Roles of Slc13a1 and Slc26a1 sulfate transporters of eel kidney in sulfate homeostasis and osmoregulation in freshwater

Tsutomu Nakada,1 Kambiz Zandi-Nejad,2 Yukihiro Kurita,1 Hisayuki Kudo,1 Vadjista Broumand,2 Charles Y. Kwon,2 Adriana Mercado,2 David B. Mount,2,3 and Shigehisa Hirose1

1Department of Biological Sciences, Tokyo Institute of Technology, Yokohama, Japan; 2Renal Division, Brigham and Women’s Hospital, Harvard Medical School, Boston; and 3Renal Division, Veterans Affairs Boston Healthcare System, Boston, Massachusetts

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Nakada, Tsutomu, Kambiz Zandi-Nejad, Yukihiro Kurita, Hisayuki Kudo, Vadjista Broumand, Charles Y. Kwon, Adriana Mercado, David B. Mount, and Shigehisa Hirose. Roles of Slc13a1 and Slc26a1 sulfate transporters of eel kidney in sulfate homeostasis and osmoregulation in freshwater. Am J Physiol Regul Integr Comp Physiol 289: R575–R585, 2005. First published March 31, 2005; doi:10.1152/ajpregu.00725.2004.—Sulfate is required for proper cell growth and development of all organisms. We have shown that the renal sulfate transport system has dual roles in euryhaline eel, namely, maintenance of sulfate homeostasis and osmoregulation of body fluids. To clarify the physiological roles of sulfate transporters in teleost fish, we cloned orthologs of the mammalian renal sulfate transporters Slc13a1 (NaSi-1) and Slc26a1 (Sat-1) from eel (Anguilla japonica) and assessed their functional characteristics, tissue localization, and regulated expression. Full-length cDNAs coding for ajSlc13a1 and ajSlc26a1 were isolated from a freshwater eel kidney cDNA library. Functional expression in Xenopus oocytes revealed the expected sulfate transport characteristics; furthermore, both transporters were inhibited by mercuric chloride. Northern blot analysis, in situ hybridization, and immunohistochemistry demonstrated robust apical and basolateral expression of ajSlc13a1 and ajSlc26a1, respectively, within the proximal tubule of freshwater eel kidney. Expression was dramatically reduced after the transfer of eels from freshwater to seawater; the circulating sulfate concentration in eels was in turn markedly elevated in freshwater compared with seawater conditions (19 mM vs. 1 mM). The reabsorption of sulfate via the apical ajSlc13a1 and basolateral ajSlc26a1 transporters may thus contribute to freshwater osmoregulation in euryhaline eels, via the regulation of circulating sulfate concentration.

freshwater adaptation; immunohistochemistry; sulfate transporter; renal proximal tubule

SULFATE IS ESSENTIAL for a variety of metabolic and cellular processes, including production of highly sulfated proteoglycans by chondrocytes, detoxification, and elimination of xenobiotics and endogenous compounds by sulfoconjugation in the liver and kidney, and biosynthesis of sulfated hormones such as gastrin and cholecystokinin (27). Mechanisms regulating the levels of plasma sulfate are therefore essential for the maintenance of normal physiology. In mammals, the regulation of sulfate homeostasis is largely determined by the kidney, with the major fraction of filtered sulfate being reabsorbed in the proximal tubule. Two sulfate transporters have been identified that are involved in this reabsorption of sulfate from the glomerular ultrafiltrate: solute carrier family 13a1 (Slc13a1; NaSi-1) and solute carrier family 26a1 (Slc26a1; Sat-1). Slc13a1 is an electrogenic Na+-dependent sulfate transporter (alternatively called Na+-SO42- cotransporter) that is located in the apical membrane of renal proximal tubule cells and mediates entry of Na+-SO42- with a stoichiometry of 3:1 (5, 24). Slc26a1 is sulfate/anion exchanger mediating SO42- efflux across the basolateral membrane in exchange for HCO3- (17). Physiological significance of this regulatory system is well established in mammals through targeted disruption of the Slc13a1 gene (9).

Physiological studies on sulfate homeostasis also have been conducted in nonmammalian systems, including those of birds (12, 40, 44, 45), bivalve (11), and fish (10, 36, 37, 43, 46–49). However, little is known concerning their molecular mechanisms. We were interested in the regulation of sulfate homeostasis in euryhaline fish, which migrate between freshwater and sulfate-rich seawater, and initiated cloning of eel orthologs of mammalian Slc13a1 and Slc26a1 as the first step toward clarification of molecular mechanisms involved in sulfate handling by euryhaline fish exposed to a wide range of environmental sulfate concentrations. Cloning of the relevant cDNAs, followed by functional analysis, Northern blot analysis, RNA in situ hybridization histochemistry, and immunohistochemistry, indicated that a transport system very similar to that of mammals is operating in freshwater eel kidneys that is rapidly and dramatically downregulated under seawater conditions. Interestingly, the eel sulfate transport system, composed of apical Slc13a1 and basolateral Slc26a1, turned out to play dual roles under freshwater conditions: 1) to prevent sulfate loss and maintain sulfate homeostasis and 2) to accumulate and retain relatively high concentrations of sulfate as an osmolyte. Our findings help explain not only the mechanism of maintaining sulfate homeostasis in freshwater but also why adult eels migrate to freshwater (32).

MATERIALS AND METHODS

Animals. Japanese eel (Anguilla japonica) were purchased from a local dealer. They were reared in a freshwater tank for 2 wk (freshwater eels). Some eels were transferred to a seawater tank and acclimated for 2 wk before use (seawater eels). The water temperature was maintained at 18–22°C. All eels were anesthetized by immersion in 0.1% ethyl m-amino-benzoate (MS-222 or tricaine) before being killed by decapitation. The tissues required for RNA extraction were...
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...dissected out, snap-frozen in liquid nitrogen, and stored at −80°C for future use. Artificial seawater (Rohtomarine) was obtained from Rei-Sea (Tokyo, Japan). Sulfate-free seawater was prepared by mixing the following chemicals (in g/100 l): 2.398 NaCl, 1.085 MgCl₂-6H₂O, 112 CaCl₂, 67 KCl, 19.6 NaHCO₃, 9.8 KBr, 4.13 SrCl₂-6H₂O, 2.66 H₂BO₃, 0.306 NaF, and 0.007 NaI. The animal protocols and procedures were approved by the Institutional Animal Care and Use Committee of Tokyo Institute of Technology and conform to the American Physiological Society’s “Guiding Principles in the Care and Use of Laboratory Animals.”

