NHE3 in an ancestral vertebrate: primary sequence, distribution, localization, and function in gills

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Submitted 24 January 2005; accepted in final form 27 June 2005


IN MAMMALIAN CELLS, Na+/H+ exchangers (NHEs) or antporters catalyze 1:1 electroneutral exchange of Na+ and H+ down their respective concentration gradients (28, 41, 46, 47, 64, 73). The first NHE was cloned from mouse cells (NHE1) (56), and it was followed by NHE3 (48, 61) and NHE2 (18, 62) from rat kidney, NHE2 is found in the cortical thick ascending limb, macula densa, distal convoluted tubules, and connecting tubules (10), and NHE3 is found predominantly in the proximal convoluted tubule and to a lesser extent in the thick ascending limb (2, 5, 6). In renal proximal tubules of mammals, apical NHE3 is functionally linked to basolateral Na+/K+/ATPase and Na+/HCO3− cotransporter in a mechanism that is responsible for the majority of Na+ and HCO3− reabsorption in the kidney (63, 65), and NHE3 null mice have markedly decreased renal HCO3− and fluid absorption, systemic acidosis, hypotension, and elevated plasma aldosterone (36, 70). The function of NHE2 in the kidney is not yet clear, and NHE2 null mice have no measurable phenotypes that suggest renal absorptive malfunction (36).

In elasmobranchs, the gills are responsible for most of systemic Na+ absorption and H+ secretion instead of the kidneys (11, 15, 25). Fittingly, similarities between epithelial cells in the gills of an elasmobranch, Atlantic stingray (Dasyatis sabina), and renal tubule cells of mammals have been discovered. For example, apical immunoreactivity for a Cl−/HCO3− exchanger (pendrin) and basolateral immunoreactivity for vacuolar H+/K+/ATPase occur in one cell type of stingray gills similar to type B intercalated cells of mammalian kidneys (25, 53). High levels of basolateral Na+/K+/ATPase immunoreactivity were observed in another gill cell type (50) that was hypothesized as the location of transepithelial Na+ absorption, and in vivo flux studies demonstrated a link between Na+ and H+ (23, 24). However, an apical mechanism of Na+ and H+ transport has not been identified, and no molecular sequence data exist for an NHE3 from an elasmobranch.

The gills of elasmobranchs provide a unique opportunity to study the roles of transporters in branchial Na+ absorption and acid secretion without having to consider any potential dual functions in Na+ and/or Cl− secretion. Unlike teleosts (the largest group of fishes), elasmobranchs have an accessory organ that is responsible for NaCl secretion in seawater (i.e., rectal gland), and their gills do not appear to be involved in salt secretion (26, 66). However, few studies have been able to take advantage of this segregation of transport functions because most elasmobranchs are large (i.e., difficult to maintain) and euryhaline species are rare (only 3% of elasmobranch species live in both seawater and freshwater) (25). The Atlantic stingray is the only North American species of elasmobranch that has permanent populations in both seawater and freshwater (7, 31, 52). It is a small elasmobranch species (adults 300–800 g) that can be...
maintained in either fresh or seawater aquaria and therefore is well suited as a model for gill ion absorption (52).

The previously known link between Na\(^+\) and H\(^+\) transport and the demonstration of Na\(^+\)/K\(^+\)-ATPase-rich cells in elasmobranch gills led us to hypothesize that an apical NHE may be expressed in the gills of elasmobranchs, where it may function in systemic Na\(^+\) absorption and H\(^+\) secretion. Therefore, the first goals of this study were to determine whether NHE2 and/or NHE3 homologs exist in the gills of the Atlantic stingray and, if so, to determine whether mRNA expression of either was altered by acclimation to freshwater or hypercapnic acidosis. Furthermore, if expression for an NHE was elevated, we also sought to localize its expression in the gills relative to Na\(^+\)/K\(^+\)-ATPase and vacuolar H\(^+\)-ATPase. In this article, we report the first complete cDNA sequence for an NHE3 ortholog in an elasmobranch, demonstrate that NHE3 protein is located on the apical side of Na\(^+\)/K\(^+\)-ATPase cells, and report the first increase in gill NHE3 expression during acclimation to low salinities.

MATERIALS AND METHODS

Animals and standard holding conditions. All procedures were approved by the University of Florida Institutional Animal Care and Use Committee. Atlantic stingrays (D. sabina, ∼154–658 g) were captured from waters surrounding Sea Horse Key, FL, in the Gulf of Mexico by using a cast net. They were transported to the University of Florida in Gainesville, FL, where they were held in a 380-liter tank maintained below 1 part per million with a biological filter, and temperature was maintained between 22 and 26°C. The room that housed the stingrays was on a 12:12-h light-dark cycle.

RT-PCR, cloning, and sequencing. Stingrays were anesthetized by an initial immersion in 150 mg/l MS-222 diluted in aquarium water. Stingrays were perfused through the conus arteriosus with cold (∼4°C) elasmobranch phosphate-buffered saline (10 mmol/l PBS was adjusted with NaCl, urea, and trimethyamine oxide to approximate the total osmolarity of stingray plasma) (52). The stingrays were then pithed, and gill filaments were removed with sterile, RNase-free tools and frozen in liquid nitrogen. Total RNA was then isolated with TRI reagent (Sigma, St. Louis, MO), and first-strand cDNA was synthesized from 2 μg of total RNA with a SuperScript II reverse transcriptase kit (Invitrogen, Carlsbad, CA), using oligo(dT) as a primer.

