JCI The Journal of Clinical Investigation

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J Clin Invest. 1993;91(5):2097-2102. https://doi.org/10.1172/JCI116433.

Research Article

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Identification of the Human NHE-1 Form of Na^+-H^+ Exchanger in Rabbit Renal Brush Border Membranes

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Abstract

To study the relation between the human Na^+-H^+ exchanger (NHE-1) and the renal brush border membrane (BBM) Na^+-H^+ exchanger, polyclonal antibodies to synthetic peptides representing a putative external (Ab-E) and an internal cytosolic domain (Ab-I) of human NHE-1 were generated in rabbits. Western immunoblot analyses indicated that both antibodies recognized a 97-kD protein in rabbit renal BBM but not basolateral membranes (BLM). Octyl glucoside–extracted rabbit renal BBM proteins also contained the 97-kD polypeptide as did a fraction eluted from an anion-exchange column with 0.2 M NaCl (fraction A). A fraction eluting between 0.2 and 0.4 M NaCl (fraction B) did not contain this protein. Prior reconstitution studies have indicated that Na^+-H^+ exchange activity is higher significantly in fraction B than fraction A. Administration of NH_4Cl for 3–7 d to rabbits, a stimulus known to increase renal BBM Na^+-H^+ exchange activity, did not result in a change in expression of the 97-kD protein in either renal BBM or BLM.

The results indicate that affinity-purified polyclonal antibodies to two separate domains of the human Na^+-H^+ exchanger recognize a 97-kD protein in rabbit renal BBM but not BLM. The dissociation between recognition of the 97-kD protein using antibodies and the majority of functional Na^+-H^+ exchange activity after chromatographic fractionation of solubilized BBM proteins and in native BBM after administration of NH_4Cl suggests that rabbit renal BBM contains more than one form of Na^+-H^+ exchanger. (*J. Clin. Invest.* 1993. 91:2097–2102.) Key words: membrane protein • intracellular pH • acidification

Introduction

In 1989, Sardet and co-workers (1) isolated and sequenced a cDNA encoding a human Na^+-H^+ exchanger. This exchanger has been called the "housekeeping" form of the Na^+-H^+ exchanger; a designation that reflects its wide distribution in tissues and its role in defense of cell pH. This exchanger, also designated NHE-1, can be differentiated on the basis of functional characteristics from the Na^+-H^+ exchanger present in the apical membrane of some transporting epithelia, such as the renal proximal convoluted tubule and the small intestine (2). Subsequently, a number of groups have reported preliminary experiments indicating that the NHE-1 cDNA hybridizes to a single mRNA species in renal cortical tissue (3–5). In initial experiments, a cDNA that is highly homologous to the housekeeping form of the Na^+-H^+ exchanger has been isolated from kidney. No studies to date, however, have examined the relative expression of this transport protein in renal tissue. The present experiments were undertaken to address this question. Affinity-purified polyclonal antibodies to synthetic peptides representing segments of putative extracellular and inner cytosolic domains of NHE-1 were generated in rabbits. These antibodies were used to study the expression of the antiporter in rabbit renal brush border membrane (BBM)[1] and basolateral membrane (BLM). In addition, detergent-solubilized BBM proteins and fractions separated by anion-exchange chromatography were also analyzed. Finally, the effect of systemic acidosis on the expression of NHE-1 was determined by quantitative immunoassays.

Methods

The methods for preparation of BBM vesicles from the rabbit kidney and the procedures for solubilizing the membrane proteins have been described extensively in recent publications (6, 7). In brief, BBMs were isolated from the cortex of the rabbit kidney by a magnesium aggregation method (6). The kidney cortex was homogenized in a medium containing the following (mM): 254 mannitol, 10 Tris, 16 Hepes, and 10 $MgSO_4$ (pH 7.5). The suspension underwent alternating low speed and high speed centrifugations. The resultant pellet was resuspended in homogenizing buffer to a final protein concentration of 10 to 20 mg/ml. Membrane proteins were extracted by mixing one part of the membrane preparation (5 mg/ml) with 1.25 parts of 8% octyl glucoside at pH 6.0 for 15 min at 0°C (7). Solubilized BBM proteins were separated by fast protein liquid chromatography (8). Solubilized membrane proteins were applied to an anion-exchange column (Mono Q HR 5/5; Pharmacia Inc., Piscataway, NJ) and batch eluted with a solution containing 50 mM Tris, 0.1 mM EDTA, 0.1 mM DTT, 2% octyl glucoside, and 0.2 M NaCl (fraction A) followed by the same buffer containing 0.4 M NaCl (fraction B) at a rate of 1 ml/min. The eluted proteins were desalted on a Sephadex-G25 column before analysis.

BLM vesicles were isolated by a method previously described (9). Kidney cortex was homogenized in the following (mM): 250 sucrose, 10 Tris, 16 Hepes (pH 7.5), and 0.1 PMSF. The homogenate was centrifuged at 2,445 *g* for 15 min and the pellet was recentrifuged for 20 min at 24,400 *g*. The resulting upper layer was resuspended in homogenizing buffer. Percoll was added to a final concentration of 8% (vol/vol) and the mixture was centrifuged for 35 min at 29,950 *g*. The fractions containing basolateral membranes as identified by the pres-

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Received for publication 5 November 1992 and in revised form 30 November 1992.

