Association of Na\(^{+}\)-H\(^{+}\) Exchanger Isoform NHE3 and Dipeptidyl Peptidase IV in the Renal Proximal Tubule*

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In an attempt to identify proteins that assemble with the apical membrane Na\(^{+}\)-H\(^{+}\) exchanger isoform NHE3, we generated monoclonal antibodies (mAbs) against affinity-purified NHE3 protein complexes isolated from solubilized renal microvillus membrane vesicles. Hybridomas were selected based on their ability to immunoprecipitate NHE3. We have characterized in detail one of the mAbs (1D11) that specifically co-precipitated NHE3 but not villin or NaPi-2. Western blot analyses of microvillus membranes and immunoelectron microscopy of kidney sections showed that mAb 1D11 recognizes a 110-kDa protein highly expressed on the apical membrane of proximal tubule cells. Immunofluorescent and immunoelectron microscopy was used to isolate the antigen against which mAb 1D11 is directed. N-terminal sequencing of the purified protein identified it as dipeptidyl peptidase IV (DPPIV) (EC 3.4.14.15), which was confirmed by assays of DPPIV enzyme activity. We also evaluated the distribution of the NHE3-DPPIV complex in microdomains of rabbit renal brush border. In contrast to the previously described NHE3-megalin complex, which principally resides in a dense membrane population (coated pits) in which NHE3 is inactive, the NHE3-DPPIV complex was predominantly in the microvillar fraction in which NHE3 is active. Serial precipitation experiments confirmed that anti-megalin and anti-DPPIV antibodies co-precipitate different pools of NHE3. Taken together, these studies revealed an unexpected association of the brush border Na\(^{+}\)-H\(^{+}\) exchanger NHE3 with dipeptidyl peptidase IV in the proximal tubule. These findings raise the possibility that association with DPPIV may affect NHE3 surface expression and/or activity.

The majority of NaCl, NaHCO\(_3\) and water filtered by the kidney is reabsorbed in the proximal tubule. Na\(^{+}\)-H\(^{+}\) exchange is the predominant mechanism for absorption of Na\(^{+}\) and secretion of H\(^{+}\) across the apical membrane of proximal tubule cells (1). Several lines of evidence indicate that NHE3 is the Na\(^{+}\)-H\(^{+}\) exchanger isoform responsible for most, if not all, apical membrane Na\(^{+}\)-H\(^{+}\) exchange activity in this segment of the nephron (2–7). This isoform thereby plays an important role in the maintenance of fluid and electrolyte balance, and its activity is regulated in response to a wide variety of acute and chronic physiologic stimuli (8–11).

The polarized expression and regulation of a transporter such as NHE3 necessarily involves interactions with other proteins. Recent studies have indicated that NHE3 is capable of binding calmodulin (12), the NHE3 regulatory factor (NHERF) (11, 13) and its homologue, exchanger-3 kinase A regulatory protein (E3KARP) (14), and the calcineurin B homologous protein (15). These interactions have generally been characterized in nonepithelial cells transfected to overexpress NHE3.

We have been investigating whether NHE3 exists in assemblies with other proteins in native kidney membranes. We previously reported that the sedimentation coefficient for NHE3 solubilized from renal membranes is greater than predicted for monomeric NHE3, indicating the presence of multimeric complexes (16). To identify proteins that are associated with NHE3 in native renal kidney membranes, we generated monoclonal antibodies (mAbs) against affinity-purified NHE3 protein complexes from solubilized microvillus membranes. The study of one of these antibodies, mAb 10A3, demonstrated that a significant pool of NHE3 exists in a complex with the scavenger receptor megalin in the kidney brush border (16). We subsequently demonstrated that NHE3 is expressed in different microdomains of the brush border and that the NHE3-megalin complex principally resides in a dense membrane compartment most likely representing coated pits (17).

We now report the detailed characterization of a second mAb (1D11) that specifically co-precipitates NHE3 from solubilized microvillus membranes isolated from rabbit renal cortex. We demonstrate that the antigen for mAb 1D11 is dipeptidyl peptidase IV (DPPIV, also known as CD26) and thus that NHE3 resides in an oligomeric complex with DPPIV in the renal proximal tubule. We also show that the NHE3-DPPIV complex is primarily located in the microvillar microdomain of the kidney brush border. These findings raise the possibility that association with DPPIV may affect NHE3 surface expression and/or activity.