Degenerate primer pairs were then designed to amplify conserved regions of vertebrate ribosomal protein L8, NHE, and Na\(^+\)/K\(^+\)-ATPase proteins (Table 1). Each PCR was performed on one-twentieth of a reverse transcriptase reaction with a FastStart Taq DNA polymerase kit (Roche Applied Science, Indianapolis, IN) in a PCR Express thermocycler (ThermoHybaid, Franklin, MA) with standard cycling parameters. PCR products were visualized by ethidium bromide staining in 1.0–1.5% agarose gels, ligated into pCR 4-TOPO vectors, and transformed into TOP10 chemically competent cells by using a TOPO TA cloning kit for sequencing (Invitrogen). Plasmid DNA was then sequenced in both directions at the Marine DNA Sequencing Facility at the Mount Desert Island Biological Laboratory (Salisbury Cove, ME).

After the sequencing of initial fragments with degenerate primers, more of the cDNA for NHE3 was cloned and sequenced by primer walking with degenerate primers. Primers 5’ NHE3 CHF1 and DS R986 were used to extend toward the 5’ end, and primers DS F1414 and 3’ NHE3 CHF3 were used to extend toward the 3’ end (Table 1). Finally, 5’ and 3’ rapid amplification of cDNA ends (RACE) was used to finish the sequence. Briefly, 5’ and 3’ RACE cDNA were prepared with a GeneRacer kit (Invitrogen) according to the manufacturer’s protocols. PCRs for 5’ RACE were completed with antisense primer 5’ RI and a sense primer that is included in the kit, and PCRs for 3’ RACE were completed with sense primers 3’ b F1 and 3’ b F2 and antisense primers that are included in the kit (Table 1). PCR, cloning, and sequencing were performed as described above, except for the use of touchdown cycle parameters and nested PCR to increase specificity. After the 5’ and 3’ ends of the NHE3 transcript were sequenced, specific primers were designed 15 bases upstream from the likely start

<table>
<thead>
<tr>
<th>Name</th>
<th>Orientation</th>
<th>Nucleotide Sequence (5’ to 3’)</th>
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<tr>
<td>L8 F1 deg*</td>
<td>Sense</td>
<td>GGA TAC ATC AAG GGA ATG GTG AAR GAY ATH AT</td>
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<td>L8 R1 deg*</td>
<td>Antisense</td>
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<td>NKA1 R1†</td>
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*Degenerate primer. †Real-time PCR primer.
codon and 44 bases after the likely stop codon to verify alignment of the complete cDNA (Table 1). PCR was performed on gill cDNA with primers NHE3 ORF F2 and NHE3 ORF R2, using the hot start version of TaKaRa ExTaq (Takara Bio, Shiga, Japan). The resulting product was cloned and sequenced as described above with sequencing primers spaced apart by ~600 bases.

**Multiplex-tissue relative quantitative PCR.** To determine whether other acid-base and/or ion transport tissues express the putative transporter mRNAs, we performed relative quantitative RT-PCR on total RNA from gill, stomach, kidney, spiral valve intestine, and rectal gland as described previously (14). Briefly, cDNA was produced from the tissues of a seawater stingray as described above, but random primers were used so that ribosomal and messenger RNA would be reverse transcribed. Nondegenerate primer pairs (Table 1) were designed for each putative transporter sequence with Oligo 6.7 (Cascade, CO) to amplify products with high efficiency (e.g., high melting temperature). To minimize the chance of amplifying contaminating genomic DNA, we designed primer pairs to include an intron-exon boundary that is conserved between vertebrate homologs (19, 32, 45). The specificity of all primer pairs used for tissue distribution was verified by sequencing PCR products. A QuantumRNA 18S internal standard primer kit (Ambion, Austin, TX) was used to control for variability in RNA quality and quantity between the different tissues tested. Multiplex PCR with primers for 18S and each putative transporter were then utilized to ensure that the reactions were terminated during the exponential phase and that the kinetics of 18S amplification approximated those of the putative transporter transcripts. Finally, the products were visualized by ethidium bromide staining in 1.5% agarose gels and photographed with Polaroid 667 film.

**Sequence analysis.** Sequence results for each initial degenerate primer pair were assembled, and the resulting contiguous amino acid translations were analyzed with the basic local alignment search tool (BLAST) on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). NHE3 fragment sequences were assembled with GeneTools software (BioTools, Edmonton, AB, Canada), and the assembled nucleotide sequence was searched for open reading frames. The predicted amino acid sequence was aligned with other full-length vertebrate NHE3 proteins using PEPTools software (BioTools). The expected locations of membrane-spanning regions and regions important for regulation of the transporter were marked and regions important for regulation of the transporter were taken from previously published reports (1, 37, 41, 72, 74). MEGA software (34) was used to make an unrooted phylogenetic tree of vertebrate NHE isoforms 1–5 with the neighbor-joining method and Poisson-corrected evolutionary distances (42). Branches were then tested for statistical significance by bootstrapping with 1,000 replicates. NHE sequence from Fugu, Danio, and Tetraodon (green pufferfish) were derived from genome databases.