1. *Abbreviations used in this paper:* Ab-E, antibody to synthetic peptide of external domain; Ab-I, antibody to synthetic peptide of internal (cytostolic) domain; BBM, brush border membrane; BLM, basolateral membrane.

ence of Na^+-K^+ ATPase activity were ultracentrifuged for 60 min at 150,000 *g*. The pellet layering on top of the Percoll was removed and resuspended in homogenizing solution to a final protein concentration of 6 to 9 mg/ml.

The purity of the membrane preparations was determined by the activity of alkaline phosphatase, a marker of the BBM, and Na^+-K^+ ATPase, a marker of BLM. These values were compared with the activities of these enzymes in homogenates of whole kidney cortex. Alkaline phosphatase activity was measured as described in Kahn et al. (10). Na^+-K^+ ATPase activity was assayed by measurement of ouabain-sensitive phosphatase activity of deoxycholate-treated samples in the presence of the following (mM): 10 KCl, 5 $MgSO_4$, 60 Tris-HCl (pH 7.8), and 6 *p*-nitrophenyl phosphate (11). Enrichment factors (enzyme activities in membranes vs. whole homogenate) for alkaline phosphatase and Na^+-K^+ ATPase averaged 9.8±1.0 and 1.4±0.6, respectively, in BBM and 1.5±0.5 and 15.2±3.4, respectively, in BLM.

Crude membrane preparations from PS120 cells (Chinese hamster lung fibroblast cells deficient in all endogenous Na^+-H^+ exchangers [12], which were kindly supplied by Dr. J. Pouyssegur, Nice, France) and rabbit brain and skeletal muscle were prepared by homogenization in iced buffer of 50 mM Tris and 0.1 mM EDTA (pH 7.5) containing 0.2 mM PMSF, 1 μg/ml antipain, 1 μg/ml aprotenin, 1 μg/ml leupeptin, and 0.1 mM DTT. The homogenate was then centrifuged at 800 *g* for 15 min. The supernatant was ultracentrifuged at 30,000 *g* for 60 min. The resultant pellet was used in Western immunoblot analysis after SDS-PAGE.

Western immunoblot analysis was performed by a modification of the method of Towbin et al. (13). The proteins were separated initially by SDS-PAGE and electrophoretically transferred to nitrocellulose. The nitrocellulose was blocked with 5% BSA in phosphate-buffered saline containing 0.1% Tween 20 (PBST) for 4 h before reacting with the antisera overnight at 4°C. The filters were washed briefly with 1% BSA-PBST. Immune complexes were detected by the addition of ^{125}I-labeled *Staphylococcus aureus* Protein A (10^6 cpm/ml) for 30 min at room temperature. The nitrocellulose filters were then washed with 50 mM Tris-HCl (pH 7.4) containing 0.25% gelatin, 0.5% Triton X-100, 0.1% SDS, 5 mM EDTA, and 150 mM NaCl for 3 h and visualized by autoradiography. Slot blot analyses was performed by direct application of BBM proteins to nitrocellulose in the presence of 25 mM phosphate-buffered (pH 6.8), 2% 2-mercaptoethanol, and 2% SDS and reacted with antibodies and Protein A as described above for the Western immunoblots. The slot blots were quantitated using laser densitometry.

Peptides corresponding to amino acids 249–256 (amino acid sequence EEIHINELLH), a putative external domain of the human Na^+-H^+ exchanger, and 787–797 (amino acid sequence SSQRIQRCLSD), a putative cytosolic domain with an added NH_2-terminal cysteine were synthesized. The peptide corresponding to the external domain of the human Na^+-H^+ exchanger was cross-linked to keyhole limpet hemocyanin in the presence of glutaraldehyde. The peptide corresponding to the internal domain of the human Na^+-H^+ exchanger was coupled to keyhole limpet hemocyanin using m-maleimidobenzoyl-*N*-hydroxysuccinimide. The peptides were then emulsified in complete Freund's Adjuvant and injected subcutaneously into rabbits. Booster injections with conjugated synthetic peptides emulsified in incomplete Freund's Adjuvant were administered at intervals of 10 to 30 d. The immunoglobulin fraction from rabbit serum was partially purified by repeated precipitations with 50% saturated ammonium sulfate and chromatography on DEAE Affi-Gel Blue to remove residual serum albumin and trace serum protease activity. Affinity-purified antibodies were prepared by chromatography on Affi-Gel 10 coupled to the original synthetic peptide using 3 M sodium thiocyanate. The affinity column–purified immunoglobulin fraction was dialyzed extensively against water to remove traces of cyanide. The sample was then lyophilized until dry and resuspended in phosphate-buffered saline containing 0.02% sodium azide to an OD^{280} reading between 1 and 2. Western immunoblots of BBM were performed using serial dilutions of the affinity-purified antibody to determine the appropriate dilution for experimental studies for each batch of antibodies. The antibody dilution used in the data presented was ≥ 1:500.