RNA isolation. Total RNA was isolated from various tissues by performing acid guanidinium thiocyanate-phenol-chloroform extraction with Isogen (Nippon Gene, Tokyo, Japan). Tissues were homogenized in Isogen (1 g tissue/10 ml Isogen) by using a Polytron tissue homogenizer, followed by chloroform extraction, isopropanol precipitation, and 75% (vol/vol) ethanol washing of precipitated RNA. The obtained RNA was dissolved in diethyl pyrocarbonate-treated water, and its concentration was measured spectrophotometrically at 260 nm.

Molecular cloning. A fragment of ajSlc13a1 cDNA (aj for A. japonica) was isolated by performing RT-PCR by using the 5'-RACE kit (Ambion, Austin, TX) according to the manufacturer’s instructions. Briefly, 1 µg of total RNA from eel kidney was first treated with calf intestinal phosphatase (CIP) in 1× CIP buffer at 37°C for 1 h. A single-stranded 5'-end sequence of cDNA, 3'-adapter and MMLV RT in 1× RT buffer with dNTPs and RNase inhibitor at 42°C for 1 h to create the first-strand cDNA. The synthesized cDNA was then subjected to two rounds of PCR by using adaptor-specific primers with gene-specific primer (GSP) 1 (5'-GATGAGGTTGGTTGAGGTGCC-3') and GSP 2 (5'-CCGATGCCTTGGTGGAGGCTTC-3'). These GSPs were designed on the basis of the amino acid sequence alignment of human (21, 23), rat (3, 28), and mouse Slc13a1 orthologs (1, 22) in addition to that found in the fugu genomic database (http://genome.jgi-psf.org/fugu6/fugu6.home.html). The amplification product of ~360 bp was subcloned into EcoRV-digested pBluescript II SK(−) (Stratagene, La Jolla, CA) and sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA) and the ABI 310 DNA sequence system (Applied Biosystems). This clone was used as a probe for Northern blot analysis of ajSlc13a1. To obtain the 3'-end sequence of cDNA, 3'-RACE was conducted with the same kit by following the manufacturer’s instructions. Briefly, 1 µg of total RNA from eel kidney was reverse-transcribed with 3'-adapter and MMLV RT in 1× RT buffer with dNTPs and RNase inhibitor at 42°C for 1 h to create the first-strand cDNA. The synthesized cDNA was then subjected to two rounds of PCR using adaptor-specific primers with gene-specific primer (GSP) 1 (5'-GATGAGGTTGGTTGAGGTGCC-3') and GSP 2 (5'-CCGATGCCTTGGTGGAGGCTTC-3'). These GSPs were designed on the basis of the amino acid sequence alignment of human (21, 23), rat (3, 28), and mouse Slc13a1 orthologs (1, 22) in addition to that found in the fugu genomic database (http://genome.jgi-psf.org/fugu6/fugu6.home.html). The amplification product of ~2,200 bp was subcloned into pBluescript II SK(−) and sequenced. To determine full-length cDNA sequences, we carried out direct sequencing of PCR products using primers that recognize the 5'-flanking region (5'-GATATCAGTTGTTGGAGTAAAG-3') and 3'-flanking region (5'-CTACITTGGACACCATTATTAGTCTGT-3') of the cDNA. For confirmation of the sequence, PCR products were subcloned into pZErO-2 (Invitrogen) and sequenced.

A fragment of ajSlc26a1 cDNA of ~900 bp was isolated by RT-PCR from eel kidney RNA with the use of sense (5'-ATH-TATTTTTTATGGGNAC-3') and antisense (5'-GTNGTGRAARCARTGNARRAA-3') degenerate primers. The PCR product was subcloned into pBluescript II SK(−) and sequenced. This clone was used as a probe for Northern blot analysis of ajSlc26a1. Full-length cDNA of ajSlc26a1 was obtained by 5'-RACE, 3'-RACE, and RT-PCR by using a strategy similar to that for ajSlc13a1 cloning as described above. The primers used were 5'-CAGATAGTGAGACGGTTAAGGC-3' (5'-RACE, GSP 1 antisense), 5'-AGCTCAGTGCGCAAGGGAGGT-3' (5'-RACE, GSP 2 antisense), 5'-TCATGTGGTTGAGGTGTTGGA-3' (3'-RACE, GSP 1 sense), 5'-GCTGGAGATCCCTTCGACCAAG-3' (3'-RACE, GSP 2 sense), 5'-TGAAGTGCGTTAGTGGATAGAG-3' (5'-flanking region, sense), and 5'-GCTTTTGCTGGTGGACTCCTCAC-3' (3'-flanking region, antisense).

Northern blot analysis. Total RNA (20 µg/lane) from a pool of various tissues of freshwater and seawater eels was electrophoresed on formaldehyde-agarose (1%) denaturing gels in 10× MOPS running buffer (20 mM MOPS, pH 7.0, 8 mM acetic acid, 1 mM EDTA) and then transferred onto Hybond-N⁺ nylon membranes (Amersham Biosciences, Piscataway, NJ) by capillary blotting. After transfer, membranes were baked for 2 h at 80°C and prehybridized for 2 h at 65°C in PerfectHyb hybridization solution (Toyobo, Osaka, Japan). The probes were labeled with [33P]dCTP (3,000 Ci/mmol) with the use of a Red-Fluor DNA labeling kit (Ambion Biosciences), and then unincorporated nucleotides were removed by passage through a Sephadex G-50 column (Amersham Biosciences). The membranes were then hybridized separately with each 33P-labeled probe in the same buffer at 68°C for 16 h. The blots were subsequently washed with increasingly stringent conditions (final wash: 1× SSC and 0.1% SDS for 30 min at 60°C). Membranes were exposed to imaging plates (Fuji Film, Tokyo, Japan) in a cassette overnight. The results were analyzed using a Fuji BAS2000 Bio-image analyzer (Fuji Film). A probe for eel β-actin (33) was used as a control to verify loading and RNA integrity.