**Salinity transfer.** Some of the tissues used for the salinity comparisons were taken from animals treated in a previous study (14). Briefly, eight stingrays were captured from Sea Horse Key and held in 100% seawater as described in Animals and standard holding conditions, except they were not fed. The stingrays were then divided into two groups: one group remained in 100% seawater (approximate concentration stable and approximately equal in the two salinities), except they were not fed. The stingrays were then divided into two groups: one group remained in 100% seawater (approximate concentration stable and approximately equal in the two salinities), except they were not fed. The stingrays were then divided into two groups: one group remained in 100% seawater (approximate concentration stable and approximately equal in the two salinities), except they were not fed. The stingrays were then divided into two groups: one group remained in 100% seawater (approximate concentration stable and approximately equal in the two salinities), except they were not fed. The stingrays were then divided into two groups: one group remained in 100% seawater (approximate concentration stable and approximately equal in the two salinities), except they were not fed. The stingrays were then divided into two groups: one group remained in 100% seawater (approximate concentration stable and approximately equal in the two salinities), except they were not fed.
sections. Alkaline phosphatase-conjugated anti-DIG antibody and its substrates (nitro blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate) were used to visualize the signal, followed by counterstaining with Kernechtrot (Muto Pure Chemicals, Tokyo, Japan). Some sections, serial to those stained with riboprobes, were labeled with an antibody that recognizes Na+/K+-ATPase, as described below.

Antibody production. A 636-bp fragment encoding the final 212 amino acids of the carboxyl tail of the putative NHE3 was subcloned into the BamHI/EcoRI sites of a pHAT10 bacterial expression vector (BD Biosciences, San Jose, CA), and the construct was transformed into Escherichia coli BL21 (codon plus). The resulting recombinant cells were grown at 27°C until the culture had an optical density of 0.7 at 600 nm. Isopropyl-

/H9252

gentamicin (Gibco) was added to a final concentration of 1 mmol/l, and incubation was continued for an additional 6 h. Cells were harvested from 4 liters of culture by centrifugation, resuspended in 150 ml of PBS containing 0.75 γ/ml lysozyme, disrupted by freeze-thaw and sonication, and centrifuged at 10,000 × g for 30 min. The recombinant protein was purified from supernatant with BD TALON metal affinity resin (BD Biosciences), followed by SDS-PAGE, and determined by densitometry.

Immunohistochemistry. Immunohistochemistry was completed on paraffin-embedded sections as described previously (13, 14), with minor modifications. Fixed tissues were dehydrated in an ethanol series and embedded in paraffin wax. Sections were cut at 6 μm and dried onto poly-L-lysine-coated slides. For gills, sections from the trailing half of the filaments were selected for immunohistochemical staining, because they contain the highest density of ion transport cells (26). Tissue sections were deparaffinized in Citrisolv (Fisher Scientific, Pittsburgh, PA) and rehydrated in an ethanol series, followed by PBS. Endogenous peroxidase activity was inhibited by incubating with 3% H2O2 for 25 min at 24°C. Nonspecific binding sites on the sections were blocked by incubating with a 1:100 dilution of normal serum (1 μg/ml bovine serum albumin, 0.09% NaN3, and 0.1% Tween 20) for 20 min.

Preliminary experiments with antisera from all four rats suggested that two were specific (R1B2 and R2B2), with R1B2 yielding the highest signal-to-background ratios. Therefore, the remaining immunostaining was conducted with R1B2. Sections were incubated with R1B2 (diluted 1:1,000 to 1:2,000 in BPB) overnight at 4°C in a humidified chamber. Negative control sections were incubated with R1B2 as described above. However, after treatment with DAB and counterstaining with Kernechtrot (Muto Pure Chemicals, Tokyo, Japan), some sections, serial to those stained with riboprobes, were labeled with an antibody that recognizes Na+/K+-ATPase (E11). Concentrations were 1:10 or 1:50 for α5 and 1:10 for E11. Detection of bound antibody was done as described above, except Vector SG, which produced a blue reaction product, was used for Na+/K+-ATPase and Vector VIP, which produced a purple reaction product, was used for vacuolar H+-ATPase.

Antibody α5 was developed by Dr. Douglas Fambrough and was obtained from the Developmental Studies Hybridoma Bank, which was developed under the auspices of the National Institute of Child Health and Human Development of the Department of Biological Sciences, University of Iowa (Iowa City, IA). It was made against the avian Na+/K+-ATPase α-subunit and binds to all isoforms. This antibody recognizes fish Na+/K+-ATPase and is now used widely for studies on fish branchial cells (e.g., Refs. 13, 14, 50). The monoclonal antibody for vacuolar H+-ATPase (E11) was a gift from Stephen Gluck (Department of Medicine, University of California, San Francisco, CA). It was made against a peptide that represents the carboxy-terminal 10 residues of the bovine kidney 31-kDa subunit of vacuolar H+-ATPase and has been used to localize vacuolar H+-ATPase in fish gills (13).

Oocyte immunohistochemistry. Female Xenopus laevis injected with human chorionic gonadotropin were purchased from Xenopus Express (Beverly Hills, FL). Oocytes were removed and collagenase dissociated as described previously (54). The full-length stingray NHE3 cDNA was subcloned into PCr 4-TOPO (Invitrogen). Capped, sense cRNA was synthesized using a PmeI linearized cDNA template and the T7 mMessage mMachine kit (Ambion). Oocytes were injected with 50 nl of stingray NHE3 cRNA (0.5 μg/μl) or water and then incubated in OR3 medium at 18°C (54). Oocytes were fixed 4–14 days after injection. The stingray NHE3 cDNA was also subcloned into pGEMHE, a Xenopus oocyte expression plasmid, as previously described (54, 71). For this cDNA, the expression plasmid did not seem to enhance protein expression (not shown).

Oocytes were washed with PBS, fixed using 4% paraformaldehyde in PBS at room temperature for 60 min, and processed for cryosectioning as described previously (57). Immunostaining was performed using 1:100 R1B2 antisera and 1:500 dilution of a donkey anti-rat-Cy2 secondary antibody. Epifluorescent images were captured using a Zeiss Axiovert 25 microscope, and multichannel images were acquired with an AxioCam digital camera and AxioVision software (Carl Zeiss, Jena, Germany) (57).