Protein concentrations were determined by the method of Lowry et al. (14). Results of experiments are presented as the mean±SEM. Gels and autoradiographs are presented as representative experiments.

Results

Synthetic peptides corresponding to a putative external domain (E) and a putative cytosolic domain (I) of the human Na^+-H^+ exchanger were used to generate antibodies in rabbits (1). In the ensuing discussion, the antibodies to these synthetic peptides are designated as Ab-E and Ab-I, respectively. As shown in Fig. 1, Ab-I detected a protein of apparent molecular mass of 110 kD, a size appropriate for NHE-1, in human red blood cells. This polypeptide was not detected using preimmune sera. Preabsorption of Ab-I with the antigen peptide reduced significantly the immunodetection of 110-kD band. Identical results in human red blood cells were obtained with Ab-E. Ab-E detected a 97-kD protein in rabbit renal BBM and a broad band at a somewhat higher apparent molecular weight in a crude membrane fraction of rabbit skeletal muscle (Fig. 2). No proteins were detected using a crude membrane fraction from rabbit brain. Ab-I also detected a 97-kD protein in rabbit renal BBM but did not detect any proteins in crude membrane fractions from rabbit skeletal muscle or brain. Fig. 3 is a representative protein stain of BBM and BLM and Western immunoblots using the two antibodies. Each of the antibodies recognized a single polypeptide of apparent molecular mass of 97 kD in BBM but not in BLM. Preimmune sera demonstrated no immunoreactivity to the 97-kD protein. In addition, rabbits immunized with keyhole limpet hemocyanin conjugated to other synthetic peptides unrelated to the NHE-1 also failed to demonstrate immunoreactivity to the 97-kD polypeptide. Fig. 4 is a Western immunoblot of BBM using both antibodies, which were preabsorbed by incubation with a 100–1,000 molar excess of the synthetic peptides. Preabsorption of Ab-E with the synthetic peptide corresponding to the external domain of the human Na^+-H^+ exchanger decreased significantly the recognition of the 97-kD proteins. By contrast, preabsorption of Ab-E with the synthetic peptide corresponding to the internal domain of the cloned Na^+-H^+ exchanger had no effect on the

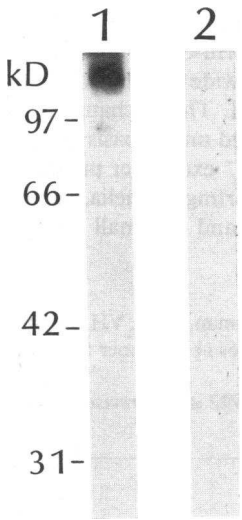

Figure 1. Representative Western immunoblots of human red blood cell proteins using Ab-I (lane *1*). Lane *2* is the same human red blood cells in which Ab-I was preabsorbed with the antigen peptide. Molecular mass standards are indicated.

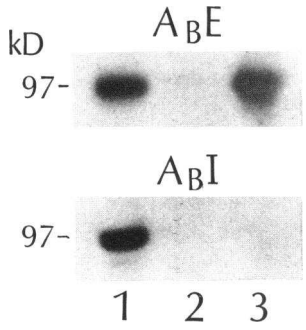

Figure 2. Representative Western immunoblots of rabbit renal BBM (lane *1*) and crude membrane fractions of rabbit brain (lane *2*) and skeletal muscle (lane *3*) using Ab-E (*top*) and Ab-I (*bottom*). Molecular mass standards are indicated.

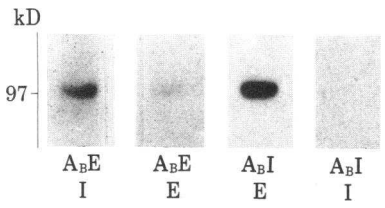

Figure 4. Representative Western immunoblots of rabbit renal BBM proteins using Ab-E and Ab-I that had been preabsorbed with the synthetic peptides representing the external (*E*) or internal (*I*) domain of the cloned Na^+-H^+ exchanger. Molecular mass standards are indicated.

immunodetection of the 97-kD polypeptide. Conversely, preabsorption of Ab-I with the synthetic peptide corresponding to the internal but not with the synthetic peptide corresponding to the external domain of the NHE-1 decreased significantly the recognition of the 97-kD protein.

The antibodies to the synthetic peptides failed to identify any bands using Western immunoblot analysis of a membrane preparation of PS120 cells. These cells derive from Chinese hamster lung fibroblasts and are deficient in all known Na^+-H^+ exchangers. To exclude the possibility that the BBM preparation was contaminated with endothelial cells, Western immunoblot was performed on BBM using a monoclonal antibody to human von Willebrand factor (Boehringer Mannheim Diagnostics, Indianapolis, IN). This antibody failed to identify any bands in BBM. Taken together, these results indicate that BBM contains a protein of apparent molecular mass of 97 kD that is recognized by two independent monospecific antibodies to the human Na^+-H^+ exchanger.

