Renal expression of novel Na\(^+/\)H\(^+\) exchanger isoform NHE8

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Na\(^+/\)H\(^+\) EXCHANGERS (NHES) are a growing family of transmembrane proteins that mediate the electroneutral exchange of Na\(^+\) for H\(^+\) (15, 24). Thus far, seven mammalian isoforms have been identified (NHE1–7) (8, 13, 14, 16, 19, 22, 27) that differ in terms of their tissue distribution, membrane localization, inhibitor sensitivity, and physiological regulation. Despite these differences, they have structural similarities that define them as a family of proteins. NHE1–7 are 660–900 amino acids in length and share a similar primary structure. Membrane topology predictions suggest that they contain an NH\(_2\)-terminal hydrophobic domain with 10–12 transmembrane spans and a COOH-terminal hydrophilic domain with no transmembrane spans.

The NH\(_2\)-terminal hydrophobic domain is most highly conserved (40–70%) and is thought to contain the putative catalytic core, whereas the COOH-terminal hydrophilic domain is less conserved (10–20%) and is thought to be important for regulation (15, 24).

In the mammalian kidney, NHE activity contributes to the maintenance of acid-base balance, volume regulation, and blood pressure control. The apical plasma membrane isoform NHE3 has a critical role in this regard. NHE3 is responsible for 50–60% of proximal reabsorption of bicarbonate and fluid (20, 25). As would be expected, NHE3-null mice have relative hypotension, metabolic acidosis, and renal salt wasting (10, 11, 20). Although NHE3 mediates most of the Na\(^+\)-dependent bicarbonate reabsorption in the proximal tubule, whether other NHE isoforms contribute to this process is uncertain. Earlier studies by our group using in vivo microperfusion of cortical proximal tubules in NHE3 null mice showed no remaining component of EIPA-inhibitable transtubular bicarbonate absorption (26). More recently, in vitro microperfusion studies of the proximal tubule with measurements of intracellular pH have revealed a large component of EIPA-inhibitable Na\(^+\)-dependent acid extrusion (−40%) across the apical membrane that persists in NHE3 or NHE3/NHE2 null mice (4). Thus this study suggested the possible presence of an NHE isoform other than NHE3 or NHE2 that contributes to apical membrane Na\(^+\)/H\(^+\) exchange in the proximal tubule.

Given the possibility of additional NHE isoforms that may play a role in renal physiology, we attempted to identify novel NHE isoforms expressed in the kidney. We report here the identification and cloning of a novel NHE isoform, NHE8, that is expressed in the kidney and is a candidate to mediate apical membrane ion transport in the proximal tubule.

MATERIALS AND METHODS

cDNA Cloning of NHE8

With the use of the rabbit NHE3 sequence as a query, a TBLASTN search of the GenBank nonredundant database identified a human mRNA that appeared to encode a novel NHE (gi no. 4589521, subsequently updated as gi no. 15503399).

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C. The gi no. 4589521 sequence appeared to be incomplete. Specifically, the first amino acid aligned with amino acids 150–200, whereas the stop codon aligned with amino acids 550–600 of known isoforms. PCR using gene-specific primers amplified the predicted 45 nucleotide product from a human kidney cDNA library, as well as mouse kidney cDNA library, confirming its expression in native renal tissue.

To ascertain the full-length mouse sequence, we utilized a combination of nested PCR, 5' rapid amplification of cDNA end (RACE) and 3' RACE. First, primers directed against vector sequences and gene-specific sites were used to perform nested PCR using a mouse kidney cDNA library (Clontech) as a template. This yielded 541 nucleotides of additional upstream sequence. However, the 5'-end of the coding sequence was still incomplete on the basis of alignment to other NHE isoforms, so 5'-RACE was performed. Total RNA was extracted from the kidneys of Black Swiss mice (TriZol), and 5'-RACE was performed (Clontech Marathon cDNA Amplification Kit, catalog no. K1802–1). This revealed an additional 78 nucleotides of sequence, which extended the open reading frame upstream by two nucleotides to include a methionine in a favorable context for translation initiation (9). This methionine was in a position analogous to the initiation methionines of other mammalian NHEs and thus likely represents the translational start site. We then used 3'-RACE to amplify a >500 nucleotide product, which consisted of 119 coding nucleotides followed by a stop codon, a 388 nucleotide 3'-untranslated region (UTR), and a poly-A tail. The stop codon aligned with the stop codon in the single, long open reading frame of the aforementioned human mRNA in the database (gi no. 4589521).

