RESEARCH ARTICLE
Molecular physiology and functional morphology of SO$_4^{2-}$ excretion by the kidney of seawater-adapted eels

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SUMMARY
Marine teleosts actively excrete SO$_4^{2-}$ and keep the plasma concentration of this ion much lower than that of environmental seawater (SW). We used the eel as a model to study the excretory mechanism of SO$_4^{2-}$ because this euryhaline species changes SO$_4^{2-}$ regulation drastically after transfer from freshwater (FW) to SW. Time-course studies showed that plasma SO$_4^{2-}$ concentration decreased 3 days after transfer of eels from FW to SW, while urine SO$_4^{2-}$ concentration increased on 1 day. Detailed analyses showed that urine SO$_4^{2-}$ concentration increased linearly from 6 h after SW transfer; however, this did not immediately translate to increased SO$_4^{2-}$ excretion because the volume of urine was decreased. We identified five SO$_4^{2-}$ transporters in the eel kidney. Three of these (Slc26a6a, Slc26a6b and Slc26a6c) are expressed in both SW- and FW-acclimated eels while Slc26a6a and Slc13a1 are expressed in SW-acclimated eels and FW-acclimated eels, respectively. We showed that changes in Slc26a6a and Slc13a1 gene expression occurred 1–3 days after SW transfer. In SW eel kidneys, immunohistochemistry using specific antisera against each transporter protein showed that Slc26a6a and Slc26a6c are localized on the apical membrane of the P1 segment of the proximal tubule, while Slc26a6b is localized on the apical membrane and Slc26a1 on the basolateral membrane of the P2 segment. The current study revealed complex molecular mechanisms of SO$_4^{2-}$ excretion in the SW eel kidney that involve segment-specific localization of multiple Slc transporters in proximal tubules and modulation of their expression in different SO$_4^{2-}$ environments. This precise regulatory mechanism may endow the eel with euryhalinity.

Key words: sulfate transporter, sulfate excretion, solute carrier 26, kidney, proximal tubule, marine teleost, Anguilla japonica.

INTRODUCTION
The SO$_4^{2-}$ content of seawater (SW) is >40-fold higher than the SO$_4^{2-}$ concentration in the plasma of marine teleosts. Thus, marine teleosts are continuously exposed to a risk of excess SO$_4^{2-}$, which enters the gills and the digestive tracts from the ingested SW. To avoid hypersulfatemia, teleosts actively excrete SO$_4^{2-}$, together with Mg$^{2+}$ and Ca$^{2+}$, primarily via the kidney (Berglund and Forster, 1958; Hickman, 1968; Marshall and Grosell, 2006). The urine of marine teleosts is isotonic to their plasma but contains concentrations of SO$_4^{2-}$, Mg$^{2+}$ and Ca$^{2+}$ that are higher than those of SW. Renal SO$_4^{2-}$ excretion is essential for SO$_4^{2-}$ homeostasis in marine teleosts (Renfro, 1999), as shown in the flounder (Beyenbach et al., 1986; Dickman and Renfro, 1986; Pelis and Renfro, 2004; Renfro et al., 1999; Renfro and Pritchard, 1982; Renfro and Pritchard, 1983), goosefish (Berglund and Forster, 1958) and killifish (Cliff and Beyenbach, 1992).