MATERIALS AND METHODS

Antibodies to NHE3—In a previous publication, we described in detail the development and characterization of monoclonal antibodies to a restricted region of the C terminus of NHE3 (18). mAb 2B9 was raised to a fusion protein (fpNHE3–702-832) that reproduced the C-terminal 131 amino acids of rabbit NHE3. This antibody was used as purified IgG from hybridoma supernatants. For immunoblotting experiments, we also used a previously characterized anti-NHE3 polyclonal

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The abbreviations used are: NHE, Na\(^{+}\)-H\(^{+}\) exchanger; NHERF, NHE regulatory factor; mAb(s), monoclonal antibody(ies); DPPIV, dipeptidyl peptidase IV; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride.
antibody raised in goats to fpNHE3—702-832 (17).

Additional Primary Antibodies—In a previous paper, we described the development and characterization of mAb 10A3 to rabbit megalin (16). A mAb (mouse IgG) to villin was purchased from AMAC, Inc. (Westbrook, Maine). A polyclonal antibody raised to the renal brush border membrane protein NaPi-IIa, NaPi-IIc, NaPi-IIe from Dr. Hein Murer and Dr. Jurg Biber, Institute of Physiology, University of Zurich-Irchel, Zurich, Switzerland (19).

Antibody Conjugates—Horseradish peroxidase-conjugated rabbit anti-goat (heavy and light chain-specific), goat anti-mouse (γ chain-specific) and goat anti-rabbit (heavy and light chain-specific) were purchased from Zymed Laboratories Inc. (San Francisco, CA) (Fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (heavy and light chain) was also purchased from Zymed Laboratories Inc. (San Francisco, CA). Immunoaffinity Chromatography for N-terminal Sequencing—In a previous paper, we described the generation of mAbs against affinity-purified NHE3 complexes as well as the enzyme-linked immunosorbent assay performed for screening the hybridoma supernatants that have been previously described in detail (16). mAbs were purified from hybridoma supernatants by affinity chromatography using protein G-Sepharose 4B (Pharmacia Biotech, Inc. Piscataway, NJ) according to manufacturer’s protocols. Purified monoclonal antibody (1–10 mg/ml) to which was added bovine serum albumin at 1 mg/ml was stored in 50% glycerol/phosphate-buffered saline at −20 °C.

Immunoprecipitation—Immunoprecipitation experiments were carried out essentially as described previously (21). Rabbit renal microvillus membranes were solubilized at 4 °C in TBS buffer, pH 7.4, containing 1% Triton X-100 and protease inhibitors as described above. The samples were subjected to either low speed (15,000 × g for 10 min) or high speed (200,000 × g for 1 h) centrifugation using a table top (Hermle™ model Z230M, National Labnet Co., Woodbridge, NJ) centrifuge or a Beckman ultracentrifuge, respectively. The pellets were resuspended in 2% SDS, 50 mM Tris-buffer, pH 6.9, and fixed tissue was processed and stained using the immunoaffinity chromatography eluate from SDS-PAGE and transferred to a PVDF membrane as above, the single major band at 110 kDa after Coomassie Blue staining was cut from the blot for N-terminal amino acid sequencing by Edman degradation at the HHMI Biopolymer Laboratory and W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University.

Immunoperoxidase—Antibody Conjugates for Immunoblotting—Antibody conjugates were prepared using a method based on Mgo− precipitation and differential centrifugation as previously described (20).

Immunoprecipitation—Immunoprecipitation experiments were carried out essentially as described previously (21). Rabbit renal microvillus membranes were solubilized at 4 °C in TBS buffer, pH 7.4, containing 1% Triton X-100 and protease inhibitors as described above. The samples were subjected to either low speed (15,000 × g for 10 min) or high speed (200,000 × g for 1 h) centrifugation using a table top (Hermle™ model Z230M, National Labnet Co., Woodbridge, NJ) centrifuge or a Beckman ultracentrifuge, respectively. 50 μg of primary antibodies were added to the supernatants, and the samples were incubated at 4 °C for 1 h. Immune complexes were collected by centrifugation at 10,000 × g for 1 h. The pellets were resuspended in 5 mg/sample of protein G-Sepharose 4B (Amersham Pharmacia Biotech). The beads were washed five times in solubilization buffer and then prepared for SDS-PAGE and immunoblotting.

SDS-PAGE and Immunoblotting—Protein samples were solubilized in SDS sample buffer, and proteins were separated by SDS-PAGE using 7.5% polyacrylamide gels according to Laemmli (22). For immunoblotting, proteins were transferred to polyvinylidene difluoride (PVDF), Immobilon-P; Millipore, Bedford, MA) from polyacrylamide gels at 500 mA for 80 min at 25 °C. The membranes were then washed five times in Blotto and incubated for 1 h with horseradish peroxidase-conjugated IgG from Zymed Laboratories Inc. Bound antibody was detected with ECL-enhanced chemiluminescence (Amersham Pharmacia Biotech) according to manufacturer’s protocols. Hyperfilm-MP (Amersham Pharmacia Biotech) exposed to membranes for 1–3 min, were developed in an x-ray film processor (100 Plus, All Pro Imaging Corp). In some experiments membranes were stripped and reprobed with additional primary antibodies (see Fig. 6A). The stripping procedure consists of incubating the PVDF membranes in 2% SDS, 50 mM Tris-buffer, pH 6.9, 100 mM β-mercaptoethanol for 60 min at 70 °C.

