Identification of renal transporters involved in sulfate excretion in marine teleost fish

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¹Department of Biological Sciences, Tokyo Institute of Technology, Yokohama, Japan; ²Physiology and Biomedical Engineering, Mayo Clinic College of Medicine, Rochester, Minnesota; and ³Shimonoseki Marine Science Museum “Kaiyukan,” Shimonoseki Academy of Marine Science, Shimonoseki, Japan

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Kato A, Chang MH, Kurita Y, Nakada T, Ogoshi M, Nakazato T, Doi H, Hirose S, Romero MF. Identification of renal transporters involved in sulfate excretion in marine teleost fish. Am J Physiol Regul Integr Comp Physiol 297: R1647–R1659, 2009. First published October 7, 2009; doi:10.1152/ajpregu.00228.2009.—Sulfate (SO4²⁻) is the second most abundant anion in seawater (SW), and excretion of excess SO4²⁻ from ingested SW is essential for marine fish to survive. Marine teleosts excrete SO4²⁻ via the urine produced in the kidney. The SO4²⁻ transporter that secretes and concentrates SO4²⁻ in the urine has not previously been identified. Here, we have identified and characterized candidates for the long-sought transporters. Using sequences from the fugu database, we have cloned cDNA fragments of all transporters belonging to the Slc13 and Slc26 families from mefugu (Takifugu obscurus). We compared Slc13 and Slc26 mRNA expression in the kidney between freshwater (FW) and SW mefugu. Among 14 clones examined, the expression of a Slc26a6 paralog (mSlc26a6A) was the most upregulated (30-fold) in the kidney of SW mefugu. Electrophysiological analyses of Xenopus oocytes expressing mSlc26a6A, mSlc26a6B, and mouse Slc26a6 (mSlc26a6A) demonstrated that all transporters mediate electrogenic Cl⁻/SO4²⁻, Cl⁻/oxalate⁻, and Cl⁻/HCO₃⁻ exchanges and electroneutral Cl⁻/formate⁻ exchange. Two-electrode voltage-clamp experiments demonstrated that the SO4²⁻-elicited currents of mSlc26a6A is quite large (~35 μA at +60 mV) and 50- to 200-fold higher than those of mSlc26a6B and mSlc26a6. Conversely, the currents elicited by oxalate and HCO₃⁻ are almost identical among mSlc26a6A, mSlc26a6B, and mSlc26a6A. Kinetic analysis revealed that mSlc26a6A has the highest SO4²⁻ affinity as well as capacity. Immunohistochemical analyses demonstrated that mSlc26a6A localizes to the apical (brush-border) region of the proximal tubules. Together, these findings suggest that mSlc26a6A is the most likely candidate for the major apical SO4²⁻ transporter that mediates SO4²⁻ secretion in the kidney of marine teleosts.

mefugu; proximal tubule; Slc13; Slc26; sulfate homeostasis; oxalate

SULFATE (SO4²⁻) IS ESSENTIAL FOR MANY BIOLOGICAL PROCESSES. Almost all vertebrate animals maintain plasma SO4²⁻ concentration at 0.2–1 mM, except the special case of the freshwater eel, which uses SO4²⁻ as a plasma osmolyte (35). In mammals, plasma [SO4²⁻] is maintained by the kidney, where SO4²⁻ is freely filtered from the blood and then reabsorbed (29, 30). The proximal tubule is the major site of active SO4²⁻ reabsorption, and the remaining SO4²⁻ (10–30%) is excreted in urine. SO4²⁻ uptake across the apical membrane is coupled to Na⁺ absorption. This coupled transport is mediated by the Na⁺/SO4²⁻ cotransporter (NaSi-1, Slc13a1) (8). The basolateral membrane of the proximal tubule exchanges SO4²⁻ for anions, such as OH⁻ and HCO₃⁻ (25), and this exchange seems to be mediated by SO4²⁻-anion transporter 1 (Sat1, Slc26a1) (30). In teleosts (modern bony fishes), plasma [SO4²⁻] is maintained at levels similar to those in mammals. In contrast to most mammals, however, marine teleosts concentrate and excrete SO4²⁻ in urine (37, 41).

The plasma of marine teleosts has ionic composition and osmolarity similar to that found in mammals and freshwater (FW) fish, i.e., hypotonic to seawater (SW). To balance passive water loss from the gills and skin, marine teleosts drink SW, absorb water, and eliminate salts. SW contains ~53 mM Mg²⁺, 27 mM SO4²⁻, 10 mM Ca²⁺, and 10 mM K⁺ as well as ~450 mM NaCl. Therefore, marine teleosts must excrete the excess ions from the ingested SW: from the gill, Na⁺, Cl⁻, and K⁺; from the intestine, Ca²⁺ and Mg²⁺; and from the kidney, Mg²⁺ and SO4²⁻ (33). Urine of marine teleosts is isotonic to the plasma but rich in Mg²⁺ and SO4²⁻, which are not secreted by the gill. Consequently, renal SO4²⁻ excretion is essential for SO4²⁻ homeostasis in marine teleosts. SW ingestion has also been reported in a number of marine mammals (36), indicating that they may have similar SO4²⁻ handling as marine teleosts. It has also been shown that the site of marine teleost SO4²⁻ secretion is the renal proximal tubule (7, 9, 44). However, the molecular mechanisms by which SO4²⁻ is secreted and concentrated in urine are not known.

To identify the transporters involved in SO4²⁻ secretion, we took a similar approach as used to identify intestinal bicarbonate transporters essential for SW adaptation (27). Specifically, we cloned all candidate SO4²⁻ transporters from the Slc13 and Slc26 families from a euryhaline pufferfish, mefugu (Takifugu obscurus) (20), with a help of database mining. We then narrowed the potential candidates by determining whether their mRNA was induced in the kidney with SW conditions. Mefugu is a species closely related to tiger puffer (T. rubripes) (57), whose whole genome was sequenced in 2002 (1). Through this systematic approach, we identified an Slc26a6 (CFEX/PAT-1) paralog (mSlc26a6A), which exhibited the highest induction in SW. The results of our functional characterization (electrophysiology) and protein localization (immunohistochemistry) lead us to propose that mSlc26a6A, an electrogenic Cl⁻/SO4²⁻ exchanger, plays a central role in the excretion of excess SO4²⁻ at the apical side of the renal proximal tubule cells of marine teleosts. This highly-efficient secretion mechanism allows marine teleosts to maintain extremely low plasma [SO4²⁻] relative to the surrounding SW.

