Subcloning, localization, and expression of the rat intestinal sodium-hydrogen exchanger isoform 8

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Xu, Hua, Rongji Chen, and Fayez K. Ghishan. Subcloning, localization, and expression of the rat intestinal sodium-hydrogen exchanger isoform 8. Am J Physiol Gastrointest Liver Physiol 289: G36–G41, 2005. First published February 24, 2005; doi:10.1152/ajpgi.00552.2004.—Apically expressed intestinal and renal sodium-hydrogen exchangers (NHEs) play a major role in Na+ absorption. Our previous studies on NHE ontogeny have shown that NHE-2 and NHE-3 are expressed at very low levels in young animals. Furthermore, single and/or double NHE-2 and NHE-3 knockout mice display no obvious abnormalities before weaning. These observations suggest that other transporter(s) may be involved in intestinal Na+ absorption during early life. The present studies were designed to clone the novel rat intestinal NHE-8 cDNA and to decipher the NHE-8 protein localization and gene expression pattern during different developmental stages. The rat NHE-8 cDNA has 2,160 bp and encodes a 575-amino acid protein. An antibody against NHE-8 protein was developed. Immunohistochemistry staining indicated apical localization of NHE-8 protein in rat intestinal epithelial cells. The apical localization of NHE-8 was also confirmed by its presence in brush-border membrane and its absence in basolateral membrane preparations. Northern blotting utilizing a NHE-8-specific probe demonstrated higher NHE-8 mRNA expression in young animals compared with adult animals. Western blot analysis revealed a similar pattern. Tissue distribution with multiple human tissue RNA blot showed that NHE-8 was expressed in multiple tissues including the gastrointestinal tract. In conclusion, we have cloned the full-length NHE-8 cDNA from rat intestine and further showed its apical localization in intestinal epithelial cells. We have also shown that NHE-8 gene expression and protein expression were regulated during ontogeny. Our data suggests that NHE-8 may play an important role in intestinal Na+ absorption during early life.

SLC9A8; intestine; ontogeny

THE SODIUM-HYDROGEN EXCHANGERS (NHEs) belong to a large family of transmembrane proteins that mediate the electroneutral exchange of Na+ for H+ (21). To date, nine NHE isoforms (NHE-1 to -9) have been identified from several species including human, rat, mice, and rabbit (1, 2, 4–6, 12, 16–20, 22, 23). These NHEs have different tissue distribution, membrane localization, inhibitor sensitivity, and physiological regulation (28).

In the small intestine, three NHE isoforms (NHE-1, NHE-2, and NHE-3) have been identified. NHE-1 is the most ubiquitous isoform and is expressed on the basolateral membrane (BLM) of intestinal epithelial cells. NHE-1 is involved in cell volume regulation as well as intracellular pH regulation (28). NHE-2 and NHE-3 are expressed on the apical membrane of intestinal epithelial cells. Both NHE-2 and NHE-3 are involved in transepithelial Na+ absorption in the mammalian gastrointestinal tract (28). Knockout of these NHEs in animals resulted in distinct phenotypes. NHE-1 knockout mice exhibit growth retardation, ataxia, seizures, and altered intracellular pH in pancreatic acinar cells (3, 7). NHE-2 knockout mice are phenotypically normal; however, they have abnormal gastric acid secretion (24). NHE-3 knockout mice have mild diarrhea and mild acidosis (14, 25).

Recently, another member of the NHE family, NHE-8, was cloned from mouse kidney (16). This isoform was found to be localized on the apical membrane of renal epithelial cells (15). However, it is not clear whether this transporter is expressed in the intestinal epithelial cells. Thus the present study was designed to clone the intestinal NHE-8 cDNA from rat and to study its localization and gene expression during maturation.

MATERIALS AND METHODS

Animals. Two-week-old (2 wk) and 3-wk-old (3 wk), and adult (120 days) male rats (Sprague-Dawley) were used. Animals were killed, and the jejunal mucosa was harvested and used for mRNA isolation and brush-border membrane (BBM) vesicle purification. All animal work was approved by the University of Arizona Institutional Animal Care and Use Committee. All experiments were repeated at least three times with different animal groups (3–4 rats/group).

