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Na^+/H^+ Exchanger Isoform 2 (NHE2) is Expressed in the Apical Membrane of the Medullary Thick Ascending Limb

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Abstract. Apical Na^+/H^+ exchangers (NHE) in the proximal tubule and medullary thick ascending limb (MTAL) display similar functions and regulation, suggesting that similar NHE isoforms are present. In the rat proximal tubule, NHE2 and NHE3 are present in the apical membrane, however, in the MTAL, NHE3, but not NHE2, mRNA has been found. In this study, the expression and subcellular localization of NHE2 in both rat and mouse MTAL were studied. To detect NHE2 mRNA, reverse transcription-polymerase chain reaction (RT-PCR) was performed in microdissected MTAL tubules using primers specific for NHE2. Analysis of PCR products with and without digestion by restriction enzymes chosen from the published NHE2 sequence gave predicted sizes. Subcloning and sequencing of the PCR product from mouse MTAL revealed 91% and 75% identity to the published NHE2 nucleotide sequence of comparable regions in rat and rabbit, respectively. Thus, NHE2 mRNA is expressed in the MTAL of mouse and rat. The subcellular localization of NHE2 was determined by immunochemistry using a specific NHE2 antibody. Immunofluorescence staining was observed in the apical, but not basolateral, membrane of MTAL of both species. In addition, anti-NHE2 antibody recognized an 85 kD protein in plasma membranes prepared from mouse and rat renal outer medulla and a MTAL cell line by Western analysis, which further support that NHE2 protein is expressed in the MTAL of both species. We conclude that NHE2 is expressed in the apical membrane of MTAL in both mouse and rat.

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Introduction

The Na^+/H^+ exchanger (NHE) is an ubiquitous plasma membrane transporter in mammalian cells. In the kidney, NHE is involved in hypertonic cell volume regulation, cell pH regulation, and salt, bicarbonate and NH_4^+ transport. At present, five NHE isoforms (NHE1, NHE2, NHE3, NHE4, and NHE5) have been identified. NHE1 is an ubiquitous isoform and has been found in almost all animal cells. NHE2, NHE3 and NHE4 are more specifically expression in various epithelia, including kidney [27]. The expression of NHE5 is limited to the brain [12]. In the kidney, NHE1 is present exclusively in the basolateral membrane of most tubule segments where it functions in hypertonic cell volume regulation and cell pH regulation (housekeeping). NHE3 is present in the apical membrane of the proximal tubule (PT) and thick ascending limb (TAL) [2, 18]. Apical NHE in PT and MTAL is involved in transepithelial salt, bicarbonate and NH_4^+ transport [7, 11, 14, 27] and it is believed that NHE3 is responsible for these functions. In contrast to NHE1 and NHE3, the functions, cellular distribution and regulation of NHE2 and NHE4 in the kidney are not clear.

Physiological studies using in vitro microperfusion techniques have demonstrated that NHE occurs in the apical membranes of both proximal tubule and medullary thick ascending limb (MTAL) cells [1, 7, 17, 22]. Apical NHE in both tubule segments participates in similar functions, including cell pH regulation and bicarbonate transport. In addition, the exchangers display similar regulation, for example, inhibition by cAMP and hyperosmolality [14, 17, 22, 23]. This suggests that the same NHE isoforms are present in the apical membranes of proximal tubule and MTAL cells. Consistent with this notion, NHE3 is expressed in the apical membrane of

both tubule segments [2, 18]. Interestingly, Azuma et al. [3] have recently shown that NHE2 mRNA is present in the proximal tubule. In addition, NHE2 is present in brush border membranes isolated from renal cortex by Western analysis [9] and localized in the apical, but not basolateral, membrane of proximal tubule by immunohistochemistry [24]. In contrast, evidence suggests that NHE2 is not expressed in the MTAL, since Borensztein et al. [5] failed to detect NHE2 message in the microdissected rat MTAL by the reverse transcription-polymerase chain reaction (RT-PCR). Thus, in the present study the expression of NHE2 in the MTAL was reassessed. We show that NHE2 is present and localized in apical membrane of both mouse and rat MTAL.