The sequence of the complete open reading frame was amplified from mouse cDNA by using primers directed against the 5'-UTR (GAACTCTGAGTGCTCGGAGAACGGCAGGTAG and the 3'-UTR (CAAGAGAAGCAGGAGGAAGGACTCGTGTAACCTG). Sequencing of this solitary PCR product revealed 100% identity to the nested PCR and RACE products and 96% identity to the corresponding sequence of mouse NHE8. PCR using sequence no. AF-482993). The sequence of the complete open reading frame was deposited in Genbank as NHE8 (GenBank accession no. AF-482993).

Northern Blot Analysis

A mouse multiple tissue Northern blot (Clontech) was probed with a 32P-labeled PCR-generated cDNA clone spanning nucleotides 3–1069 of the mouse NHE8 coding region, which shares only 30% sequence identity to known NHE isoforms. The Northern blot was prehybridized in Church-Gilbert solution containing 40% formamide and 100 μg/ml denatured salmon sperm DNA for 24 h at 42°C. The blot was hybridized for 24 h at 42°C in the same solution containing 2 x 106 counts per min · ml1 of either the 32P-random prime-labeled NHE8 probe or a similarly labeled β-actin probe as an RNA loading control. After hybridization, high-stringency washes were performed by washing with 0.1 x SSC, 0.5% SDS, for 30 min at 65°C.

Preparation of Fusion Protein Antibodies

To generate polyclonal antibodies specific for NHE8, a maltose-binding fusion protein that contained the COOH-terminal 89 amino acids of human NHE8 (MBP-hNHE8_t89) was constructed. These 89 COOH-terminal amino acids share <25% amino acid sequence identity with NHE1–7 and were thus likely to provide isofrom-specific antibodies. The human sequence was used as a template because at the time of antibody preparation, the COOH end of the mouse sequence was unknown. The mouse and human cDNA sequences were subsequently found to be 93% identical in this region.

In brief, primers with engineered restriction sites (EcoRI and XbaI) were used to amplify a PCR product from the human kidney cDNA library (Clontech). The resultant PCR product, which contained the terminal 267 coding nucleotides + stop codon of NHE8, was cloned into the EcoRI/XbaI sites of the pMal-C2 vector (New England Biolabs). The resulting pMal-C2/NHE8 vector was transfected into Top10F (Invitrogen) competent cells, and the MBP/hNHE8_189 fusion protein was expressed and purified according to manufacturer recommendations (New England Biolabs).

Rabbits were immunized with the MBP-hNHE8_189 by Pocono Rabbit Farm (Canadensis, PA). Antisera were negatively purified by passage through an MBP lysate-affinity column. Sepharose G fast-flow beads with immobilized lysate of cells expressing MBP were prepared per manufacturer's recommendations (Amersham). The negatively purified antibodies were then dialyzed in PBS-glycerol, concentrated by centrifugation through Centricon-30 filters (Amicon), and stored at −20°C.

Transient Expression of NHE8 in COS-7 Cells

Rabbit NHE1, rat NHE2, rabbit NHE3, and rat NHE4 cDNAs had been previously subcloned into pBluescript KS(+) (17). For eukaryotic expression, both NHE1 and NHE3 cDNAs were each subcloned into the EcoRI/Xhol sites of pcDNA3. NHE2 cDNA was subcloned into the NotI/Xhol sites of pcDNA3, and NHE4 cDNA was subcloned into the Xhol/BamH I sites of pcDNA3. The mouse NHE8 PCR product containing the complete open reading frame was initially inserted into pcR2.1TOPO (Invitrogen) and then subsequently subcloned into the EcoRI site of pcDNA3.1, and orientation was confirmed by sequencing.

COS-7 cells were grown in DMEM/high-glucose medium with 10% FCS, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37°C in 5% CO2/95% air. Cells were transfected with cDNAs by use of LipofectAMINE 2000 per manufacturer's protocol (Invitrogen). Two hours after transfection, transfection complexes were removed and fresh medium ± 50 μg/ml tunicamycin were added. Cells were assayed for protein expression by Western blot analysis ~36 h posttransfection.

