Cloning of rainbow trout SLC26A1: involvement in renal sulfate secretion

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The purpose of this study was 1) to molecularly identify the SLC26A6 anion exchangers involved in sulfate/anion exchange in the kidney of rainbow trout, 2) to determine localization of the cloned SLC26A6 trout homolog in the trunk kidney with transmission electron microscopy and light microscopy, and 3) to demonstrate the involvement of the cloned SLC26A6 transport in sulfate transport.

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MATERIALS AND METHODS

Experimental Animal and Tissue Preparation

All experiments were conducted under protocol number 215507, which was approved by the University of Alberta and the Biosciences Animal Policy and Welfare Committee under the directions provided by the Canadian Council for Animal Care in Canada.

Preparations for molecular identification, tissue expression, and transmission electron microscopy. Adult rainbow trout (Oncorhynchus mykiss) of either sex were obtained from Alberta Trout Growers and held in 450-liter indoor tanks with flowing dechlorinated Edmonton tap water (1.00 mM Na, 1.05 mM Ca, 0.54 mM SO4), in the aquatic facility of the Department of Biology, University of Alberta (Edmonton, Alberta, Canada). The water temperature was maintained at 11–14°C. The fish were fed daily with commercial fish pellets. Photoperiod was maintained similar to the natural photoperiod in Edmonton, Alberta, Canada.

Some of the rainbow trout were acclimated gradually (~30, 50, 70, and 90% seawater, for 1 wk each) to full-strength seawater (510 mM Na, 9.38 mM Ca, 24.6 mM SO4), made using Instant Ocean salts (Aquarium Systems, Mentor, OH) in dechlorinated Edmonton tap water, and were maintained at least for 1 wk in 190-liter circulating indoor holding tanks. The experimental animals in seawater and freshwater were terminally anesthetized with ethyl-n-aminozoblate (1.0 g/l, MS-222), and tissues were immediately dissected out, rapidly frozen in liquid N2, and stored at −80°C for later analysis. For transmission electron microscopy (TEM), small pieces of trunk kidney of both freshwater and seawater fish (n = 3 each) were sampled from the middle part of trunk kidney, fixed in 2% paraformaldehyde (PFA)-2% glutaraldehyde in 0.1 M phosphate buffer (PB; pH 7.4) for 2 h, and postfixed in 1% osmium tetroxide in 0.1 M PB for 1 h.

Preparations for in situ hybridization and immunocytochemistry. Adult rainbow trout (O. mykiss) from Sagami suisan (Kanagawa, Japan) were held in a 1,000-liter tank with circulating dechlorinated Tokyo tap water (0.96 mM Na, 0.63 mM Ca2+) in the aquatic facility of the Department of Agriculture, University of Tokyo (Tokyo, Japan). The water temperature was maintained at 11°C, and the fish were fed daily with commercial fish pellets. Some of them were acclimated to full-strength seawater (478 mM Na, 12.57 mM Ca2+), as mentioned above, and maintained at least for 1 wk. The trunk kidneys from both freshwater and seawater fish (n = 3 each) were fixed in 4% PFA in 0.1 M PB for 24 h. The trunk kidneys were then cut into small pieces (~5 mm) across the axis, dehydrated in ethanol, and embedded in Paraplast. Serial sections cut at 4-μm thickness were divided into two groups and mounted on Matsunami adhesive silane (MAS)-coated slides (Matsunami Glass, Osaka, Japan) for in situ hybridization and immunocytochemistry.

cDNA Cloning

Total RNA was extracted using Trizol reagent (Invitrogen) from frozen trunk kidney and gill filaments of seawater rainbow trout. Poly(A)+ RNA purified with an Oligotex mRNA minikit (Qiagen) was treated with the cMaster RTplusPCR system (Eppendorf) to obtain cDNA. Polymerase chain reactions were performed using Taq DNA polymerase (Eppendorf). The resulting products were ligated into a pCR2.1 vector (Invitrogen) and the nucleotide sequences were determined in an automated DNA sequencer (3100 Genetic Analyzer; PerkinElmer/Applied Biosystems) in the Molecular Biology Service Unit (Department of Biology, University of Alberta). The sequences were analyzed using GeneTool 2.0 software (BioTools; www.bio tools.com).