Expression of ajSlc26a1 and ajSlc13a1 in Xenopus laevis oocytes. The ajSlc26a1 and ajSlc13a1 constructs in pZErO-2 were linearized at their 3'-end with the use of EcoRI and BamH1 restriction enzymes, respectively, and cRNAs were transcribed in vitro using T7 RNA polymerase and mMESSAGE mMACHINE kit (Ambion). Oocytes were surgically collected from anesthetized animals under 0.17% tricaine and incubated for 1–2 h with shaking in ND96 without chloride (in mM: 100 NaCl, 2 KCl, 1 MgCl₂, and 5 HEPES-Tris, pH 7.4) and 2 mg/ml collagenase A (for chemical defolliculation), after which oocytes were washed with ND96 with calcium (in mM: 96 NaCl, 2 KCl, 2 CaCl₂, 1 MgCl₂, and 5 HEPES-Tris, pH 7.4) four times and incubated in ND96 solution for 24 h. Stage V-VI defolliculated oocytes (13) were then injected with 50 nl of water or 0.5 µg/µl of the cRNA (25 ng/oocyte) solution with the use of a Nanoliter-2000 injector (World Precision Instruments, Sarasota, FL). Oocytes were incubated at 16°C in ND96 supplemented with 2.5 mM sodium pyruvate and 5 mg/100 ml gentamicin for 48–72 h. The incubation medium was changed every 24 h, including the day of the uptake experiment. All the uptake solutions had 10 µCi/ml [35S]ICN Biomedicals, Costa Mesa, CA).

Oocytes injected with ajSlc26a1 cRNA were placed in an uptake solution either without chloride (in mM: 100 N-methyl-D-glucamine (NMDG)-glucanate, 2 potassium glucanate, 1 calcium glucanate, 1 magnesium glucanate, 10 HEPES-Tris, and 1 K₂SO₄) or with chloride (in mM: 100 choline chloride, 2 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES-Tris, and 1 K₂SO₄) for the 1-h uptake. Inhibiting substances included DIDS (1 mM), HgCl₂ (100 µM), potassium thiocyanate (5 mM), olofuranoside (5 mM), collagenase A (for chemical defolliculation), after which oocytes were placed in a medium lacking sodium and chloride (in mM: 100 NMDG-glucanate, 2 potassium glucanate, 1 calcium glucanate, 1 magnesium glucanate, 10 HEPES-Tris) for 3 h before uptake studies were performed. For chloride uptake, oocytes were placed in an uptake medium containing (in mM) 90 NMDG-glucanate, 2 potassium glucanate, 1 calcium glucanate, 1 magnesium glucanate, 10 HEPES-Tris, and 10 [35S]Cl⁻/KCI.

Oocytes injected with ajSlc13a1 were placed in an uptake solution either with sodium (in mM: 100 sodium glucanate, 2 potassium AJP-Regul Integr Comp Physiol • VOL 289 • AUGUST 2005 • www.ajpregu.org
glucuronate, 1 calcium gluconate, 1 magnesium gluconate, 10 HEPES-Tris, and 1 K$_2$SO$_4$ or without sodium (in mM: 100 NMDG-gluconate, 2 potassium gluconate, 1 calcium gluconate, 1 magnesium gluconate, 10 HEPES-Tris, and 1 K$_2$SO$_4$) for 1 h. Uptakes were performed in the presence and absence of HgCl$_2$ (100 µM), potassium thiosulfate (5 mM), oxalate (5 mM), or selexane (5 mM).

After uptake, the oocytes were washed three times in ice-cold uptake solution (without radioisotope) and individually dissolved in 10% SDS. Tracer activity was then determined using scintillation counting. The uptake experiments all included 10–25 oocytes in each experimental group, statistical significance was defined as two tailed ($P < 0.05$), and results are reported as means ± SE.

**Antibody production and specificity.** A 245-bp fragment encoding a part of an intracellular domain (amino acid residues 179–259) of ajSlc13a1 and a 303-bp fragment encoding a part of the intracellular COOH terminus (amino acid residues 603–702) of ajSlc26a1 were subcloned into the BambHI/EcoRI sites of the bacterial expression vector pHAT10 (BD Biosciences Clontech, Palo Alto, CA). The recombinant proteins were purified with Talon metal affinity resins (BD Biosciences) according to the manufacturer’s instructions. Briefly, BL21 cells transformed with pHAT-ajSlc13a1 or pHAT-ajSlc26a1 were used to inoculate 1.5 liters of Luria-Bertani broth containing 100 µg/ml ampicillin. The cultures were harvested at an A$_{600}$ of 0.5 at 37°C, and protein expression was induced by adding isopropyl-1-thio-D-galactopyranoside to a final concentration of 1 mM for 3 h at 37°C. The cells were harvested from the cultures by centrifugation and resuspended in 20 ml of extraction/wash buffer, disrupted by freezing-thawing and sonication. After centrifugation (10,000 g at 4°C), supernatants and pellets were saved as water-soluble and -insoluble fractions, respectively. In the case of pHAT-ajSlc13a1, the recombinant protein was purified from supernatant using Talon metal affinity resin. In the case of pHAT-ajSlc26a1, however, the recombinant protein was recovered in the pellet and was solubilized by resuspension in solution containing 8 M urea, 50 mM phosphate, and 300 mM NaCl, pH 7.6, for 2 h at 4°C. The mixture was then centrifuged at 10,000 g for 40 min to remove any insoluble materials. Urea-solubilized recombinant ajSlc26a1 was purified using Talon metal affinity resin. After purification, ajSlc13a1 and ajSlc26a1 were dialyzed against saline at 4°C. Polyclonal antibodies to ajSlc13a1 and ajSlc26a1 were prepared in New Zealand White rabbits by injecting ~200 µg of purified recombinant proteins, emulsified with the adjuvant TiterMax Gold (CytRx; 1:1), intramuscularly at multiple sites. The rabbits were injected three times at 1-mo intervals and bled 7 days after the third immunization.

