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Localization of the Na^+/H^+ exchanger isoform NHE-3 in rabbit and canine kidney

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Abstract

The distribution and subcellular localization of Na^+/H^+ exchanger isoform NHE-3 was studied in rabbit and canine kidney using polyclonal antibodies to an NHE-3 fusion protein. Western blot analyses were performed against microsomal, brush-border, and basolateral membranes isolated from rabbit kidney cortex, outer medulla, and inner medulla. Immunoblots indicated that NHE-3 antibody recognized a strong band with 95–100 kDa molecular mass in cortical microsomes. Subcellular localization studies showed that NHE-3 was expressed in brush-border membranes of kidney cortex. Expression of NHE-3 in the medullary regions was studied by immunoblot analysis of NHE-3 antibody against the microsomal membranes from the outer and inner medulla. NHE-3 antibody specifically labelled a 95–100 kDa protein in outer but not inner medulla. Subcellular localization studies demonstrated that NHE-3 is localized to the brush-border membranes of the outer medulla. Immunoblot analysis against brush-border membranes from canine kidney cortex and outer medulla demonstrated the presence of an 83–90 kDa protein. The above experiments suggest that NHE-3 in rabbit kidney is a 95–100 kDa protein and is expressed in brush-border membranes of the cortex and outer medulla. The canine kidney NHE-3 is a 83–90 kDa protein and is expressed in brush-border membranes of the cortex and outer medulla. Based on its subcellular localization, we conclude that NHE-3 may be involved in vectorial Na^+ and HCO_3^- transport and pH_o regulation.

Keywords: Sodium ion–proton exchanger; Isoform NHE-3; Localization; (Rabbit kidney); (Canine kidney)

1. Introduction

The amiloride-sensitive Na^+/H^+ exchanger mediates an electroneutral process that plays a key role in the regulation of intra- and extracellular pH. This exchanger is located in the plasma membranes of nearly all mammalian cells [1,2]. In the kidney, the Na^+/H^+ exchanger has been identified on the apical and/or basolateral membranes in most nephron segments including the proximal tubule [3], the loops of Henle [4–8], the distal tubule [9], and the cortical and medullary collecting duct [10–16].

The majority of filtered bicarbonate is reabsorbed in the kidney proximal tubule via the luminal Na^+/H^+ exchanger and basolateral Na^+/HCO_3^- cotransporter acting in series [1]. A basolateral Na^+/H^+ exchanger has also been identified in kidney proximal tubule cells and is presumed to be mostly involved with cell pH regulation [17]. Several studies have demonstrated the luminal Na^+/H^+ exchanger is adaptively upregulated in certain pathophysiologic states including metabolic acidosis [18–21], respiratory acidosis [22], and potassium depletion [23]. This would result in enhanced HCO_3^- reabsorption in proximal tubules. A recent study have shown the basolateral Na^+/H^+ exchanger is also upregulated in metabolic acidosis [24].

Based on biochemical and pharmacological properties, several isoforms of the Na^+/H^+ exchanger have been identified in several tissues including kidney

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[25,26]. Recently, a human growth factor-activatable Na^+/H^+ exchanger (NHE-1) was cloned and sequenced from fibroblast cells [27]. Based on the deduced amino acid sequence, NHE-1 has a relative molecular mass of 91 kDa. Studies of the cell distribution of NHE-1 in the kidney have shown the exchanger is predominantly localized to the basolateral membranes of proximal tubule, thick ascending limb, and distal convoluted tubules [17]. Antibodies directed against an NHE-1 synthetic peptide localized the exchanger to a brush-border membrane fraction eluted from an anion exchange (Mono Q) column [28]. This fraction, however, was found to contain minimal levels of Na^+/H^+ exchange activity [28]. Using an NHE-1 cDNA probe under low stringency hybridization conditions, three structurally related isoforms of Na^+/H^+ exchanger have been cloned and sequenced [29–31]. Northern blot analyses demonstrated these exchangers, designated NHE-2, NHE-3, and NHE-4, are expressed in the kidney [29–31]. NHE-2, NHE-3, and NHE-4 cDNAs encode proteins with predicted molecular masses of 92, 93 and 82 kDa, respectively [29–31]. The mRNA for Na^+/H^+ exchanger isoform NHE-3 is expressed specifically in the kidney and intestine, with the kidney cortex having the most abundant levels [30]. It has been suggested that NHE-3 might be involved in Na^+ absorption in the kidney and the intestine [30]. Two recent studies suggest that NHE-3 is localized to the luminal membranes of proximal tubule and small intestine [32,33].