In humans, plasma SO$_4^{2-}$ concentration has been used as a marker of renal failure in conditions such as uremia (Holmes et al., 1960). Because of increasing clinical significance in renal failure, the mammalian kidney has been extensively studied in terms of SO$_4^{2-}$ regulation as well as the regulation of other ions (Berglund and Sorbo, 1960). The SO$_4^{2-}$-retaining system is dominant in mammals to enable them to survive in the SO$_4^{2-}$-deficient, terrestrial environment. However, the filtered load of SO$_4^{2-}$ exceeds the capacity of maximal tubular reabsorption and the excess is excreted into the urine (Mudge et al., 1973; Lin and Levy, 1983). Recently, several SO$_4^{2-}$ transporters have been identified in mammals, and all belong to the solute carrier (Slc) superfamily, which consists of 47 families (He et al., 2009). Two SO$_4^{2-}$ transporters, named NaSi-1 (Slc13a1) and Sat-1 (Slc26a1), have been well studied and are suggested to be involved in SO$_4^{2-}$ reabsorption. Slc13a1 is known to be a Na$^+$/SO$_4^{2-}$ cotransporter located on the apical or brush border membrane of the epithelial cells lining the renal proximal tubule and the small and large intestine of mammals (Besseghir and Roch-Ramel, 1987; Silberg et al., 1995; Byeon et al., 1998; Markovich, 2001). Slc26a1 is known to be a multifunctional anion exchanger located on the basolateral membrane of epithelial cells that have Slc3a1 on the apical side (Bissig et al., 1994; Karniski et al., 1998; Krick et al., 2009). Slc26a6 has also been localized to the apical membrane of the renal proximal tubule in mammals, which exchanges several anions: oxalate/SO$_4^{2-}$, Cl$^{-}$/formate, Cl$^{-}$/oxalate, oxalate/formate, oxalate/oxalate, Cl$^{-}$/HCO$_3^{-}$ and Cl$^{-}$/OH$^{-}$ (Knauf et al., 2001; Jiang et al., 2002; Xie et al., 2002; Thomson et al., 2005).

The renal proximal tubule has been suggested as an important site for SO$_4^{2-}$ regulation in marine teleosts (Renfro and Pritchard, 1982; Dickman and Renfro, 1986; Cliff and Beyenbach, 1992). Though the information about SO$_4^{2-}$ transporters in marine teleosts is limited, Katoh and colleagues identified Slc26a1 in the renal proximal tubule of SW-acclimated rainbow trout (Katoh et al., 2006). More recently, Katoh and coworkers identified Slc26a6a in the renal proximal tubule of pufferfish (Takifugu obscurus) and showed that the expressed protein transports SO$_4^{2-}$ (Katoh et al., 2009). In
freshwater (FW) fish, Nakada and colleagues showed that Slc26a1 and Slc13a1 are located in the proximal tubule of the eel kidney and play important roles in renal SO$_4^{2-}$ reabsorption (Nakada et al., 2005).

We recently showed that the kidney is a major site of SO$_4^{2-}$ excretion in SW eels using $^{35}$SO$_2$O as a tracer (T.W. and Y.T., unpublished). We also found that SO$_4^{2-}$ regulation changes dramatically in eels after transfer from FW to SW. We then attempted to identify the transporters responsible for the regulation of SO$_4^{2-}$ excretion and examined their gene expression in the kidney after SW transfer. Finally, in an attempt to delineate the whole regulatory system of SO$_4^{2-}$ excretion in the SW eel kidney, SO$_4^{2-}$ transporters were localized using specific antisera raised against the transporter proteins.

MATERIALS AND METHODS

Animals

Japanese eels, _Anguilla japonica_ (Temminck and Schlegel 1847), weighing 192±5 g (N=45), were purchased from a local dealer. They were kept in a FW tank at 18°C for at least 2 weeks before use (FW eel). Some eels were transferred to a SW tank and acclimated at 18°C for at least 2 weeks before use (SW eel). The major ionic composition of FW (tap water) and SW were as follows: [Na$^+$], 1.0 mmol l$^{-1}$; [Cl$^-$], 0.5 mmol l$^{-1}$; [Ca$^{2+}$], 0.25 mmol l$^{-1}$; [Mg$^{2+}$], 0.5 mmol l$^{-1}$; and [SO$_4^{2-}$], 0.3 mmol l$^{-1}$ in FW; and [Na$^+$], 450 mmol l$^{-1}$; [Cl$^-$], 525 mmol l$^{-1}$; [Ca$^{2+}$], 10 mmol l$^{-1}$; [Mg$^{2+}$], 50 mmol l$^{-1}$; and [SO$_4^{2-}$], 30 mmol l$^{-1}$ in SW. All experiments including fish maintenance were approved by the Committee of Animal Experiments at the University of Tokyo.