Specificities of the antisera were established by Western blotting of the protein extracts of HEK-293T cells exogenously expressing ajSlc13a1 or ajSlc26a1 (data not shown). The fragments corresponding to the open reading frames of ajSlc13a1 and ajSlc26a1 were subcloned into the pcDNA3 vector. HEK-293T cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich Japan, Tokyo, Japan) containing 10% fetal bovine serum (Invitrogen Japan, Tokyo, Japan), 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were transfected with the ajSlc13a1, ajSlc26a1, or mock plasmid by using Lipofectamine 2000 (Invitrogen Japan) according to the manufacturer’s instructions. The cells were washed three times with PBS and solubilized with Laemmli buffer. The cell lysates were separated by SDS-PAGE using a 7.5% polyacrylamide gel and electroblotted onto a polyvinylidene difluoride membrane. Non-specific binding was blocked with 5% nonfat skim milk in TBST (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature. The membranes were incubated with anti-ajSlc13a1, anti-ajSlc26a1, or preimmune serum at 1:10,000 dilution overnight at 4°C. After being washed with TBST, membranes were then reacted with horse-radish peroxidase-conjugated donkey anti-rabbit IgG at 1:30,000 dilution for 1 h at room temperature. The bound secondary antibody was visualized by enhanced chemiluminescence detection using ECL-Plus reagent (Amersham Bioscience) according to the manufacturer’s instructions. Two specific protein bands of 60 and 120 kDa were stained with anti-ajSlc13a1 antiserum, which correspond to monomeric and dimeric forms of ajSlc13a1. Similarly, anti-ajSlc26a1 antiserum visualized 80-kDa monomer and 170-kDa dimer bands of ajSlc26a1 on Western blotting of extracts of HEK-293 cells expressing ajSlc26a1. The sizes of ajSlc13a1 and ajSlc26a1, determined by Western blotting, are consistent with those predicted from the coding sequences of their cDNAs.

**In situ hybridization.** Kidneys from anesthetized freshwater eels perfused and fixed with 10% buffered neutral formalin (Muto Pure Chemicals, Tokyo, Japan) were harvested, embedded in paraffin, and sectioned (4 µm). The following DNA templates were used for the preparation of digoxigenin (DIG)-labeled riboprobes: a 431-bp fragment of ajSlc13a1 cDNA (nucleotides 215–645) and a 489-bp fragment of ajSlc26a1 cDNA (nucleotides 1831–2319). DIG RNA labeling mix (Roche Diagnostics, Mannheim, Germany) was used for synthesizing DIG-labeled sense and antisense probes. Alkaline phosphatase-conjugated anti-DIG antibody and nitro blue tetrazolium/ bromochloroindolyl phosphate substrates were used to visualize the signal, followed by counterstaining with Kernechtrot (Muto Pure Chemicals).

**Immunohistochemistry.** Kidneys from freshwater eel were fixed in 0.1 M phosphate buffer, pH 7.4, containing 4% (wt/vol) parafomaldehyde for 1 h at 4°C. After incubation in 0.1 M phosphate buffer, pH 7.4, containing 20% (wt/vol) sucrose for 16 h at 4°C, specimens were frozen in Tissue Tek OCT compound on a cryostat holder. Sections (6 µm) were prepared in −20°C cryostat and mounted on (3-aminopropyl)triethoxysilane-coated glass slides and air dried for 1 h. After being washed with PBS, sections were incubated for 2 h at room temperature with 2.5% (vol/vol) normal goat serum and then overnight at 4°C with anti-ajSlc13a1 antisemur (1:10,000) or anti-ajSlc26a1 antisemur (1:10,000). Sections were then washed with PBS and treated with methanol containing 0.3% (vol/vol) H$_2$O$_2$ for 20 min at room temperature. After being washed with PBS, the specimens were treated with biotinylated secondary antibody (1:2,000) and avidin-peroxidase conjugate using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Finally, bound antibodies were visualized by using 3,3'-diaminobenzidine tetrahydrochloride in 50 mM Tris-HCl, pH 7.4, and counterstaining with hematoxylin.

**Measurement of serum ionic composition.** The blood was collected from the ventricle of anesthetized eel and allowed to clot; the serum was then prepared by centrifugation and stored at −40°C for future use. The serum osmolarity was measured using the cryoscopic method (38). Concentrations of Na$^+$, K$^+$, and Cl$^-$ were measured using the established electrode methods (25). Ca$^{2+}$ and Mg$^{2+}$ concentrations were measured using the o-cresolphthalein complexone method (7) and xylidine blue method (26), respectively. These measurements were conducted by SRL Laboratories (Tokyo, Japan). The concentration of SO$_4^{2-}$ was measured using the ion chromatographic method (6) with PIA-1000 (Shimazu, Kyoto, Japan) and serially diluted serum samples (5 µl). The standard curve was made by using defined concentrations of K$_2$SO$_4$.