The purpose of the current investigation was to examine the presence and subcellular localization of NHE-3 isoform in rabbit and canine kidney. These objectives were accomplished by immunoblot analysis using antibodies generated against an NHE-3 fusion protein. We found that NHE-3 is expressed in the brush-border membranes of cortex and outer medulla of rabbit and canine kidney.

2. Materials and methods

2.1. Membrane vesicles preparation

Male New Zealand white rabbits were killed by intravenous sodium pentobarbital administration. Brush-border membrane (BBM) vesicles were isolated from renal cortex by a Ca^{2+} aggregation method [34] as employed previously [21,23]. Brush-border membranes from outer or inner medulla were isolated according to Chang et al. [35]. Basolateral membrane (BLM) vesicles were isolated from renal cortex, outer or inner medulla by differential and Percoll gradient centrifugation [36] as employed previously [37–39]. Microsomal membranes were prepared according to Grassl et al. [36]. Canine renal brush-border membrane vesicles were isolated by a Ca^{2+} aggregation method as employed before [40]. Protein concentration was determined by the bicinchoninic acid (BCA) method [41] according to the manufacturer's protocol (Pierce, Rockford, IL). Membrane fractions from the cortex and outer medulla were assayed for specific activity of the brush border alkaline phosphatase and for the basolateral Na^+,K^+-ATPase to determine the degree of purification. The purification of brush-border membrane vesicles relative to the initial homogenate, assessed by alkaline phosphatase, was 8–10-fold for the cortex and 6–8-fold for the outer medulla. Brush-border membranes and basolateral membranes from the inner medulla were assayed for the specific activity of Na^+,K^+-ATPase to assess the degree of cross contamination or purification, respectively. The brush-border membranes isolated from the inner medulla had 2–3-fold enrichment in Na^+,K^+-ATPase suggesting mild degree of basolateral cross-contamination. The purification of basolateral membrane vesicles relative to the initial homogenate, assessed by Na^+,K^+-ATPase, was 7–11-fold for the cortex, outer medulla, and inner medulla.

2.2. Preparation of NHE-3 fusion protein

A gluthathione-*S*-transferase (GST) fusion protein was prepared from the C-terminal 121 amino acids (amino acids 528–648) of the NHE-3 protein encoded by a rat NHE-3 cDNA [33,45]. Briefly, the $BstY1_{1815}$–$BstY1_{2181}$ fragment was ligated into *Bam*HI restriction site of the pGEX-3X vector generating an in frame fusion to GST. The correctness of the reading frame of the NHE-3 fusion protein was verified by Sanger dideoxy DNA sequencing. NHE-3 fusion protein was induced in *Escherichia coli* and purified from the Triton-insoluble fraction by SDS-PAGE. The excised gel piece containing the fusion protein band was electroeluted and concentrated.

2.3. Immunization with the NHE-3 fusion protein

NHE-3 fusion protein was emulsified in complete Freund's Adjuvant and injected subcutaneously into two rabbits. Booster injections were carried out at 4 weeks intervals using incomplete Freund's Adjuvant. Animals were bled 10 days after each booster injection.

2.4. Preabsorption of the immune serum

NHE-3 fusion protein was coupled to a cyanogen bromide-activated Sepharose 4B column (purchased from Pharmacia, NJ). Immune serum was equilibrated with the column for 1 h at room temperature, eluted by centrifugation, and saved until used. The concentration of immunoglobulins was the same in control and pre-

absorbed immune serum as assayed by spectrophotometry following affinity purification with Protein-A Sepharose column (0.107 in control vs. 0.113 mg/ml in preabsorbed immune serum).