Cell culture. Human intestinal epithelial (Caco-2) cells were purchased from American Type Culture Collection and cultured according to American Type Culture Collection guidelines. Cells were cultured at 37°C in a 95% air-5% CO2 atmosphere and passaged every 72 h. Media and other reagents used for cell culture were purchased from Irvine Scientific (Irvine, CA).

Construction of intestinal cDNA libraries. mRNA was purified from jejunal mucosa of 3-wk rats using the FastTrack mRNA purification kit (Invitrogen; Carlsbad, CA). Two rat intestinal cDNA libraries, 5′ RNA ligase-mediated (RLM) rapid amplification of cDNA ends (RACE) and 3′ RLM RACE cDNA libraries, were constructed using FirstChoice RLM RACE kit (Ambion; Austin, TX). The third rat intestinal cDNA library was also constructed using SuperScript II reverse transcriptase (Invitrogen) in the presence of oligo (dT)15.

Cloning of rat NHE-8 cDNA. A BLASTN search against the mouse NHE8 cDNA sequence (GenBank accession no. AF482993) (16) was conducted. The search identified two predicted rat sequences (GenBank accession nos. XM230865 and XM342585) with high homology to the mouse renal NHE-8 cDNA sequence. The longer sequence (XM230865) is composed of 1,251 bp and is homologous with the mouse NHE-8 cDNA from 554 to 1,731 bp. The shorter sequence (XM342585) is 558 bp and is homologous with mouse NHE-8 cDNA from 8 to 523 bp. Comparison of the two rat sequences to the mouse cDNA sequence showed that neither rat sequence was a full-length sequence.

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transcript. To confirm whether the two predicted sequences were derived from the same NHE-8 gene, we utilized 5′ RACE and 3′ RACE to amplify 5′ untranslated region (UTR) and 3′ UTR. Using primers directed against the linker sequence and NHE-8-specific RACE to amplify can be found in GenBank with accession no. AY496958.

NHE, sodium-hydrogen exchanger; RACE, rapid amplification of cDNA ends.

Table 1. PCR primers for amplifying rat intestinal NHE-8 cDNA

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′ → 3′)</th>
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<tbody>
<tr>
<td>5′ RACE NHE-8-specific primer</td>
<td>CTCCTCGTGTGGGCTTGCTTC</td>
</tr>
<tr>
<td>3′ RACE NHE-8-specific primer</td>
<td>TGAGACAGAGAGACGTCC</td>
</tr>
<tr>
<td>Full NHE-8 forward primer</td>
<td>AAGATGCGGAGAGATTCACC</td>
</tr>
<tr>
<td>Full NHE-8 reverse primer</td>
<td>GGTCCTGCTGTGGTCACGA</td>
</tr>
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Production of NHE-8 antiserum. Rabbit polyclonal antibodies were raised against synthetic peptides designed from the NH2 terminus (PILPVQTEQAAQQEQE) and COOH terminus (EDARARRR-SKKDVNL) of the rat NHE-8 protein. Peptides were conjugated to KLH and coinjected into New Zealand White rabbits (Invtogen). The rabbits received multiple immunogenic boosts over a several-week period and were bled periodically throughout this process. The anti-peptide antibody titer was determined by an ELISA and used to measure the antigenic properties of the animal bleeds. All serum was aliquoted and stored at −20°C.

Western blot analysis. BBM and BLM were isolated from rat jejunal mucosa with the methods described previously by our laboratory (10, 11). Protein (40 μg) was loaded on SDS-PAGE gels and subjected to Western blot analysis. A: immunoblot with preimmune and NHE-8 serum at a 1:3,000 dilution with BBM protein. B: antigen blocking experiments using NH2- or COOH-terminal antigenic peptides to block NHE-8 antisera (1 mg/ml for 16 h at 15°C) before reacting with BBM proteins on the membrane. C: detection of Na+-K+ ATPase in BBM or BLM proteins. A 1:125 dilution of the Na+-K+ ATPase α-subunit antiserum was used (Affinity BioReagents, Golden, CO). Note that Na+-K+/ATPase is a BLM-specific protein. D: detection of NHE-8 in BBM or BLM proteins. BBM, BLM; BBM vescicle; BBMV, BLM vescicle.