In earlier studies from this laboratory, evidence has been advanced that Na^+-H^+ exchange activity can be demonstrated in octyl glucoside–extracted renal BBM proteins assayed after reconstitution of these proteins into artificial lipid vesicles (7). In addition, fractionation of the solubilized membrane proteins by anion-exchange chromatography indicated little Na^+-H^+ exchange activity in a protein fraction eluting with 0.2 M NaCl (fraction A) (8). By comparison, most of the Na^+-H^+ exchange activity was present in a more anionic fraction eluting between 0.2 and 0.4 M NaCl (fraction B). Fig. 5 is a representative Western immunoblot of BBM, total solubilized proteins, fraction A, and fraction B. The 97-kD polypeptide was recognized by both antibodies in BBM, total solubilized proteins, and fraction A. However, neither antibody demonstrated any significant immunoreactivity with proteins in fraction B; a fraction that contains the majority of the Na^+-H^+ exchange activity as determined by a reconstitution assay. Identical results were obtained when the above experiments were performed in the presence of a variety of protease inhibitors (0.1 mg/ml L-1-chloro-3[4-tosylamido]-4-phenyl-2-butanone [TPCK], 0.1 mg/ml L-1-chloro-3-[4-tosylamido]-7-amino-2-hepatone-HCl [TLCK], and 1 μg/ml of antipain, leupeptin, and aprotenin).

On the basis of earlier experiments that demonstrated an increase in the activity of the BBM Na^+-H^+ exchanger and, more recently, an increased expression of the mRNA for the human Na^+-H^+ exchanger in rat kidney after administration of NH_4Cl, additional studies were performed in rabbits that had received NH_4Cl in drinking water for 3–4 d (15, 16). The administration of NH_4Cl was associated with a higher serum concentration of Cl^- of 108.5±0.8 as compared with 101.2±0.8 mM/dl in controls ($P < 0.05$) and a lower concentration of HCO_3^- (19.8±1.1 as compared with 26.5±0.7 mM/dl in controls [$P < 0.05$]). No proteins were recognized using either antibody in the BLM of control or experimental animals. As determined by the use of Ab-I (Fig. 6), there was no significant difference in the amount of the 97-kD polypeptide in the BBM in experimental animals ($n = 4$) as compared with controls ($n = 3$). Identical results were obtained using Ab-E. The ratio of enrichment factors for alkaline phosphatase and Na^+-K^+ ATPase in BBM from control and experimental animals averaged 0.94±0.09 (P = NS) and 1.04±0.06 (P = NS), respectively. The ratio of enrichment factors in BLM in control and experimental animals was 0.97±0.04 (P = NS) for alkaline phosphatase and 0.96±0.07 (P = NS)) for Na^+-K^+ ATPase.

Discussion

Preliminary evidence has been advanced to suggest that renal tissue contains a Na^+-H^+ exchanger that is highly homologous to a recently cloned human Na^+-H^+ exchanger (1, 3–5). No

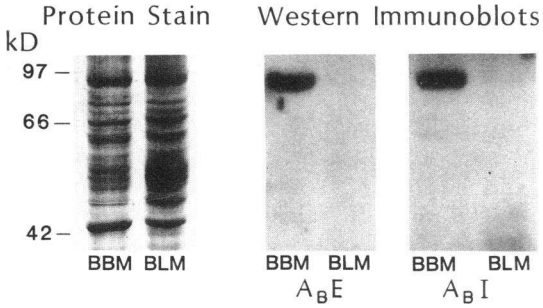

Figure 3. Representative protein stain and Western immunoblots of rabbit renal BBM and BLM proteins separated by SDS-PAGE. Coomassie blue stains are shown on the left. Western immunoblots using Ab-E and Ab-I are shown on the right. Molecular mass standards are indicated.

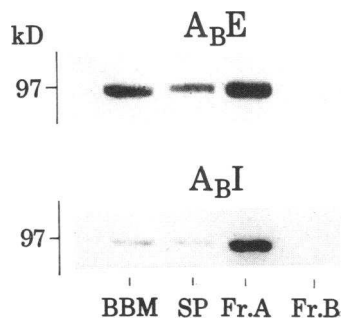

Figure 5. Representative Western immunoblots of rabbit renal BBM proteins, octyl glucoside–extracted BBM proteins (*SP*), and solubilized protein fractions eluting from an anion-exchanger column with 0.2 M NaCl (*Fr.A*) or between 0.2 and 0.4 M NaCl (*Fr.B*) using Ab-E (*top*) or Ab-I (*bottom*). Molecular mass standards are indicated.

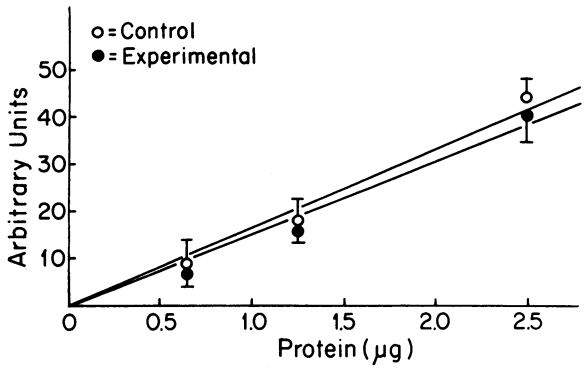

Figure 6. Quantitative slot blot analysis of rabbit renal BBM proteins of control animal ($n = 3$) (*open circles*) and animals receiving NH_4Cl (*experimental, closed circles*) ($n = 4$) using Ab-I. Results are expressed as the mean±SEM. Protein quantities (μg) are graphed as a function of laser densitometer measurements expressed in arbitrary units.

previous information is available, however, to indicate the level of expression of the protein in renal tissue or its membrane location. The relation between this housekeeping form of the Na^+-H^+ exchanger and the functionally well-characterized renal BBM Na^+-H^+ exchanger also has not been established. To begin an examination of these questions, synthetic peptides representing putative external and cytosolic (internal) extramembranous domains of human NHE-1 were used to generate monospecific antisera in rabbits.