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R1647
MATERIALS AND METHODS

Animals. Mefgu T. obscurus (200–380 g) were purchased from a local dealer and reared in tanks containing 150 liters brackish water (3–14% diluted SW) until use. For FW samples, mefgu were transferred to 150-liter FW tanks and held for 8–9 days before sample collection. For SW samples, mefgu in FW tanks were transferred to 150-liter SW tanks and acclimated for 8–9 days. The water temperature was maintained at 18–22°C. All fish were anaesthetized by immersion in 0.1%ethyl m-amino benzoate (MS-222, tricaine) before being killed by decapitation. The tissues required for RNA extraction were dissected, snap-frozen in liquid nitrogen, and stored at −80°C until use. Artificial SW (Rohto-Marine) was obtained from Rei-Sea (Tokyo, Japan). The animal protocols and procedures were approved by the Institutional Animal Care and Use Committee of Tokyo Institute of Technology (mefgu) or Mayo Clinic (Xenopus) and conform to the American Physiological Society’s guiding principles in the care and use of laboratory animals (1a).

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

Molecular cloning. Complementary DNA was reverse-transcribed using random hexamers and the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Fragments of mefgu Slc26a6A and Slc26a6B were isolated by RT-PCR from fugu intestine RNA with primers (Table 1 and S1) that were designed based on the fugu genomic database (http://genome.jgi-psf.org/fugu6/fugu6.home.html). The PCR products were subcloned into pBluescript II SK(+) and sequenced. These clones were used as probes for Northern blot analysis.

Table 1. List of primers used for PCR amplification

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Northern blot analysis. Total RNA (20 μg/lane) from a pool of various tissues of FW and SW mefgu was electrophoresed on formaldehyde-agarose (1%) denaturing gels in 10× MOPS running buffer (20 mM MOPS, pH 7.0, 8 mM sodium acetate, 1 mM EDTA) and then transferred onto Hybond-N + nylon membranes (GE Healthcare, 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 32P-dCTP (3,000 Ci/mmol) with the use of a Ready-To-Go DNA labeling kit (GE Healthcare) and the unincorporated nucleotides were removed by passing the reaction mixture through a Sephadex G-50 column (GE Healthcare). The membranes were then hybridized separately with each 32P-labeled probe in the same buffer at 68°C for 16 h. The blots were subsequently washed under increasingly stringent conditions (final wash: 1× SSC and 0.1% SDS for 30 min at 60°C). Membranes were exposed to an imaging plate (Fujifilm, Tokyo, Japan) in a cassette overnight. The results were analyzed using a Fuji BAS2000 Bio-Image Analyzer (Fujiﬁlm). A probe for mefgu β-actin was used as a control to verify loading and RNA integrity.

Real-time PCR. Expression of mfSlc26a6A, mfSlc26a6B, mfSlc26a6C, mfSlc26a1, and mfSlc13a1 was quantified by real-time PCR. Total RNAs were extracted from the kidney of mefgu acclimated to SW and FW (n = 5 for each group) and reverse-transcribed into cDNA using oligo(dT) primer and the SuperScript III First-Strand Synthesis System (Invitrogen). Multiplex real-time PCR was performed for quantification of mfSlc26a6A, mfSlc26a6B, mfSlc26a6C, mfSlc26a1, and mfSlc13a1 mRNA expression, with amplification of GAPDH as an endogenous control. Reactions were performed with the SYBR Green method using SYBR Premix Ex Taq II Kit (Takara Bio, Otsu, Japan) on a Thermal Cycler Dice Real-Time System (Takara Bio) and calculated using the Relative Standard Curve method. mRNA concentrations of mfSlc26a6A, mfSlc26a6B, and mfSlc26a1 were normalized to GAPDH levels. Experiments were performed in duplicate. Data were expressed as the means ± SE. Significant differences at P < 0.01 were determined by two-sample Student’s t-test, assuming unequal variance.

Immunohistochemistry. Kidneys from SW mefgu were fixed in 0.1 M phosphate buffer, pH 7.4, containing 4% (wt/vol) paraformaldehyde 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 a −20°C cryostat, mounted on (3-aminopropyl)trimethoxysilane-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 antisera and preimmune sera at a 1:1,000 (immunofluorescence method) dilution. After being incubated with antisera and preimmune sera, sections were washed with PBS and then incubated for 1 h at room temperature with a cocktail of Alexa Fluor 488-conjugated anti-rabbit IgG (1:2,000; Invitrogen), Alexa Fluor 546-conjugated anti-mouse IgG (1:2,000; Invitrogen), and Hoechst 33342 (100 ng/ml; Molecular Probes). Fluorescence was detected using a charge-coupled device camera (Carl Zeiss, Oberkochen, Germany).