Time-course changes in SO$_4^{2-}$ concentration in body fluids after transfer to SW

To examine the time course changes in SO$_4^{2-}$ concentration in plasma, urine and rectal fluid, FW eels (N=9) were directly transferred to full-strength SW. On days 0, 1, 3, 7 and 14 after transfer, eels were lightly anesthetized in 0.1% (w/v) tricaine methanesulphonate (Sigma, St Louis, MO, USA), and 100 μl of blood was collected from the caudal vein into a chilled syringe; bladder urine and rectal fluid were collected by syringe via inserted tubes (PE10, Clay Adams, Franklin Lakes, NJ, USA). Blood was transferred to a chilled tube and centrifuged at 10,000 g for 5 min at 4°C to separate plasma. After sampling on day 14, eels were killed by excess exposure to saturated 2-phenoxethanol (Wako Pure Chemical Industries, Osaka, Japan), and the kidneys were immediately dissected out, frozen in liquid nitrogen and stored at ~80°C for later analysis.

Short-term changes in urine volume and SO$_4^{2-}$ concentration

FW eels (N=5) were anesthetized in 0.1% (w/v) tricaine methanesulphonate for 15 min and cannulated with polyethylene tubes (0.5 mm i.d., 0.8 mm o.d.) into the ventral aorta and urinary bladder. After surgery, eels were placed in plastic troughs with circulating, aerated water at 18°C. After more than 18 h of recovery, circulating water in the trough was changed from FW to SW by a three-way stopcock. Total bladder urine, and 30 μl of blood via the aortic tube, were collected for plasma analysis at 0, 1, 3, 6, 12 and 24 h after the salinity change.

SO$_4^{2-}$ concentrations in plasma and urine were measured using ion chromatography (AV10, Shimadzu, Kyoto, Japan). An anion-exchange column (IC-A3, Shimadzu), was used for the measurement of SO$_4^{2-}$. Standard curves for SO$_4^{2-}$ were made using commercial standard solutions (Shimadzu). Concentration was estimated from a regression line calculation using Class-VP version 6.12 software (Shimadzu). Inter- and intra-assay coefficients of variation were 2.53% and 3.89%, respectively.

Identification of SO$_4^{2-}$ transporters in the kidney

To examine the tissue distribution of SO$_4^{2-}$ transporters, the gills, esophagus, stomach, anterior intestine, posterior intestine, rectum, kidney and urinary bladder were collected from FW and SW eels (N=3 each). The kidney was also collected from eels 1, 3, 7 and 14 days after SW transfer (N=4 each). Tissue samples were immediately frozen in liquid nitrogen and kept at ~80°C until use. For immunohistochemical analyses, a sample of kidney from both FW and SW fish (N=3 each) was fixed in 4% paraformaldehyde in 0.1 mol l$^{-1}$ phosphate-buffered saline (PBS), pH 7.4 for 24 h.

Total RNA was isolated from various tissues using acid guanidinium thiocyanate–phenol–chloroform extraction with Isogen (Nippon Gene, Tokyo, Japan). Tissues were homogenized in Isogen (100 mg ml$^{-1}$) by Micro smash$^{	ext{TM}}$ MA-100 (TOMY, Tokyo, Japan), followed by chloroform extraction, isopropanol precipitation, and 75% (v/v) ethanol washing of precipitated RNA. The RNA obtained was dissolved in diethyl pyrocarbonate (DEPC)-treated water, and its concentration was measured spectrophotometrically at 260, 280 and 300 nm using a spectrophotometer (DU-640, Beckman, Fullerton, CA, USA).

Sample tissues were used to identify the _Slc_ genes that were expressed abundantly in the kidney and other tissues and to compare gene expression between FW and SW fish. Time-course analysis of gene expression (1, 3, 7 and 14 days following SW transfer) was also performed using cDNA obtained from the kidney. A 1 μg sample of total RNA from each tissue was reverse-transcribed using the SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). PCR was performed using gene-specific sense and antisense primer combinations (Table 1) based on the _Slc_ sequences deposited by Nakada and colleagues in the DDBJ/EMBL/GenBank database (Nakada et al., 2005); _Slc13a1_ (AB111926), _Slc26a1_ (AB111927), _Slc26a3_ (AB111930), _Slc26a6a_ (AB084425), _Slc26a6b_ (AB111928), _Slc26a6c_ (AB111929), β-Actin (AB074846) was used as an internal control. PCR amplification was performed under the following conditions: 94°C for 1 min, then 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, and finally 72°C for 5 min.