Fig. 2. Characterization of NHE-8 antibodies. Brush-border membrane (BBM) and basolateral membrane (BLM) proteins were isolated from jejunal mucosa of 3-wk-old and adult rats. Protein (40 μg) was loaded on SDS-PAGE gels and subjected to Western blot analysis. A: immunoblot with preimmune and NHE-8 serum at a 1:3,000 dilution with BBM protein. B: antigen blocking experiments using NH2- or COOH-terminal antigenic peptides to block NHE-8 antisera (1 mg/ml for 16 h at 15°C) before reacting with BBM proteins on the membrane. C: detection of Na+-K+ ATPase in BBM or BLM proteins. A 1:125 dilution of the Na+-K+ ATPase α-subunit antiserum was used (Affinity BioReagents, Golden, CO). Note that Na+-K+/ATPase is a BLM-specific protein. D: detection of NHE-8 in BBM or BLM proteins. BBM, BLM; BBM vescicle; BBMV, BLM vescicle.
anti-rabbit IgG; Molecular Probes, Eugene, OR) at a 1:400 dilution and visualized by confocal microscopy (MRC-1024ES laser scanning confocal; Bio-Rad) equipped with a Nikon TE-300 research grade microscope using the HQ-598-40 emission filter and an excitation wavelength of 568 nm. Images were captured with identical settings on the laser scanning confocal microscope.

RNA purification and Northern blot analyses. mRNA was isolated from rat jejunal mucosa using the FastTrack mRNA purification kit (Invitrogen), and 10 μg of mRNA were utilized for Northern blot analyses. The full-length rat NHE-8 cDNA was radiolabeled as the probe for NHE-8 mRNA detection. Northern blot analyses were performed under high-stringency washing conditions as described previously (8). 1B15 (encoding rat cyclophilin) cDNA (13) specific probes were used as internal standards for quantitating NHE-8 gene expression. Blots were exposed to a phosphorimaging screen, and band intensities were determined with Quantity One Software (FX Molecular Imager; Bio-Rad). NHE-8 gene expression levels were estimated by taking the ratio of hybridization intensities of NHE-8 mRNA over 1B15 mRNA. The experiment was repeated with mRNA isolated from three different animal groups.

NHE-8 expression in human tissues. mRNA was purified from Caco-2 cells using the Micro FastTrack mRNA purification kit (Invitrogen). Reverse transcription reaction was performed to make first cDNA using conditions described previously (26). An 855-bp fragment of the human NHE-8 cDNA was PCR amplified with the primers designed at 616- to 1,470-bp region (GenBank accession no. KIAA0939). PCR products were subcloned into pCR2.1-TOPO vectors and were sequenced on both strands. The human NHE-8 cDNA fragments were radiolabeled and used as probes for Northern blot. The human NHE-8 gene expression was analyzed using a multiple human tissue RNA blot (Human Multiple Tissue Expression Array; Clontech) under high-stringency washing conditions, as described previously.

Statistical analysis. ANOVA post hoc tests (StatView 5.0.1, SAS Institute; Cary, NC) were used to compare values of the experimental data. P values of <0.05 were considered significant.

RESULTS

cDNA sequences of the rat intestinal NHE-8. 5′ RACE and 3′ RACE identified a 30-bp sequence in front of the translational start codon of NHE-8 and a 403-bp sequences downstream of the translational stop codon of NHE-8, respectively. Sequence data showed that this rat intestinal NHE-8 cDNA has 2,160 bp and encodes a putative protein of 575 amino acids with open reading frame from 31 to 1,755 bp. Hydrophathy analysis by Simple Modular Architecture Research Tool (http://smart.ox.ac.uk) suggests that this novel rat intestinal NHE-8 protein spans 11 transmembrane domains and has multiple posttranslational modification sites for glycosylation (Fig. 1). This rat intestinal NHE-8 cDNA sequence has been deposited in GenBank with accession no. AY496958.