Degenerate PCR primers F1 and R1 (Table 1 and Fig. 1) were designed on the basis of homology to sequences from previously determined human and mouse SLC26A members in GenBank and BLAST searches on the web site for puffer fish (http://fugu.biology.qmul.ac.uk/) and salmon (GRASP, http://web.ncbi.nlm.nih.gov/grasp) to obtain a partial cDNA fragment of a rainbow trout SLC26A anion exchanger. After an initial denaturation at 96°C for 2 min, 35 cycles of PCR were performed, each consisting of 55-s denaturation at 94°C, 30-s annealing at 55°C, and 90-s extension at 72°C. Gene-specific primers F2 and R2 (Table 1 and Fig. 1) were designed for 3′ and 5′ rapid amplification of cDNA ends (RACE) methods, respectively. The 3′ RACE System for Rapid Amplification of cDNA Ends software (Invitrogen) and 5′ RACE System for Rapid Amplification of cDNA Ends software (version 2.0; Invitrogen) were used according to the supplier’s instructions for 3′ RACE and 5′ RACE with the specifically designed primers mentioned above, respectively.

To confirm the nucleotide sequence obtained using the 5′ and 3′ RACE methods, we performed three PCR reactions with the gene-specific primers F4 and R5 (Table 1 and Fig. 1). One of the sequencing reactions was obtained by placing a transposon containing universal priming sites into DNA at random locations using the GPS-1 Genome Priming System (New England Biolabs, Beverly, MA). According to the first sequence obtained using the GPS-1, sequence primers F3, F6, F7, R3, R6, and R7 were designed for further sequencing reactions.

Sequence Analysis of Cloned SLC26A Anion Exchanger

The hydropathy profile of the deduced amino acid sequence was analyzed using PepTool version 2.0 software (BioTools) according to the algorithm of Kyte and Doolittle. Predicted topology of the membrane domain of the anion exchanger was derived using the web servers PredictProtein (www.predictprotein.org), SOSUI (http://sosui.proteome.bio.tuat.ac.jp/~sosui/proteome/sosuiframe0.html), and HMMPtop (www.enzim.hu/hmmpot/). The phylogenetic relationship between amino acid sequences of the cloned SLC26A and previously found human homologs was analyzed using the ClustalW program on the DNA Data Bank of Japan web site (www.ddbj.nih.go.jp). We used a neighbor-joining bootstrap method (1,000 times repeated) with gaps ignored, and the tree was figured using TreeView Win32 software (http://taxonomy.zoology.gla.ug/kod/treewied.html).

Northern Blot Analysis

The brain, white muscle, intestine, gill, eye, head kidney, trunk kidney, heart, liver, and blood were removed from seawater-adapted rainbow trout (n = 3), and total RNA was isolated from these tissues as described above. Total RNA (20 μg) was electrophoresed on a 1.2% formamide-agarose gel and transferred to nylon membrane (Hybond-N; Amersham Biosciences). A cDNA corresponding to nucleotides 1473–2121 in SLC26A was labeled with [α-32P]dCTP (Amersham Biosciences) with the Random Primers DNA labeling system (Invitrogen) and then purified using the QAquick nucleotide removal kit (Qiagen). The membrane was hybridized with the radioisotope-labeled probe (24 × 105 cpm/ml).

Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
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<tr>
<td>F1</td>
<td>TGG(G/C)CC(T/G)A(G/C)(A/G/C)A(G/T)TACA</td>
</tr>
<tr>
<td>F2</td>
<td>TAGGCGAGCCAATCCGCC</td>
</tr>
<tr>
<td>F3</td>
<td>TGGCGTGAATGTTATACAGG</td>
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<td>F4</td>
<td>AGCGGAGCTGACATGATTCCG</td>
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<td>F5</td>
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<td>F7</td>
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in 1% BSA, 0.5 M NaH₂PO₄, 1 mM EDTA, and 7% SDS at 65°C for 24 h, and the membrane was exposed to a screen for 2 wks. Specific bands were visualized using a PhosphorImager (Molecular Dynamics).