Immunohistochemical analyses

Fixed kidney samples were washed twice in 70% ethanol and then embedded in Paraplast (Kendall, Mansfield, MA, USA). Sections (6 μm) were sequentially rehydrated through a graded ethanol series and non-specific signal was blocked with 0.6% hydrogen peroxide (H$_2$O$_2$) in methanol for 30 min at room temperature and 2% normal goat serum in PBS (NGS-PBS for Slc13a1 and Slc26 proteins) or 2% normal horse serum (NHS-PBS for _β_–actin) containing 0.01% sodium azide for 30 min at room temperature. Sections were then incubated with antisera against Slc13a1, Slc26a1, Slc26a6a, Slc26a6b and Slc26a6c diluted 1:10000 with NGS-PBS or against _β_–actin diluted 1:500 with NHS-PBS for 48 h at 4°C, and stained with elite avidin–biotin–peroxidase complex kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer’s instructions. The signal was visualized by exposure
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Table 1. Primers for expression analyses

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Name</th>
<th>Sense primer</th>
<th>Antisense primer</th>
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<tr>
<td>Slc13a1</td>
<td>ajslc13a1S1</td>
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<tr>
<td>β-actin</td>
<td>B-ACT-S1</td>
<td>5'-CTCCCTGGAGAAGACTGAGCC-3'</td>
<td>5'-GACGGAGATTTGGCCTAGG-3'</td>
</tr>
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Statistical analysis

Changes in SO$_4^{2-}$ concentration after SW transfer were analyzed by Dunnett’s test. All analyses were performed using the statistical software KyPlot 5.0 (Kyens, Tokyo, Japan). Significance was defined as *P<0.05, **P<0.01 and ***P<0.001. All results are expressed as means ± s.e.m.

RESULTS

Time-course changes in SO$_4^{2-}$ regulation after SW transfer

Plasma SO$_4^{2-}$ concentration tended to increase 1 day after SW transfer but decreased abruptly after day 3, suggesting an activation of SO$_4^{2-}$ excretory mechanisms after this time point (Fig. 1A). The decrease in plasma SO$_4^{2-}$ concentration continued until 7 days after transfer, when levels appeared to stabilize. Urine SO$_4^{2-}$ concentration increased rapidly at first before decreasing. Despite this decrease, urine SO$_4^{2-}$ levels after SW transfer remained significantly higher than in FW (Fig. 1B). Rectal fluid SO$_4^{2-}$ concentration increased 1 day after SW transfer but to a smaller extent than the change in urine SO$_4^{2-}$ concentration (Fig. 1C). Urinary SO$_4^{2-}$ excretion showed a pattern similar to the change in urine SO$_4^{2-}$ concentration, but the levels 3 days

NaHSO$_3$:HCl:water of 6:5:100) for 3 min, and then counterstained with hematoxylin.

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Urine SO$_4^{2-}$ detected in the negative tissues shown in Fig. 1, plasma SO$_4^{2-}$ concentration did not change significantly 1 day after SW transfer, but urine SO$_4^{2-}$ concentration increased profoundly. In order to examine the changes occurring within 1 day of transfer, we measured plasma and urine SO$_4^{2-}$ concentration and volume for 24 h. Plasma SO$_4^{2-}$ concentration increased slightly after 1 h, but returned to the FW level, where it remained thereafter (Fig. 2A). In contrast, urine SO$_4^{2-}$ concentration stayed low for 6 h and increased linearly thereafter, reaching a 100-fold increase after 24 h, suggesting that the reabsorptive mechanism of the FW-type kidney was turned off after 6 h (Fig. 2B). Urine volume decreased immediately to 1/10 that of FW eels within 2 h of SW transfer and was maintained at the low level for 24 h (Fig. 2B). Urinary SO$_4^{2-}$ excretion decreased slightly after 1 h and increased linearly thereafter (Fig. 2C).

**Short-term changes in plasma and renal SO$_4^{2-}$ regulation**

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**Tissue distribution of Slc transporter genes**

Eel Slc genes, except for Slc26a3, were abundantly expressed in the kidney, followed by the intestine, with low levels of expression in the urinary bladder (Fig. 3). The Slc26a3 gene was expressed almost exclusively in the digestive tract. The gills did not express any of the Slc genes abundantly. The Slc13a1 gene was expressed abundantly in the FW eel kidney while Slc26a6a was expressed abundantly in the SW eel kidney. Even after an additional 10 cycles, no signal was detected in the negative tissues shown in Fig. 3 (data not shown).