Western blotting. Preliminary Western blots with antisera R1B2 failed to label a single band, and therefore antisera R1B2 was affinity purified with recombinant antigen using a Pierce Seize primary immunoprecipitation kit (Rockford, IL). Briefly, an affinity column was generated by coupling 100 μl of Stingray NHE3 protein to an AffiGel-10 column (Bio-Rad). Oocytes were incubated with the affinity column, and the bound NHE3 proteins were collected. The affinity-purified antibody was eluted with Pierce Gentle Elution Buffer. The affinity-purified antibody was used for Western blot analysis.

Immunoblots were prepared from Ringer-perfused gill tissue by using a procedure modified from Ohara et al. (14). Briefly, tissues were homogenized in buffer [250 mmol/l sucrose, 30 mmol/l NaCl, pH 7.2] with a mechanical homogenizer for 30 s at maximum speed on ice. Homogenates were then centrifuged at 10,000 × g for 10 min at 4°C to remove debris and whole nuclei. The total protein concentration was determined with the Pierce BCA assay, and Laemmli loading buffer was added (35). Protein samples (40 μg) were loaded and run in 7.5% Tris-HCl precast polyacrylamide gels (Bio-Rad, Hercules, CA) and then transferred onto polyvinylidene difluoride (PVDF) membranes. A separate
that was 75.4 and 91.4% identical to Na
ported the amplification of a
internal control primers was observed for all tissues, at roughly
1). In all cases, the 315-bp product expected from the 18S
decreasing order) spiral valve, kidney, gill, and stomach (Fig.
product was most abundant for rectal gland, followed by (in
315-bp product from the 18S ribosomal RNA were
molecular weight markers were run in the lanes to the
sequence (AATAAA) is 43 nucleotides upstream from a polyA
end of the cDNA. A transcript cleavage
end of the cDNA obtained by RACE,

tide open reading frame that codes for an 837-amino acid
tail. PCR with primers just upstream from the start codon and
likely in-frame stop codon (TAA) is 159 nucleotides
mRNA in epithelial transport tissues. After 30 cycles of PCR
with the putative stingray NHE2- and NHE3-specific primers
(NHE2 F1–NHE2 R1 and NHE3 F1–NHE3 R1), the expected
428-bp NKA1 fragment was amplified, in decreasing order,
from rectal gland, spiral valve, kidney, gill, and rectal gland. Faint bands for NHE2 and NHE3 were also visible for stomach, when lanes were overloaded (unpublished observation). The 428-bp NKA1 fragment was amplified, in decreasing order, from rectal gland, spiral valve, kidney, gill, and stomach. 18S was amplified approximately equally from cDNA of all tissues. One hundred-base pair ladder molecular weight markers were run in the lanes to the left and right of samples. NKA1, Na+/K+-ATPase α1; s. valve, spiral valve; r. gland, rectal gland.

RESULTS

Molecular identification of putative transporters. In initial
PCR reactions, oligonucleotide primers NHE F2 deg and NHE
R1 deg supported the amplification of ~667-bp products from
stingray gill cDNA that matched the size expected from ver-
ebrate NHE nucleotide alignments. After several clones had
been sequenced, it was clear that the two degenerate primers
amplified three stingray NHE homologs (GenBank accession
nos. AY626248, AY626249, and AY626250) that were 74.7,
69.5, and 77.1% identical to human NHE1 (BC012121), NHE2
(NM_003038), and NHE3 (NM_004174), respectively. BLAST e-values for the putative stingray NHE2 and NHE3
sequence fragments were <10−75 for mammalian NHE2 and
NHE3, respectively.

Oligonucleotide primers NK2-F1 deg and P-R1 deg sup-
ported the amplification of a ~700-bp product (AY652417)
that was 75.4 and 91.4% identical to Na+/K+-ATPase subunit
α1 from human (NM_000701) and torpedo ray (Torpedo
californica; X02810), respectively. BLAST e-values for the
putative stingray Na+/K+-ATPase subunit α1 sequence frag-
ment were also <10−75 for the mammalian ortholog.

Distribution of putative transporters. Multiplex RT-PCR
with cDNA from gill, stomach, kidney, spiral value intestine,
and rectal gland was conducted to determine the distribution of
putative stingray NHE2, NHE3, and Na+/K+-ATPase α1
mRNA in epithelial transport tissues. After 30 cycles of PCR
with the putative stingray NHE2- and NHE3-specific primers
(NHE2 F1–NHE2 R1 and NHE3 F1–NHE3 R1), the expected
470- and 431-bp products were the most abundant for spiral
valve intestine, followed by gill and kidney (Fig. 1). Faint bands were also visible for rectal gland and stomach when gel
lanes were overloaded (unpublished observation). After 32
cycles of PCR with the putative stingray Na+/K+-ATPase α1-specific primers (NKA F1–NKA R1), the expected 428-bp
product was most abundant for rectal gland, followed by (in
decreasing order) spiral valve, kidney, gill, and stomach (Fig.
1). In all cases, the 315-bp product expected from the 18S
internal control primers was observed for all tissues, at roughly
equivalent levels (Fig. 1).