2.5. SDS-PAGE and immunoblot analysis

Membrane fractions (200 μg/lane) were solubilized and subjected to a vertical slab SDS-PAGE according to the Laemmli protocol [42]. Proteins were elctrophoretically transferred to nitrocellulose at 200 mA for 15 h. Strips of nitrocellulose were blocked in 0.1% Tween 20/phosphate-buffered saline (PBST) and then incubated with 10 μl of serum diluted at 1:400 in PBST for 2 h. The excess antibody was washed with PBST and the antigen-antibody complex was treated with alkaline phosphatase-conjugated goat anti-rabbit IgG diluted at 1:1000 in PBST. The nitrocellulose filters were developed with Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) dissolved in 70 or 100% *N,N*-dimethylformamide (DMF).

2.6. Materials

Alkaline phosphatase-conjugated anti-rabbit IgG and BCIP were purchased from Boehringer Mannheim (Indianapolis, IN). Nitrocellulose filters and NBT were purchased from Sigma (St.Louis, MO). CNBr-activated Sepharose 4B was purchased from Pharmacia (Piscataway, NJ). Sodium dodecyl sulphate (SDS), acrylamide, and *N,N'*-methylenebisacrylamide were purchased from Bio-Rad (Hercules, CA).

3. Results

We first studied the expression of NHE-3 in crude microsomal membranes isolated from rabbit kidney cortex. Fig. 1 (left lane) demonstrates that the immune serum containing NHE-3 antibody detected a strong and diffuse band with an apparent molecular mass of 95–100 kDa in cortical microsomal membranes. To determine the specificity of immunodetection of the 95–100 kDa protein, the NHE-3 antibody was depleted from the immune serum by preabsorption with the fusion protein. Such treatment should block specific labelling of NHE-3 in membranes subjected to Western blot analysis. As demonstrated in Fig. 1 (right lane), the immunodetection of the 95–100 kDa protein was significantly blocked in the presence of preabsorbed immune serum. We have studied the specificity of the NHE-3 antibody in the Na^+/H^+ exchange-deficient PS120 cells prior to and after transfection with the NHE-3 cDNA, and also in the NHE-1 overexpressing PS127 cells. The results indicate that NHE-3 antibody is highly specific and does not react with NHE-1 [45]. Taken together, these experiments illustrate the specificity of the NHE-3 antibody.

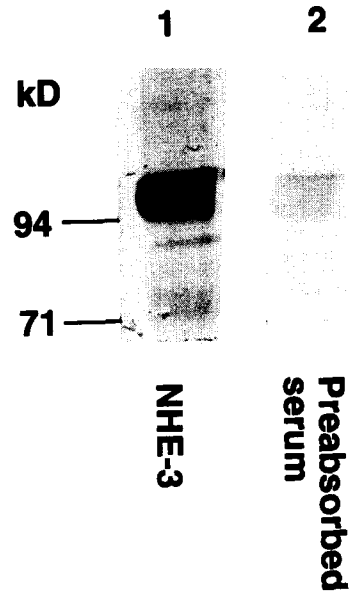

Fig. 1. Representative immunoblots of rabbit renal cortical microsomes using: NHE-3 antiserum (left lane) and antiserum equilibrated with NHE-3 fusion protein column(right lane).