The amino acid sequence used to produce Ab-I differs between the rabbit and the human sequences in 1 amino acid position whereas that used to produce Ab-E is identical between the species (17). Both Ab-E and Ab-I detected a protein of appropriate molecular weight in human red blood cells. Ab-I detected a 97-kD polypeptide in rabbit renal BBM but did not identify any proteins in crude membrane fractions of rabbit skeletal muscle or brain. Ab-E also detected a 97-kD band in BBM but, by contrast to Ab-I, detected a broad band at ~ 97 kD in rabbit skeletal muscle. The recognition of a band on Western immunoblots of rabbit skeletal muscle by Ab-E but not Ab-I may indicate that the amino acid difference in the peptide sequence used to generate Ab-I is a critical antigenic determinant. The reason for the failure of both antibodies to detect NHE-1 in brain is not known but may be related to the level of expression of the transporter in brain tissue.

One striking result of the present studies was the demonstration that both antipeptide antibodies to human NHE-1 recognized a 97-kD protein in BBM of the rabbit. Preimmune sera contained no antibodies to the 97-kD protein and preabsorption of the antisera with the specific peptide antigen prevented immunologic recognition of the 97-kD polypeptide in BBM. The findings that antibodies raised to different domains recognize a protein of the same apparent molecular weight provides presumptive evidence that the antibodies recognize the same protein. Western immunoblots were performed using a membrane preparation from PS120 cells, which are Chinese hamster lung fibroblasts deficient in all Na^+-H^+ exchangers. Neither of the antisynthetic peptide antibodies recognized any polypeptides in the membranes of these cells. In preliminary experiments, BBM and BLM of LLC-PK_1 cells, cells known to contain a Na^+-H^+ exchanger highly homologous to that cloned by Sardet and co-workers (1, 3), were prepared by density gradient fractionation in sorbitol. Ab-I detected a doublet band of an approximate molecular mass of > 90 kD in both membrane fractions. A recent report by Sabolic et al. (18) using rat BBM and BLM indicates that both membrane preparations may be contaminated with endothelial cell membranes. Several observations, however, would indicate that the present results are not the consequence of contamination of the membrane preparations with endothelial cells. The antibodies used in the present studies did not react with any proteins in BLM. A monoclonal antibody to von Willebrand factor also failed to react with BBM proteins. Taken together, it is suggested that rabbit renal BBM contains a 97-kD antigen with epitopes common to the human Na^+-H^+ exchanger.

In preliminary histocytochemical studies, the antipeptide antibodies failed to detect proteins in any tubule segments. The reason for the differences in the ability to detect specific proteins using histochemical techniques versus Western immunoblot analyses is not known but similar disparities have been documented in other systems using other antibodies. It is possible that the level of expression of antigen is too low to be detected by histochemical methods. A preliminary report by Biemesderfer et al. (19) using an antibody generated against a fusion protein to NHE-1 indicated no reactivity to BBM of the rabbit kidney by indirect immunofluorescence. Some reactivity was seen in the BLM of juxtamedullary proximal tubules and in more distal nephron segments. These results contrast with those of the present experiments. The reasons for this seeming disparity are not known. Different antigen peptides were used in the two studies. In addition, antibodies to the fusion protein were raised in guinea pigs in the experiments of Biemesderfer et al. (19) whereas the antipeptide antibodies used in the present studies were raised in rabbits. Finally, in preliminary indirect immunocytochemistry experiments in the kidney of the rat using a similar, but not identical fusion protein, no labeling of either BBM or BLM of any nephron segments was detected. Of interest, only the vascular smooth muscle was labeled by the antibody to the fusion protein. This distribution of immunofluorescence was also seen using Ab-I. Clearly, additional studies will be required to clarify these results.

Neither Ab-E or Ab-I reacted with proteins in rabbit renal BLM. The failure of the antibodies to identify a protein of molecular weight appropriate to NHE-1 in the BLM of the rabbit perhaps is not a surprising finding. Studies by others indicate that isolated renal BLM do not demonstrate Na^+-H^+ exchange activity (20). As noted above, preliminary immunohistochemical studies in the rabbit using an antibody to a fusion protein of NHE-1 suggest that this transporter is localized to BLM of segments of juxtamedullary proximal convoluted tubules and in more distal nephron segments (19). A recent study by Krapf and Solioz (21) using reverse transcription and the polymerase chain reaction indicate that the mRNA for NHE-1 is expressed in S1 and S2 segments of juxamedullary nephrons in the kidney of the rat. The mRNA is expressed only weakly in midcortical proximal tubule nephrons, is not expressed in superficial proximal tubules, but is expressed in more distal nephron segments. In the present studies, the membranes were prepared from the superficial cortex of the rabbit kidney. If the distribution of the transporter in the kidney of

the rabbit is similar to that of the rat, it would appear that the amount of NHE-1 protein in our BLM preparation is too low to detect.