**Sulfate Injections**

Adult rainbow trout (~250 g) of either sex, taken from holding facilities at the University of Alberta, were injected with 150 μl of 1 M Na₂SO₄ under anesthesia with MS-222 and placed in 190-liter tanks after their recovery. At 0 h (n = 8, initial controls), 3 h (n = 7), 12 h (n = 8), or 24 h after injection (n = 8), fish were overdosed with MS-222, and the trunk kidneys and blood were sampled. The experiments were performed with fewer than 10 fish in a tank, and feeding was stopped before the injections and during the experiments. Total RNA were isolated from the tissues, and Northern blot analysis was performed using 10 μg of total RNA, as described above. The hybridized signals and ethidium bromide-stained 18S ribosomal RNA bands were quantified using ImageQuant (Molecular Dynamics) and Scion Image beta-4.0.2 (http://www.scioncorp.com/), respectively. To correct for differences in RNA sample loading, the quantitation of SLC26A1 mRNA in each sample was given by the ratio of the hybridized signal to 18S ribosomal RNA. Significant differences were examined using one-way ANOVA, followed by Dunnett’s test, which compares the value to that of the initial control group (0 h).

**Transmission Electron Microscopy**

After dehydration in ethanol, the samples were transferred to propylene oxide and embedded in Spurr’s resin. Ultrathin sections were cut with a diamond knife, mounted on grids, stained with uranyl acetate and lead citrate, and observed with TEM (JEM1010; JEOL, Tokyo, Japan).

**In Situ Hybridization**

We utilized in situ hybridization to determine the cellular localization of the cloned SLC26A anion exchanger in the trunk kidney. These three specifically designed oligonucleotide antisense probes (44 bases each, corresponding to nucleotide sites 734 –778, 1395–1439, and 1785–1829) were labeled with digoxygenin (DIG) by using the DIG oligonucleotide tailing kit (2nd generation; Roche). To hybridize the probes to specific mRNA fragments, the deparaffined sections were incubated sequentially with:

1. 0.01 M phosphate-buffered saline (PBS, pH 7.4),
2. 0.2 N HCl for 15 min,
3. 2%/ml protease K in PBS for 30 min at 37°C,
4. 4% PFA in 0.1 M PB for 10 min for postfixation,
5. PBS for 1 min at room temperature,
6. 0.2% glycine in PBS for 15 min (twice),
7. distilled water (DW) for 1 min for washing,
8. 40% formamide in 2/SSC for 2 h for prehybridization,
9. DIG-labeled probes (1/2g/ml each) diluted in hybridization buffer (40% formamide, 2/SSC, 20 mM Tris•HCl, pH 7.6, 1/1000 Denhardt’s, 0.1% Tween 20, 25/250 g/ml calf thymus DNA, and 10% dextran sulfate) overnight at 37°C,
10. 2/SSC for 15 min,
11. 1/2SSC for 15 min at 40°C (twice),
12. 2/SSC for 15 min, and
13. PBS for a few minutes. The sections that possessed the hybridized mRNA were sequentially incubated to detect the signals with:

1. 2% normal goat serum (NGS) in PBS for 30 min,
2. anti-DIG-gold (Roche) diluted 1:30 with PBS containing 1% BSA for 30 min at room temperature,
3. PBS for 5 min, and
4. DW for 3 min (5 times). The gold particles that existed at the localization of hybridized probes were emphasized

**Fig. 1.** Nucleotide sequence of the cDNA encoding the SLC26A1 from rainbow trout kidney and the deduced amino acid sequence. Designed primer sites are shown highlighted, and potential N-linked glycosylation sites are circled. The amino acid positions in boxes are the SLC26A transporter signature (amino acid residues 71–92) and the STAS domain (amino acid residues 516–644), respectively.
with a silver-enhancing kit (British BioCell International) after incubation for 15 min at 20°C. The sections were dehydrated in series of ethanol and xylene, mounted with Permount, and observed under a light microscope (Nikon) equipped with differential interference contrast optics. In addition, serial sections were stained with periodic acid/Shiff (PAS; WAKO Chemical, Osaka, Japan) to examine the general morphology of the trunk kidney and counterstained with hematoxylin.