Isolation of Rat Brush-Border Membranes

Renal brush-border membranes (BBMs) were isolated from rat renal cortex by magnesium aggregation and differential centrifugation as described previously (2). Specific activity of the apical membrane marker γ-glutamyl transpeptidase was assayed as described (21), and average enrichment was 10–12. Protein concentration was assayed by the method of Lowry. Membranes were stored at −70°C.

In Situ Hybridization

Riboprobe generation. The terminal 269 coding nucleotides of mouse NHE8 were amplified by using primers sense GCAACCAACTCTGCTGTCATCCTC and antisense GAA- CAACCTCTGCTGTCATCCTC. This PCR product was first cloned into pcR2.1TOPO and then cut out and subcloned into the EcoRI site of pBluescript SK(+). In situ hybridization was performed by using 33P-labeled riboprobes as described previously (23). Antisense or sense riboprobes were tran-
scribed from a linearized pBluescript plasmid by using [α-<sup>33</sup>P]UTP and T3 or T7 RNA polymerases, respectively. Adult mouse kidneys were fixed in 4% paraformaldehyde and embedded in paraffin. Then, 5-μm-thick sections were cut and mounted on Vectabond-coated slides. Sections were deparaffinized with xylene and rehydrated with graded ethanol, hybridized overnight with radiolabeled antisense or sense riboprobes, and then washed as described previously. Sections were dipped in Ilford K5D emulsion, exposed in the dark at 4°C for 21 days, and developed with Kodak D-19. Slides were counterstained with hematoxylin and eosin. Bright-field and dark-field microscopy was performed with a Leica M420 microscope and a Leica DMR microscope, and images were obtained by using an Optronics Magnafire digital camera.

**RESULTS**

Aliquots of cells or kidney membranes were solubilized in sample buffer, and the proteins were separated by SDS-PAGE using 7.5% polyacrylamide gels. For immunoblotting, proteins were transferred to polyvinylidene difluoride (Immobilon-P, Millipore) at 500 mA for 5 h at 4°C with a Transphor transfer electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA) and stained with Ponceau S to check the transfer. For immunoblotting studies, polyvinylidene difluoride membranes were incubated first in blocking buffer and then washed in Blotto (5% nonfat dry milk in PBS and 1% Tween, pH 7.4) for 1 h to block nonspecific binding of the antibody, followed by overnight incubation with the primary antibody. All primary antibodies were used at a 1:1,000 dilution. These antibodies included mouse anti-NHE1 (4E9) (18), rabbit anti-NHE2 (Chemicon), mouse anti-NHE3 (3H3, provided by Dr. D. Biemesderfer, Yale University), mouse anti-NHE4 (11H11) (17) and rabbit anti-NHE8. The membranes were then washed in Blotto and incubated for 1 h with the appropriate secondary antibody (horseradish peroxidase-conjugated IgG, Zymed Laboratories, San Francisco, CA) diluted 1:2,000 in Blotto. Membranes were then washed with Blotto several times followed by a final wash in PBS. Bound antibody was detected using the ECL chemiluminescence system (Amersham) according to the manufacturer’s protocol.

**SDS-PAGE and Immunoblotting**

As described in MATERIALS AND METHODS, a database search identified a partial length cDNA encoding a novel NHE isoform, and RACE PCR was used to obtain the complete coding sequence from mouse kidney cDNA. As shown in Fig. 1, the sequence encodes a predicted protein of 576 amino acids, which we have named NHE8. A database search has also identified the human ortholog, labeled as KIAA0939 protein, using the ClustalW multiple sequence alignment program (www.ebi.uk/clustalw/). The proteins are 96% identical at the amino acid level. With the use of Prosite (http://us.expasy.org/prosite/) and ScanSite (http://scansite.mit.edu/) programs, both proteins are predicted to have four potential N-glycosylation sites (G), a PKC-epolin phosphorylation site (PKC), an SH2-binding domain (SH2), an SH3-binding domain (SH3), an ERK-binding domain (ERK1 binding), and a type III PDZ-Dlg-zona occludens-1 binding region (PDZ). *, identical amino acid residues; ., conserved amino acid substitution; ,, semiconserved amino acid substitution.

