in Triton X-100 and C12E8 (Fig. 1, panel B). Such detergent insolubility of membrane proteins has frequently been shown to result from specific protein-protein (cytoskeletal) (14) or protein-lipid (31) interactions.

Sucrose Velocity Gradient Centrifugation—Studies of Na+/H+ exchangers expressed in cell culture have shown them to form homomultimeric assemblies (32). Also, since the data presented in Fig. 1 suggested that NHE3 may interact with other brush border proteins, we next sought to estimate the size of the native renal Na+/H+ exchanger. To this end, we determined the sedimentation coefficients of NHE1 and NHE3 using sucrose velocity gradient centrifugation (Fig. 2). In these studies, the high speed Triton X-100 supernatant, prepared as described for Fig. 1, was applied to the top of 5–25% sucrose gradients, and the samples were centrifuged for 12 h as described under “Materials and Methods.” The distribution of the Na+/H+ exchangers (NHE1 and NHE3) in the gradient was determined by probing immunoblots of the fractions with isoform-specific antibodies. Their sedimentation coefficients were calculated and compared with standard proteins with known S values (Fig. 2). NHE1 sedimented as a single discrete peak with a sedimentation coefficient of approximately s20,w = 6.5. In contrast, NHE3 had a very broad distribution with a significantly higher sedimentation coefficient. These data suggest that NHE3 exists in multimeric assemblies under these conditions. Moreover, the broad distribution of NHE3 in the gradient suggests that these assemblies are heterogeneous.

Affinity Isolation of the Native NHE3 Transporter Complex and Preparation of Monoclonal Antibodies—In order to evaluate further the possibility that NHE3 exists in assemblies with other proteins, we generated mAbs to the native NHE3 complex as a strategy to identify proteins that specifically associate with the Na+/H+ exchanger. The NHE3 transporter complex, solubilized with C12E8, was isolated using an affinity column prepared with mAb 2B9 raised to the carboxyl terminus of rabbit NHE3 (9). Mice were immunized and boosted with the column eluate purified from approximately 1 mg of rabbit brush border membrane vesicles. Hybridomas were prepared using standard protocols, and their supernatants were screened using successive assays that included ELISA, immunoblotting, immunocytochemistry, and immunoprecipitation. Our goal was to first identify mAbs that were capable of immunoprecipitating native NHE3. Once these were identified, each antibody was characterized in order to determine if it was specific for NHE3 or an associated protein. Of the approximately 180 hybridomas that were positive by ELISA to rabbit
brush border membranes, we identified 10 mAbs that stained the brush border of the proximal tubule by immunocytochemistry and that immunoprecipitated NHE3 from Triton X-100 or C12E8-solubilized brush border membrane vesicles.

In order to identify mAbs that were directed to NHE3, we performed immunoblotting experiments using microsomes prepared from LAP cells that had been transfected with NHE3 or other isoforms of NHE as controls (6, 7). Only one of the 10 mAbs (3G11) labeled NHE3 by immunoblotting (data not shown). This mAb also cross-reacted with NHE1, indicating that its epitope is shared between the two isoforms. Of the remaining 9 mAbs, one mAb (10A3) has been characterized in detail, and these studies are presented below.

Characterization of mAb 10A3—Although mAb 10A3 immunoprecipitated NHE3 (see Fig. 5), when used for immunoblotting it labeled a very large molecular weight protein that migrated by SDS-PAGE above the 200-kDa standard near the top of the 7.5% gel (Fig. 3, panel A). When used for immunocytochemistry, this mAb also exhibited a unique staining pattern. As seen by immunoelectron microscopy in Fig. 3, panel B, although some staining was detected on microvilli, most of the staining was restricted to the coated pit region of the brush border of proximal tubules. No staining was observed in the controls (data not shown). These observations raised the possibility that the 10A3 antigen may be the putative scavenger receptor megalin, which is located most abundantly in the coated pit region of the renal brush border (33) and which has a predicted molecular mass of over 500 kDa (34).