Tissue Preparation for Immunocytochemistry—Rabbit kidneys were perfused with pyridoxal phosphate fixative containing 2% paraformaldehyde, 75 mM phosphate buffer, pH 7.4, and fixed tissue was processed and stained using the immunoperoxidase method exactly as described previously (23). Ultrathin sections of Epon-embedded tissue were examined using a Zeiss 910 electron microscope.

Immunofluorescence Chromatography for N-terminal Sequencing—An immunofluorescence chromatography matrix was prepared by covalently coupling mAb 1D11 to protein A-Sepharose CL-4B (Pharmacia) through a rabbit anti-mouse IgG bridge (24). Microvillus membrane vesicles (1 mg) solubilized in 1% Triton X-100, 0.7 μg/ml pepstatin A, 0.5 μg/ml leupeptin, 40 μg/ml phenylmethylsulfonyl fluoride and 1 mM K2EDTA in phosphate-buffer saline were incubated with the immunofluorescence chromatography eluate. The supernatant was washed until salt wash buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 0.1% Triton X-100, pH 7.4, 20 °C), four times with RIPA buffer (20 mM Tris-Cl, 0.5% Triton X-100, 1% deoxycholate, 0.1% SDS, 150 mM NaCl, 2.5 mM Na2EDTA, pH 7.4, 4 °C), and finally twice more with salt wash buffer. Bound antigen was eluted by incubating the matrix with Immunofluorescence elution buffer (50 mM glycine, 4% HCl, 0.1% Triton, pH 2.7) for 2 min at 4 °C. The eluate was immediately neutralized with 1 M Tris base. This was repeated four times, and the eluates were then subjected to neutralization. After separation of the immunofluorescence chromatography eluate by SDS-PAGE and transfer to a PVDF membrane as above, the single major band at 110 kDa after Coomassie Blue staining was cut from the blot for N-terminal amino acid sequencing by Edman degradation at the HHMI Biopolymer Laboratory and W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University.

Immunofluorescence Chromatography for Enzyme Assays—Microvillus membranes (1 mg) were solubilized and subjected to immunofluorescence chromatography as above. Fifty μl of each fraction (5% of initial volume) were saved for subsequent Western blot analysis and enzyme assays. Subsequently, 5% of the total volume of the microvillus membranes after incubation with the column (flow-through), as well as the eluate from the column, were concentrated to 50 μl for subsequent assays. Thus, all samples used for Western blot analysis and enzyme assays (see Fig. 5) were matched for initial content of microvillus membranes. Of the 50-μl samples of each fraction (5% of total yield), 15 μl were used for SDS-PAGE and immunoblotting, and 15 μl were used for each of the two enzyme assays. Both dipeptidyl peptidase IV (25) and γ-glutamyl transpeptidase (26) activities were measured by measuring the release of p-nitroanilino from the hydrolysis of glycylproline p-nitroanilide tosylate and γ-glutamyl p-nitroanilide (Sigma), respectively. For the DPPIV assay, 15 μl of each fraction obtained from the immunofluorescence column were added to 1 ml reaction mixture containing 1.5 mM glycylproplyl-p-nitroanilide tosylate and 75 mM glycine/NaOH buffer, pH 8.7. The mixture was incubated for 30 min at 37 °C and terminated by the addition of 3 ml of 0.1 M acetate buffer, pH 4.2. For the γ-GTP assay, 15 μl of each fraction were added to 2 ml of reaction mixture containing 4.2 mM L-γ-glutamyl-p-nitroanilide, 52.5 mM glycylglycine, 10.5 mM MgCl2 in 50 mM ammodiel buffer, pH 9.3, and incubated for 5 min at 25 °C. Determination of p-nitroaniline released enzymatically was based on the absorbance at 320 nm.

Preparation of Dense Membrane Vesicles from Rabbit Renal Cortices—Post mitochondrial microsomes were prepared and separated on 15–25% OptiPrep™ gradients essentially as described previously (17). 1-ml fractions were manually collected from the top and stored at −70 °C. Dense vesicles, which are enriched in megalin, were pooled from fractions 5–7 in the gradient (17).