Quantitative real-time PCR. The mRNA expression of
NHE3 and Na+/K+-ATPase α1 in the gills from freshwater-acclimated stingrays was 116 and 61% greater than in gills
from seawater stingrays, respectively (Fig. 2A). Alternatively,
there was no difference in expression of NHE2 between the
two salinities. There was also no difference in expression of
mRNA for any of the transporters in the gills from normocap-
nic and hypercapnic stingrays after either 2 or 4 h of exposure
(Fig. 2B). The relative expression level of NHE3 and Na+/K+-
ATPase α1 mRNA were well correlated in the gills of seawater
control stingrays (r² = 0.758, slope = 0.46, and P < 0.0001)
(Fig. 2C). Alternatively, the relative expression level of NHE2
mRNA was not correlated to either NHE3 or Na+/K+-ATPase
α1 (unpublished observations).

Molecular identification of NHE3. Because NHE3 expres-
sion was greater in freshwater-acclimated stingrays than in
seawater stingrays, we completed the cloning and sequencing of
NHE3 so that it could be further characterized. The com-
plete putative stingray NHE3 cDNA (accession no. AY626250) contains 2,744 nucleotides with a 2,511-nucleo-
tide open reading frame that codes for an 837-amino acid
protein (Fig. 3). The likely start codon (ATG) is 75 nucleotides
from the 5′ end of the cDNA obtained by RACE, and a likely in-frame stop codon (TAA) is 159 nucleotides
upstream from the 3′ end of the cDNA. A transcript cleavage
sequence (AATTTA) is 43 nucleotides upstream from a polyA
tail. PCR with primers just upstream from the start codon and
just downstream from the stop codon amplified a product of the expected 2,606 base pairs, and sequencing of this product verified alignment of the complete cDNA. The complete stingray protein grouped with other vertebrate NHE3 homologs in phylogenetic analysis (Fig. 4). In separate trees that included human NHE6–8, NHE1–5 formed one group and NHE6–8 formed another distantly related group (unpublished observation).

In situ hybridization and immunological analysis. Antisense RNA probes 1 and 19 labeled specific epithelial cells in the filament of seawater stingrays (Fig. 5), and no labeling was observed when sections were incubated with the corresponding sense probes (Fig. 5A). Staining of serial sections demonstrated that riboprobes for NHE3 labeled cells that are immunoreactive for Na+/H+–ATPase (Fig. 5, D and E).

The anti-stingray NHE3 antiserum, RIB2, reacted strongly with a subpopulation of epithelial cells in stingray gills (Fig. 6A). In seawater control stingrays, staining was limited to the apical side of cuboidal epithelial cells in the filament and at the base of lamellae (Fig. 6A). No staining was observed when sections were incubated with BPB, preimmune serum, or R1B2 antibody preincubated with antigen (Fig. 6B). We stained for NHE3 and Na+/K+–ATPase or NHE3 and vacuolar H+–ATPase in the same gill sections to determine whether NHE3 immunoreactivity colocalized with either of the ATPase ion pumps. NHE3 immunoreactivity was always in the same cells as Na+/K+–ATPase immunoreactivity (Fig. 6C). Within these double-labeled cells, Na+/K+–ATPase immunoreactivity was confined to the basolateral region and NHE3 staining was always in the apical region (Fig. 6C). Alternatively, NHE3 immunoreactivity was never in the same cells as vacuolar H+–ATPase immunoreactivity (Fig. 6D).

Antiserum R1B2 labeled Xenopus oocytes that were injected with stingray NHE3 cRNA but not oocytes injected with water (Fig. 7, A–D). Most of the staining was intracellular (Fig. 7D), suggesting that additional proteins and/or signals are required to locate the majority of stingray NHE3 to the plasma membrane (Fig. 6). The affinity-purified R1B2 antibody labeled the 25-kDa recombinant stingray NHE3 carboxyl tail protein in Western blots (Fig. 7E). The affinity-purified antibody also produced the same immunohistochemical labeling as antiserum (not shown) and labeled a single 87-kDa protein on Western blots of stingray gill proteins (Fig. 7F). Preincubation of the antibody with excess antigen blocked this labeling (Fig. 7F). There were marked qualitative differences in the location and abundance of NHE3-immunoreactive cells between the
Fig. 3. Amino acid alignment of the putative stingray NHE3 with NHE3 from other vertebrates (labeled by genus). Gaps (dashes) were introduced to maintain alignment. All amino acids that are identical or similar to those of the human NHE3 are shaded (Blosum 62 scoring matrix with the following amino acid groups considered similar: DN, EQ, ST, KR, FYW, and LIVM). Putative transmembrane domains are labeled M1 to M11, and an additional hydrophobic region is marked with a bold dashed line. Serine phosphorylation sites of mammalian NHE3 that partially mediate the inhibitory effects of cAMP are marked with a blue P and are conserved in all homologs (41). A region known to interact with NHE regulatory factors 1 and 2 (NHERF1 and 2) and partially mediate the inhibitory effects of cAMP (72) is marked with an orange line and is well conserved in all homologs. A terminal carboxyl region of mammalian NHE3 known to mediate endocytosis of NHE3-containing vesicles is marked with a red line (1). Phosphatidylinositol 3-kinase (PI3) activation promotes exocytosis of NHE3 via the same region (1). This region is well conserved in the mammals, mostly conserved in stingray, but less conserved in dace (Tribolodon, teleost). Finally, a region known to mediate serum stimulation of NHE3 activity is marked with a green line and is well conserved in all the vertebrates except dace (37). GenBank accession nos. are as follows: Homo, NP_004165; Rattus, NP_036786; Didelphis, Q28362; Dasyatis, AAT45738; and Tribolodon, BAB83083.
ELASMOBRANCH GISSLs, WHERE IT MAY FUNCTION IN SYSTEMIC Na+

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DISCUSSION

Our study is the first to use molecular and immunological techniques to demonstrate that an NHE3 homologous to that of mammalian renal proximal tubules and intestines is present in elasmobranch gills, where it may function in systemic Na+ absorption and H+ secretion. These conclusions are based on our molecular identification of an NHE3 ortholog in stingray gills, demonstration of greater mRNA expression of NHE3 in freshwater than in seawater, and localization of NHE3 mRNA and protein in Na+ /K+ -ATPase-rich cells.