Materials and Methods

Tubule Microdissection

The experiments were performed in male Sprague-Dawley rats (70–160 g) and male CD mice (25- to 35-day-old) that had free access to water and standard laboratory chow until anesthesia. Our protocol was modified from Yu et al. [25]. Animals were anesthetized with 50 mg/kg ip Nembutal and the left kidney was perfused in situ with 10-ml ice-cold bicarbonate-free Dulbecco's modified Eagle's medium (DMEM; GIBCO) containing 1 mg/ml type I collagenase (digestion solution). Coronal slices were cut and incubated in this solution at 30°C, bubbled with 100% O_2 for 20 min and then washed three times in collagenase- and bicarbonate-free DMEM solution containing 0.1% bovine serum albumin (Sigma) (dissection solution) and placed on ice. MTALs were microdissected from the inner stripe of the outer medulla as previously described [17] in dissection solution. After dissection, tubules were transferred to a wash dish containing fresh dissection solution, captured on polylysine-coated glass microbeads (0.5 mm diameter, Thomas Scientific), and transferred to a 0.5 ml Eppendorf tube. Four beads, each with an adherent tubule 0.4 to 0.6 mm long, were pooled in a single tube. Bead-tubules were rinsed three times with 10 μl of dissection solution containing 2 U/μl RNase inhibitor (Boehringer-Mannheim) and solubilized with 10 μl of 2% Triton X-100 containing 2 U/μl RNase inhibitor. To exclude contamination from exogenous source, along with each series of tubules dissected at a single session, beads taken from the wash dish, but with no tubules attached, was processed in parallel [25].

Reverse Transcription (RT) and Polymerase Chain Reaction (PCR)

Samples were reversed transcribed *in situ* by adding to each tube a RT mix to make up a total volume of 20 μl. Each tube contained: 0.5 μg oligo (dT) primer, 200 U of Superscript MuMLV RT (GIBCO BRL), 0.5 mM dNTP mix, 10 mM dithiothreitol, 100 mM Tris-HCl (pH 8.4), 50 mM KCl and 2.5 mM $MgCl_2$. Tubes were incubated for 1 hr at 42°C and then the reaction was terminated by heating to 95°C for 5 min. After RT, each reaction tube was centrifuged briefly to pellet the beads and then the solution was transferred to a 0.2 ml thin-wall PCR tube.

For PCR, a PCR mix was added to the PCR tube to make up a total volume of 100 μl. Each PCR reaction tube contained: 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM dNTP, 2.5 U Taq DNA polymerase (promega)/7 μM anti-Taq polymerase antibody (Clontech) mixture, 100 pmole of each NHE2 primer. Anti-Taq polymerase was used as an alternative to ''hot start'' PCR to optimize the PCR reaction. The tubes were placed in the DNA Thermal Cycler (Perkin Elmer GeneAmp PCR system 2400), which was programmed to execute the following protocol: 94°C 4 min (initial melt); 35 cycles of 94°C 1 min, 60°C 1 min, 72°C 1.5 min; and then 72°C 7 min (final extension). NHE2 primers for the PCR reactions encode a sequence of 312 bp in the cytoplasmic domain of NHE2 (amino acids 690–803). This sequence is specific for NHE2 as described by Borensztein et al. [5]. Primer 1 (antisense) was 5′-CCTTGGTGGGGGCTTGGGTG-3′ defined by bases 2546–2565 and primer 2 (sense) was 5′-GCAGATGGTAATAGCAGCGA-3′ defined by bases 2254–2273.

For PCR product analysis, the PCR samples were fractionated on 2% agarose gels stained with ethidium bromide.

Cloning and Sequencing of PCR Products

PCR products were purified using a PCR purification kit (Qiagen) and ligated into a plasmid vector pCR using TA cloning system according to the instructions specified by the manufacturer (Invitrogen). Plasmid DNA was prepared from recombinant colonies and PCR products were sequenced using the dideoxynucleotide chain termination method (Sequenase version 2.0 DNA Sequencing Kit, United States Biochemical).