RESULTS

Immunoprecipitation of NHE3 by mAb 1D11—In a previous paper, we described the generation of mAbs against affinity-purified NHE3 protein complexes (16). Hybridomas were selected based on ability to immunoprecipitate NHE3. One mAb (10A3) was characterized in detail and was found to be directed at megalin. Studies with mAb 10A3 revealed that a significant pool of renal brush border NHE3 resides in oligomeric complexes with megalin (16).

In the present study we characterize a second mAb, 1D11, that was also generated against affinity-purified NHE3 protein complexes and was found to immunoprecipitate NHE3. We first evaluated whether 1D11 is directed to NHE3 or to an associated protein. The left half of Fig. 1 shows the results of an immunoblotting experiment in which rabbit renal microvillus membranes were subjected to SDS-PAGE, transferred to a PVDF membrane, and probed with mAb 1D11, a mAb against NHE3, and a polyclonal antibody to another brush border membrane protein, the Na/Pi cotransporter. mAb 1D11 was found to label a 110-kDa protein that is distinct from the 80-kDa monomeric form of NHE3.
We next verified whether mAb 1D11 specifically co-precipitates NHE3. Shown in the right half of Fig. 1 are the results of an immunoprecipitation experiment in which rabbit renal microvillus membrane proteins solubilized in 1% Triton X-100 were immunoprecipitated with mAb 1D11. The immune complexes were prepared for immunoblotting and probed with mAb 1D11, an anti-NHE3 mAb and anti-NaPi-2 antibody. Both the 110-kDa 1D11 antigen and NHE3 were co-precipitated by mAb 1D11. In contrast, mAb 1D11 did not co-precipitate NaPi-2, indicating that the interaction between the 1D11 antigen and NHE3 is specific.

In the studies above, immunoprecipitation was performed using solubilized microvillus membrane proteins after clearing by centrifugation at 15,000 × g for 10 min. But we have previously reported that a significant amount of NHE3 can 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 in the solubilized extract resides in large, insoluble aggregates (16). Co-localization of NHE3 and the 1D11 antigen in such insoluble aggregates could in principle account for their apparent co-precipitation from the low-speed supernatants of detergent-solubilized renal microvillus membranes in Fig. 1. Arguing against this possibility is the lack of co-precipitation of another brush border membrane protein, NaPi-2. Nevertheless, to verify that NHE3 and the 1D11 antigen could also be co-precipitated from the truly soluble supernatant, we performed additional immunoprecipitation experiments (Fig. 2) using both the low speed (15,000 × g) and high speed (200,000 × g) supernatants of Triton X-100 solubilized renal microvillus membranes. These supernatants were precipitated either with a mAb to NHE3, mAb 1D11, or a mAb to the microvillar core protein villin. The immunocomplexes were prepared for immunoblotting and probed with a polyclonal Ab to NHE3. Shown in Fig. 2, mAb 1D11 immunoprecipitates NHE3 from truly solubilized microvillus membranes but does not precipitate villin.

Although mAb 1D11 specifically immunoprecipitates NHE3, we were unable to demonstrate that the anti-NHE3 mAb 2B9 co-precipitates the 110-kDa 1D11 antigen (not shown). Of interest in this regard, we had previously found that when NHE3 is complexed with megalin, the C-terminal epitope for anti-NHE3 mAb 2B9 is blocked from antibody binding (16). We therefore evaluated whether 1D11 also immunoprecipitates a pool of NHE3 not recognized by anti-NHE3 mAb 2B9. To this end, serial immunoprecipitation was performed three times with mAb 2B9, resulting in progressive depletion of the NHE3 available for binding to this antibody, as shown in Fig. 3. To determine whether there was any remaining NHE3 that was complexed with the 1D11 antigen and that mAb 2B9 was not able to recognize, a fourth immunoprecipitation was performed from the same sample using mAb 1D11. As seen in Fig. 3, an appreciable amount of NHE3 that could not be precipitated by anti-NHE3 mAb 2B9 was co-precipitated by mAb 1D11. These findings indicate that the C-terminal epitope for binding of mAb 2B9 to NHE3 is occluded when NHE3 is complexed with the 110-kDa 1D11 antigen.

Characterization of 1D11 Antigen—Having demonstrated that the 110-kDa 1D11 antigen and NHE3 are specifically associated, our first step in the characterization of the 1D11 antigen was to determine the subcellular sites of its expression in proximal tubule cells. As seen by immunoelectron microscopy in Fig. 4, mAb 1D11 stained the brush border of proximal tubule cells. In contrast to the predominantly coated pit staining previously observed for anti-megalin mAb 10A3 (16), mAb 1D11 very strongly labeled the microvillar domain of the brush border, although staining of the intermicrovillar pit regions was also detected. The findings in Fig. 4 indicate that the 110-kDa 1D11 antigen is a brush border membrane protein.