Expression of mfSlc26a6A, mfSlc26a6B, and mfSlc26a6 in Xenopus oocytes and electrophysiology. The entire coding regions of mfSlc26a6A, mfSlc26a6B, and mfSlc26a6 cDNAs were inserted to the pGEMHE Xenopus laevis expression vector as described previously (27, 56). The plasmids were linearized with NotI or NheI, and cRNAs were transcribed in vitro using the T7 mMESSAGE mMACHINE kit (Ambion, Austin, TX). X. laevis oocytes were dissociated with collagenase as previously described (45) and injected with 25 nl of water or a solution containing cRNA at 0.4–1 μg/μl (10–25 ng/oocyte), using a Nanoject-II injector (Drummond Scientific, Broomall, PA). Oocytes were incubated at 16°C in OR3 media (45), and studied 3–6 days after injection.
Ion-selective microelectrode analysis. To measure the intracellular chloride concentration ([Cl\^-\]), of Xenopus oocytes, a Cl\^- ion-selective microelectrode was prepared with Cl\^- -ionophore I, cocktail A (cat. no. 24902; Fluca Chemicals, Buchs, Switzerland) and used as previously described (6, 46). [Cl\^-\], was measured as the difference between the Cl\^- electrode and a KCl voltage electrode impaled into the oocyte, and membrane potential (V_m) was measured as the difference between the KCl microelectrode and an extracellular calomel. [Cl\^-\], electrodes were calibrated using 10 and 100 mM NaCl, followed by a test of the specificity by using 100 mM NaHCO_3 and a point calibration in ND96 (pH 7.5). ND96 contained 96 mM NaCl, 2 mM KCl, 1 mM MgCl_2, 1.8 mM CaCl_2, and 5 mM HEPES (pH 7.5). In solutions with 0 mM Cl\^- (OCI-ND96), Cl\^- was replaced with gluconate. For solutions containing SO_4^{2-}, 10 mM NaCl or sodium gluconate was replaced with 5 mM disodium sulfate (Na_2SO_4) and 2.5 mM choline chloride or choline gluconate. For solutions containing oxalate, 0.75 ml of 133 mM disodium oxalate (Na_2C_2O_4) were mixed with 99.25 ml ND96 or 0Cl-ND96 just before use, because ND96-containing oxalate formed precipitates of calcium oxalate after storage of several days. For solutions containing formate, 5 mM NaCl or sodium gluconate was replaced with 5 mM sodium formate (NaHCOO). For CO_3^-/HCO_3^- -equilibrated solutions, 33 mM NaHCO_3 was replaced with 33 mM NaCl or sodium gluconate and the solutions were bubbled with 5% CO_2/95% O_2 during the experiments. All solutions were titrated to pH 7.5 at room temperature using NaOH and had an osmolality of 195–200 mosmol/kg H_2O.

Two-electrode voltage clamp analyses. Current-voltage (I-V) relationships of cRNA or water-injected oocytes in the presence of test anions were analyzed as previously described (50). In brief, an oocyte was perfused with ND96, clamped at a holding potential (V_h) of -60 mV and then perfused with ND96 containing 70 mM Cl\^- (70Cl-ND96). After that, the oocyte was perfused with 70Cl-ND96 containing 0.2 mM SO_4^{2-}, 70Cl-ND96 containing 1 mM SO_4^{2-}, 70Cl-ND96 containing 5 mM SO_4^{2-}, and 70Cl-ND96 containing 15 mM SO_4^{2-}. At each change of solution, oocyte was perfused in solution until the current (I) was stabilized, and the I-V relation was then recorded. In this way, sulfate-elicted currents were measured by addition of 0.2, 1, 5, and 15 mM SO_4^{2-} in 70Cl-ND96 or addition of 0.2, 1, 5, 15, and 48 mM SO_4^{2-} in ND96 containing 20 mM Cl\^- (20Cl-ND96) and were calculated as I_{SO_4}\ - I_{so_4} (Oxalate) currents were measured by addition of 0.2 and 1 mM oxalate in 70Cl-ND96 or 20Cl-ND96 and calculated as I_{oxalate} - I_{oxalate}. Formate currents were measured by addition of 1 and 5 mM formate in 70Cl-ND96 or 20Cl-ND96 and calculated as I_{formate} - I_{formate}. HCO_3^- currents were measured by addition of 33 mM HCO_3^-/5% CO_2 in ND96 containing 67.8 mM Cl\^- or 20Cl-ND96 and calculated as I_{bicarbonate} - I_{bicarbonate}.

To prepare 70Cl-ND96 containing 15 mM SO_4^{2-}, 33.6 mM NaCl was replaced with 15 mM Na_2SO_4, 3.6 mM sodium gluconate, and 7.5 mM choline gluconate or N-methyl-D-glucamine gluconate. To prepare 20Cl-ND96 containing 48 mM SO_4^{2-}, 96 mM NaCl was replaced with 48 mM Na_2SO_4, 12.4 mM choline chloride, and 11.6 mM choline gluconate or N-methyl-D-glucamine gluconate. OCI-ND96 was prepared by replacing Cl\^- with gluconate, and 70Cl-ND96 and 20Cl-ND96 were prepared by mixing OCI-ND96 with ND96 containing 103.6 mM Cl\^- . The test solutions with differing SO_4^{2-} concentrations were prepared by mixing 70Cl-ND96 with 70Cl-ND96 containing 15 mM SO_4^{2-} or 20Cl-ND96 with 20Cl-ND96 containing 48 mM SO_4^{2-}. Other solutions containing oxalate, formate, and HCO_3^- were prepared as described above. All solutions were titrated to pH 7.5 at room temperature using NaOH and had an osmolality of 195–200 mosmol/kg H_2O.

The oocyte currents were recorded with an OC-720C voltage clamp (Warner Instruments, Hamden, CT), filtered at 2–5 kHz, digitized at 10 kHz, and recorded using Pulse software (HEKA, Lambrecht, Germany) as previously described (10). Experiments involving low or no Cl\^- used 3 M KCl-agar bridges. The data were analyzed using the PulseFit program (HEKA). For periods when I-V protocols were not being run, oocytes were clamped at a V_h of -60 mV, and current was constantly monitored and recorded at 1 Hz. I-V protocols consisted of 200-ms steps from V_h in 20 mV steps between -140 and +60 mV. Data were expressed as means ± SE. Significant differences at P < 0.01 or P < 0.05 were determined by two-sample Student’s t-test assuming unequal variance.

RESULTS

Identification of candidate sulfate transporters by database mining. The database of The Human Genome Organisation Nomenclature Committee provides a list of 46 solute transporter families and 360 transporter genes (http://www.genenames.org/ and http://www.bioparadigms.org/slc/menu.asp)(14). Members of the solute carrier families SLC13 (31) and SLC26 (34) have sulfate transport activities. We therefore searched the T. rubripes genome sequence for homologs of the human SLC13 and SLC26 family members and identified 14 candidate sequences in the fugu genome (Table 2). Since the members of the Slc26 family can also act as HCO_3^- transporters, they had already been characterized in our previous study that identified intestinal HCO_3^- transporters upregulated in SW mefugu (27). Thus, the present study extends this analysis to the kidney and SO_4^{2-} transport kinetics of these Slc26 family members.