To determine the subcellular localization of NHE-3 in the cortex, brush-border and basolateral membranes were isolated and subjected to immunoblot analysis. Fig. 2A (left lane) demonstrates that the immune serum containing NHE-3 antibody detected a strong and diffuse band with an apparent molecular weight of 95–100 kDa in cortical brush-border membranes. As shown in Fig. 2A (right lane), NHE-3 antiserum reacted faintly with basolateral membranes isolated from kidney cortex. A 70 kDa protein was also occasionally detected with NHE-3 immune serum. The immunodetection of the 70 kDa protein was not consistent, suggesting the presence of a proteolytic breakdown product of the 95–100 kDa protein. Fig. 2B is a Western blot analysis examining the specificity of the NHE-3 immunue serum in brush-border membranes. As shown in this experiment, immunodetection of the 95–100 kDa protein by NHE-3 immune serum (Fig. 2B, lane 1) was significantly blocked in the presence of the antiserum that was equilibrated with a NHE-3-GST fusion protein column (Fig. 2B, lane 2). To examine the specificity of the NHE-3 antiserum further, GST was induced in bacteria using the parent pGEX vector and utilized to prepare a GST affinity column. Equilibration of the immune serum with a GST affinity column did not block the labelling of the 95–100 kDa protein (Fig. 2B, lane 3). Taken together, these results illustrate the anti NHE-3 specificity of the immune serum.

As shown in Fig. 2A (right lane), NHE-3 antibody detected a faint band in basolateral membranes iso-

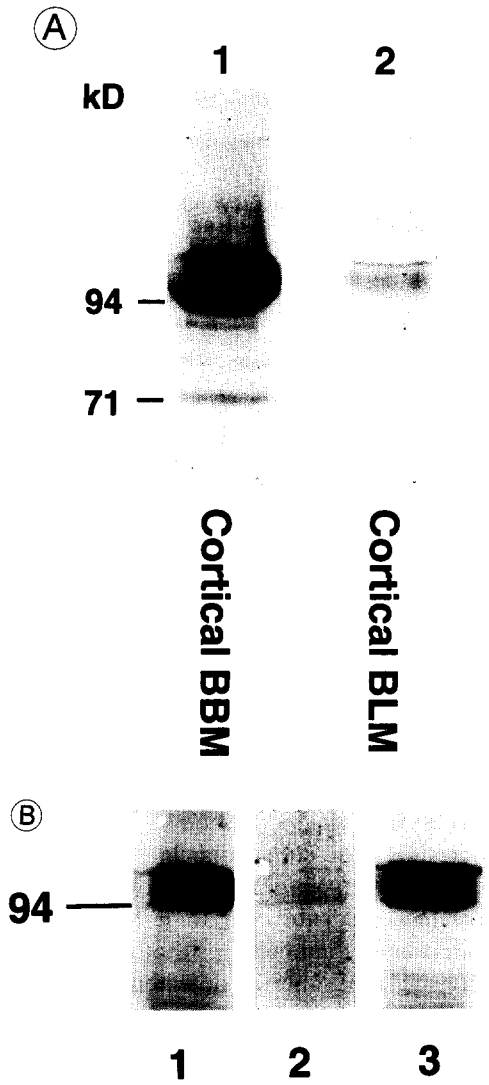

Fig. 2. (A) Representative immunoblots of rabbit cortical BBM (left lane) and BLM proteins (right lane) using NHE-3 antibody. (B) Representative immunoblots of rabbit renal brush-border membranes using: NHE-3 antiserum (lane 1), antiserum equilibrated with NHE-3 fusion protein column (lane 2) and antiserum equilibrated with GST column (right 3).

lated from kidney cortex. The faint band could be due to either low abundance of NHE-3 and/or cross-contamination by brush-border membranes in the basolateral membranes. To assess the degree of cross contamination of basolateral membranes with brush-border membranes, if any, basolateral membrane proteins were tested for the presence of the Na/glucose cotransporter by immunoblot analysis. The Na/glucose cotransporter is exclusively present in brush-border membranes [46] and, therefore, immunodetection of this cotransport in basolateral membranes would strongly argue for the presence of cross-contamination in basolateral membrane vesicles. As shown in Fig. 3 the Na/glucose cotransport antibody detected a faint band in cortical basolateral membranes (right lane) as compared to a very strong and diffuse band in brush-border membrane fraction (left lane). These results strongly suggest that the NHE-3 detected in basolateral membranes is most likely due to contaminating brush-border membranes. The degree of cross-contamination of basolateral membranes with brush-border membranes, as assessed by relative abundance of the Na/Glut cotransporter, correlates very well with the amount of NHE-3 in the basolateral membranes in Fig. 2A.