To isolate the protein against which mAb 1D11 is directed, immunoaffinity chromatography was carried out by use of a column to which mAb 1D11 was covalently bound. The bound antigen from solubilized microvillus membranes was eluted using a low pH buffer. The eluate was concentrated, subjected to SDS-PAGE and transferred to a PVDF membrane. The single major protein was cut from the blot and submitted for N-terminal sequencing. The following sequence was obtained: MVXKLLG. A data base search indicated that nine of nine of the identified amino acids matched human, mouse, and rat dipeptidyl peptidase IV (DPPIV, EC 3.4.14.5).
Given the possibility that DPPIV may have been a contaminant in the 110-kDa region of the blot submitted for N-terminal amino acid sequencing, we performed measurements of enzymatic activity to verify that the major protein band isolated on the mAb 1D11 immunoaffinity column actually represents DPPIV. Solubilized microvillus membranes were incubated with the 1D11 immunoaffinity column. Samples of the solubilized microvillus membrane starting material (SM) applied to the column, the flow-through (FT) representing solubilized microvillus membranes after incubation with the column, and the eluate from the column were compared both for the presence of the 110-kDa 1D11 antigen by Western blotting (Fig. 5A) and for enzymatic activity (Fig. 5B). As illustrated in Fig. 5, the 1D11 antigen was completely depleted from the flow-through and was recovered in the eluate. These findings correlated with virtually complete depletion of DPPIV enzymatic activity from the flow-through with recovery in the eluate. In contrast, activity of another brush border membrane enzyme, γ-glutamyltransferase, was not depleted in the flow-through and was not recovered in the eluate from the 1D11 immunoaffinity column. These results confirm that the 1D11 antigen is DPPIV.

Distribution of the NHE3-DPPIV Complex in Microdomains of Rabbit Renal Brush Border—In view of our previous findings that NHE3 is expressed in different microdomains of the apical membrane of proximal tubule cells and that the NHE3-megalin complex principally resides in a dense membrane compartment most likely representing coated pits (17), it was of interest to similarly examine in which microdomain the NHE3-DPPIV complex is expressed. For this purpose, we performed both immunoblotting (Fig. 6A) and immunoprecipitation (Fig. 6B) experiments using microvillus membrane vesicles (MMV) isolated by divalent cation aggregation (20), and dense membrane vesicles (DV) isolated by density fractionation of total renal cortical membranes (17). The immunoblotting experiments were performed using equal amounts of protein from either microvilli or dense vesicles to analyze semi-quantitatively the expression of DPPIV in these sub-compartments. Illustrated in Fig. 6A, megalin is more highly expressed in dense vesicles compared with microvilli, villin is predominantly expressed in microvilli, and NHE3 is equally distributed between these two microdomains as previously reported (17). We observed that DPPIV is much more abundant in microvilli than in dense vesicles, confirming previous findings that DPPIV is a protein of the microvillar microdomain (23).

To examine the relative abundance of the NHE3-DPPIV complex in these two membrane compartments, we then performed immunoprecipitation with mAb 1D11 and analyzed the precipitates for the presence of NHE3 by Western blotting. Shown in Fig. 6B, although NHE3 abundance was essentially the same in solubilized microvillus membrane vesicles (MMV) and dense vesicles (DV), a greater quantity of NHE3 was co-precipitated by mAb 1D11 from microvilli compared with dense vesicles. These results contrast with previous findings indicating that the NHE3-megalin complex is most abundant in the dense vesicles, which likely represent intermicrovillar coated pits (17).
The findings in Fig. 6 suggest that the pools of NHE3 associated with DPPIV and megalin have distinct patterns of distribution. To further explore the relationship between these pools of NHE3, we performed serial immunoprecipitation experiments using mAbs 1D11 (anti-DPPIV) and 10A3 (anti-megalin). Shown in Fig. 7A, serial precipitation with mAb 1D11 led to progressive depletion of the amount of NHE3 that co-precipitates with DPPIV. To determine whether there was any remaining NHE3 that was not associated with DPPIV but was associated with megalin, a fourth immunoprecipitation was performed from the same sample using anti-megalin mAb 10A3. As seen in Fig. 7A, a substantial amount of NHE3 that could not be precipitated by anti-DPPIV mAb 1D11 was co-precipitated by mAb 10A3, indicating that a pool of NHE3 exists that is complexed with megalin but not with DPPIV. Similarly, shown in Fig. 7B, after serial immunoprecipitation by anti-megalin mAb 10A3, an additional quantity of NHE3 was precipitated by anti-DPPIV mAb 1D11, indicating that a pool of NHE3 exists that is complexed with DPPIV but not with megalin. Finally, we found that precipitation of megalin from solubilized microvillus membranes by anti-DPPIV mAb 1D11 was only faintly detected, and precipitation of DPPIV by anti-megalin mAb 10A3 was not detected at all (data not shown). Taken together, the findings in Figs. 6 and 7 indicate that the pools of NHE3 complexed with megalin and DPPIV are largely distinct and preferentially reside in different microdomains of the apical membrane of proximal tubule cells.