**RESULTS**

**Molecular properties of ajSlc13a1 and ajSlc26a1.** We isolated an eel homolog of mammalian Slc13a1 by using RT-PCR of freshwater eel kidney total RNA and sequence information for rat (29), human (21), and mouse Slc13a1 cDNA (1), in addition to relevant sequence for the fugu genome. The complete coding sequence was obtained by 5'- and 3'-RACE and deposited in the DDBJ/EMBL/GenBank database (accession no. AB111926). The isolated cDNA was ~2.3 kb, including 30 bp of 5'-untranslated region (UTR) and 380 bp of 3'-UTR. The coding region predicts a protein of 619 amino acid residues with a calculated molecular mass of 68 kDa (Fig. 1A). The
protein contains putative phosphorylation sites (protein kinase A: Ser\textsuperscript{170}, protein kinase C: Ser\textsuperscript{6}, Ser\textsuperscript{262}, Ser\textsuperscript{348}, Ser\textsuperscript{54}, Thr\textsuperscript{360}, and Thr\textsuperscript{445}) and an N-glycosylation site (Asn\textsuperscript{159}) (Fig. 1, A and D). As expected, the sequence is most closely related to those of known Slc13a1 orthologs among the members of the sulfate permease family. For example, it shares high amino acid sequence identity with Slc13a1 from rat (54%), mouse (53%), human (54%), zebrafish (58%), fugu (55%), and African clawed frog (48%) (Fig. 1A). In addition, the consensus sequence for Na\textsuperscript{+}-coupled cotransporters (Prosite PS01271), known as the “Na\textsuperscript{+}-sulfate signature,” also is present in ajSlc13a1 (Fig. 1D). Furthermore, ajSlc13a1 is placed in the Slc13a1 cluster by a phylogenetic analysis (Fig. 1B) and is therefore termed the eel gene ajSlc13a1. Hydrophobicity analysis of the ajSlc13a1 protein predicted a membrane topology with 11 putative transmembrane spans, an intracellular NH\textsubscript{2}-terminal domain, and an extracellular COOH-terminal tail (Fig. 1, C and D). The number of predicted transmembrane spans varied from 8 (29) to 13 (2) depending on computer programs used for prediction. Because the rabbit Slc13a2 has been confirmed experimentally to contain an odd number of transmembrane spans (35, 54), we tentatively assumed an 11-transmembrane model for ajSlc13a1.

The ajSlc26a1 ortholog was cloned by taking a similar approach, using RT-PCR and 5\textprime/-3\textprime/-RACE reactions. The isolated cDNA was 3.2 kb long, including 0.2 kb of 5\textprime/-UTR and 0.9 kb of 3\textprime/-UTR. It has high sequence homology to trout (75%), fugu (70%), mouse (52%), and human (52%) Slc26a1 (Fig. 2A) and encodes a membrane protein of 702 amino acid residues with a calculated molecular mass of 77 kDa. The protein contains putative protein kinase C phosphorylation sites (Thr\textsuperscript{38}, Thr\textsuperscript{112}, and Thr\textsuperscript{354}) and N-glycosylation sites (Asn\textsuperscript{159}, Asn\textsuperscript{165}, and Asn\textsuperscript{170}). Hydrophobicity analysis of ajSlc26a1 predicted a membrane topology with 12 putative transmembrane spans and intracellular NH\textsubscript{2}-terminal and COOH-terminal tails (Fig. 2, C and D). Like Slc26a1 of other species, ajSlc26a1 contains the following three motifs characteristic of members of the Slc26 sulfate/anion exchanger family (34): the Prosite Slc26 sulfate transporter signature (PS01130) and sulfate transporter and anti-sigma factor domain (STAS; Pf01740) (Fig. 2D). These characteristics, in addition to its position in the phylogenetic tree (Fig. 2B), confirm that ajSlc26a1 is indeed the eel ortholog of mammalian Slc26a1; its sequence information has been deposited in the DDBJ/EMBL/GenBank database (accession no. AB111927).

**Kidney- and freshwater-specific expression.** Via Northern blot analysis, we next determined the tissue distribution of ajSlc13a1 mRNA using total RNA preparations from eight different tissues of freshwater and seawater eels. A single transcript with the expected size of ~3 kb was detected very specifically in the kidney of freshwater eel (Fig. 3, top). Furthermore, kidney was the major site of expression for ajSlc26a1 mRNA; a strong hybridization signal of ~3.5 kb was detected in freshwater kidney and a weak signal in seawater kidney (Fig. 3, bottom). Low but detectable levels of the ajSlc26a1 message also were observed in the posterior intestine.

Figure 4A depicts the time course of changes in the message levels of ajSlc13a1 and ajSlc26a1 when eels were transferred from seawater to freshwater or vice versa. Both ajSlc13a1 and ajSlc26a1 transcript levels increased gradually during adaptation of eels to freshwater but declined very rapidly after their transfer to seawater (Fig. 4A).

**Salinity-dependent regulation of message levels of ajSlc13a1 and ajSlc26a1.** To determine whether the above-mentioned marked changes in the expression levels of ajSlc13a1 and ajSlc26a1 mRNA are dependent on the sulfate concentrations of freshwater (<2 mM) and seawater (~26 mM), we prepared sulfate-supplemented freshwater (FW\textsuperscript{+}/sulfate) and sulfate-free seawater (SW\textsuperscript{−}/sulfate) and measured their effects on the message levels of the two sulfate transporters. Unexpectedly, large changes were observed in their levels in the kidney when freshwater eels were transferred to SW\textsuperscript{−}/sulfate, whereas no significant difference was observed in the degree of changes between seawater and SW\textsuperscript{−}/sulfate (Fig. 4B). These results indicate that ajSlc13a1 and ajSlc26a1 mRNA levels in the kidney are mainly regulated in a salinity-dependent manner, rather than in response to variation in ambient sulfate.