**Antibodies**

The antiserum (NAK121) was raised in a rabbit against a synthetic peptide corresponding to a part of the highly conserved region of the chum salmon Na\(^+\)-K\(^+\)-ATPase α-subunit (20). The antibody used to detect the vacuolar-type H\(^+\)-ATPase (V-ATPase) was the affinity-purified antibody from a polyclonal antiserum raised in a rabbit against a synthetic peptide. The antigen region was designed based on the highly conserved region in the A-subunit of killifish V-ATPase (21).

**Western Blot Analysis for Antibody to Na\(^+\)-K\(^+\)-ATPase and V-ATPase**

Western blot analysis was performed according to the method described by Tresguerres et al. (51) with some minor modifications. Frozen trunk kidney samples were weighed, pulverized in 1:10 wt/vol of ice-cold homogenization buffer (250 mM sucrose, 1 mM EDTA, 30 mM Tris, 100 mg/ml PMSF, and 2 mg/ml pepstatin, pH 7.4) in a porcelain grinder on ice, and sonicated for 20 s. Debris was removed by centrifugation (4,200 g for 15 min at 4°C), and membranes were pelleted by centrifugation at 20,800 g for 60 min at 4°C. The resulting pellets were resuspended in homogenization buffer (4,200 g for 15 min at 4°C), and membranes were pelleted by centrifugation at 20,800 g for 60 min at 4°C. The resulting pellets were resuspended in homogenization buffer, and an aliquot was saved for BCA protein determination at 4°C. The resulting pellets were resuspended in homogenization buffer (4,200 g for 15 min at 4°C), and membranes were pelleted by centrifugation at 20,800 g for 60 min at 4°C. The resulting pellets were resuspended in homogenization buffer, and an aliquot was saved for BCA protein determination at 4°C. The resulting pellets were resuspended in homogenization buffer (4,200 g for 15 min at 4°C), and membranes were pelleted by centrifugation at 20,800 g for 60 min at 4°C. The resulting pellets were resuspended in homogenization buffer, and an aliquot was saved for BCA protein determination at 4°C.

**Ion Concentration Measurements**

Na\(^+\) and Ca\(^2+\) concentrations were measured using an atomic absorption spectrophotometer (Perkin Elmer 3300), and SO\(_4\)\(^2-\) concentrations were examined by ion chromatography (DX 500 system; Dionex, Sunnyvale, CA).

**RESULTS**

**cDNA Cloning and Sequence Analysis of SLC26A Anion Exchanger**

RT-PCR was performed with trunk kidney and gill filament RNA for the degenerate primers F1 and R1 (Table 1 and Fig. 1). A single PCR product with the expected length (~1,000 bp) was obtained only from the trunk kidney, not from the gill filaments. After 3’ and 5’ RACE, a full-length cDNA encoding the SLC26A exchanger (3,258 bases) was cloned from trunk kidney, and we obtained the deduced amino acid sequence (693 amino acids) of the putative SLC26A transporter (Fig. 1). The cDNA sequence has been deposited in the database (GenBank accession no. AY512964), and the nucleotide sequence and amino acid sequence showed 52% of identity from human SLC26A1 (Sat-1). The rainbow trout homolog of SLC26A1 has potential N-glycosylation sites as shown in Fig. 1. Bioinformatics analysis for conserved protein domains suggests that the cloned trout SLC26A1 has conserved domains typical of previously discovered SLC26A anion exchangers. These include the SLC26A transporter signature (PS01130, amino acid residue 71–92), the sulfate transporter family conserved amino acid sequence (pfam00916, amino acid residue 176–486), the sulfate transporter and anti-sigma factor antagonist (STAS) domain (pfam01740, amino acid residue 516–644), and the anti-sigma regulator factor SpolIAA (COG1366, amino acid residue 586–640) (Fig. 1).