**DISCUSSION**

In the present study we have shown that mAb 1D11 directed to DPPIV co-precipitates NHE3 from solubilized microvillus membranes isolated from rabbit kidney, suggesting that NHE3 and DPPIV are associated in an oligomeric complex. Our finding that anti-DPPIV mAb 1D11 co-precipitates neither NaPi-2 nor villin from solubilized microvillus membranes indicates the specificity of the interaction between NHE3 and DPPIV.

DPPIV, also known as CD26, is a plasma membrane protein expressed in many types of epithelial and nonepithelial cells (27). It is a highly specific serine protease that cleaves N-terminal dipeptides from peptides with a penultimate proline or alanine (28). Through this action, it is able to degrade and inactivate several peptides, including glucagon-like peptide-1, or peptide YY, substance P and α-neuropeptide Y, substance P and β chemokines such as eotaxin, stromal derived factor-1, and RANTES (regulated on activation normal T-cell expressed and secreted) (29). In addition to its catalytic activity, DPPIV also functions as a binding protein. At the T-cell surface, DPPIV binds to the soluble extracellular enzyme adenosine deaminase (30), and DPPIV is closely involved in mediating cell entry of human immunodeficiency virus (31). There is also evidence for a signaling role for DPPIV/CD26 in T-cell activation (32). The kidney is a principal site of expression of DPPIV, where it is one of the major brush border membrane proteins (28). However, its physiological role in kidney tissue other than as a peptidase remains unclear.

Interestingly, recent studies have revealed that other proteases are capable of regulating the activity of membrane transporters in kidney and other tissues. For example, a 329-amino acid protein has been cloned that belongs to the serine protease family and activates the amiloride-sensitive epithelial sodium channel by an indirect effect not involving direct proteolysis of the channel subunits (33). Initially isolated from Xenopus kidney epithelial cells (A6 cells), this channel-activating protease-1 has a mammalian homologue that regulates epithelial sodium channel activity in kidney collecting duct cells (34). Activation of the epithelial sodium channel by the

**FIG. 6. Distribution of the NHE3-DPPIV complex in the microdomains of rabbit renal brush border.** A, equal quantities (20 μg of protein) of microvillus membrane vesicles (MMV) prepared by divalent cation aggregation and dense vesicles (DV) prepared by density fractionation of postmitochondrial microsomes, were analyzed by immunoblotting. Blots were probed with antibodies to megalin (mAb 10A3), DPPIV (mAb 1D11), NHE3 (goat polyclonal Ab) and villin (mAb). B, equivalent quantities (100 μg) of solubilized microvillus membrane vesicles (MMV) and dense vesicles (DV) were immunoprecipitated with anti-DPPIV mAb 1D11 (1D11 IP). The immune complexes, as well as samples of the starting material, were prepared for immunoblotting, and the blot was probed with a polyclonal goat antibody to NHE3.

**FIG. 5. 1D11 antigen is dipeptidyl peptidase IV.** Solubilized renal microvillus membranes were passed through an immunoaffinity column with immobilized mAb 1D11. Samples of the starting material (SM), unbound material (flow-through, FT), and the column eluate were matched for initial content of microvillus membranes. A, mAb 1D11 was used to probe a Western blot of the different fractions obtained from the 1D11 affinity column. B, activities of DPPIV and γ-glutamyl transpeptidase (γ-GTP) were assayed on the fractions. Results are expressed as mean absorbance at 380 nm ± S.E. for n = 3.
serine protease prostasin, which is probably an orthologue of channel-activating protease-1, has also been reported (35). Activation of chloride and potassium channels by trypan acting upon the proteinase-activated receptor-2 has been described in pancreatic and collecting tubule cells (36, 37). In view of this emerging evidence for regulation of transporters by proteases, it is possible that DPPIV may play a similar role in modulating the activity of NHE3. Indeed, although we found that preincubation with the DPPIV inhibitor diprotin A had no effect on NHE3 activity in rabbit renal microvillus membrane vesicles (results not shown), we have observed in preliminary studies that diprotin A inhibits NHE3 activity in OK cells, a proximal tubule cell line (38).