**Functional properties.** The functional and pharmacological characteristics of ajSlc13a1 and ajSlc26a1 were determined by heterologous expression in *X. laevis* oocytes, using published protocols (53). The sulfate uptake by ajSlc13a1-injected oocytes was not statistically higher than that of control water-injected oocytes in the absence of sodium (Fig. 5A). However, there was a 10-fold increase in sulfate uptake, from 23.5 to 261.2 pmol/oocyte\textsuperscript{-1}\textperiodcentered h\textsuperscript{-1} (P < 0.000002) in ajSlc13a1-expressing oocytes in the presence of 100 mM Na\textsuperscript{+} (Fig. 5A). There also was a modest but statistically significant Na\textsuperscript{+}-dependent sulfate uptake in water-injected cells (from 17.1 to 29.8 pmol/oocyte\textsuperscript{-1}\textperiodcentered h\textsuperscript{-1}, P < 0.0005), consistent with the expression of an endogenous Slc13a1 or Slc13a4 ortholog in *Xenopus* oocytes. The effect of different inhibitors on the sulfate uptake by ajSlc13a1-injected oocytes is depicted in Fig. 5A and is similar to that in previous studies with the rat and human orthologs (21, 31). Whereas all of the studied compounds inhibit Na\textsuperscript{+}-dependent sulfate uptake, this effect is
most robust with HgCl₂. This finding may have further implications, because mercury is known as an environmental toxin (see Discussion).

As shown in Fig. 5B, ajSlc26a1 mediates sulfate transport in the absence of extracellular Cl⁻ and Na⁺ (27.7 vs. 4.9 pmol/oocyte⁻¹.h⁻¹ in water-injected oocytes, P < 0.0002). Sulfate uptakes in ajSlc26a1-expressing oocytes were not affected by substitution of NMDG-glucuronate with 100 mM sodium glutonate (data not shown). However, sulfate uptakes increased to 45.8 pmol/oocyte⁻¹.h⁻¹ (P < 0.0007) in the presence of 100 mM extracellular choline chloride (Fig. 5B), similar to published values for mouse, human, and rat Slc26a1 (22, 42, 52, 53) (see also Discussion). Whereas ajSlc26a1 was sensitive to 1 mM DIDS in the absence of extracellular Cl⁻, the addition of 100 mM choline chloride increased sensitivity to this inhibitor (18.9 vs. 9.3 pmol/oocyte⁻¹.h⁻¹ with or without Cl⁻, respectively, P < 0.00015). As we previously reported for mouse Slc26a1 (see also Discussion), we did not find that ajSlc26a1 directly transports 3⁴Cl⁻ (Fig. 5C, with mouse Slc26a6 as a positive control). Like ajSlc13a1, ajSlc26a1 is sensitive to cis-inhibition by extracellular thiosulfate, oxalate, and selenite (Fig. 5D). Furthermore, as reported for rat Slc26a1, ajSlc26a1 is also highly sensitive to 100 µM HgCl₂ (45.3 vs. 14.0 pmol/oocyte⁻¹.h⁻¹ in the presence of HgCl₂, P < 0.00008) (Fig. 5E).

Polarized colocalization of ajSlc13a1 and ajSlc26a1 in renal proximal tubule cells. Because Northern blotting indicated that the ajSlc13a1 and ajSlc26a1 genes are almost exclusively expressed in freshwater eel kidney, we performed RNA in situ hybridization to determine their expression patterns in the kidney at the cellular level. Clear labeling was detected in proximal tubule cells of freshwater eel kidney with both ajSlc13a1 and ajSlc26a1 cRNA probes (Fig. 6A). Colocalization of ajSlc13a1 and ajSlc26a1 in renal proximal tubule cells was confirmed by immunostaining of two serial sections (Fig. 6B). Immunohistochemistry further demonstrated apical localization of ajSlc13a1 and basolateral localization of ajSlc26a1 (Fig. 6B). Consistent with the results of Northern blot analysis, little or no significant staining was obtained when sections of seawater eel kidney were stained with the antisera, suggesting downregulation of the two sulfate transporters also at the protein level (data not shown). Specificities of the antisera were established by Western blot analysis (data not shown).

Elevated levels of serum sulfate in freshwater eels. When we measured the serum concentration of sulfate in freshwater and seawater-injected eels, we found that sulfate concentration was significantly higher in seawater-injected eels (39.2 ± 1.5 pmol/ml) than in freshwater-injected eels (12.3 ± 1.1 pmol/ml), suggesting that the eels were capable of actively transporting sulfate across the apical membrane of renal proximal tubule cells in freshwater. These findings are consistent with previous reports showing that sulfate transporters are expressed in renal proximal tubule cells (27, 42, 52, 53).}

**Fig. 2. Structural features and phylogenetic tree of ajSlc26a1.** A: alignment of amino acid sequences of ajSlc26a1 and members of the Slc26a1 family of other species. Accession numbers are as follows: ajSlc26a1, A. japonica Slc26a1 (AB111927); omSlc26a1, Oncorhynchus mykiss Slc26a1 (AB111927); trSlc26a1, T. rubripes Slc26a1 (hypothetical protein from scaffold 281 of the fugu genome database); hSlc26a1, human Slc26a1 (AF297659); and mSlc26a1, mouse Slc26a1 (AF349043). Bold lines indicate TM spans of ajSlc26a1 predicted from the Kyte-Doolittle hydropathy plot (20). Wavy underline indicates the region used as antigen. Asterisks and pound signs indicate putative PKC phosphorylation sites and putative N-glycosylation sites, respectively. B: phylogenetic tree of ajSlc26a1 and Slc26 family members of other species. The phylogenetic tree was constructed using the ClustalW computer program. The scale bar represents a genetic distance of 0.1 amino acid substitutions per site. aj, A. japonica; h, human; r, rat; tr, fugu (T. rubripes); om, trout (O. mykiss); and dr, zebrafish (D. rerio). Accession nos. are as follows: ajSlc26a1 (AB111927), omSlc26a1 (AY512964), hSlc26a1 (AF297659), slc26a1 (AF349043), hSlc26a2 (U14528), rSlc26a2 (O70531), ajSlc26a3 (AB111930), hSlc26a4 (O45511), rSlc26a4 (AF167412), hSlc26a5 (AF523354), rSlc26a5 (AJ303732), drSlc26a5 (AY278118), hSlc26a6 (AF279265), ajSlc26a6 (AB084425), hSlc26a6b (AB111928), hSlc26a6c, (AB111929), hSlc26a7 (AF331521), hSlc26a8 (AF331522), and hSlc26a11 (NM_173626). C: Kyte-Doolittle hydropathy plot of ajSlc26a1. The plot was constructed using the GENETYX-MAC computer program. D: secondary structure model of ajSlc26a1 with putative 12 TM spans. STAS, sulfate transporter anti-sigma factor. Bold lines indicate the regions used as antigens.
seawater eels, we obtained an unexpected result. The serum sulfate concentration was much higher in freshwater eels (19 mM, or 38 meq) than in seawater eels (1 mM, or 2 meq) (Table 1). This increase was accompanied by a marked decrease in Cl\(^-\) concentration in freshwater eel serum (from 120 mM to 80 mM) to maintain plasma osmolarity within a physiological range. This fact suggests, as discussed below in detail, dual physiological roles for the sulfate transport system composed of Slc13a1 and Slc26a1: maintenance of sulfate homeostasis and osmoregulation.