**Time-course analyses of Slc gene expression after SW transfer**

Expression of Slc13a1 decreased slightly 1 day after SW transfer, and the signal disappeared after day 3 (Fig. 4). Conversely, Slc26a6a transcript was detectable after 1 day, and increased gradually for 2 weeks. The expression of other Slc genes that were detected in the eel kidney consistently did not show changes for 14 days after SW transfer (Fig. 4).

**Morphological identification of proximal tubule segments in eel kidney**

It has been suggested that the renal tubules of European eel (Olivereau and Olivereau, 1977) and of Japanese eel (Teranishi and Kaneko, 2010) can be divided into the proximal tubule and distal tubule based on luminal staining by PAS. Proximal tubules were further divided into the P1 and P2 segments by the signal intensity derived from the length and density of the brush border membrane. In this study, the proximal P1 and P2 segments and distal tubule were identifiable by PAS staining (Fig. 5A). The P1 and P2 segments were also distinguishable by the density of the luminal immunohistochemical signal using an antisera against β-actin (Fig. 5B). PAS staining and β-actin immunoreactivity were stronger in P1 segments than in P2 segments. A schematic drawing of the eel proximal nephron is shown in Fig. 5C.

**Immunohistochemical identification of Slc proteins in eel kidney**

We used the relative abundance of immunoreactive β-actin in the luminal brush border membrane to distinguish the P1 and P2 segments of proximal tubules (Fig. 6A). In serial sections of SW eel kidney, immunoreactive Slc26a6a and Slc26a6c were localized on the apical membrane of the P1 segment of proximal tubules (Fig. 6C,E). In contrast, immunoreactive Slc26a6b was located in the apical membrane (Fig. 6D) and Slc26a1 in the basolateral membrane of the P2 segment of the same tubules (Fig. 6B). No
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Because SO$_4^{2-}$ is an important anion for body functions such as mucus formation and regulation of enzyme activity, its regulation has been the focus of intensive research particularly in the field of mammalian physiology (for review, see Markovich, 2001). In these studies, the mechanisms for SO$_4^{2-}$ absorption and retention have been investigated but little is known about how animals get rid of excess SO$_4^{2-}$. This is partly because the land is generally a SO$_4^{2-}$-deficient environment. Therefore, marine teleosts, which live in an environment with high levels of SO$_4^{2-}$, provide a unique opportunity to investigate the mechanism of SO$_4^{2-}$ excretion. In particular, euryhaline teleosts are excellent models for analyzing the mechanism of SO$_4^{2-}$ regulation because they maintain SO$_4^{2-}$ homeostasis in both SO$_4^{2-}$-deficient FW and SO$_4^{2-}$-rich SW by reversing regulation from absorption/retention to excretion. In the present study, we showed that the eel, a euryhaline teleost, switches on excretory mechanisms and switches off reabsorptive mechanisms in the kidney within 3 days of SW transfer. This process involves regulation of multiple SO$_4^{2-}$ transporters localized on different (apical or basolateral) sides of epithelial cells in different segments of renal proximal tubules.

**SO$_4^{2-}$ regulation by the kidney after SW transfer**

Eels are unique among euryhaline teleosts because they exhibit a higher plasma SO$_4^{2-}$ concentration in FW than in SW, which is the opposite situation to that for other euryhaline teleosts (Nakada et al., 2005) (T.W. and Y.T., unpublished). In the initial part of this study, therefore, we examined the time course of changes in SO$_4^{2-}$ regulation after transfer of eels from FW to SW. We found that at 3 days after SW transfer SO$_4^{2-}$ regulation had switched from absorption/retention to excretion, with plasma and urine SO$_4^{2-}$ concentration stabilizing (Fig. 1). SO$_4^{2-}$ ions are actively secreted from the renal proximal tubules of marine teleosts (Renfro and Pritchard, 1983; Dickman and Renfro, 1986; Cliff and Beyenbach, 1992); therefore, the lower plasma SO$_4^{2-}$ concentration in SW eels may be due to up-regulated SO$_4^{2-}$ excretion by the kidney.