The apical brush border of proximal tubule cells is composed of two microdomains, the microvilli and the intermicrovillar coated pits (39). We previously demonstrated that NHE3 is equally abundant in microvillus membrane vesicles and in a population of non-microvillar dense membrane vesicles that likely represent the intermicrovillar coated pits (17). Moreover, we identified a pool of NHE3 that is associated in oligomeric complexes with the scavenger receptor megalin and found that NHE3-megalin complexes are principally concentrated in the intermicrovillar coated pit microdomain of the renal brush border where NHE3 is inactive (17). In the present study, we have found that the NHE3-DPPIV complex is more abundant in microvilli, where NHE3 is active, than in intermicrovillar coated pits. Serial immunoprecipitation experiments using mAbs 1D11 (anti-DPPIV) and 10A3 (anti-megalin) have revealed that the pools of NHE3 complexed with DPPIV and megalin are largely distinct. A third pool of NHE3 can be defined by precipitation with anti-NHE3 mAb 2B9, which co-precipitates neither megalin nor DPPIV because its epitope is free of association with megalin or DPPIV. We have previously shown that intermicrovillar NHE3, much of which is complexed with megalin, is inactive (17). The relative contributions of “free” NHE3 and DPPIV-associated NHE3 to the measured Na\(^-\)/H\(^+\) exchange activity in renal microvillus membrane vesicles remain to be defined. Also unknown is the relationship of any of these three forms of NHE3 to the regulatory proteins NHERF (11, 13) and E3KARP (14).

Thus, taking our present and previous results together, there are at least three forms of NHE3 in the renal brush border: NHE3-megalin complexes that predominately reside in the intermicrovillar microdomain (17), NHE3-DPPIV complexes that are mainly present in the microvilli, and NHE3 that is also largely microvillar but is free of association with megalin or DPPIV. We have previously shown that intermicrovillar NHE3, much of which is complexed with megalin, is inactive (17). The relative contributions of “free” NHE3 and DPPIV-associated NHE3 to the measured Na\(^-\)/H\(^+\) exchange activity in renal microvillus membrane vesicles remain to be defined. Also unknown is the relationship of any of these three forms of NHE3 to the regulatory proteins NHERF (11, 13) and E3KARP (14). To date, we have been unable to demonstrate co-precipitation of NHERF or E3KARP from renal microvillus membranes by anti-NHE3 mAb 2B9 or anti-megalin mAb 10A3 under the conditions we have used for membrane solubilization. However, a substantial fraction of renal brush border NHE3 is detergent insoluble (16). Since NHERF and E3KARP bind the cytoskeletal protein ezrin (40–42), it is possible that in native kidney tissue they predominantly associate with NHE3 that is detergent-insoluble and hence is not available for immunoprecipitation under the conditions of our experiments.

In conclusion, we have demonstrated an unexpected association of Na\(^-\)/H\(^+\) exchanger isoform NHE3 with dipeptidyl peptidase IV in microvillus membranes isolated from proximal tubule cells. Inhibition of DPPIV reduces NHE3 activity in a proximal tubule cell line (38). Given that DPPIV is a multifunctional protein with known activities as a peptidase, binding protein, and signaling molecule, our findings raise the possibility that association with DPPIV regulates NHE3 by directly or indirectly affecting its surface expression and/or activity.