Characterization of rat NHE-8 antibodies. Small intestinal BBM and BLM proteins were purified from 3-wk and adult

Fig. 3. Localization of NHE-8 transporter protein in rat intestine. Intestinal tissue sections were prepared from 3-wk-old rats and reacted with preimmune serum (A) or NHE-8 antiserum (B–D) at 1:500 dilution for 60 min. Immunohistochemical staining results were analyzed by MRC-1024ES laser scanning confocal microscopy.
but significantly decreased in adult rats (0.38 ± 0.03 band to that of the 1B15 band) was similar in 2- and 3-wk rats (indicated by the ratio of the signal intensities of the NHE-8 band at a size of 1 kb). Hybridization pattern from Northern blot analysis showed that intestinal NHE-8 mRNA abundance was significantly reduced in adult rats. NHE-8 mRNA abundance clearly showed that intestinal NHE-8 mRNA abundance was a size of 1 kb. Hybridization pattern from Northern blot analysis. As shown in Fig. 2, a single immunoreactive band was partially blocked by N- and C-terminal antigenic peptides (Fig. 2B). Furthermore, Na\(^+\)-K\(^+\)-ATPase protein was recognized only in BBM protein but not in BBM protein (Fig. 2C), demonstrating the purity of the membrane protein preps. In contrast, NHE-8 protein was detected only in BBM proteins (Fig. 2D). Immunohistochemical analysis of rat intestine using this antibody showed specific recognition of the NHE-8 protein only on the apical membrane of the intestinal epithelium (Fig. 3, B–D). The preimmune serum did not react with any proteins in the rat intestine (Fig. 3A).

**Intestinal NHE-8 gene expression during maturation.** mRNA was isolated from jejunal mucosa of different age group rats, and 10 μg of mRNA was fractionated by gel electrophoresis, blotted, and hybridized with rat NHE-8 and 1B15 cDNA-specific probes. NHE-8 probes recognized a hybridization signal at ~4.0 kb, and 1B15 probes recognized a hybridization signal at ~1.0 kb. A: typical experiment. B: phosphoimage analysis of rat intestinal NHE-8 mRNA abundance in different age rats. Results are means ± SE from 3 separate experiments. *P < 0.02 for 2- and 3-wk-old rats vs. adult (Ad) rats.

**Intestinal NHE-8 protein expression during maturation.** BBM protein was purified from 3-wk and adult rat jejunum, and 40 μg of protein were used for Western blot analysis. Results showed that NHE immunoreactive protein abundance (indicated by the ratio of optical densities of the NHE-8 band to that of the β-actin band) was 0.79 ± 0.128 for 3-wk rats and 0.35 ± 0.078 for adult rats (Fig. 5). NHE-8 protein expression was reduced significantly by ~56% in adult animals (n = 3; P < 0.01).

**NHE-8 tissue distribution analyses.** The NHE-8 mRNA expression pattern was analyzed using the human tissue RNA blots (Human Multiple Tissue Expression Array). Hybridization utilizing the 76-human tissue blot showed that NHE-8 gene expression is present in many tissue types (data not shown). Furthermore, Northern blot analysis on a 12-human tissue RNA blot identified two transcripts at ~4.0 and 7.0 kb in heart, lung, skeletal muscle, intestine, kidney, liver, and placenta (Fig. 6).

**DISCUSSION**

The NHEs are a growing family of transmembrane proteins that mediate the electroneutral exchange of Na\(^+\) for H\(^+\) (21). NHEs play important roles in various cellular functions and have distinct patterns of tissue/cell expression and membrane localization. In the small intestine, three NHEs (NHE-1, NHE-2, and NHE-3) have been cloned and characterized. NHE-1 is expressed on the BLM of intestinal epithelial cells and plays a crucial role in cell volume as well as intracellular pH regulation (21, 28). Conversely, NHE-2 and NHE-3 are localized on the apical membrane of intestinal epithelial cells and play important roles in the intestinal Na\(^+\) absorption (28).