Tissue Preparation and Immunohistochemistry

Animals were anesthetized with ip Nembutal, and the kidneys were perfusion-fixed as described previously [4, 24]. In brief, a cannula was inserted into the descending aorta distal to the renal arteries and the kidneys perfused retrograde first with normal saline at 37°C, followed by fixative containing 4% paraformaldehyde and 4% sucrose in PBS, pH 7.4. Blocks of tissues were postfixed overnight in the same fixative at 4°C, washed with 0.1 M aqueous NH_4Cl solution for 30 min, and cryoprotected by incubation in 2.3 M sucrose in PBS for 1 hr. The tissue blocks were next snap-frozen in OCT compound (Miles Laboratory) with liquid nitrogen-cooled isopentane.

Crysections were cut and mounted on glass slides. Sections were first incubated with 1% SDS in PBS for 5 min for antigen retrieval as suggested by Brown et al. [6]. In the following steps, 0.2% TritonX-100 was added to the PBS to permeablize the tissues. After removal of SDS by washing in PBS, the sections were blocked with 20% donkey serum in PBS for 20 min, and then incubated with the primary antibody (antisera against NHE2, 1:50 dilution). After 2 hr incubation the sections were washed three times with fresh PBS followed by incubation of donkey CY5-conjugated anti-rabbit IgG (secondary antibody, 1:400 dilution) for 1 hr and rinsed with fresh PBS. The sections were examined with a Zeiss Axiovert 100TV inverted microscope coupled to a MRC-1000 confocal scanning unit (Biorad) equipped with krypton-argon laser, three photomultiplier detectors, and one transmitted light detector. Antisera against NHE2 (AB597) was raised in rabbit against a fusion protein of glutathione-S-transferase and the last 87 amino acids of NHE2 peptide [20].

Cell Membrane Preparation and Western Analysis

Freshly obtained rat and mouse cortex, inner stripe of outer medulla and cultured cells [PS120 fibroblast [9] and MTAL cell line [13]] grown confluent in 10 cm-petri dish were lysed with 10 mM Tris, 2 mM $MgCl_2$, 2 mM EDTA, 2 mM EGTA, pH 7.4 containing 250 mM sucrose, 2 μg/ml aprotinin, 2.5 μg/ml leupeptin, 2 μg/ml pepstatin, 1 mM o-

phenathroline and 1 mM phenylmethylsulfonyl fluoride. Lysed cells were then homogenized with a glass Dounce homogenizer (25 to 30 strokes) and a 26-gauge needle and centrifuged at 800 *g* for 15 min at 4°C. The supernatant was then subjected to a high-speed centrifugation for 1 hr at 40,000 × *g* at 4°C to obtain a crude cell membrane fraction. The crude cell membranes were resuspended in water for sodium dodecylsufate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western analysis.

For western analysis, crude cell membranes were resolved by 10% SDS-PAGE and electrotransferred to nitrocellulose membranes (Hybond ECL, Amersham). The membranes were subsequently blocked with 5% milk. Antiserum (1:500 dilution) against NHE2 (AB597) was used. Immunodetection was by enhanced chemiluminescence using horseradish peroxidase-conjugated goat anti-rabbit antibody (Amersham).

Results

Detection of NHE2 mRNA in the Mouse and Rat MTAL

As shown in Fig. 1*A*, an amplification product of the predicted size of 312 bp was detected in MTAL tubules and the kidneys (positive control) of both mouse and rat. No PCR products were obtained when reverse transcriptase was omitted from the RT reaction (negative control). The identity of the PCR products as specific for NHE2 was assessed by restriction analysis of the PCR product using enzymes chosen based on the published rat NHE2 sequence [21] and by sequence analysis of PCR product. As shown in Fig. 1*B*, digestion of PCR products from the MTAL tubules and kidneys of both mouse and rat with the restriction enzyme, Mbol, revealed two fragments of the predicted sizes, 81 and 231 bp. Digestion of PCR products with Sau3Al also gave two major fragments of predicted sizes of 81 and 231 bp (*data not shown*). Fig. 2*A* shows that the nucleotide sequence of the mouse MTAL PCR product was 91% and 75% identical to the published NHE2 sequence of the corresponding region in rat [21] and rabbit [19], respectively. At the amino acid level, as shown in Fig. 2*B*, the mouse MTAL NHE2 PCR product was 91% identical to the corresponding region of rat NHE2 and 77% identical to the rabbit NHE2. These results indicate that NHE2 mRNA is expressed in MTAL of mouse and rat.