Additional studies were undertaken to examine the relation between recognition of the 97-kD protein by the antibodies and functional expression of Na^+-H^+ exchange activity in the BBM. Earlier studies from this laboratory have indicated that octyl glucoside–extracted BBM proteins demonstrate Na^+-H^+ exchange activity when reconstituted into artificial lipid vesicles (7). Given that the starting material was renal BBM and that the regulation of this solubilized exchanger by specific protein kinases was similar to that observed in native apical membrane vesicles, it has been assumed that the reconstituted activity represents the activity of the apical membrane form of the exchanger (22–25). Fractionation of renal BBM solubilized proteins by anion-exchange chromatography indicated that only the more anionic proteins, those eluting between 0.2 and 0.4 M NaCl (fraction B), demonstrate significant Na^+-H^+ exchange activity (8). The fraction eluting at lower salt concentrations (fraction A) had little transport activity. As determined by Western immunoblot analysis using both antibodies, the 97-kD protein was present in solubilized BBM proteins and in fraction A but not in fraction B. Thus, there is a dissociation between the majority of transport activity and protein recognition by the antibodies in detergent-solubilized BBM proteins fractionated by ion-exchange chromatography. The precise explanation for these findings is unclear but it might suggest that rabbit BBM contains at least two types of Na^+-H^+ exchangers; one being the well-characterized BBM exchanger, the other being the NHE-1 form cloned by Sardet et al. (1). There are, however, alternative explanations that could be considered. It is possible that fraction A, the fraction demonstrating little Na^+-H^+ exchange activity, contains naturally occurring inhibitors of the transporter. It is also possible, albeit unlikely, that despite the presence of protease inhibitors in the fractionation experiments the proteins in fraction B had undergone cleavage to smaller fragments that still express transport activity but no longer are recognized by either antibody. The location of the peptide sequences chosen for antibody preparation in relation to the overall primary structure of the human housekeeping form of the Na^+-H^+ exchanger makes this possibility unlikely. On the basis of these considerations, we favor the tentative conclusion that rabbit renal BBM contains more than one form of Na^+-H^+ exchanger.

Systemic metabolic acidosis has been demonstrated to increase the maximum velocity of the BBM Na^+-H^+ exchanger (15). Krapf et al. (16) reported recently that administration of NH_4Cl to rats results in an increase in the steady state mRNA expression of the NHE-1 form of the Na^+-H^+ exchanger in the renal cortex as assayed by Northern and slot blot hybridization analysis. As determined by quantitative dot blot analysis, however, NH_4Cl administration did not affect the expression of Na^+-H^+ exchanger protein present in the BBM of the rabbit kidney. These results would appear to indicate that the demonstrated increase in BBM Na^+-H^+ exchange activity after systemic acidosis is not the consequence of an increase in the amount of the 97-kD polypeptide representing the NHE-1 exchanger. These findings also support the previous suggestion that the BBM contain two or more types of Na^+-H^+ exchangers. If this is the correct interpretation, it is recognized that the functional significance of the form or forms of Na^+-H^+ exchanger(s) recognized by the antibodies in rabbit BBM and the relation between this transporter and the BBM Na^+-H^+ exchanger remains to be determined. Several explanations may be considered for the apparent discrepancy between the results of the present experiments using antibodies and those of Krapf et al. (16) using a cDNA to NHE-1. First, there may be differences between species; studies comparable to those of Krapf et al. (16) have not been reported in rabbits. Second, Northern hybridization analysis does not provide precise tissue localization. It is possible, for example, that renal cortex of rat contains nontubule cells that have a high concentration of the mRNA for the human Na^+-H^+ exchanger. Finally, the demonstration that mRNA levels are altered does not provide direct information on the efficiency of translation of the protein in question.

Recently, Yamashita and Kawakita (26) reported results of experiments using solubilized bovine BBM and polyclonal antibodies to synthetic peptides representing domains of the NH_2- or COOH-terminal ends of NHE-1. The peptide antigens used in this study differed from those used in the present communication. The antibody to the COOH- but not the NH_2-terminal peptide recognized a 110-kD polypeptide in bovine BBM. As determined by a reconstitution assay, there appeared to be a correlation between Na^+-H^+ exchange activity and recognition of the 110-kD polypeptide on Western immunoblot analysis of chromatographic fractions of the solubilized proteins. These findings are not consistent with the findings of the present experiments. Aspects of the experiments of Yamashita and Kawakita (26), however, render them difficult to interpret. No evidence was provided that transport was occurring into the vesicular space of the reconstituted proteoliposomes; that is, binding of sodium to the protein or lipid components of the proteoliposomes was not excluded. These experiments did not address the possibility that the uptake of sodium was the consequence of electrodiffusion due to generated potential differences and no data on the transport of hydrogen ions were provided. Thus, it was not established rigorously that the measured uptake of sodium represented Na^+-H^+ exchanger activity. The proposed correlation between transport activity and protein expression also is difficult to interpret given the fact that transport activity in the fractionation studies was expressed per volume rather than per mg protein and that there appeared to be a relation between transport activity and protein recovery. Finally, the source of the renal BBM, the detergent, and the lipids used for formation of the proteoliposomes in these experiments differed from that of previous and the present experiment reported from our laboratory.