NHE-8, a new NHE family member, was cloned recently from mouse kidney and shown to be expressed on the apical side of renal epithelial cells (15, 16). It is, however, unclear whether NHE-8 is expressed in the small intestine. A BLASTN
search against the mouse renal NHE-8 cDNA recognized two predicted rat sequences that showed high homology with mouse NHE-8 cDNA. To identify the 5’ and 3’ cDNA sequences of rat NHE-8, 5’ RACE and 3’ RACE were conducted based on the BLASTN search results. The full-length rat NHE-8 cDNA was then PCR amplified from a rat intestinal cDNA library. The cloned rat intestinal NHE-8 cDNA contains a 2,160-bp sequence that encodes a putative protein of 575 amino acid residues. The rat NHE-8 protein has 11 transmembrane domains and multiple potential posttranslational modification sites for glycosylation. At the nucleotide sequence level, rat intestinal NHE-8 cDNA possesses a 94.7% identity with the mouse NHE-8 cDNA and an 88.6% identity with the human NHE-8 cDNA. At the amino acid level, rat NHE-8 protein is 99.7% homologous with mouse NHE-8 protein and 96.4% homologous with human NHE-8 protein. The overall homology of NHE-8 cDNA sequence with other NHEs is <30%.

To study NHE-8 protein expression and localization, NHE-8 antibody was raised by coinjecting two peptides designed at the N- and C-terminal of rat NHE-8 protein. Western blot analysis showed that the NHE-8 antibody recognized a single 65-kDa protein band from rat intestinal BBM protein. The antibody was partially blocked by its antigenic N- or C-peptide, suggesting that both the N- and C-terminus peptides of the rat NHE-8 protein could stimulate the antibody production. Further studies with BLM protein showed that K⁺-Na⁺-ATPase was highly detected in BLM protein but not in BBM protein, suggesting that our BBM protein does not have BLM contamination. NHE-8 is strongly detected in BBM protein, indicating the apical expression of NHE-8 in intestinal epithelia. Immunohistochemistry staining confirmed antibody recognition of NHE-8 protein on the apical side of the intestinal epithelial cells. Our protein localization results are in agreement with the previously published observations seen in the mice, which shows the presence of NHE-8 on the apical membrane of mouse kidney (15, 16). It is interesting to note the size difference between rat intestinal NHE-8 protein and mouse renal NHE-8 protein. In our study, rat NHE-8 protein was found to be ~65 kDa, whereas mouse NHE-8 protein is ~85 kDa (15, 16). This size difference is likely attributed to different posttranslational modulation between species and tissues.

Studies on NHE-8 tissue distribution show that NHE-8 is not only present in the intestine but in the kidney, as well as in other tissues such as skeletal muscle and liver. These observations are similar to other studies that showed NHE-8 mRNA expression in the skeletal muscle and kidney (19).

Northern blot results showed that NHE-8 expression was higher in 2- and 3-wk rats and lower in adult rats. This expression pattern differs from that of intestinal NHE-2 and NHE-3. The highest expression of NHE-2 and NHE-3 is seen in 6-wk-old rats and adult rats, and the lowest expression is observed in 2-wk rats. In 2-wk rats, NHE-2 and NHE-3 protein expression is <22% of adult expression (9, 10). In contrast, NHE-8 protein abundance in adult is ~44% of 3-wk expression. These results suggest that the intestinal NHE-8 protein might play a possible role of Na⁺ absorption during early development. Further evidence from NHE-8 knockout mice also supports this hypothesis. In NHE-3 knockout mice, an amiloride-sensitive Na⁺ transporter presents in the intestinal Na⁺ absorption, and this transporter partially compensates for intestinal Na⁺ absorption in NHE-3 knockout animals (14). Although the function of NHE-8 protein has not been completely characterized (15, 16), our observations strongly suggest a possible role of NHE-8 in the intestinal Na⁺ absorption during early development.

In summary, we have cloned the full-length rat intestinal NHE-8 cDNA and characterized the NHE-8 antibody. The NHE-8 protein is apically expressed in the intestinal epithelial cells, and its expression is regulated through development. NHE-8 is a predominant NHE isoform apically expressed in the intestine in young animals. The protein localization and expression pattern in the small intestine suggest a role of NHE-8 as a transporter involved in early life intestinal Na⁺ absorption.

GRANTS
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REFERENCES