In order to test this hypothesis, we evaluated whether mAb 10A3 and a well characterized anti-megalin mAb (DC6) (35) would immunoprecipitate the same protein from solubilized renal microsomes. Indeed, as indicated by the solid arrow in Fig. 4, both 10A3 and DC6 immunoprecipitated the same protein that was visualized on the immunoblot by staining with both antibodies. This large (>200 kDa) protein was not precipitated by a control mAb (anti-villin). The smaller protein bands observed in the lower part of the blot (open arrow in Fig. 4) were due to staining by the anti-mouse IgG secondary antibody of the heavy chains of the primary antibodies (including the control) used for precipitation. The data in Fig. 4 confirm that mAbs 10A3 and DC6 are both directed at the same renal protein, namely megalin.

Specificity of NHE3-Megalin Association—During hybridoma screening, mAb 10A3 had been selected based on its ability to co-precipitate NHE3 from the low speed (15,000 × g for 10 min) supernatants of Triton X-100-solubilized renal microsomes. But, as demonstrated earlier (Fig. 1), a significant amount of NHE3 could be pelleted from detergent-solubilized membranes when centrifuged at high speed for a longer time (200,000 × g for 1 h), indicating that some of the transporter is found in large, insoluble aggregates. Co-localization of NHE3 and megalin in such insoluble aggregates might account for their apparent co-precipitation from the low speed supernatants of detergent-solubilized renal membranes. Therefore, it was important to verify that NHE3 and megalin could also be co-precipitated from the truly soluble supernatant after high speed centrifugation. In addition, because megalin is thought to be a multi-ligand receptor (36–41), we considered the possibility that solubilized megalin might associate with multiple renal membrane proteins nonspecifically. Accordingly, it was important to test whether other brush border proteins could be co-precipitated with megalin.

To address these issues, we performed immunoprecipitation experiments (shown in Fig. 5) using both the low speed and

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**Fig. 3. Characterization of mAb 10A3.** mAb 10A3 was used for immunoblotting (panel A) and immunocytochemistry (panel B) in rabbit kidney. Rabbit renal microsomes (100 μg) were separated by SDS-PAGE and prepared for immunoblotting (panel A). mAb 10A3 stained primarily a high molecular mass (>200 kDa) protein. Panel B shows the 10A3 antigen localized at the level of the electron microscope using the immunoperoxidase method. Shown here is the apical region of two proximal tubule cells. The electron dense reaction product is localized to the microvilli, the coated pit region of the brush border, and within the endosome-like compartments (arrows). Magnification × 16,000.

**Fig. 4. The 10A3 antigen is megalin.** Rabbit renal microsomes (100 μg) were solubilized in TBS containing 1% Triton X-100. After clearing by centrifugation at 15,000 × g, the supernatants were subjected to immunoprecipitation using either mAb 10A3, DC6, or anti-villin (negative control). The immune complexes were separated by SDS-PAGE and prepared for immunoblotting. The blots were stained with either mAb 10A3 or DC6.
The peak of NHE3 precipitated with anti-carboxyl-terminal antibody binding.

High speed supernatants of Triton X-100-solubilized renal microsomes. In addition to antibodies to NHE3 (mAb 2B9) and megalin (mAb 10A3), we used antibodies to the microvillar core protein villin and the brush border Na⁺,P⁻ cotransporter NaPi-2, or a mAb to the microvillar core protein villin. Immune complexes, as well as a sample (100 μg) of rabbit renal cortical microsomes (lane M), were prepared for immunoblotting, and the blot was probed successively with a polyclonal antibody to NHE3 that was bound to megalin and that mAb 2B9 could not detect, an additional immunoprecipitation was performed from the same sample using mAb 10A3. As seen in the 4th lane of this figure, a significant amount of NHE3 (that could not be precipitated by anti-NHE3 mAb 2B9) was co-precipitated with megalin.

In order to test directly this hypothesis (Fig. 6), we subjected an aliquot of solubilized BBMV to repeated precipitation with anti-NHE3 mAb 2B9 (1st three lanes). After three precipitations, all of the NHE3 available for binding to this mAb was removed. Then, in order to determine if there was any remaining NHE3 that was bound to megalin and that mAb 2B9 could not detect, an additional immunoprecipitation was performed from the same sample using mAb 10A3. As seen in the 4th lane of this figure, a significant amount of NHE3 (that could not be precipitated by anti-NHE3 mAb 2B9) was co-precipitated with megalin.