Selection of candidate clones by Northern blot analysis. Partial cDNA clones for the 14 candidate transporters predicted by database mining (5 Slc13 family members and 9 Slc26 family members, Table 2 and Fig. 1A) were obtained by RT-PCR by using RNA preparations from kidney of SW mefugu, sequenced, and used as probes for Northern blot analysis. Among the candidates, eight clones were expressed in the mefugu kidney: mfSlc13a1 (scaffold 3041), mfSlc13a5 (scaffold 1026), mfSlc26a1 (scaffold 281), mfSlc26a5 (scaffold 3467), mfSlc26a6A (scaffold 1951), mfSlc26a6B (scaffold 216), mfSlc26a6C (scaffold 1034), and mfSlc26a11 (scaffold 591). Notably, only the mRNA level of mfSlc26a6A was strongly induced by SW (Fig. 1, B

![Table 2. Tiger puffer (Takifugu rubripes) cDNA clones that are homologous to SLC13 and SLC26 family members](http://ajpregu.physiology.org/)

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The result of a BLAST search at the web site http://genome.jgi-psf.org/fugu/fugu1.home.html showed that there are 5 Slc13 clones and 9 Slc26 clones.
and C), making it the most likely candidate for renal SO$_4^{2-}$ excretion. This result was confirmed by quantitative real-time PCR, which demonstrated that the expression of mfSlc26a6A in the kidney of SW mefugu is 30 ± 2.9 times higher than those of FW mefugu (Fig. 1C, $P < 0.01$, $n = 5$).

mfSlc26a6A and mfSlc26a6B are electrogenic Cl$^-$/SO$_4^{2-}$ exchangers with distinct activities. Oocytes were injected with mfSlc26a6A, mfSlc26a6B, and mouse Slc26a6 (mSlc26a6) cRNAs, and their cytosolic chloride concentration ([Cl$^-$]) was monitored in response to exposure to Cl$^-$-free medium (Fig. 2A). Mouse Slc26a6 (mSlc26a6) was used as a positive control. Exposure to 5 mM SO$_4^{2-}$ elicited a hyperpolarization ($-23.2 ± 2.6$ mV, $n = 3$, for mfSlc26a6A; $-4.5 ± 1.1$ mV, $n = 3$, for mfSlc26a6B; and $-5.3 ± 1.4$ mV, $n = 3$, for mSlc26a6) but not in control (water-injected) oocytes. In oocytes expressing mfSlc26a6A, mfSlc26a6B, and mSlc26a6, Cl$^-$ removal caused marked reduction of [Cl$^-$]$_i$ ($-29.6 ± 5.1$ μM/s, $n = 3$, for mfSlc26a6A; $-10.6 ± 3.3$ μM/s, $n = 3$, for mfSlc26a6B; and $-10.3 ± 4.1$ μM/s, $n = 3$, for mSlc26a6) and a marked hyperpolarization ($-91.9 ± 10.6$ mV, $n = 3$, for mfSlc26a6A; $-68.5 ± 12.3$ mV, $n = 3$, for mfSlc26a6B; and $-81.2 ± 9.0$ mV, $n = 3$, for mSlc26a6). Readdition of Cl$^-$ elicited depolarization and recovery of [Cl$^-$], $P < 0.01$, $n = 3$, for mSlc26a6). These results indicate that both Slc26a6A and Slc26a6B mediate electrogenic Cl$^-$/SO$_4^{2-}$ exchange. The electrophysiological parameters determined here for the positive control, mSlc26a6, are within ranges equivalent to those reported previously (56).

To determine whether the activities of mfSlc26a6A, mfSlc26a6B and mSlc26a6 differed, we analyzed I-V relationships using two-electrode voltage-clamp. SO$_4^{2-}$ concentration (0.2, 1, 5, 15, and 48 mM) elicited outwardly rectifying currents in oocytes expressing mfSlc26a6A, mfSlc26a6B, and mSlc26a6 (Fig. 2B and Fig. 3), and these data also support the electrogenic nature of Cl$^-$/SO$_4^{2-}$ exchange. Low Cl$^-$ concentration (20 mM) reduced the rectification of Slc26a6A and yielded nearly linear I-V curves. Remarkably, the SO$_4^{2-}$ current of mfSlc26a6A oocytes was much greater than those of mfSlc26a6B and mSlc26a6 oocytes (Fig. 2B and Fig. 4). For example, the currents of
mfSlc26a6A oocytes were approximately +35 μA at +60 mV (50 to 200 times larger than those of mfSlc26a6B and mSlc26a6 oocytes; P < 0.01, n = 3–6 for each condition). SO$_4^{2-}$ steady-state kinetics were calculated from the Michaelis-Menten equation (Fig. 5). The $I_{\text{max}}$ value of mfSlc26a6A for SO$_4^{2-}$ (63.8 ± 4.0 μA) was 86 to 100 times higher than those of mfSlc26a6B and mSlc26a6 (0.63 ± 0.18 μA for mfSlc26a6B and 0.74 ± 0.19 μA for mSlc26a6) when [Cl$^-$_out] is 70 mM (P < 0.01, n = 4 for each condition) and is 10 to 15 times greater than those of the others when [Cl$^-$_out] is 20 mM (47.6 ± 5.0 μA for mfSlc26a6A; 4.9 ± 0.37 μA for mSlc26a6; and 3.2 ± 0.71 μA for mSlc26a6; P < 0.01, n = 3–6 for each condition). mfSlc26a6A also showed approximately twofold higher SO$_4^{2-}$ affinity than mfSlc26a6B and mSlc26a6 when [Cl$^-$_out] was 70 mM ($K_m$ = 5.3 ± 0.9 mM for mfSlc26a6A; 11.7 ± 6.6 mM for mSlc26a6; and 12.7 ± 5.9 mM for mSlc26a6). The SO$_4^{2-}$ affinity was ~15-fold greater affinity than mSlc26a6B and mSlc26a6 when [Cl$^-$_out] was 20 mM ($K_m$ = 1.7 ± 0.8 mM for mfSlc26a6A; 29.5 ± 9.2 mM for mSlc26a6; and 19.4 ± 10.2 mM for mSlc26a6). However, reduction of [Cl$^-$_out] from 70 mM to 20 mM did not change $I_{\text{max}}$ (mfSlc26a6A), did reduce $K_m$ (mfSlc26a6A) threefold, but increased both $I_{\text{max}}$ and $K_m$ of mfSlc26a6B and mSlc26a6.