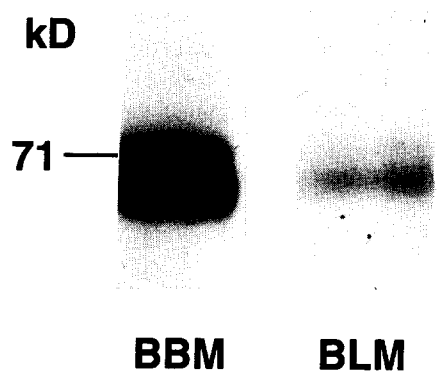

Fig. 3. Representative immunoblots of rabbit cortical BBM (left lane), and BLM proteins (right lane) using Na/Glut antibody.

The results of the studies in Figs. 1 and 2 demonstrate that NHE-3 is localized to rabbit renal cortical brush-border membranes. However, several investigators have identified Na^+/H^+ exchange activity in various nephron segments located in the medullary region [12–16]. Therefore, we next examined the distribution of NHE-3 in the medulla. Fig. 4 is a representative Western blot of outer or inner medullary microsomal

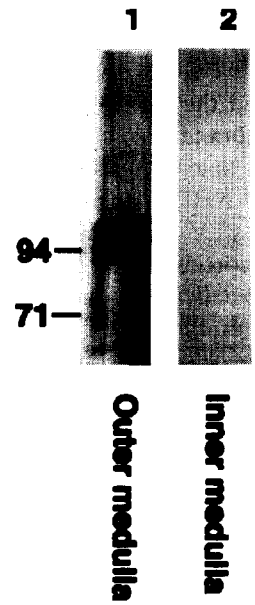

Fig. 4. Representative immunoblots of rabbit renal microsomal membranes from outer medulla (left lane) and inner medulla (right lane) using NHE-3 antibody.

membranes analyzed by NHE-3 antiserum. As shown in Fig. 4, NHE-3 antibody reacted with a 95–100 kDa protein in the microsomal membranes isolated from outer medulla (left lane) but not those from the inner medulla (right lane), suggesting absence of expression of this exchanger in the inner medulla. To determine the subcellular localization of NHE-3 in the outer medulla, brush-border or basolateral membranes from this renal segment were analyzed by immunoblotting using the NHE-3 antiserum. As shown in Fig. 5 (left lane), the NHE-3 specific antibody detected a relatively strong band with an apparent molecular weight of 95–100 kDa in the outer medullary brush-border membranes. In contrast, as shown in Fig. 5 (right lane), the NHE-3 antibody only reacted faintly with a 95–100 kDa band in medullary basolateral membranes. As seen in the cortex, this faint band most probably represents cross-contamination of brush-border membrane proteins in the basolateral membrane fraction.

To examine the distribution of NHE-3 in the canine kidney, brush-border membranes were isolated from canine renal cortex and outer medulla, and studied by immunoblot analysis. Fig. 6 (left lane) demonstrates that the immune serum containing NHE-3 antibody detected a diffuse band with an apparent molecular weight of 83–90 kDa in canine cortical brush-border membranes and only faintly a protein of similar molecular weight in outer medullary brush-border membranes (Fig. 6, right lane). Preabsorption of the immune serum with fusion protein blocked the immunodetection of the 83–90 kDa protein (data not shown). A 74 kDa protein was also occasionally detected with NHE-3 immune serum.

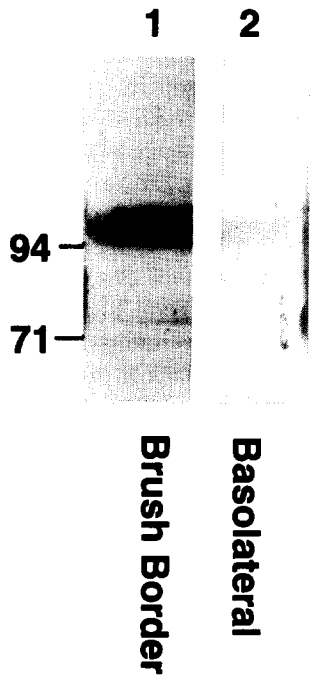

Fig. 5. Representative immunoblots of rabbit renal outer medullary BBM (left lane) and BLM proteins (right lane) using NHE-3 antibody.