Experiments published after the present studies were initiated by Orlowski and co-workers (27) in the rat and Tse and co-workers (28) in the rabbit indicate that there is a family of Na^+-H^+ exchangers that bear some homology to NHE-1. Of particular interest is the NHE-3 isoform cloned by Tse et al. (28), which localizes to the kidney and selected portions of the small intestine suggested on the basis of functional studies to express BBM-type Na^+-H^+ transport activity. This isoform contains possible phosphorylation sites for a number of protein kinases known to regulate the activity of this transporter. Although additional information is required, this isoform is a candidate for the renal BBM Na^+-H^+ exchanger. The proposed amino acid sequence of NHE-3 is nearly identical to the amino acid domain of NHE-1 used to generate Ab-E in the

present experiments. The amino acid sequence used for Ab-I, however, differs significantly. If it is accepted that rabbit renal proximal tubules do not contain NHE-1, as suggested by in situ hybridization studies in the rat, and do contain NHE-3, a fact yet to be established, it remains possible that additional isoforms of Na^+-H^+ exchangers will be identified.

In summary, the results of the present experiments using monospecific antibodies to two separate domains of the recently cloned human NHE-1 provide evidence for the presence of epitopes of this Na^+-H^+ exchanger in rabbit BBM. The dissociation between recognition of the 97-kD protein using antibodies and the majority of functional Na^+-H^+ exchange activity after chromatographic fractionation of solubilized BBM proteins and in native BBM after administration of NH_4Cl suggests that rabbit renal BBM contains more than one form of Na^+-H^+ exchanger.

Acknowledgments

We acknowledge the secretarial skills of Ms. Ann West. Drs. L. Mandel and B. Doctor, Duke University Medical Center (Durham, NC) performed the preliminary immunocytochemical studies. Dr. Jacques Pouyssegur, Centre de la Recherche Scientifique, Parc Valrose (Nice, France) kindly supplied the PS120 cells. Charles Hensley, Ph.D., Department of Physiology, University of Southern California School of Medicine (Los Angeles, CA) prepared the membrane fractions from the LLC-PK_1 cells. The contribution of these collaborators is acknowledged.

These studies were supported by the National Institutes of Health grants DK37319 and DK01374 and by funds from Research Service, Department of Veterans Affairs.

References

1. Sardet, C., A. Franchi, and J. Pouyssegur. 1989. Molecular cloning, primary structure and expression of human growth factor-activatable Na^+-H^+ antiporter. *Cell.* 56:271–280.

2. Haggerty, J. G., N. Agarwal, R. F. Reilly, E. A. Adelberg, and C. W. Slayman. 1988. Pharmacologically different Na-H antiporters on the apical and basolateral surfaces of cultured kidney cells (LLC-PK_1). *Proc. Natl. Acad. Sci. USA.* 85:6797–6801.

3. Reilly, R. F., F. Hildebrant, D. Biemesderfer, C. Sardet, J. Pouyssegur, P. Aronson, C. Slayman, and P. Igarashi. 1991. cDNA cloning and immunolocalization of a Na^+/H^+ exchanger in LLC-PK_1 renal epithelial cells. *Am. J. Physiol.* 261 (*Renal Fluid Electrolyte Physiol. 30*):F1088–F1094.

4. Pearce, D., R. Krapf, F. C. Rector, and T. L. Reudelhuber. 1990. Cloning of a partial length rat kidney cDNA homologous to the human Na/H antiporter. *Kidney Int.* 37:233. (Abstr.)

5. Hildebrant, F., R. F. Reilly, C. Sardet, J. Pouyssegur, C. Slayman, P. Aronson, and P. Igarashi. 1990. Cloning of a cDNA encoding a rabbit renal Na^+-H^+ exchanger. *Kidney Int.* 37:539. (Abstr.)

6. Kahn, A. M., G. M. Dolson, M. K. Hise, S. C. Bennett, and E. J. Weinman. 1985. Parathyroid hormone and dibutyryl cyclic AMP inhibit Na^+-H^+ counter transport in brush border membrane vesicles isolated from a suspension of rabbit proximal tubules. *Am. J. Physiol.* 248 (*Renal Fluid Electrolyte Physiol. 17*):F212–F281.

7. Weinman, E. J., S. Shenolikar, E. J. Cragoe, Jr., and W. P. Dubinsky. 1988. Solubilization and reconstitution of renal brush border Na^+-H^+ exchanger. *J. Membr. Biol.* 101:1–9.

8. Weinman, E. J., D. Steplock, G. Bui, N. Yuan, and S. Shenolikar. 1990. Regulation of the renal Na^+-H^+ exchanger by cAMP dependent protein kinase. *Am. J. Physiol.* 258 (*Renal Fluid Electrolyte Physiol. 27*):F1254–F1258.