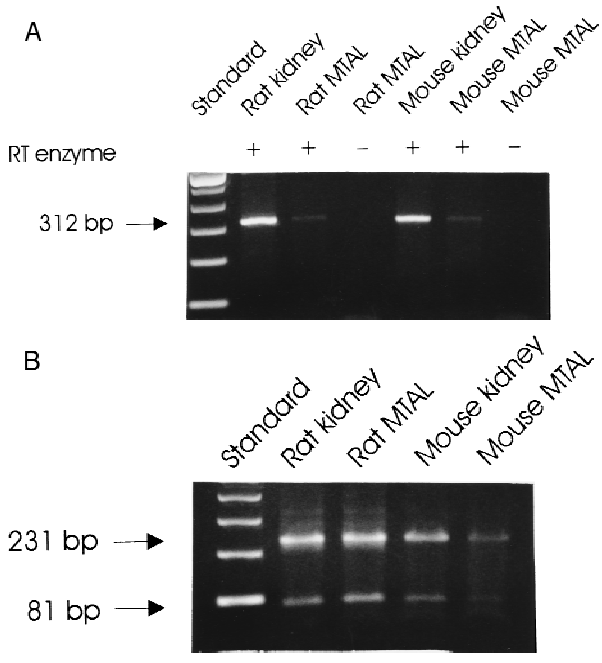

Fig. 1. Amplification and restriction analysis of NHE2 RT-PCR products from mouse and rat whole kidney and MTAL. PCR products with (*B*) and without (*A*) restriction enzyme digestion were electrophoresed on 2% agarose gel and stained with ethidium bromide. (*A*) NHE2 PCR products amplified from microdissected rat and mouse MTAL tubules. Each reaction was performed in the presence (+) and absence (−) of RT. Positive controls were performed using 1 μg total RNA from whole kidney homogenate. (*B*) NHE2 PCR products from mouse and rat whole kidney and MTAL tubules digested with a restriction enzyme, Mobl. Molecular weight standards were 100 bp ladder from GIBCO-BRL. *See* text for abbreviations.

Subcellular Localization of NHE2 in the Mouse and Rat MTAL

Recently, Hoogerwerf et al. [9] employed Western blot analysis and immunochemistry to demonstrate that NHE2 is present exclusively in the brush border, but not basolateral, membrane in human and rat intestine. In addition, NHE2 was found in the brush border membranes isolated from rat renal cortex [3]. In contrast, Soleimani et al. [16] showed that NHE2 is expressed only in the basolateral membrane in a mouse inner medullary collecting duct (IMCD) cell line. These results indicate that the membrane localization of NHE2 may vary depending on the tissue and cell type. Therefore, we examined the subcellular localization of NHE2 in the MTAL by immunochemistry. Figure 3 shows immunostaining of MTAL at the level of the inner stripe of the outer medulla in rat kidneys using a specific anti-NHE2 antiserum (*see* Refs. 9 and 19 for characterization of anti-NHE2 antibody). The staining was localized to the apical, but not basolateral, membrane of MTAL. Consistent with demonstration of NHE2 in brush border membrane of cortex by Western analysis by Hoogerwerf et al. [9], we also found NHE2 staining in apical membrane of proximal tubule [24]. No staining was detected in outer medullary collecting duct (OMCD). Identical staining pattern was also observed in the mouse MTAL (*data not shown*)

To obtain additional evidence that NHE2 protein is expressed in the MTAL, Western analysis using an anti-NHE2 antiserum (AB597) was performed on cell membranes isolated from renal cortex, outer medulla and a cell line derived from mouse MTAL [13]. In the present