We sought to examine further these pools of NHE3 by estimating their sedimentation coefficients using sucrose velocity gradient centrifugation. We predict that NHE3 that is assembled with megalin will have a greater sedimentation coefficient than “free” NHE3. Therefore, we subjected the high speed supernatant of Triton X-100-solubilized microsomes to sedimentation through 5–25% sucrose gradients. Fractions were collected across the gradients, and one-half of each fraction was subjected to immunoprecipitation with either mAb 2B9 or mAb 10A3. The presence of NHE3 in the resulting immune complexes was analyzed by immunoblotting, as shown in Fig. 7. The peak of NHE3 precipitated with anti-carboxyl-terminal antibody binding was associated with megalin. The immune complexes were washed, prepared for immunoblotting, and the blots probed for NHE3 using an anti-NHE3 polyclonal antibody. NHE3 (arrow) appears as an 80-kDa band.

megalin even from the high speed, well solubilized supernatant indicates that the association between the two proteins does not result from their co-localization in large insoluble membrane aggregates. Moreover, the observation that neither villin nor NaPi-2 co-precipitated with megalin indicates that the NHE3-megalin association does not reflect promiscuous binding of solubilized megalin to brush border proteins nonspecifically.

**NHE3 Exists in Megalin-bound or Megalin-free States**—Although Fig. 5 shows specific NHE3-megalin interaction, it also demonstrates that when our anti-NHE3 mAbs directed to the carboxyl-terminal 131 amino acids are used to immunoprecipitate NHE3, we cannot co-immunoprecipitate megalin. One explanation for these data is that renal brush border NHE3 exists in two forms that are structurally different. One form is not associated with megalin and is available for binding (immunoprecipitation) with our anti-NHE3 mAbs. A second form is associated with megalin in such a way that the carboxyl-terminal epitopes of the Na⁺/H⁺ exchanger are blocked from antibody binding.
understanding of the renal function of this very large (38), vitamin B12 complexes (40), and calcium (43), a complete binding various ligands including plasminogen (49), albumin Ca2+ data show that the interaction of NHE3 with megalin is not megalin required Ca2+ reported ligands for megalin, including that of the receptor-protein in the kidney (43). In fact, binding of most of the proteins due its proposed protein scavenger function (38). To ad-

rect interactions with other poorly soluble cellular elements that NHE3 and megalin were co-precipitated because of indirect artifact. Specifically, we wanted to rule out the possibilities that NHE3 and megalin were co-precipitated because of indi-

Discussion

In this study we have shown that the renal brush border Na+/H+ exchanger (NHE3) exists as part of at least two distinct oligomeric units. In one form the Na+/H+ exchanger has a sedimentation coefficient of 9.6 S and can be immuno-precipitated by specific mAbs to carboxyl-terminal epitopes (amino acids 702–832) within the transporter. A second form of NHE3 has a sedimentation coefficient of 21 S and cannot be immuno-precipitated by the carboxyl-terminal mAbs. This second form of the Na+/H+ exchanger is part of a molecular complex which includes the brush border protein megalin.

Megalin, also called gp330 and Heymann nephritis antigen, was first identified by Kerjaschki and Farquhar as the autoantigen for Heymann nephritis in 1982 (45). These early studies, showing megalin to be concentrated in the clathrin-coated pits of the renal brush border, suggested that it might function as a receptor in the proximal tubule (Ref. 46; for reviews see Refs. 36 and 47). This notion has been supported by molecular cloning studies that show megalin to be a member of the low density lipoprotein receptor gene family (34, 48). However, although in vitro studies have shown megalin to be capable of binding various ligands including plasminogen (49), albumin (38), vitamin B12 complexes (40), and calcium (43), a complete understanding of the renal function of this very large (>500 kDa) glycoprotein is still incomplete.