Other transport activities of mfSlc26a6A and mfSlc26a6B. Mammalian Slc26a6 transports several anions such as HCO$_3^-$, SO$_4^{2-}$, formate, and oxalate (18, 23, 56). We have shown that mfSlc26a6A and mfSlc26a6B are electrogenic Cl$^-$/HCO$_3^-$ exchangers (27) and electrogenic Cl$^-$/SO$_4^{2-}$ exchangers (above). Therefore, we next tested whether mfSlc26a6A and mfSlc26a6B transported oxalate and formate and compared their activities to those of mSlc26a6.

Exposure of the oocytes to 1 mM oxalate in the presence of normal-bath Cl$^-$ (104 mM) did not affect the [Cl$^-$_in]; it did elicit a small hyperpolarization (~6.1 ± 1.2 mV, n = 3, for mfSlc26a6A; ~4.5 ± 1.4 mV, n = 3, for mfSlc26a6B; and ~1.4 ± 0.2 mV, n = 3, for mSlc26a6) but not in control oocytes (Fig. 6A). In oocytes expressing mfSlc26a6A, mfSlc26a6B, and mSlc26a6, Cl$^-$ removal in the presence of 1 mM oxalate caused marked reduction of [Cl$^-$_in] (~18.2 ± 6.0 μM/s, n = 3, for mSlc26a6A; ~9.9 ± 2.8 μM/s, n = 3, for

**Fig. 2. SO$_4^{2-}$ transport mediated by mfSlc26a6A, mfSlc26a6B, and mouse Slc26a6 (mSlc26a6).** A: representative traces of intracellular Cl$^-$ concentration ([Cl$^-$_i]) and membrane potential ($V_m$) of oocytes injected with mfSlc26a6A (blue), mfSlc26a6B (red), mSlc26a6 (black), or water (gray) are shown. In the continuous presence of 5 mM SO$_4^{2-}$, the Cl$^-$/SO$_4^{2-}$ exchange activities were monitored as changes in [Cl$^-$_i], and $V_m$ when extracellular Cl$^-$ was removed (grey shading) and readded. Results for solution changes from 70 mM Cl$^-$/ND96 (70Cl$^-$/ND96) with 70Cl$^-$/ND96 containing 5 mM SO$_4^{2-}$ are indicated by white boxes; results for solution changes to Cl$^-$-free solution (0 Cl$^-$) are indicated by gray boxes. Numbers below [Cl$^-$_i], traces are the initial rates of changes in [Cl$^-$_i] (μM/s). B: current-voltage (I-V) relationships of oocytes expressing mfSlc26a6A (blue), mfSlc26a6B (red), and mSlc26a6 (black), and control oocytes (gray) in the presence of 5 mM SO$_4^{2-}$ and 70 mM Cl$^-$ (solid line) or 5 mM SO$_4^{2-}$ and 20 mM Cl$^-$ (dotted line) (holding potential, −60 mV). Right: modified version of the left axis in which the vertical axis has been expanded to show weak activities of mfSlc26a6B and mSlc26a6. Values are means ± SE, n = 3–6. Sulfate-elicited currents were calculated as $I_{\text{Sulf}}$ = $I_{\text{no Sulf}}$. 

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mfSlc26a6B; and $-27.5 \pm 7.6 \mu M/s, n = 3$, for mSlc26a6) and a marked hyperpolarization ($-61.5 \pm 8.4 mV, n = 3$, for mfSlc26a6A; $-65.2 \pm 3.4 mV, n = 3$, for mfSlc26a6B; and $-59.9 \pm 6.1 mV, n = 3$, for mSlc26a6). Cl$^{-}$ readdition elicited a depolarization and recovery of [Cl$^{-}$]. Control oocytes did not show any of these responses with Cl$^{-}$ removal and readdition.

These results indicate that the mefugu and mouse Slc26a6 paralogs mediate electrogenic Cl$^{-}$/oxalate$^{2-}$ exchange.

Likewise, exposure to 5 mM formate (normal-bath Cl$^{-}$) did not affect either [Cl$^{-}$], or $V_m$ of oocytes expressing mfSlc26a6A, mfSlc26a6B, and mSlc26a6 (Fig. 6B). Cl$^{-}$ removal (with bath formate) caused marked reduction of [Cl$^{-}$], ($-121 \pm 14 \mu M/s$,

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**Fig. 3.** SO$_4^{2-}$ dose response: I-V relationships. I-V curves from oocytes expressing mfSlc26a6A (A), mfSlc26a6B (B), and mouse Slc26a6 (C) and water-injected oocytes (D) in the presence of various SO$_4^{2-}$ concentrations (holding potential, $-60 mV$) are shown. Results in the presence of 70 and 20 mM Cl$^{-}$ are shown top and bottom, respectively. Values are means ± SE, $n = 3–6$. Sulfate-elicited currents were calculated as $I_{(sulfate)} - I_{(no\ sulfate)}$.

**Fig. 4.** Comparison of currents elicited by HCO$_3^-$, oxalate, and SO$_4^{2-}$ in oocytes expressing mfSlc26a6A, mfSlc26a6B, and mSlc26a6. The relative currents at $+60 mV$ compared with the mean ± SE of the currents of mfSlc26a6A oocytes at the same conditions were calculated and are shown, $n = 3–6$. The solution conditions are indicated at the bottom. *$P < 0.01$; **$P < 0.05$. **
results indicate that mSlc26a6A, mSlc26a6B, and mSlc26a6 mediate electroneutral \( \text{Cl}^- \)/formate exchange.