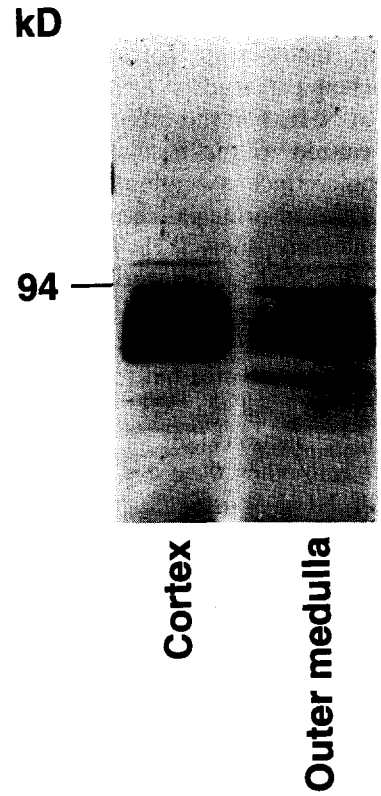

Fig. 6. Representative immunoblots of canine renal brush-border membranes from cortex (left lane) and outer medulla (right lane) using NHE-3 antibody.

The results of the above experiments suggest that rabbit renal NHE-3 is a 95–100 kDa polypeptide that is expressed in brush-border membranes of cortex and outer medulla. The canine renal NHE-3 is an 83–90 kDa protein that is largely localized to the brush-border membranes of cortex.

4. Discussion

Recent cloning studies have demonstrated the existence of four structurally related Na^+/H^+ exchanger isoforms [29–31]. These isoforms, designated NHE-1, -2, -3, and -4 have been cloned and sequenced [29–31]. The cDNAs for these isoforms encode proteins with predicted molecular masses of 91, 92, 93 and 82 kDa, respectively [29–31]. Tissue distribution studies demonstrate that all these exchanger isoforms are expressed in the kidney. Northern blot analysis suggest that in rat kidney NHE-3 mRNA is the most abundant and NHE-4 mRNA is the least abundant [29–31]. However, it is difficult to draw any firm conclusion about the abundance of the isoform based on these Northern blot data. These experiments were done on whole kidney

mRNAa and as such regional distribution of the exchangers, i.e., medulla vs. cortex, was not studied. Immunocytochemical studies evaluating the cellular distribution of NHE-1 in the rabbit kidney have shown that it is localized to the basolateral membranes of proximal tubule, thick ascending limb, distal convoluted tubule, and in principal cells of the cortical and medullary collecting ducts [17]. The highest level of staining was detected in the distal convoluted tubule and the thick ascending limb of Henle's loop. A recent study found that NHE-1 is present in brush-border membranes of kidney cortex but found it to be a minor component of the total Na^+/H^+ exchange activity in these membrane fractions [28].

Polyclonal antibodies were developed to a C-terminal region of NHE-3 using fusion protein (see the experimental procedures). The peptide sequence chosen from NHE-3 had a low degree of homology to NHE-1, -2, and -4. The results of immunoblot analyses demonstrated this Na^+/H^+ exchanger isoform was distinctly expressed in different regions of the kidney (Figs. 1,2,4,5). The NHE-3 antiserum intensely recognized a polypeptide with an apparent molecular mass of 95–100 kDa, a size appropriate for NHE-3. Subcellular localization of NHE-3 demonstrated this exchanger was present in brush-border membranes of kidney cortex. This membrane fraction of kidney cortex corresponds to the apical domain of the proximal tubule cells and suggest NHE-3 is the isoform present in proximal tubule cells.