Because our finding of an association between NHE3 and megalin was unexpected, we sought to exclude carefully the possibility that our observation was a result of experimental artifact. Specifically, we wanted to rule out the possibilities that NHE3 and megalin were co-precipitated because of indirect interactions with other poorly soluble cellular elements such as the cytoskeleton and/or resulted from promiscuous nonspecific binding of megalin to solubilized brush border proteins due its proposed protein scavenger function (38). To ad-

dress these concerns, we demonstrated (see Figs. 5 and 7) that NHE3 could be co-precipitated with megalin even from the high speed supernatant of Triton X-100-solubilized renal microsomes. These are conditions that should pellet any insoluble cytoskeletal elements. The interaction of NHE3 and megalin did not appear to result from a general protein scavenger function of megalin since we were unable to detect this association with other cytoskeletal (villin) or membrane (NaPi-2) proteins of the brush border.

The interaction of NHE3 with megalin may represent a novel class of binding to megalin. The extracellular domain of megalin contains four putative ligand-binding domains, each of which consists of cysteine-rich repeats that are characteristic of the low density lipoprotein receptor gene family (34). To date, the best characterized binding partner of megalin is receptor-associated protein, which binds with high affinity in a Ca2+-
dependent manner and competes with all previously known ligands for megalin (35). Recent studies by Orlando and co-workers (35) have identified the second cluster of ligand-binding repeats (specifically in the region of amino acids 1111–1210) as the probable binding domain for receptor-associated protein and many, if not most, of the known ligands. The fact that in our study the interaction of NHE3 with megalin is not Ca2+-dependent suggests that this interaction may be mediated through a different binding domain within megalin. The fact that epitopes within the carboxyl-terminal hydrophilic domain of NHE3 (amino acids 702–832) are blocked in this complex raises the possibility that this region of NHE3 represents part of the domain mediating association with megalin itself or linking protein(s).

Although both NHE-RF and E3KARP are thought to interact with NHE3, the direct relationship of these proteins with either NHE3 or megalin in kidney is not known. Lamprecht and co-workers (18) have suggested that these proteins function as adapters that link NHE3 to cytoskeletal elements such as ezrin. Since ezrin binds protein kinase A type II and since NHE3 is phosphorylated by protein kinase A, these authors propose that such linkage is a mechanism whereby protein kinase A is brought into close proximity to NHE3. In future studies, it will be important to determine if NHE-RF and/or E3KARP are part of either the 9.6 S or 21 S pools of NHE3.

The fact that there is a significant pool of NHE3 in the

![Fig. 7. Sucrose velocity gradient centrifugation identifies two pools of NHE3.](image_url)

![Fig. 8. Association of NHE3 and megalin is not Ca2+-dependent.](image_url)
proximal tubule that is inaccessible to antibodies raised to regions of the carboxyl terminus of the transporter raises questions regarding previously published studies that immunocolocalized NHE3 in the kidney (7, 9, 11). Since all of these studies utilized antibodies raised to the carboxyl terminus of NHE3, there is a distinct possibility that these studies did not detect all of the NHE3 present in the proximal tubule. In particular, the inaccessibility of available anti-NHE3 antibodies to detect NHE3 associated with megalin may explain the absence of detectable staining for NHE3 in coated pits and coated vesicles in previous studies (9). A complete description of the localization of NHE3 in the proximal tubule will require immunocytochemical studies using antibodies that are capable of binding both free and megalin-bound forms of the brush border Na+/H+ exchanger. Such future studies will be important for evaluating the possibility that association with megalin is involved in the described regulation of NHE3 activity by endocytosis in proximal tubule cells (50, 51).