To compare the transport activities of mSlc26a6A, mSlc26a6B, and mSlc26a6 in more detail, we analyzed I-V relationships in the presence of formate or oxalate. In the presence of 20 mM \( \text{Cl}^- \) and were calculated as \( I_{\text{SO}_4^{2-}} - I_{\text{no SO}_4^{2-}} \). Maximum current \( (I_{\text{max}}) \) and Michaelis-Menten constant \( (K_m) \) values as a function of \( V_m \) are shown. Values are means ± SE, \( n = 3–6 \). \( I_{\text{max}} \) and \( K_m \) values as a function of \( V_m \) are shown in B and C, respectively.
Fig. 6. Formate and oxalate transport mediated by mfSlc26a6A, mfSlc26a6B, and mSlc26a6. A and B: representative traces of intracellular Cl⁻ concentration ([Cl⁻]i) and Vm of oocytes injected with mfSlc26a6A (blue), mfSlc26a6B (red), mSlc26a6 (black), and water (gray) are shown. In the continuous presence of 1 mM oxalate (A) and 5 mM formate (B), the Cl⁻/oxalate and Cl⁻/formate exchange activities were monitored as changes in [Cl⁻]i, and Vm when extracellular Cl⁻ was removed (grey shading) and read. Results for solution changes from 70 mM-Cl⁻-ND96 (70Cl-ND96) to 70Cl⁻-ND96 containing 1 mM oxalate or 5 mM formate are indicated by white boxes, and results for solution changes to Cl⁻-free solution are indicated by gray boxes.

C–F: I-V relationships of oocytes in the presence of 1 mM oxalate (C), 0.2 mM oxalate (D), 5 mM formate (E), or 33 mM HCO₃⁻/5% CO₂ (F) (holding potential, −60 mV). Values are means ± SE, n = 3–4. Currents elicited with oxalate, formate, or HCO₃⁻ were measured in the presence of 70 and 20 mM Cl⁻ (solid and dotted lines, respectively) and calculated as I(oxalate) = I(no oxalate), I(formate) = I(no formate), and I(bicarbonate) = I(no bicarbonate).
presence of 1 mM (data not shown) or 5 mM (Fig. 6E) formate, oocytes expressing mfSlc26a6A, mfSlc26a6B, and mSlc26a6 did not display currents different than water-injected oocytes. Moreover, these same Slc26a6 oocytes did have obvious I-V responses when tested for SO$_4^{2-}$ transport. These data further support the electroneutral nature of Cl$^-$/HCO$_3^-$/formate exchange by these Slc26a6-transporters. In contrast, oxalate (0.2 or 1 mM) elicited outwardly rectifying currents in oocytes expressing mfSlc26a6A, mfSlc26a6B, and mSlc26a6 (Fig. 6, C and D). Likewise, these data indicate the electrogenic nature of Cl$^-$/HCO$_3^-$/oxalate exchange of these Slc26a6-transporters. In all cases, the currents were enhanced at lower Cl$^-$ concentration (20 mM) and higher oxalate concentration (1 mM). The I-V curves of oocyte expressing mfSlc26a6A, mfSlc26a6B, and mSlc26a6 were almost the same, except for one exception. Only for the case of 20 mM Cl$^-$ and 1 mM oxalate at +60 mV did oocytes expressing mfSlc26a6A show 1.4 to 2.0 times larger current than oocytes expressing mfSlc26a6B and mSlc26a6 (Fig. 4, P < 0.05, n = 3). I-V relationships in the presence of 5% CO$_2$/33 mM HCO$_3^-$ were also analyzed; these are shown in Fig. 6F.

Functional analyses of mfSlc26a6C. Oocytes were injected with mfSlc26a6C cRNA, and their cytosolic chloride concentrations and pH were monitored in response to exposure to Cl$^-$-free medium in the presence of HCO$_3^-$, SO$_4^{2-}$/HCO$_3^-$, formate, and oxalate. However, oocytes expressing mfSlc26a6C showed no changes in [Cl$^-$], pH, or V_m (data not shown). These results suggest that mfSlc26a6C is not an anion exchanger, and further analyses are necessary to determine the function of this protein.

**DISCUSSION**

Marine teleosts maintain body fluid homeostasis by drinking SW and eliminating excess ions. Sulfate (SO$_4^{2-}$) is the second most abundant anion in SW, present at an average of ~27 mM. It is estimated that the rate of SO$_4^{2-}$ ingestion through SW drinking in marine fish is 30–150 μmol·kg$^{-1}$·h$^{-1}$ (33). This ingestion rate is much greater than the catabolic generation of SO$_4^{2-}$ from the sulfur-containing amino acids (estimated to be 4 to 13 μmol·kg$^{-1}$·h$^{-1}$ under fasting conditions) (22, 55). Marine teleosts must therefore continually eliminate this ex-

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**Immunohistochemical localization of mfSlc26a6A in the SW mefugu kidney.** Sections of the kidney of SW mefugu were analyzed by immunofluorescence. Three types of tubules (proximal tubule, distal tubule, and collecting duct) are present in mefugu kidney (20), and each type of tubule is distinguished by anti-Na$^+$-K$^+$-ATPase staining (20, 28). Costaining of mfSlc26a6A and Na$^+$-K$^+$-ATPase showed that mfSlc26a6A exists in the apical membrane of proximal tubule epithelial cells (Fig. 7, C and F), through which SO$_4^{2-}$ is thought to be secreted (40, 44). Additionally, mfSlc26a6A also localizes to the brush-border region of the proximal tubules, which are stained with phalloidin (Fig. 7, G and H). Phalloidin binds to actin filaments, and strongly stains a well-developed apical brush border of proximal tubules (20). No such signals were observed when the tissues were stained with preimmune serum as a negative control. The specificity of these antibodies was established by specific staining of mammalian culture cells (COS7) exogenously expressing the antigens (27).

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**Fig. 7.** Immunohistochemistry of mfSlc26a6A in the kidney of SW-acclimated mefugu. A–F: sections of mefugu kidney were stained with anti-mfSlc26a6A (green), anti-rat Na$^+$-K$^+$-ATPase (red), and Hoechst 33258 (nucleus, blue). Scale bars: 50 μm. P, proximal tubule; D, distal tubule. G and H: sections of kidney were stained with anti-mfSlc26a6A (green), phalloidin (red), and Hoechst (blue). Bars: 50 μm.
cess SO$_4^{2-}$ through the kidney to keep the plasma [SO$_4^{2-}$] at a normal physiological concentration (~2 mM). Similar handling of sulfate may occur in other marine vertebrates that drink SW, including certain species of marine mammals (36). It is known that the bladder urine [SO$_4^{2-}$] is 40–80 mM in marine teleosts, which is created by SO$_4^{2-}$ secretion from epithelial cells of the renal proximal tubules (33). However, the transporter(s) involved in the epithelial secretion of SO$_4^{2-}$ have not been determined. In this study, by using genome resources of Takifugu and its related euryhaline species mefugu, we identified candidate transporters for SO$_4^{2-}$ in the kidney of marine teleosts.