**DISCUSSION**

Over the last decade there have been major advances in our understanding of the molecular and cellular basis for the regulation of sulfate homeostasis in mammals through the identification (1, 3, 21, 22, 29, 42), molecular and functional characterization (5, 28, 39), and cellular and subcellular localization studies (8, 17, 24) of two sulfate transporters, Slc13a1 and Slc26a1 (Fig. 7) (for a review, see Ref. 27). In the present study we applied a similar approach to the euryhaline eel system, cloning and characterizing the eel orthologs Slc13a1 and Slc26a1. We thus have established that a very similar sulfate-transporting system is operative in renal proximal tubule cells of freshwater eels, as schematically summarized in Fig. 7. In freshwater conditions where sulfate is not readily available, sulfate ions appear to be reabsorbed from the glomerular filtrate to the blood, to a large extent by a combined action of Slc13a1 and Slc26a1, via the mechanisms illustrated in Fig. 7. This model is supported by the robust expression of these genes in the proximal tubule, as observed with Northern blotting (Fig. 3), in situ hybridization (Fig. 6A), and immunohistochemistry (Fig. 6B), in addition to the functional attributes of the expressed transporters (Fig. 5).

The renal sulfate transport system of the eel seems to play important roles not only in maintaining sulfate homeostasis but also in osmoregulation in freshwater habitats. Surprisingly, serum concentrations of sulfate were much higher in freshwater eels (19 mM, or ~38 meq) than in seawater eels (1 mM, or ~2 meq) (Table 1). This increase was accompanied by a marked decrease in Cl\(^-\) concentration in freshwater eel serum (from 120 mM to 80 mM) to maintain plasma osmolarity within a physiological range. This fact suggests, as discussed below in detail, dual physiological roles for the sulfate transport system composed of Slc13a1 and Slc26a1: maintenance of sulfate homeostasis and osmoregulation.

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The renal sulfate transport system of the eel seems to play important roles not only in maintaining sulfate homeostasis but also in osmoregulation in freshwater habitats. Surprisingly, serum concentrations of sulfate were much higher in freshwater eels than in seawater eels, and the increment was attenuated by a concomitant decrease in chloride levels (Table 1). These compensatory changes of sulfate and chloride ions appear to be an efficient strategy for freshwater adaptation, because the absorption of chloride from freshwater to maintain body fluid osmolarity is energetically costly compared to the renal reabsorption of sulfate ions derived, for example, from metabolism of sulfur-containing substances. Indeed, it is known that Cl\(^-\) influx rates in eels are very low (4, 15, 16, 18); for
example, in the European eel *A. anguilla*, it has been reported to be only 0.05 mmol/L h⁻¹ compared with 116 mmol/L h⁻¹ in the rainbow trout (16). Given such an extremely low rate of Cl⁻ uptake, the mechanism for maintaining ionic homeostasis in freshwater eels was something of a mystery before the current study was performed. The above-mentioned osmoregulatory role of the *ajSlc13a1* and *ajSlc26a1* sulfate transporters is further consistent with the observation that their expressions were strongly induced even in sulfate-supplemented freshwater (Fig. 4B). Furthermore, we observed rapid downregulation of the two transporters upon transfer to seawater, even when sulfate free (Fig. 4B); we hypothesize that this is necessary for achieving, upon seawater transfer, an immediate lowering of serum sulfate concentrations from 26 mM to ~1 mM, to compensate for passive accumulation of chloride ions under seawater conditions. In addition to the osmoregulatory role of sulfate in freshwater, it is tempting to speculate that sulfate, a multivalent anion, tends to hold water and help prevent desiccation, enabling eels to move a considerable distance over land and colonize isolated ponds.

Differences in the degree and kinetics of induction or down-regulation of *ajSlc13a1* and *ajSlc26a1* are noteworthy (Fig. 4). The apical *ajSlc13a1* exhibited more rapid and complete down-regulation than the basolateral *ajSlc26a1* when eels were transferred from freshwater to seawater. This differential regulation of expression is consistent with the previous notion that apically located Slc13a1 represents the major target for known regulatory factors such as 1,25(OH)₂ vitamin D₃ (14), thyroid hormones (51), and glucocorticoids (50).