Analysis of short-term changes after SW transfer showed that plasma SO$_4^{2-}$ concentration slightly increased after 1 h but returned to FW levels after 3 h (Fig. 2). This transient increase may be due to increased SO$_4^{2-}$ uptake by the gills, because $^{35}$SO$_4^{2-}$ uptake from the environment was accelerated in FW eels compared with that in SW eels (T.W. and Y.T., unpublished). The intestine may also contribute to the transient increase in SO$_4^{2-}$ plasma concentration as vigorous drinking occurs immediately after SW transfer in eels, which could lead to increased intestinal SO$_4^{2-}$ absorption (Hirano,
The urine SO$_4^{2-}$ concentration increased after 6 h, but total excretion did not increase because of the immediate decrease in urine volume after SW transfer (Fig. 2C).

SO$_4^{2-}$ transporters involved in SO$_4^{2-}$ excretion by the kidney

Among eel homologs of SO$_4^{2-}$ transporters, four genes (Slc26a6a, Slc26a6b, Slc26a6c and Slc26a1) were shown to be expressed significantly in the kidney of SW eels. Further, Slc26a6a was expressed only in the SW eel kidney, which indicates a role in SO$_4^{2-}$ excretion in SW. The Slc26a6a gene has also been identified in the euryhaline pufferfish and its expression is up-regulated in SW (Kato et al., 2009). Slc26a6a has the highest SO$_4^{2-}$ transport activity among the Slc26a6 family members, and it excretes SO$_4^{2-}$ in exchange for Cl$^-$ in the kidney (Kato et al., 2009) but secretes HCO$_3^-$ in exchange for Cl$^-$ in the intestine (Kurita et al., 2008). In contrast, Slc13a1 was expressed only in the FW eel kidney as shown previously by Nakada and colleagues (Nakada et al., 2005). The time-course study of the transporter genes revealed that the disappearance of Slc13a1 transcripts and appearance of Slc26a6a transcripts occurred 3 days after SW transfer, which is synchronous with the time course of changes in plasma and urine SO$_4^{2-}$ concentration after SW transfer.

Expression of the Slc26a1 gene was unchanged in the eel kidney after SW transfer, but in rainbowtrout expression of the gene was slightly increased 3 h after SO$_4^{2-}$ injection (Katoh et al., 2006). Slc26a1 is responsible for both excretion (current study) and reabsorption (Nakada et al., 2005) of SO$_4^{2-}$ in SW and FW kidney of eels, respectively. It seems that the Slc26a6 genes have diversified into three types (a–c) in the teleost and all are involved in the excretion of SO$_4^{2-}$ in the marine teleost kidney.

In contrast to the obvious down-regulation of the Slc13a1 gene in the eel kidney after SW transfer, as shown in a previous study (Nakada et al., 2005), the expression of the Slc13a1 gene is unaltered in the kidney of SW-adapted pufferfish (Kato et al., 2009). This result suggests physiological regulation such as phosphorylation of transporter activity in addition to transcriptional regulation. In mammals, phosphorylation inhibitors reduced SO$_4^{2-}$ transport activity of these transporters (Markovich, 2000). Tunicamycin (an N-glycosylation inhibitor) also reduced SO$_4^{2-}$ uptake by Slc13a1 and Slc26a1 in Xenopus oocytes (Markovich, 2001) and site-directed mutagenesis of the putative N-glycosylation sites of rat Slc13a1 and Slc26a1 reduced SO$_4^{2-}$ transport activity in Xenopus oocytes (Li and Pajor, 2003).
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Kato and colleagues suggested that Na⁺/K⁺-ATPase located at the basolateral membrane of tubular cells may serve as a driving force for the electrogenic Cl⁻/SO₄²⁻ exchanger (Slc26a6a) by increasing the intracellular negative membrane potential (Kato et al., 2009). Ura and colleagues (Ura et al., 1996) and Teranishi and Kaneko (Teranishi and Kaneko, 2010) observed immunoreactive Na⁺/K⁺-ATPase on the basolateral membrane of renal proximal tubular cells in SW-acclimated eels. Thus, a similar driving force of SO₄²⁻ transport may function in the proximal tubule of SW eels.