REFERENCES

1. Tse, C. M., Brant, S. R., Walker, M. S., Pouyssegur, J., and Donowitz, M. (1992) J. Biol. Chem. 267, 9384–9386
2. Tse, C. M., Levine, S. A., Yun, C. H., Montrose, M. H., Little, P. J., Pouyssegur, J., and Donowitz, M. (1993) J. Biol. Chem. 268, 11917–11924
3. Sardet, C., Franchi, A., and Pouyssegur, J. (1989) Cell 56, 273–280
4. Reilly, R. F., Hildebrandt, F., Biemesderfer, D., Sardet, C., Pouyssegur, J., Aronson, P. S., Slayman, C. W., and Igarashi, P. (1991) Am. J. Physiol. 261, F1088–F1094
5. Orlofski, J., Kandasamy, R. A., and Shull, G. E. (1992) J. Biol. Chem. 267, 9331–9339
6. Pizzonia, J. A., Biemesderfer, D., Abu-Alfa, A. K., Wu, M.-S., Exner, M., Isenring, P., Igarashi, P., and Aronson, P. S. (1998) Am. J. Physiol. 275, F510–F517
7. Biemesderfer, D., Pizzonia, J., Abu-Alfa, A., Exner, M., Reilly, R., Igarashi, P., and Aronson, P. S. (1998) Am. J. Physiol. 265, F736–F742
8. Biemesderfer, D., Reilly, R. F., Exner, M., Igarashi, P., and Aronson, P. S. (1992) Am. J. Physiol. 263, F833–F840
9. Biemesderfer, D., Rutherford, P. A., Nagy, T., Pizzonia, J. H., Abu-Alfa, A. K., and Aronson, P. S. (1997) Am. J. Physiol. 273, F289–F299
10. Wu, M. S., Biemesderfer, D., Caplan, M., and Forbush, B. D. (1988) Kidney Int. 33, 899–913
11. Amemiya, M., Loffing, J., Lutscher, M., Kaissling, B., Murer, H., and Aronson, P. S. (1993) Exp. Nephrol. 5, 490–497
12. Dudoit, B., Robine, S., Huet, C., Sahhuqillo-Merino, C., Blair, L., Coudrier, E., and Louvard, D. (1987) J. Biol. Chem. 265, 359–369
13. Levi, M., Lutscher, M., Sorribas, V., Custer, M., Arar, M., Kaissling, B., Murer, H., and Biener, J. (1994) Am. J. Physiol. 267, F900–F908
14. Lowry, O. H., Rosebrough, N. H., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
15. Copeland, C. S., Doms, R. W., Bolzau, E. M., Webster, R. G., and Helenius, A. (1986) J. Cell Biol. 103, 1179–1191
16. Griffith, O. M. (1989) Techniques of Preparative Zonal, and Continuous Flow Ultracentrifugation, Beckman Instruments, Palo Alto, CA
17. Schneider, C., Newman, R. A., Sutherland, D. R., Asser, U., and Greaves, M. F. (1992) J. Biol. Chem. 257, 10766–10769
18. Kasgarian, M., Biemesderfer, D., Caplan, M., and Forbush, B. D. (1985) Kidney Int. 28, 899–913
19. Biemesderfer, D., DeGray, B., and Aronson, P. S. (1998) J. Biol. Chem. 273, 12391–12396
20. Loemlil, U. K. (1970) Nature 227, 680–685
21. McLean, W., and Nakane, P. P. (1973) J. Histochem. Cytochem. 22, 1077–1083
22. Biemesderfer, D., Dekan, G., Aronson, P. S., and Farquhar, M. G. (1992) Am. J. Physiol. 262, F55–F67
23. Simons, K. and Ikonen, E. (1997) Nature 387, 569–572
24. Fafournoux, P., Noel, J., and Pouyssegur, J. (1994) J. Biol. Chem. 269, 2589–2596
25. Kerjaschki, D., Sherkey, D. J., and Farquhar, M. G. (1984) J. Cell Biol. 98, 1591–1596
26. Saito, A., Pietromonaco, S., Lee, A. K., and Farquhar, M. G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9725–9729
27. Biemesderfer, D., DeGray, B., and Aronson, P. S. (1998) J. Biol. Chem. 273, F900–F907
28. Laemmli, U. K. (1970) Nature 227, 680–685
29. McClean, W., and Nakane, P. P. (1973) J. Histochem. Cytochem. 22, 1077–1083
30. Biemesderfer, D., Dekan, G., Aronson, P. S., and Farquhar, M. G. (1992) Am. J. Physiol. 262, F55–F67
31. Simons, K. and Ikonen, E. (1997) Nature 387, 569–572
32. Fafournoux, P., Noel, J., and Pouyssegur, J. (1994) J. Biol. Chem. 269, 2589–2596