REFERENCES
1. Alpern, R. J. (1990) Physiol. Rev. 70, 79–114
2. Biemesderfer, D., Pizzonia, J., Abu-Alfa, A., Exner, M., Reilly, R., Igarashi, P., and Aronson, P. S. (1993) Am. J. Physiol. 265, F736–F742
3. Amemiya, M., Loffing, J., Lemmel, M., Kaissling, B., Alpern, R. J., and Moe, O. W. (1995) Kidney Int. 48, 1206–1215
4. Wu, M. S., Biemesderfer, D., Giebisch, G., and Aronson, P. S. (1996) J. Biol. Chem. 271, 32749–32752
5. Schultheis, P. J., Clarke, L. L., Meneton, P., Miller, M. L., Soleimani, M., Gawai, L. R., Riddle, T. M., Duffy, J. J., Doetschman, T., Wang, T., Giebisch, G., Aronson, P. S., Lorenz, J. N., and Shull, G. E. (1998) Nat. Genet. 19, 262–265
6. Wang, T., Yang, C. A., Abati, T., Schultheis, P. J., Shull, G. E., Giebisch, G., and Aronson, P. S. (1999) Am. J. Physiol. 277, F298–F302
7. Chen, J. Y.; still, M. Lee, M. G.; Schultheis, P. J., Shull, G. E.; Mueller, S., and Baum, M. (2000) J. Clin. Invest. 105, 1141–1146
8. Alpern, R. J., Yama, Y., Yano, A., Horie, S., Miller, R. T., Moe, O. W., and Preisig, P. A. (1990) J. Lab. Clin. Med. 123, 137–140
9. Paillard, F. J. Exp. Nephrol. 5, 277–284
10. Moe, O. W. (1999) J. Am. Soc. Nephrol. 10, 2412–2425
11. Weinman, E. J., Minkoff, C., and Shenolikar, S. (2000) Am. J. Physiol. Renal Physiol. 279, F295–F309
12. Wakabayashi, S., Ikeda, T., Noel, J., Schmitt, B., Orlofski, J., Pouysegur, J., and Shigekawa, M. (1995) J. Biol. Chem. 270, 26490–26495
13. Weinman, E. J., Stepelock, D., Donowitz, M., and Shenolikar, S. (2000) Biochemistry 39, 6123–6129
14. Yun, C. H., Oh, S., Zizak, M., Stepelrock, D., Tsao, S., Tse, C. M., Weinman, E. J., and Donowitz, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 3010–3015
15. Pang, T. Su, X., Wakabayashi, S., and Shigekawa, M. (2001) J. Biol. Chem. 276, 17367–17372
16. Biemesderfer, D., Nagy, T., DeGray, B., and Aronson, P. S. (1999) J. Biol. Chem. 274, 17518–17524
17. Biemesderfer, D., DeGray, B., and Aronson, P. S. (2001) J. Biol. Chem. 276, 2 D. Biemesderfer and P. S. Aronson, unpublished observations.
18. 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.
19. Levi, M., Lotscher, V., DeGray, B., and Aronson, P. S. (1998) *J. Biol. Chem.* 273, 12391–12396.

20. Biemesderfer, D., DeGray, B., and Aronson, P. S. (1998) *Am. J. Physiol.* 262, F55–F67.
21. Laemmli, U. K. (1970) *Nature* 227, 680–685.
22. Schneider, C., Newman, R. A., Sutherland, D. R., Asser, U., and Greaves, M. F. (1982) *J. Biol. Chem.* 257, 10766–10769.

23. Mentlein, R. (1999) *Regul. Pept.* 85, 9–24.
24. Kameoka, J., Tanaka, T., Nojima, Y., Schlossman, S. F., and Morimoto, C. (1993) *Science* 261, 466–469.
31. Ohtsuki, T., Teuda, H., and Morimoto, C. (2000) *J. Dermatol. Sci.* 22, 152–160.
32. Morimoto, C., and Schlossman, S. F. (1998) *Immunol. Rev.* 161, 55–70.
33. Vallet, V., Chraibi, A., Gaeggele, H. P., Horisberger, J. D., and Rossier, B. C. (1997) *Nature* 389, 607–610.
34. Vuagniaux, G., Vallet, V., Jaeger, N. F., Pfister, C., Benz, M., Farman, N., Courtois-Country, N., Vandewalle, A., Rossier, B. C., and Hummler, E. (2000) *J. Am. Soc. Nephrol.* 11, 828–834.
35. Adachi, M., Kitamura, K., Miyoshi, T., Nariyko, T., Iwashita, K., Shiraishi, N., Nonoguchi, H., and Tomita, K. (2001) *J. Am. Soc. Nephrol.* 12, 1114–1121.
36. Bertog, M., Letz, B., Kong, W., Steinhoff, M., Higgins, M. A., Bielfeld-Ackermann, A., Promter, E., Bunnell, N. W., and Körbmann, C. (1999) *J. Physiol.* 521, 3–17.
37. Nguyen, T. D., Mosley, M. W., Steinhoff, M., Okolo, C., Koh, D. S., and Bunnell, N. W. (1999) *J. Clin. Invest.* 103, 261–269.
38. Girardi, A. C. C., Knauf, F., and Aronson, P. S. (2000) *J. Am. Soc. Nephrol.* 11, 4A.
39. Rodman, J. S., Seidman, L., and Farquhar, M. G. (1986) *J. Cell Biol.* 102, 77–87.
40. Reczek, D., Berrymen, M., and Bretscher, A. (1997) *J. Cell Biol.* 139, 169–179.
41. Yun, C. H., Lamprecht, G., Forster, D. V., and Sidor, A. (1998) *J. Biol. Chem.* 273, 25856–25863.
42. Lamprecht, G., Weinman, E. J., and Yun, C. H. C. (1998) *J. Biol. Chem.* 273, 29972–29978.
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