Molecular identification of stingray NHE2/4 and NHE3. The phylogenetic analysis of our full-length sequence with available NHE sequences demonstrates that it is likely an elasmobranch ortholog of mammalian NHE3 (human SCL9A3). The apparent size of 87 kDa on Western blots (Fig. 7B) is the same size as NHE3 in rat (30) and matches the predicted size of stingray NHE3 minus a predicted NH2-terminal signal peptide (SignalP 3.0) (20). Our stingray sequence is the earliest NHE3 ortholog sequenced to date, and the high homology of the stingray and mammalian NHE3s (>70% identical amino acids) suggests that NHE3 has changed very little in the elasmobranchs during evolution. Unexpectedly, the stingray NHE3 grouped closer to the mammalian NHE3s than the teleost NHE3s in phylogenetic analysis (Fig. 4). Teleosts, which include Fugu, Tetraodon, Danio, and Tribolodon, are in the sister group to the tetrapod lineage (including mammals) and share a more recent common ancestor with mammals than elasmobranchs (43). Therefore, the divergence of the teleost NHE3 sequences is contrary to the well-established phylogeny of major vertebrate groups and suggests that NHE3 evolved rapidly in the teleosts since they separated from the tetrapod lineage more than 400 million years ago (17). The activity of NHE3 in mammals is regulated by phosphorylation, protein trafficking, and regulatory proteins via the carboxyl, hydrophilic region (41). Interestingly, most of the regulatory, carboxyl regions of stingray and mammalian NHE3s are well conserved (Fig. 3), suggesting that the elasmobranch NHE3 can be regulated by similar pathways and that these control mechanisms were needed early in vertebrate evolution. Unexpectedly, the stingray NHE3 grouped closer to the mammalian NHE3s than the teleost NHE3s in phylogenetic analysis (Fig. 4). Teleosts, which include Fugu, Tetraodon, Danio, and Tribolodon, are in the sister group to the tetrapod lineage (including mammals) and share a more recent common ancestor with mammals than elasmobranchs (43). Therefore, the divergence of the teleost NHE3 sequences is contrary to the well-established phylogeny of major vertebrate groups and suggests that NHE3 evolved rapidly in the teleosts since they separated from the tetrapod lineage more than 380 million years ago (43). Most of the divergence has occurred in the regulatory carboxyl tail of teleost NHE3 (Fig. 3), suggesting that teleosts may regulate NHE3 activity by different cellular

Fig. 4. Phylogenetic tree of cell surface NHE homologs (NHE1–5) demonstrating that NHE3 forms 2 subgroups, 1 that includes the teleosts and another that includes mammals and elasmobranchs. Database searches were used to find NHE3 orthologs in the teleosts, Fugu, Tetraodon, and Danio. The tree was constructed using the neighbor-joining method with Poisson correction, and numbers indicate bootstrap values for 1,000 replicates. All homologs are labeled by genus. GenBank accession nos. are as follows, from top to bottom: NHE1: Homo, NP_003038; Amphiuya, AAD33928; Oncorhynchus, Q01345; Pseudopleuronectes, AAA44956; Cyprinus, CAB45232; and Anguilla, CAB45085. NHE2/4: Homo, NP_003039; Rattus, P48763; Mus, NP_796058; Rattus, NP_775121; and Tetraodon, scaffold 14781; NHE3: Fugu, Genescan_5486; Tetraodon, scaffold 7101; Danio, AL954769; Tribolodon (Osorezan dace), BAB83083; Rattus, NP_036786; Mus, XP_127434; Didelphis (opossum), Q28362; Homo, NP_004165; Oryctolagus, P26432; and Dasyatis, AAT45738; NHE5: Tetraodon, NP_620213; Homo, NP_004585; and Tetraodon (green pufferfish), scaffold 14611. Bar represents 10% replacement of amino acids per site.
signaling events than those used in mammals and elasmobranchs. For example, although three serine phosphorylation sites are conserved between teleosts and mammals, much of the region needed for control of endocytosis and exocytosis (1) has diverged in teleosts (Fig. 3).

Interestingly, our data suggest that NHE2 and NHE4 of mammals diverged recently, after the division of the teleost and tetrapod lineages (Fig. 4). In phylogenetic analyses of sequence fragments that included our putative stingray NHE2 sequence fragment, mammalian NHE2 and NHE4 always grouped together, separately from the stingray and teleost homologs (unpublished observations). Consequently, even though the fish homologs are slightly more homologous with NHE2 (~50% identical amino acids) than with NHE4 (~40% identical amino acids), they should be named NHE2/4, because they may have characteristics that are a blend of both isoforms. In mammals, NHE4 is expressed in the basolateral membranes of renal cells that lack detectable levels of NHE1 activity, where it may regulate intracellular pH and volume, and NHE2 is expressed in the apical membranes of renal and gastrointestinal cells, where it may participate in secretory functions (46). Further work is needed to characterize NHE2/4 in fishes to provide a reference point to understand the recent divergence of function and sequence between mammalian NHE2 and NHE4.