**Localization of SO₄²⁻ transporters in SW eel kidney**

The teleosts, which make up more than half of the total vertebrate species, are an incredibly diverse group (Nelson, 2006). This variation is reflected in the kidney morphology, with some marine fish lacking distal tubules and even glomeruli (Berglund and Forster, 1958). However, generally, the kidney of euryhaline fish is well developed and possesses all nephron segments. It has been shown that the eel possesses two different segments in the proximal tubule in addition to the neck segment, which exhibit different cellular morphology (Olivereau and Lemoine, 1968; Olivereau and Olivereau, 1977; Teranishi and Kaneko, 2010). The most distinct among them is the density of the brush border membrane, which can be visualized by β-actin immunostaining (Fig. 5B). The more dense the brush border the greater the amount of glycoprotein, which can be visualized by PAS staining (Fig. 5A).

Our immunohistochemical study showed that the SO₄²⁻ transporters Slc26a6a, Slc26a6b, Slc26a6c and Slc26a1 exist in the proximal tubules of the SW eel kidney, indicating their role in the active excretion of SO₄²⁻. Slc26a6a may be particularly important because (1) the P1 segment is thought to be the site of divalent ion secretion in marine teleosts (Beyenbach, 1995), (2) up-regulation of the Slc26a6a gene occurred after transfer of eels to SW and (3) Slc26a6a possesses the greatest SO₄²⁻-transporting activity of in pufferfish (Kato et al., 2009). We also showed that Slc26a6c colocalizes with Slc26a6a on the apical membrane of the cells in the P1 segment. However, we could not detect a counterpart transporter on the basolateral side that transports SO₄²⁻ from the blood into the tubular cells. In the P2 segment, however, Slc26a6b and Slc26a1 are localized on the apical and basolateral side, respectively, in the SW eel kidney (Fig. 6). Therefore, these transporters are most likely to be involved in SO₄²⁻ excretion in this segment. Slc26a6a is also localized on the apical side of the renal proximal tubular cells in the pufferfish (Kato et al., 2009), but a counterpart on the basolateral side has not yet been determined.

In mammals, immunohistochemical studies have shown that Slc26a6 is localized on the apical side of the renal proximal tubule (Knauf et al., 2001; Thomson et al., 2005), while Slc26a1 is localized on the basolateral membrane (Karniski et al., 1998). They are assumed to work together to excrete SO₄²⁻ in the same proximal tubular cells (Markovich and Aronson, 2007), but colocalization in the same cells has not yet been demonstrated. The apical location of Slc26a6a and basolateral location of Slc26a1 have been reported in the renal proximal tubule of two teleost species [rainbow trout (Kato et al., 2006) and pufferfish (Kato et al., 2009)], but colocalization of the two transporters in the same cells has not been demonstrated in these studies. Therefore, the current immunohistochemical study is the first to show the detailed localization of the four possible SO₄²⁻ transporters most likely to be responsible for SO₄²⁻ excretion in the SW eel kidney.

From the data obtained in this study, we propose a model for renal SO₄²⁻ excretion (Fig. 7). The apical Slc26a6a is most likely to be responsible for active renal SO₄²⁻ excretion in the SW eel kidney as this gene is expressed only in SW eels. Basolateral Slc26a1 may also be involved, but it is localized in a different segment of the proximal tubule. As a counterpart of Slc26a1 to achieve SO₄²⁻ transport from the blood into the tubular lumen, we identified Slc26a6b in the P2 segment. However, we could not identify the counterpart of Slc26a6a and Slc26a6c, which must be located on the basolateral side of the P1 segment. Slc26a2, Slc26a3, Slc26a7, Slc26a8, Slc26a9 and Slc26a11 are possible SO₄²⁻ transporters in mammals (Mount and Romero, 2004), and we could isolate Slc26a2 cDNA in the eel. However, the expression level of Slc26a2 was too low for it to have a significant function in the SW eel kidney (data not shown). In the eel, Slc26a3 was not expressed in the kidney (Figs 3 and 4). Other Slc26 genes have not yet been cloned in eels. Once their counterparts are identified, we can delineate the whole picture of the mechanism of SO₄²⁻ excretion in SW eel kidney, which hopefully represents the system of marine teleost fish in general.

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