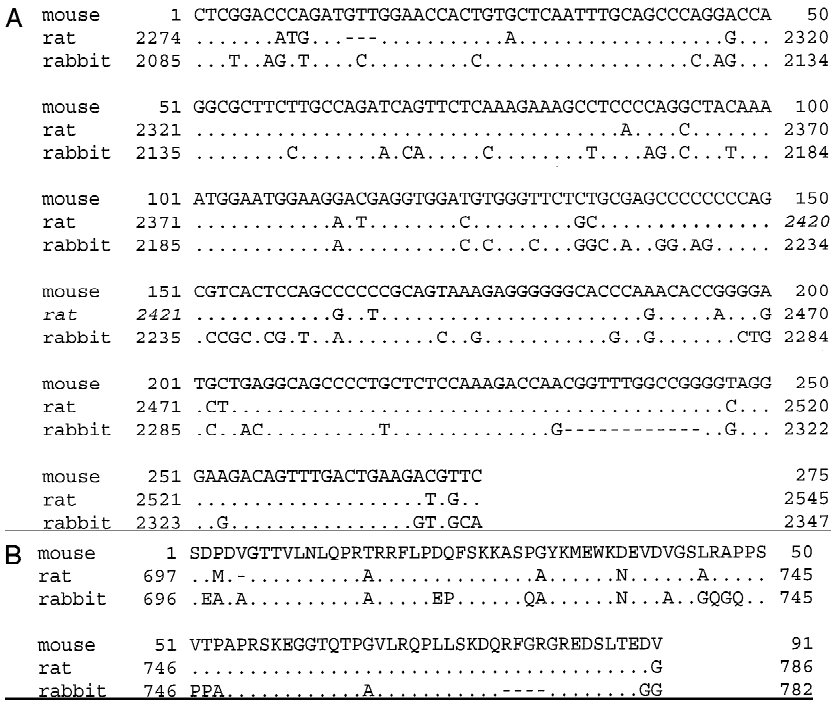

Fig. 2. Sequence comparison of NHE2 PCR products cDNA from mouse MTAL with reported NHE2 cDNA for rat and rabbit (*A*) nucleotide sequences. (*B*) Deduced amino acid sequences. Identical and deleted nucleotides and amino acids are indicated by dots and dashes, respectively, in the sequence of rat and rabbits. Numbers represent positions derived from published sequences for rat and rabbit NHE2.

study, we used an MTAL cell line counterpart but not its native to demonstrate the presence of NHE2 protein in MTAL cells. This was because currently available techniques used to extract native MTAL cells either yield insufficient cells (in vitro microdissection technique) or sufficient cells that are contaminated with other renal medullary cell types (tubule suspension technique) for Western analysis. The MTAL cell line used in the present study has previously been shown to retain many characteristics, such as expression of bumetanide-sensitive NA^+:K^+:$2Cl^-$ cotransporter and uromodulin, of its native counterpart [13]. In addition, using RT-PCR technique we have found that this cell line, like its native counterpart, expresses NHE1, NHE2 and NHE3, but not NHE4 mRNA (*data not shown*). Hoogerwerf et al. [9] have previously shown that AB597 antiserum recognized NHE2 as an 85 kD protein in the rat renal cortex. In accordance with their findings, we found that this antiserum recognized an 85 kD protein in the mouse renal cortex, but not in the NHE-deficient cell line (PS120) (Fig. 4*C*). As shown in Fig. 4*A*, AB597 antiserum detected a 85 kD band in cell membranes of mouse and rat outer medulla and the MTAL cell line. When this antiserum was preabsorbed with GST/NHE2 fusion protein, these 85 kD bands were not detected. Thus, these results confirm that NHE2 protein is expressed in the MTAL of both mouse and rat.

Discussion

In this study, we demonstrate by RT-PCR that NHE2 mRNA is present in MTAL tubules microdissected from both the mouse and rat. In addition, NHE2 protein is present in the MTAL and localized to the apical, but not basolateral, membrane of MTAL by Western analysis and immunohistochemistry, respectively.