Database mining demonstrated that fish have three paralogs for SLC26a6: mSlc26a6A, mSlc26a6B, and mSlc26a6C. The commonly studied mammals have a single gene for Slc6a6 that generates two splice variants named Slc26a6A and Slc26a6B, and they are expressed in various tissues including the intestine and kidney. In vitro, Slc26a6 mediates exchange of various anions such as sulfate (SO$_4^{2-}$), chloride (Cl$^-$), iodide (I$^-$), formate (HCOO$^-$), oxalate ([COO$^-$_2]), hydroxyl ion (OH$^-$), and bicarbonate (HCO$_3^-$). Comparative analyses between wild-type and Slc26a6$^{-/-}$ mice have demonstrated that Slc26a6 mediates oxalate-stimulated NaCl absorption and contributes to apical membrane Cl$^-$/base exchange in the kidney proximal tubule (53), to HCO$_3^-$ secretion in the duodenum (53) and pancreatic duct (52), and to intestinal secretion of oxalate that reduces the plasma [oxalate] and frequency of urinary stones (17). In SW-acclimated mefugu, we found that the expression of mSlc26a6A is dramatically increased in the kidney (Fig. 1), as well as in the intestine (27). HCO$_3^-$ secretion in the intestine is stimulated in marine teleosts; this facilitates the precipitation of Ca$^{2+}$ and Mg$^{2+}$ for rectal excretion (12, 13, 54). We have proposed that mSlc26a6A and mSlc26a6B are the strongest candidates for the transporters responsible for HCO$_3^-$ secretion by the intestine (27). In this study, we also propose that mSlc26a6A is the strongest candidate for SO$_4^{2-}$ secretion by the marine teleost kidney. Both results suggest that mSlc26a6A is one of the key molecules involved in SW acclimation of fish.

The mSlc26a6A clone displayed extremely large SO$_4^{2-}$ currents when expressed in Xenopus oocytes, i.e., the SO$_4^{2-}$-elicited current is 50–100 times larger than those measured from mSlc26a6B or mSlc26a6. Immunohistochemical analyses demonstrated that the mSlc26a6A protein localizes to the apical membrane of proximal tubules, where marine teleosts secrete SO$_4^{2-}$. Furthermore, among all SO$_4^{2-}$ transporters belonging to the Slc13 and Slc26 families in the Takifugu genome, mSlc26a6A exhibited the largest induction in the SW mefugu kidney. The mRNA expression level in the kidney of SW mefugu is 2.3- and 140-fold greater than the other apical SO$_4^{2-}$ transporters (mSlc26a6B and mSlc13a1), respectively. These findings strongly suggest that mSlc26a6A mainly conducts SO$_4^{2-}$ at the apical proximal tubule membrane of marine teleosts and is the most likely candidate for the long-sought renal SO$_4^{2-}$ secretor of the marine teleost kidney (Fig. 8A).
For the secretion of \( \text{SO}_4^{2-} \) into the primary urine, which contains high concentrations of \( \text{SO}_4^{2-} \) (up to 80 mM), a powerful driving force is necessary. Since the exchange of \( \text{Cl}^-/\text{SO}_4^{2-} \) causes a decrease in the intracellular negative charge, the intracellular negative charge maintained by the sodium pump (\( \text{Na}^+-\text{K}^+\)-ATPase) will drive the electrogenic \( \text{Cl}^-/\text{SO}_4^{2-} \) exchange. Namely, as shown in Fig. 8A, mSlc26a6aA is an electrogenic \( \text{Cl}^-/\text{SO}_4^{2-} \) exchanger, indirectly driven by \( \text{Na}^+-\text{K}^+\)-ATPase. Because of the difficulty of measuring \( [\text{SO}_4^{2-}]_{\text{in}} \) in oocytes, we were unable to determine the stoichiometry of \( \text{Cl}^-/\text{SO}_4^{2-} \) exchange in the present study; if we assume a 1:1 stoichiometry, we can theoretically calculate the mSlc26a6aA currents based on the following equation (Table 3).

\[
\Delta \mu_{\text{mSlc26a6a}} = \Delta \mu_{\text{Cl}^-/\text{SO}_4^{2-}} = R T \ln \left( \left[ \text{Cl}^- \right]_{\text{in}} / \left[ \text{Cl}^- \right]_{\text{out}} \right) + (1) \cdot F V_m
\]

\[
- \left( R T \ln \left( [\text{SO}_4^{2-}]_{\text{in}} / [\text{SO}_4^{2-}]_{\text{out}} \right) + (-2) \cdot F V_m \right)
\]