The distribution of NHE2/4 and NHE3 mRNA in stingrays is consistent with absorptive functions in epithelial tissues. Although little functional data are available for the spiral valve intestine, the high expression of NHEs in this tissue suggests that it may be responsible for ion and fluid absorption similar to that in mammalian small intestines. Although we previously demonstrated that the kidneys of Atlantic stingrays made no contribution to whole animal net acid secretion (11), the high expression in this tissue suggests a NaHCO₃-reabsorptive function similar to that in mammalian kidneys.
Mechanisms of acid secretion and Na\(^+\) absorption. Pharmacological, immunological, and molecular studies have suggested two potential mechanisms of Na\(^+\) absorption and H\(^+\) secretion in fish gills: 1) an apical vacuolar H\(^+\)-ATPase electrically linked to Na\(^+\) absorption via an apical Na\(^+\)/H\(^+\) channel and 2) electroneutral exchange of Na\(^+\) and H\(^+\) via proteins of the NHE family (16, 21, 40). On the basis of thermodynamic considerations of ion gradients, it has been suggested that an apical NHE is not possible in freshwater, where the Na\(^+\) concentration is usually <1 mmol/l (39). Alternatively, the vacuolar H\(^+\)-ATPase/Na\(^+\) channel mechanism was favored because it is a primary active mechanism and because subunits of the proton pump were localized to the apical side of branchial epithelial cells of some teleosts in freshwater (Onchorynchus mykiss, O. kisutch, and Oreochromis mossambicus) (38, 49, 59, 68, 69). However, more recent studies on elasmobranchs (25, 51, 60) and even a teleost (Fundulus heteroclitus) (33) have demonstrated that vacuolar H\(^+\)-ATPase is in the basolateral membranes of gill epithelia in at least some fishes, where they may have a role in base secretion and Cl\(^-\) absorption instead of acid secretion and Na\(^+\) absorption.

Our demonstration of greater NHE3 mRNA expression and immunoreactivity in freshwater-acclimated stingray gills relative to seawater stingray gills suggests that an NHE can absorb Na\(^+\) from a hypoionic environment. Our clear apical localization of NHE3 in Na\(^+\)/K\(^+\)-ATPase-rich cells is the first dem-
onstration of NHE immunoreactivity in a freshwater elasmobranch and strongly supports this predicted function (Figs. 6, 8, and 9). Presumably, high levels of Na\(^+/\)K\(^+/\)ATPase activity would be required in the same cell to lower intracellular Na\(^+\) concentration enough to allow Na\(^+\) entry from the environment. Our demonstration of increased Na\(^+/\)K\(^+/\)ATPase mRNA and immunoreactivity confirms earlier findings of greater Na\(^+/\)K\(^+/\)ATPase activity and protein levels in freshwater than in seawater (50). A recent study demonstrated apical NHE3 immunoreactivity in the gills of freshwater Osorezan dace (Tribolodon hakonensis), a unique teleost that lives in a highly acidic environment (29). That study demonstrated a large increase in NHE3 mRNA and protein expression when dace were transferred from neutral to acidic water, suggesting a function in systemic acid secretion. However, NHE3 expression was not compared with higher salinities, and therefore the effect of salinity on dace NHE3 is unknown.

Before the availability of full-length fish cDNA sequences, a single, heterologous antibody for NHE3 (1380) was used to detect immunohistochemical reactivity in three teleosts (O. mykiss, Pseudolabrus tetrious, and Periophthalmodon schlosseri) and two elasmobranchs (Mustelus antarcticus and Squantina australis) (21, 22, 67). Unfortunately, the cellular (pavement or mitochondrion-rich cell) and subcellular (apical, cytoplasmic, or basolateral) localization was variable between species, and therefore further work with homologous antibodies is needed before definitive conclusions can be made about NHE3 localization in those species. In retrospect, there is a low degree of sequence identity between the carboxyl region of rabbit NHE3 used to generate antibody 1380 and the corresponding regions of the fish proteins (29/85 amino acids for NHE3 of dace and 45/85 amino acids for NHE3 of stingray) that may explain some of the variability in staining. An antibody generated against an internal region of rat NHE3 (666) was used to detect immunoreactivity on Western blots of gill proteins from an elasmobranch (Raja erinacea), but localization was not achieved (12).
In previously published experiments, we demonstrated that in vivo net acid excretion rates from seawater stingrays increased to their highest values (5 times control excretion rates) during the first 4 h of hypercapnia and that blood pH decreased to its lowest value after 2 h of hypercapnia (11). In the current study, there were no repeatable changes in expression of NHE2/4, NHE3, or Na+/K+ -ATPase during these time intervals. This lack of an increase in mRNA expression for these transporters during hypercapnia suggests that either 1) acid secretion is accomplished via other transporters that we have not considered or 2) one or more of these transporters are involved but that stimulation of acid secretion is via posttranslational regulation during an acute acidosis such as hypercapnia (11). H+/K+ -ATPases and vacuolar H+-ATPase (27, 44) are the only other H+-transporters known to mediate epithelial acid secretion in vertebrates. However, in a previously published study of the same animals, we demonstrated that H+ /K+ -ATPase α1 mRNA expression was also unaffected by 

Fig. 8. Representative light micrographs of Atlantic stingray gill sections from seawater (A), freshwater-acclimated (B), and freshwater stingrays (C) incubated with antiserum R1B2 (brown). The location of immunoreactive cells was different in the 3 groups, ranging from mostly in the filament in seawater stingrays to mostly in the lamellae in freshwater stingrays. Immunoreactivity was in the apical region of cells in all salinity treatments (A–C). Scale bars, 50 μm.