### Table 1. Serum ionic composition of freshwater and seawater eels

<table>
<thead>
<tr>
<th>Type of Eel</th>
<th>n</th>
<th>Serum Osmolarity, mosM</th>
<th>Na⁺, meq</th>
<th>K⁺, meq</th>
<th>Ca²⁺, meq</th>
<th>Mg²⁺, meq</th>
<th>Cl⁻, meq</th>
<th>SO₄²⁻, meq</th>
</tr>
</thead>
<tbody>
<tr>
<td>FW eel</td>
<td>4</td>
<td>293.3 (11.6)</td>
<td>165.3 (8.3)</td>
<td>&lt;4.2 (1.0)</td>
<td>3.5 (0.3)</td>
<td>1.2 (0.4)</td>
<td>78.7 (4.6)</td>
<td>37.5 (5.6)</td>
</tr>
<tr>
<td>SW eel</td>
<td>4</td>
<td>338.7 (17.0)</td>
<td>169.3 (2.8)</td>
<td>5.6 (0.9)</td>
<td>3.1 (0.1)</td>
<td>1.8 (0.6)</td>
<td>125.3 (2.8)</td>
<td>1.9 (0.03)</td>
</tr>
</tbody>
</table>

Values are means (SD). Freshwater eels show a marked increase in sulfate (SO₄²⁻) concentration and a concomitant decrease in Cl⁻ concentration to maintain the appropriate ionic balance to keep serum osmolarity in a physiological range. Eels were acclimated to freshwater (FW) or seawater (SW) for 2 wk.

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**Fig. 6.** In situ hybridization and immunohistochemistry of *ajSlc13a1* and *ajSlc26a1* in freshwater eel kidney. Left (a, c, e, and g), low-magnification images; right (b, d, f, and h), high-magnification images of boxed areas in corresponding images at left. A: proximal tubule cell expression of *ajSlc13a1* and *ajSlc26a1* mRNA. Arrow in c points to brown melanin and hemosiderin granules present in the intertubular hematopoietic tissue of teleost kidney. B: colocalization of *ajSlc13a1* and *ajSlc26a1* in renal proximal tubule cells. Polarized localization of *ajSlc13a1* (apical) and *ajSlc26a1* (basolateral) is also shown in f and h. D, distal tubule; G, glomerulus; and P, proximal tubule. Scale bars, 20 μm.

**Fig. 7.** Model of renal sulfate reabsorption system in freshwater eel. The sulfate transporters *ajSlc13a1* and *ajSlc26a1* are located in the apical and basolateral membranes, respectively, of proximal tubule cells. Driven by the Na⁺ gradient created by Na⁺-K⁺-ATPase, the apical *ajSlc13a1* transports the luminal sulfate anion into the cells. The increment of sulfate anion concentration generates an outward sulfate gradient, leading to sulfate exit across the basolateral membrane. Stoichiometries of the ions transported by *ajSlc13a1* and *ajSlc26a1* have not been determined but are supposed to be the same as those determined for the mammalian counterparts (5). BBM, brush-border membrane; PTEC, proximal tubule epithelial cell; TJ, tight junction.
mammalian Scl26a1 orthologs function as robust oxalate exchangers (17, 42, 53) such that persistent expression of ajScl26a1 in seawater may reflect other roles in the homeostasis of this and/or other anions. The salinity-dependent regulation of expression of ajScl13a1 and ajScl26a1 is an interesting phenomenon for which the molecular mechanisms have yet to be clarified.

Heterologous expression of both ajScl13a1 and ajScl26a1 in Xenopus oocytes revealed the expected functional attributes, i.e., Na\(^+\)-dependent and Na\(^+\)-independent transport of sulfate, respectively. Scl26a1 is unique among the 10 mammalian Scl26 paralogs (34) in that Cl\(^-\) and other monovalent anions (halides, formate, and lactate) dramatically enhance sulfate and oxalate transport (53). We found a similar activating effect of extracellular Cl\(^-\) on ajScl26a1 sulfate transport (Fig. 5C), although in contrast to the results of others (22, 42, 52), we did detect statistically significant sulfate transport by ajScl26a1-injected (Fig. 5B) and mouse Scl26a1-injected oocytes (53) in the absence of Cl\(^-\). Furthermore, we found an absence of direct Cl\(^-\) transport in oocytes expressing ajScl26a1; although this finding is consistent with our prior data for mouse Scl26a1, it differs from data reported by Markovich and colleagues (22, 42), who found that the mouse and human orthologs mediate Cl\(^-\) transport in oocytes. Notably, the “cis-activation” of extracellular Cl\(^-\), consistent across several reports (17, 22, 42, 52, 53), is not consistent with direct SO\(_4^{2-}\)/Cl\(^-\) exchange, in which one would expect the Cl\(^-\) inhibition that is characteristic of other Scl26 exchangers that mediate both SO\(_4^{2-}\) and Cl\(^-\) exchange. It also is reported in several species that oxalate and SO\(_4^{2-}\) exchange in basolateral membrane vesicles of the renal proximal tubule, likely mediated by Scl26a1 (17), is not sensitive to extravesicular Cl\(^-\) (19, 41, 44). In summary, although we do not rule out a direct role for Scl26a1 in Cl\(^-\) transport, we favor a modulatory effect of this anion on divalent anion exchange; regardless of the mechanism(s) of anion exchange mediated by Scl26a1, this appears to be a conserved attribute of this transporter in mammals and eel.

Finally, we found that mercury (HgCl\(_2\)) is a potent inhibitor of both ajScl13a1 and ajScl26a1. Similar data have been reported for the mammalian orthologs in the context of detailed study of their differential sensitivity to a number of heavy metals. The mechanisms of this sensitivity are not known but may differ for rat Slc13a1 and Slc26a1 (30, 31). Regardless, the shared sensitivity of the eel orthologs indicates that this environmental toxin has the potential to almost completely inhibit transepithelial sulfate transport in the proximal tubule of euryhaline fish.

Perspectives

Eels have a fascinating life cycle. They breed in the sea, migrate to freshwater to grow, and spend most of their lives in freshwater before returning to the sea to spawn. The efficient sulfate recycling system reported in this article may be advantageous to eels in a race for survival in freshwater; by reducing the serum Cl\(^-\) concentration required for freshwater survival, at ~80 mM in eels (Table 1) vs. ~120 mM in freshwater fish (16), this system reduces the cost of maintaining anion homeostasis in freshwater by ~30%. In this context, our findings may provide a molecular basis for the life cycle of eels by explaining how this species survives in freshwater with a minimum of Cl\(^-\) absorption.

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REFERENCES

SULFATE TRANSPORTERS IN FRESHWATER EEL KIDNEY