where \( R \) is the gas constant, \( T \) is the absolute temperature, \( F \) is the Faraday constant, \( \ln \) is the natural logarithm, \( V_m \) is the membrane potential, and \( \Delta \mu_{\text{ion}} \) is the electrochemical potential difference (Joules/mole). When we use this equation for the model of the proximal tubule of SW fish, we can estimate the ratio of \( [\text{SO}_4^{2-}] \) in the primary urine and the cytoplasm of the proximal tubule as \( [\text{SO}_4^{2-}]_{\text{out}} / [\text{SO}_4^{2-}]_{\text{in}} \) values. At the condition of equilibria, the calculated \( [\text{SO}_4^{2-}]_{\text{out}} / [\text{SO}_4^{2-}]_{\text{in}} \) values are 167, 112, and 75 when \( V_m \) is -80, -70, and -60 mV, respectively, under the following assumptions: 1) \( [\text{Cl}^-]_{\text{out}} \) is 140 mM, which is similar to plasma \( [\text{Cl}^-] \) of SW-acclimated mefugu (20); 2) \( [\text{Cl}^-]_{\text{in}} \) is 20 mM, which is similar to cytosolic \( [\text{Cl}^-] \) of mammalian proximal tubular cells (5, 16, 24); and 3) the temperature is 293 K (20°C). These thermodynamic calculations suggest that mSlc26a6aA can concentrate \( \text{SO}_4^{2-} \) in urine up to 40 mM when the \( [\text{SO}_4^{2-}]_{\text{in}} \) is 0.24 to 0.53 mM. These results of thermodynamic calculations fit well with the hypothetical model for the secretion of \( \text{SO}_4^{2-} \) by the proximal tubule (Fig. 8A). In this calculation, the value of \( [\text{SO}_4^{2-}]_{\text{out}} / [\text{SO}_4^{2-}]_{\text{in}} \) increases with higher values of \( [\text{Cl}^-]_{\text{out}} / [\text{Cl}^-]_{\text{in}} \) and lower values of \( V_m \). Therefore 1) high \( V_m \) (negative inside) produced by \( \text{Na}^+-\text{K}^+\)-ATPase; 2) low cytoplasmic \( [\text{Cl}^-] \), which is possibly secreted by chloride channel(s); and 3) high cytoplasmic \( [\text{SO}_4^{2-}] \) of ~1 mM, which is possibly supplied by basolateral Slc26a1 are sufficient for the apical secretion of \( \text{SO}_4^{2-} \) by mSlc26a6aA. To definitively establish its physiological role in the renal sulfate secretion of marine teleosts, it will be necessary to demonstrate that the mSlc26a6aA can fulfill the necessary function in vivo. It is worth mentioning, however, that our electrophysiological data obtained using the *Xenopus* oocyte expression system clearly demonstrate that mSlc26a6aA has the ability to excrete sulfate under conditions relevant to fluids in the renal tubules of teleost fish (sulfate being the dominant anion with \( \text{Cl}^- \) present).

In FW fish, \( \text{SO}_4^{2-} \) is absorbed by the renal proximal tubule for \( \text{SO}_4^{2-} \) homeostasis. Not all \( \text{SO}_4^{2-} \) is secreted, as low levels of \( \text{SO}_4^{2-} \) are necessary for biosyntheses of sulfated extracellular matrix proteins such as chondroitin sulfate and keratan sulfate. Recently, Nakada et al. (35) have demonstrated that the apical \( \text{Na}^+/\text{SO}_4^{2-} \) cotransporter Slc13a1 (NaS1-1) and basolateral \( \text{Cl}^-/\text{SO}_4^{2-} \) exchanger Slc26a1 (Sat-1) are upregulated in the eel kidney during FW acclimation, and they proposed a model of \( \text{SO}_4^{2-} \) absorption by the FW teleosts kidney (35) (Fig. 8B). However, this model has not been confirmed in mefugu because the expression of mSlc13a1 was relatively low and was not induced in the kidney of FW-acclimated mefugu (Fig. 1, B and C). In SW fish, we have proposed that mSlc26a6aA is the apical \( \text{Cl}^-/\text{SO}_4^{2-} \) exchanger for \( \text{SO}_4^{2-} \) secretion by the kidney. Future studies should seek to identify the molecular entity that encodes the basolateral transporter mediating \( \text{SO}_4^{2-} \) entry to proximal tubule cells from blood vessels. The strongest candidate for this is Slc26a1; we base this claim on the following observations. The expression of renal Slc26a1 is 1) upregulated in FW eel but is detectable in SW eel at significant levels (35); 2) upregulated in rainbow trout when \( \text{Na}_2\text{SO}_4 \) is injected (21); and 3) abundantly expressed in both FW and SW mefugu (Fig. 1, B and C). These findings indicate that Slc26a1 may have a role in basolateral, renal \( \text{SO}_4^{2-} \) transport in both FW and SW fish. Thus, it is likely that lower intracellular \( \text{SO}_4^{2-} \) concentrations are achieved by Slc26a6aA function in SW (Fig. 8A), and higher intracellular \( \text{SO}_4^{2-} \) concentrations are achieved by Slc13a1 function in FW (Fig. 8B). The regulated action of these transporters causes a \( \text{SO}_4^{2-} \) concentration gradient across basolateral membranes, which in turn allows \( \text{SO}_4^{2-} \) to move in opposite directions in SW and FW conditions, yet still using the same basolateral Slc26a1 protein.

The striking \( \text{SO}_4^{2-} \) activity differences between mSlc26a6aA and mSlc26a6b or mSlc26a6 are noteworthy, i.e., mSlc26a6aA \( \text{SO}_4^{2-} \) currents are 10-fold higher than the other Slc26a6 transporters. In general, oocytes expressing ion transporters exhibit currents between several hundred nA to several μA, and those expressing ion channels exhibit currents between several dozen to 100 μA (6). Therefore, \( \text{SO}_4^{2-} \)-elicited current of mSlc26a6aA is as high as that of ion channels or a transporter with a very high turnover number such as CIC-4 or CIC-5 (38, 48). Interestingly, this high activity is specific for \( \text{SO}_4^{2-} \), as the mSlc26a6aA currents elicited by other anions (\( \text{HCO}_3^- \) and oxalate) are much lower, i.e., comparable to those of mSlc26a6b and mSlc26a6. Further analyses on chimeric transporters of mSlc26a6aA, mSlc26a6b, and mSlc26a6 could reveal a domain or motif that bears the \( \text{SO}_4^{2-} \)-specific activity of mSlc26a6aA.

### Perspectives and Significance

Marine teleosts avoid dehydration by continuously drinking SW, which contains high concentrations of \( \text{Ca}^{2+} \) and \( \text{SO}_4^{2-} \).
The major portions of the Ca\(^{2+}\) and SO\(_4\(^{2-}\) \) burdens acquired from drinking SW are eliminated by intestinal precipitation of Ca\(^{2+}\) with bicarbonate and by renal excretion of sulfate, respectively, both of which involve Slc26a6A transport (27). Consistent with its broad ion specificity, Slc26a6A acts as a bicarbonate transporter in the intestine, as demonstrated in our previous work (27), and a sulfate (SO\(_4\(^{2-}\) \) ) transporter in the kidney (present study) depending on the surrounding ionic conditions. It therefore seems that the substrate promiscuity of a single gene product, Slc26a6A, is an effective strategy for conditions. It therefore seems that the substrate promiscuity of a single gene product, Slc26a6A, is an effective strategy for conditions. It therefore seems that the substrate promiscuity of a single gene product, Slc26a6A, is an effective strategy for conditions. 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