In contrast to the results of the present study, Borensztein et al. [5] did not detect NHE2 mRNA by RT-PCR in microdissected rat MTAL. In both studies, rats of the same strain and gender and with similar weights were used. In addition, identical specific NHE2 PCR primers and similar PCR parameters were employed. However, the RT-PCR protocol used in the present study may have been more sensitive than that employed by Borensztein et al. [5]. First, we used a larger amount of total RNA for each RT reaction. In the present study, total RNA was collected from 2 mm of MTAL as compared to 1 mm of MTAL in the study by Borensztein et al. [5]. In addition, in our study total RNA was extracted by solubilizing MTAL tubules with Triton X-100 and used directly without further purification while in the study by Borensztein et al., total RNA was extracted from pooled MTAL tubules using the microadapted method of Chomczynski and Sacchi [5] which involves an RNA purification step that might decrease the yield of RNA. Finally, the PCR reaction in the present study was optimized by employing anti-Taq polymerase antibody, an alternative method to ''hot-start'' PCR. It is likely that the NHE2 gene expression is low in the rat MTAL, and thus, only detected by optimized RT-PCR used in the present study.

Although the nucleotide and amino acid sequence of mouse NHE2, NHE3 and NHE4 are currently not

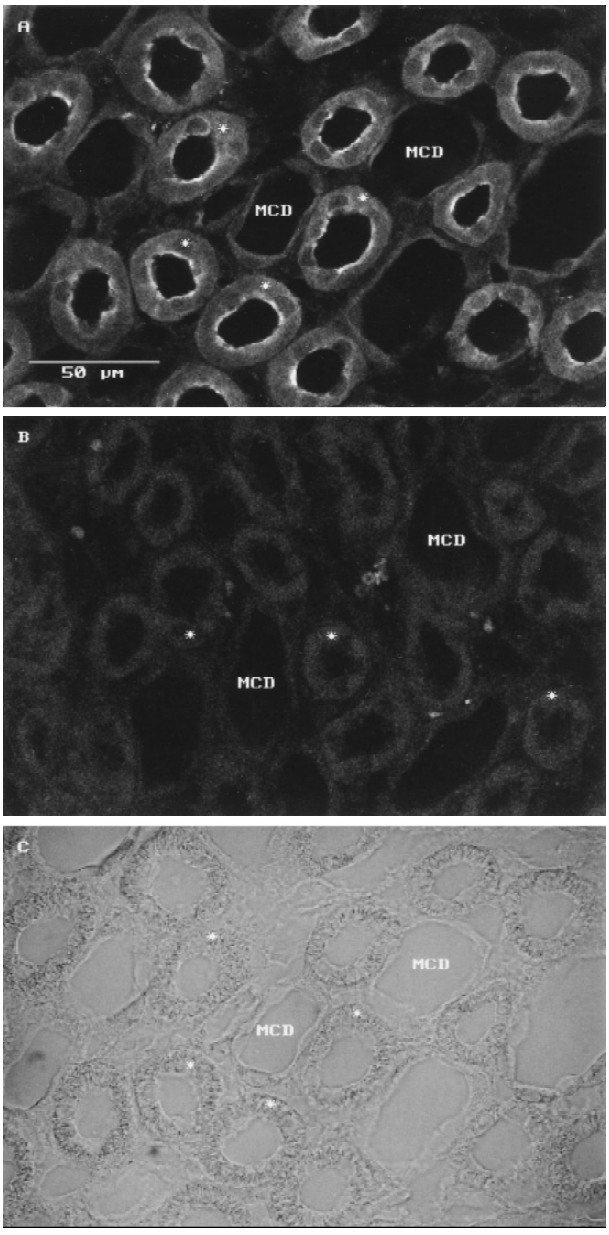

Fig. 3. Indirect immunofluorescence (*A* and *B*) and phase contrast (*C*) microscopy showing NHE2 distribution in MTAL and $OMCD_i$ cells in inner stripe of outer medulla of rat. Cryosections of fixed rat kidney at the level of the inner stripe of the outer medulla were stained with rabbit anti-NHE2 immune serum with (*B*) or without (*A*) preabsorption with NHE2 fusion protein. Antiserum was detected with CY5-conjugated donkey anti-rabbit IgG. NHE2 protein is detected in the apical, but not basolateral, membrane of MTAL (*) and $OMCD_i$ (MCD).