Hydropathy plot analysis (Fig. 3) predicts that NHE8 has an ~450 amino acid NH<sub>2</sub>-terminal hydrophobic domain with 10–12 transmembrane domains followed by a COOH-terminal hydrophilic tail. This pattern is similar to that seen with mammalian NHE1–7, as modeled by NHE3, except that NHE8 has a shorter COOH-terminal hydrophilic tail (~100 compared with 200–350 amino acids). Interestingly, the more closely related D. melanogaster “NHE1” also has a relatively short hydrophilic tail domain (Fig. 3).

The expression of NHE8 in mouse tissues was evaluated by Northern blot analysis (Fig. 4). Mouse NHE8 is ubiquitously expressed, with the highest levels in...
the kidney as well as testis, skeletal muscle, and liver. The predominant transcript in most organs is 4.2 kb in length, with a 2.2-kb transcript also detected in the organs with highest levels of expression. However, in testis the predominant transcript is slightly larger at 4.4 kb.

To study the expression of NHE8 protein, a rabbit polyclonal antibody was developed against the COOH-terminal hydrophilic tail of human NHE8. To verify the isoform specificity of the antibody, it was used to probe Western blots prepared from COS-7 cells transfected with NHE1, NHE2, NHE3, NHE4, and NHE8 cDNAs. As shown in Fig. 5A, the anti-NHE8 predominantly labeled a protein of an apparent molecular mass of 85 kDa in cells transfected with NHE8 but not in cells transfected with the other four NHE isoforms confirming NHE8-isoform specificity. Expression of NHE1, NHE2, NHE3, and NHE4 was confirmed by use of specific antibodies directed against each isoform (Fig. 5B).

The major form of NHE8 detected in transfected COS-7 cells has an apparent molecular mass of 85 kDa, significantly larger than its predicted 64-kDa molecular mass. We suspected this discrepancy was due to glycosylation, because protein sequence analysis identified four potential N-glycosylation sites at Asn8, Asn15, Asn181, or Asn485 (Fig. 1). To investigate this possibility, we treated NHE8-transfected COS-7 cells with tunicamycin to prevent N-glycosylation of newly synthesized proteins. Tunicamycin treatment collapsed the 85- and 60-kDa NHE8 bands to a lower 52-kDa band, confirming N-glycosylation (Fig. 6). In contrast, tunicamycin failed to induce a shift in apparent molecular mass of NHE3, confirming previous findings that NHE3 is not glycosylated (5). The migration of unglycosylated NHE8 with an apparent molecular mass less than its predicted molecular mass is consistent with the behavior of other NHE proteins subjected to SDS-PAGE (17).

To determine whether NHE8 protein is expressed in native rat kidney, we used the anti-NHE8 antibody to probe Western blots prepared from isolated BBM. As illustrated in Fig. 7A, the predominant BBM protein labeled by the antibody had an apparent molecular mass of ~85 kDa, identical to that labeled in solubilized COS-7 cells transfected with NHE8 cDNA (COS-7/NHE8).

To confirm that NHE8 is an apical membrane protein, the abundance of NHE8 was compared in equal
aliquots (100 µg protein) of rat renal cortical whole homogenate and purified BBMs that were isolated by divalent cation precipitation and differential centrifugation. As shown in Fig. 7B, the enrichment of NHE8 in BBMs compared with the starting homogenate was similar to that observed for NHE3, indicating that NHE8 is either a brush-border protein as is NHE3 (1, 3) or resides in a membrane compartment that copurifies with BBMs.

Unfortunately, the anti-NHE8 antibody did not work well for immunocytochemistry, so we utilized in situ hybridization to determine the cellular localization of NHE8. A short riboprobe directed against the terminal 269 nucleotides of NHE8 was generated and used to probe mouse kidney sections. This region was chosen because it has the least sequence similarity to other NHE isoforms and thus was unlikely to cross-hybridize with known isoforms. Hybridization of NHE8 antisense riboprobes to sagittal sections of adult kidney (Fig. 8A) showed that the message is expressed most intensely in a region corresponding to the outer stripe of the outer medulla. In addition to the strong signal in the outer stripe, a signal of moderate intensity is present diffusely throughout the cortex (Fig. 8B). No NHE8 expression is evident in the inner stripe or the inner medulla. Figure 8C shows NHE8 expression in the majority of tubules in the outer stripe as well as in the tubules surrounding the juxtedudillary glomeruli. This pattern of expression in the majority of tubules surrounding glomeruli strongly suggests expression in proximal tubules. No message is present within the glomeruli. Higher magnification of the outer stripe (Fig. 8D) shows silver grains corresponding to NHE8 message expression present within tubules possessing a brush border (arrows), confirming expression in proximal tubules. Hybridizations performed with the control probe (sense) revealed no significant hybridization signal (not shown). Thus NHE8 is highly expressed in the proximal tubules in the outer stripe of the outer medulla, with significant but lower expression in the proximal tubules in the cortex.