The NHE-3 antiserum also recognized a protein of molecular size appropriate for NHE-3 in the brush-border membranes of outer medulla suggesting the presence of NHE-3 in this renal region. Localization of NHE-3 to any specific nephron segment in the outer medulla, however, will be difficult since proximal S3, limbs of Henle, and outer medullary collecting duct are contained in outer medullary region. Nevertheless, one important conclusion from these studies was that NHE-3 appears only in brush-border membranes of certain nephron segments. Staining with NHE-3 antibody did not detect any reactivity in the microsomal membranes of the inner medulla, suggesting a lack of expression of NHE-3 in the inner medulla. The majority of the cells in the inner medullary region belong to the inner medullary collecting duct cells. Several functional studies have identified a Na^+/H^+ exchanger in the basolateral membranes of inner medullary collecting duct cells [14–16]. These studies suggested the luminal membranes of the inner medullary collecting ducts cells do not contain any sodium-dependent acid-base transporter, consistent with the absence of Na^+/H^+ exchange activity in these membrane fractions [14–16]. The expression of the NHE-3 in the cortex and outer medulla (the segments that demonstrate Na^+/H^+ exchange on their luminal membrane) and its absence in the inner medulla (where Na^+/H^+ exchange is absent on the luminal membranes) suggest that NHE-3 is a brush-border membrane isoform.

A recent investigation examining the distribution of NHE-3 in rabbit kidney localized this exchanger to the brush-border membranes of proximal tubules [32]. The NHE-3 had an apparent molecular mass of ~80 kDa [32]. The NHE-3 in the present experiments was found to have a molecular mass of ~95–100 kDa. The reason for the difference in the mobility of the NHE-3 exchanger in these two studies is not clear. One possibility is that the gel systems in these two studies might be different. Another plausible explanation is that NHE-3 might be present in more than one isoform. Possibilities like alternative splicing of the encoding cDNA could result in more than one isoform. As such, the antibody that was used in those experiments [32] could recognize a different protein than the current experiments. Definitive answer will come from sequencing the immunoprecipitated proteins utilizing the respective antibodies.

The NHE-3 cDNA in rat or rabbit encodes a protein with a molecular mass of ~92 kDa [29, 30]. The rabbit NHE-3 in the current studies was found to have a molecular weight of 95–100 kDa (Figs. 1, 2). Pretreatment of rabbit brush-border membranes with glycopeptidase F resulted in increased mobility of the exchanger to ~91 kDa suggesting that rabbit NHE-3 is a glycoprotein(data not shown). The mobility of NHE-3 in canine kidney, however, was not affected by glycopeptidase F pretreatment (data not shown). These results suggest that NHE-3 is differentially glycosylated in rabbit and canine kidney.

The distinct distribution and subcellular localization of NHE-3 suggest this isoform performs a unique function. The presence of NHE-3 in the luminal membranes of kidney cortex suggest the exchanger is likely responsible for HCO_3^- reabsorption and extracellular pH regulation. NHE-3 is also expressed in the outer medulla. Of the nephron segments that are contained in the outer medulla, both descending and ascending limb of Henle demonstrate significant Na^+/H^+ exchange activity on their luminal membranes and are involved in vectorial transport of HCO_3^-. Based on the results of the above experiment (Figs. 4 and 5), we propose that NHE-3 is the Na^+/H^+ exchanger isoform expressed in the luminal membranes of descending and/or ascending limb of Henle. Definitive cellular localization of NHE-3 in the outer medulla by immunocytochemistry could answer this question. However, repeated attempts in our laboratories in performing immunocytochemical studies with NHE-3 specific polyclonal antibodies have not been successful.

In conclusion, the results of the above experiments demonstrate the NHE-3 isoform is expressed in multiple nephron segments in a distinct and specific pattern.

NHE-3 is a Na^+/H^+ exchanger isoform that is expressed in the brush-border membranes of kidney cortex and outer medulla and is absent in the inner medulla. NHE-3 was found to be differentially glycosylated in rabbit and canine kidney. Based on its localization, we conclude that NHE-3 may be involved in vectorial Na^+ and HCO_3^- transport and pH_o regulation.

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