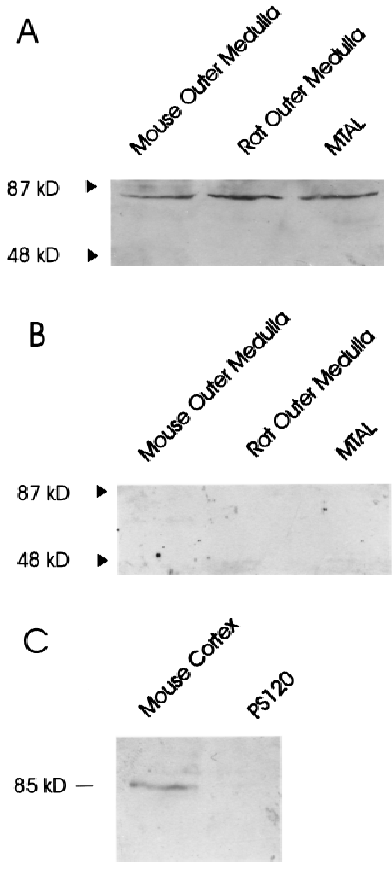

Fig. 4. (*A*) Western analysis using anti-NHE2 antiserum (AB597)(1:500 dilution) on membrane proteins from mouse renal outer medulla (100 μg/lane), rat renal outer medulla (100 μg/lane) and MTAL cell line (50 μg/lane). (*B*) Antiserum was preincubated with fusion protein prior to labeling. (*C*) Western analysis using AB597 antiserum (1:1000 dilution) on membrane proteins from mouse renal cortex and PS120 cell (NHE-deficient).

known, it is unlikely that anti-NHE2 antiserum (AB597) will cross react with other NHE isoforms in the mouse. First, mouse NHE1 has recently been cloned and sequenced and found to be highly identical to rat NHE1 (>85% in amino acid sequence) (Shiela Bell, University of Cincinnati, *personal communication*), but not to other rat NHE isoforms. Second, by Western blot analysis (using specific NHE1 and NHE3 antisera kindly provided by O. Moe, University of Texas Southwestern and E. Cheng, University of Chicago, respectively), we found that the size of NHE1 and NHE3 in the mouse kidney [18] and MTAL cell line (*data not shown*) are 100 kD and 100–105 kD, respectively, which is clearly distinct from the 85 kD protein recognized by the anti-NHE2 antiserum used in this study.

Among NHE isoforms, NHE2 is most closely related to NHE4. However, it is unlikely that the NHE we detected in the apical membrane of MTAL is NHE4. First, the amino acid sequence against which anti-NHE2 antibody was raised shares no homology with that of NHE4 [9, 20]. Second, NHE4 mRNA was not detected by specific RT-PCR in microdissected MTAL by both Borensztein et al. [5] and us (*data not shown*). Third,

data from several other studies suggest that NHE4 is expressed only in the basolateral membranes of the collecting duct [27]. The absence of NHE2 staining in the apical membrane of both rat and mouse OMCD is consistent with previous observations [8] that NHE is not functionally expressed in the apical membrane of this tubule segment.

As discussed previously, it was not until recently, NHE3 was believed to mediate all apical NHE functions in the proximal tubule and thick ascending limb. The demonstration that NHE2, which is structurally and pharmacologically distinct from NHE3 [26], is present in the apical membrane of proximal tubules by other investigators [3, 9, 24] and of MTAL in this study suggests that these two NHE isoforms may perform different or complimentary apical NHE functions. Alternatively, apical NHE functions may be mediated by NHE2 or NHE3 under different physiological conditions. Consistent with this hypothesis, it has recently been shown that rat NHE2 and NHE3 expression are stimulated and inhibited, respectively, by external hyperosmolality in NHE-deficient fibroblasts [10]. Further studies are required to delineate the relative expression of these two isoforms in MTAL under different physiology conditions.

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