Fig. 9. Representative light micrographs of Atlantic stingray gill sections from seawater (A), freshwater-acclimated (B), and freshwater stingrays (C) incubated with antiserum R1B2 (brown) and antibody α5 (blue). R1B2 immunoreactivity was always in the apical region of cells with basolateral Na+/K+ -ATPase, regardless of salinity treatment (A–C). Scale bars, 50 μm.

Fig. 10. Model of hypothesized ion and acid-base transport mechanisms in the gills of Atlantic stingrays based on previous immunocytochemical results (50, 51, 53) and the quantitative PCR and localization results of the current study. There are 2 populations of cells that appear to be specialized for transepithelial ion and acid-base transport: A-type mitochondrion-rich cells (A MRC) and B-type mitochondrion-rich cells (B MRC). Electrogenic transport is indicated with unequal arrow weights. Solid arrows indicate facilitated transport, and broken arrows indicate diffusion. V, vacuolar H+-ATPase; PDN, pendrin-like anion exchanger.
that vacuolar H^+-ATPase is localized to the basolateral membranes of elasmobranchs (25, 51, 60) and therefore cannot secrete acid directly into environmental water. Although our data did not identify a transporter that was upregulated during acidosis, they did suggest that NHE3 might be the most abundant putative acid transporter that we measured. For example, by using quantified plasmid DNA for our standard curves, we were able to calculate that the relative cDNA transcript copies per unit volume of total gill cDNA were 334.8 ± 28.4, 73.2 ± 36.1, and 1.0 ± 0.2 for NHE3, NHE2/4, and H^+K^+-ATPase α1, respectively. Although these values may not strictly reflect mRNA abundance in native gill tissue because of variable reverse transcription rates (58), they and the apical localization of NHE3 protein do lead us to hypothesize that NHE3 may be the largest quantitative contributor to acid secretion.

We were unable to determine the potential function of NHE2/4 in the gills, because its expression did not change with either salinity or hypercapnic manipulations. Similarly, NHE3 is clearly responsible for the majority of NaHCO_3 reabsorption in mammalian renal tubules, but the exact functions of NHE2 in mammalian renal tubules are still being determined (46). Although NHE2 knockout mice have no measurable renal absorptive dysfunctions (36, 70), a recent study showed that NHE2 is important for bicarbonate reabsorption in mammalian distal nephron segments under conditions of high distal bicarbonate delivery (4).

**Stingray gill model.** Our group previously identified a subpopulation of mitochondrion-rich cells in the gills of elasmobranchs that express high levels of Na^+/K^+-ATPase in the basolateral membranes (50). The current study is the first to use homologous antibodies to demonstrate apical immunoreactivity for NHE3 in these cells. Together with the high homology of our stingray NHE3 to mammalian NHE3 and the increased expression in freshwater, the colocalization of apical NHE3 and basolateral Na^+/K^+-ATPase strongly suggest that this cell functions for Na^+ absorption and H^+ secretion similarly to renal proximal tubule cells of mammals (3). In this scenario, Na^+/K^+-ATPase in the basolateral membrane creates a low intracellular Na^+ concentration that provides a gradient for Na^+ to enter through the apical membrane via NHE3 (Fig. 10). In addition, a yet to be identified basolateral mechanism for HCO_3^- efflux toward the blood can lower the intracellular pH and help make apical Na^+/H^+ exchange favorable.

The mechanism for Cl^- absorption/HCO_3^- secretion was suggested by previous studies that used heterologous antibodies to localize pendrin immunoreactivity to the apical side of vacuolar H^+-ATPase-rich cells (25, 53). In this cell, we predict that vacuolar H^+-ATPase alkalizes the intracellular space by removing H^+ across the basolateral membrane (Fig. 10). The alkaline environment then generates intracellular HCO_3^- that is secreted across the apical membrane in exchange for Cl^- via a putative pendrin-like anion exchanger. This scenario is similar to B-type intercalated cells of mammalian renal collecting ducts (55).

In summary, we have sequenced the first putative NHE3 ortholog from an elasmobranch, which dates the origin of NHE3 to at least before the division of bony and cartilaginous fishes. The stingray NHE3 shares a high degree of homology with mammalian NHE3 and is expressed in tissues that are expected to absorb Na^+.

NHE3 mRNA increased in the gills during acclimation to low environmental salinity and that NHE3 protein is expressed exclusively in the apical side of mitochondrion-rich cells that have high levels of basolateral Na^+/K^+-ATPase. Taken together, these data are strong evidence that the mechanism for Na^+ absorption and acid secretion in the gills of elasmobranchs is similar to the mechanism of NaHCO_3 reabsorption in the renal proximal tubules of mammals.

**ACKNOWLEDGMENTS**

We thank Michael Myamoto and Andrew Diamanduros for assistance with phylogenetic analysis of the NHE homologs. We also thank Kristina Choe and Henry Coulter for assisting in the collection of stingrays.

**GRANTS**

This material is based on work supported by National Science Foundation Grants IBN-0089943 (to D. H. Evans), IBN-0414327 (to K. P. Choe), and IBN-0111073 (to J. B. Claiborne), by Ministry of Education, Culture, Sports, Science, and Technology, Japan Grants 14104002 (to S. Hirose) and 16710145 (to A. Kato), and by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-56218 (to M. F. Romero). An East Asia and Pacific Summer Institute Program fellowship from the Japan Society for the Promotion of Science was also critical to completing this study.

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