Fig. 5. Immunoblot to verify specificity of NHE8 antibody. A: NHE8 antibody labels a protein in COS-7 cells transfected with NHE8 but not in COS-7 cells transfected with NHE1, NHE2, NHE3, or NHE4. B: blotting controls to verify NHE1, NHE2, NHE3, or NHE4 protein expression in the correspondingly transfected COS-7 cells.

Fig. 6. Immunoblot to demonstrate that NHE8 is N-glycosylated. Lanes 1 and 2: NHE3-transfected COS-7 cells treated with and without tunicamycin and probed for NHE3. Lanes 3 and 4: NHE8-transfected COS-7 cells treated with and without tunicamycin and probed for NHE8.

Fig. 7. Immunoblots to demonstrate that NHE8 protein is expressed in rat kidney and copurifies with cortical brush-border membranes (BBMs). A: COS-7 cells transfected with NHE8 (COS-7/NHE8) run in parallel with rat kidney BBMs and probed for NHE8. B: 100 µg of rat kidney cortical whole homogenate as well as 100 µg of BBMs purified from the whole homogenate were run in parallel and probed for NHE8 (lanes 1 and 2) and for NHE3 (lanes 3 and 4), which is known to localize to BBM.
DISCUSSION

NHE8 is the newest member of a growing family of mammalian Na+/H+ exchangers. Sequence analysis suggests that NHE8 is the mammalian ortholog of an ancient invertebrate isoform of unknown function. Northern blot demonstrates that NHE8 is ubiquitously expressed in multiple mouse tissues. In the kidney, NHE8 copurifies with BBMs isolated from renal cortex, suggesting apical membrane localization. In situ hybridization demonstrates NHE8 message in the proximal tubule. Given these results, NHE8 is a candidate to mediate apical membrane transport in the proximal tubule.

Although we have not yet been successful in functionally expressing NHE8, recent studies suggest possible roles for NHE8 in proximal tubular transport physiology. For example, one possibility is that NHE8 is an EIPA-sensitive Na+/H+ exchanger and accounts for the EIPA-inhibitable Na+-dependent acid extrusion that takes place across the apical membrane of proximal tubule cells in NHE3/NHE2 null mice (4). Another possibility is that NHE8 is an EIPA-resistant Na+/H+ exchanger and helps mediate the appreciable rate of bicarbonate absorption that persists and is resistant to inhibition by EIPA, baflomycin, or SCH-28080 in the proximal tubule of NHE3 null mice (26). Alternatively, it is possible that the primary function of NHE8 is to mediate an ion transport process other than Na+/H+ exchange. For example, Na+/NH4+ exchange has been described to take place across the apical membrane of proximal tubule cells (7, 12), and it is possible that this transport activity is mediated by NHE8 rather than NHE3. Finally, because NHE isoforms are known to exist as stable dimers (6), it is possible that NHE8 associates with NHE3 and modulates its transport function. Further studies are presently under way to investigate these possibilities.

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Fig. 8. In situ hybridization of NHE8 riboprobe on adult mouse kidney sections. A: bright-field (left) and dark-field (right) images of a sagittal mouse kidney section showing NHE8 signal in a region corresponding to the outer stripe of the outer medulla. Magnification, ×6.3. B: bright-field (left) and dark-field (right) images showing brightest signal in the outer stripe as well as a diffuse NHE8 signal in the cortex. Magnification, ×23.3. C, cortex; OS, outer stripe of the outer medulla; IS, inner stripe of the outer medulla; IM, inner medulla. C: dark-field image showing tubular localization of NHE8 in the outer stripe of the outer medulla (OS OM) and cortex (c). g, Glomeruli; IS OM, inner stripe of the outer medulla. Magnification, ×35. D: higher magnification (×927) of the outer stripe of the outer medulla showing NHE8 localization in proximal tubules identified by the presence of a brush border (arrow). PT, proximal tubule; CD, collecting duct.
REFERENCES