9. Kahn, A. M., S. Branham, and E. J. Weinman. 1984. Mechanism of L-malate transport in rat renal basolateral membrane vesicles. *Am. J. Physiol.* 246 (*Renal Fluid Electrolyte Physiol. 15*):F779–F784.

10. Kahn, A. M., S. Branham, and E. J. Weinman. 1983. Mechanism of urate and p-aminohippurate transport in rat renal microvillus membrane vesicles. *Am. J. Physiol.* 245 (*Renal Fluid Electrolyte Physiol. 14*):F151–F158.

11. Ahmed, K., and J. D. Judah. 1964. Preparation of lipoproteins containing cation-dependent ATPase. *Biochim. Biophys. Acta.* 93:603–613.

12. Wakabayahi, S., P. Fafournoux, C. Sardet, and J. Pouyssegur. 1992. The Na^+/H^+ antiporter cytoplasmic domain mediates growth factor signals and controls "H^+-sensing". *Proc. Natl. Acad. Sci. USA.* 89:2424–2428.

13. Towbin, H., T. Staehlein, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

14. Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.

15. Tsai, C.-J., H. E. Ives, R. J. Alpern, V. J. Yee, D. G. Warnock, and F. C. Rector. 1984. Increased V_{max} for Na/H antiporter activity in proximal tubule brush border vesicles from rabbits with metabolic acidosis. *Am. J. Physiol.* 247 (*Renal Fluid Electrolyte Physiol. 16*):F339–F343.

16. Krapf, R., C. Lynch, X.-P. Xi, T. L. Reudelhuber, J. Pouyssegur, and F. C. Rector. 1991. Expression of rat renal Na/H antiporter mRNA levels in response to respiratory and metabolic acidosis. *J. Clin. Invest.* 87:747–751.

17. Ming Tse, C., A. I. Ma, V. W. Yang, A. J. M. Watson, S. Levine, M. H. Montrose, J. Potter, C. Sardet, J. Pouyssegur, and M. Donowitz. 1991. Molecular cloning and expression of a cDNA encoding the rabbit ileal villus basolateral membrane Na^+/H^+ exchanger. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:1957–1987.

18. Sabolic, I., O. Culic, S. Lin, and D. Brown. 1992. Localization of ect-ATPase in rat kidney and isolated renal cortical membrane vesicles. *Am. J. Physiol.* 262 (*Renal Fluid Electrolyte Physiol. 31*):F217–F228.

19. Biemesderfer, D., R. F. Reilly, M. Exnaer, F. Hilderbrandt, P. Igarashi, and P. S. Aronson. 1991. Immunochemical characterization of an epithelial Na^+-H^+ exchanger. *J. Am. Soc. Nephrol.* 2:696. (Abstr.)

20. Krapf, R., R. J. Alpern, F. C. Rector, and C. A. Berry. 1987. Basolateral membrane Na/base cotransport is dependent on CO_2/HCO_3 in the proximal convoluted tubule. *J. Gen. Physiol.* 90:833–853.

21. Krapf, R., and M. Solioz. 1991. Na/H antiporter mRNA expression in single nephron segments of rat kidney cortex. *J. Clin. Invest.* 88:783–788.

22. Weinman, E. J., S. Shenolikar, and A. M. Kahn. 1987. cAMP-associated inhibition of Na^+-H^+ exchanger in rabbit kidney brush-border membranes. *Am. J. Physiol.* 252 (*Renal Fluid Electrolyte Physiol. 21*):F19–F25.

23. Weinman, E. J., W. P. Dubinsky, and S. Shenolikar. 1988. Reconstitution of cAMP-dependent protein kinase regulated renal Na^+-H^+ exchanger. *J. Membr. Biol.* 101:11–18.

24. Weinman, E. J., and S. Shenolikar. 1986. Protein kinase C activates the renal apical membrane Na^+-H^+ exchanger. *J. Membr. Biol.* 93:133–139.

25. Weinman, E. J., W. P. Dubinsky, K. Fischer, D. Steplock, Q. Dinh, L. Chang, and S. Shenolikar. 1988. Regulation of reconstituted renal Na^+-H^+ exchanger by calcium dependent protein kinases. *J. Membr. Biol.* 103:237–244.

26. Yamashita, T., and M. Kawakita. 1992. Reconstitution of Na^+/H^+ antiporter of bovine renal brush-border membrane into proteoliposomes and detection of a 110 kDa protein cross-reactive with antibodies against a human Na^+/H^+ antiporter partial peptide in antiport-active fractions after partial fractionation. *J. Biochem.* 113:162–167.

27. Orlowski, J., R. A. Kandasamy, and G. E. Shull. 1992. Molecular cloning of putative members of the Na/H exchanger gene family. *J. Biol. Chem.* 267:9331–9339.

28. Tse, C.-M., S. R. Brant, S. Walker, J. Pouyssegur, and M. Donowitz. 1992. Cloning and sequencing of a rabbit cDNA encoding and intestinal and kidney-specific Na^+/H^+ exchanger isoform (NHE-3). *J. Biol. Chem.* 267:9340–9346.