Figure 2 shows the hydropathy analysis of the predicted amino acid sequences of the cloned SLC26A1 rainbow trout homolog. The hydropathy profile shows the possible transmembrane domains of this homolog. Programs from three different servers, PredictProtein, SOSUI, and HMMPtop, yield highly divergent models of the rainbow trout homolog of the SLC26A that included 10, 11, and 10 transmembrane domains, respectively. The PredictProtein program derived the most reasonable membrane topology, suggesting 10 transmembrane domains with intracellular NH\(_2\)- and COOH-terminal ends. This is in congruence with current predicted structures of mammalian SLC26A family members. These characteristics, in addition to its position in the phylogenetic tree (Fig. 3), confirm that cloned SLC26A1 is the rainbow trout homolog of mammalian SLC26A1 (Sat-1).

**Tissue Distribution of SLC26A1 mRNA**

Tissue distribution of the rainbow trout homolog of SLC26A1 anion exchanger was examined by Northern blot
analysis with total RNA from seawater-adapted rainbow trout tissues. As shown in Fig. 4A, hybridized signals were detected as a major band of ~3.3 kb and were found in the trunk kidney but not in other tissues examined. It was confirmed that the size of the mRNA was in agreement with that of the cDNA for SLC26A1 rainbow trout homolog.

**SLC26A1 mRNA Expression After Excess Sulfate Injection**

Plasma SO$_4^{2-}$ concentration also showed a transient increase ($P < 0.01$) at 3 h after the injection but was rapidly restored by 24 h to a level that was not significantly different from the level at 0 h (initial control) (Fig. 5A). Correspondingly, SLC26A1 mRNA expression level in trunk kidney became significantly ($P < 0.01$, ~2.5 times) elevated at 3 h after Na$_2$SO$_4$ injection, followed by a decrease to the initial expression level by 12 h (Fig. 5B).

**Ultrastructure of Kidney Tubules**

In rainbow trout trunk kidney, tubules were classified into three clearly differentiated fine structures: the first (PI) and second segments (PII) of proximal tubules and distal (DS) tubules (Fig. 6, A–F). Although every cell in these three tubules has a rich population of mitochondria and basolateral tubular system as common fine structures, these three segments are characterized by the shape of their mitochondria. Cells in PI and DS segments have longer mitochondria than those in PII cells (Fig. 6, B, D, and F). In addition, the apical membranes of the cells in PI segments are equipped with long and numerous brush borders pointing into the tubular lumen, whereas cells in PII segments have less extended brush borders. Cells in DS have distinctly less developed brush borders compared with PI and PII segments. The PI segment is signalized by pinocytotic vesicles and lysosomes in the subapical region of the cells. These PI tubule cells also have small electrondense dots just below the brush borders. DS segments were distinguished from PI and PII segments by the extended tubular system in the cytoplasm, which seemed lattice like (Fig. 6, E and F). The tubular system in the cytoplasm was confined to the basolateral region of the cells in PI and PII segments (Fig. 6, B and D). There were no remarkable differences in the fine structure between tissues from seawater (not shown) and freshwater trout (Fig. 6).

**Western Blot Analysis**

Because the antibodies used for immunocytochemistry are heterologous, it is important to demonstrate specificity in rainbow trout. The specificities of the antibodies were tested in kidney by performing Western blot analysis. The antibody to
V-ATPase recognized two major bands, both of ~70 kDa (Fig. 7A). The antiserum to Na\(^+/K^+\)-ATPase recognized a major protein band with a size of ~100 kDa (Fig. 7B).

**Specific Localization of SLC26A Anion Exchanger in the First Segment of Proximal Tubules**

The three probe regions were chosen because they have sufficient GC content to serve as a probe and were unlikely to cross-hybridize to any other mRNA. In both seawater- and freshwater-adapted rainbow trout trunk kidney, hybridization of SLC26A1 anion exchanger probes to a section of the trunk kidney showed that the mRNA is expressed most intensely in a region corresponding to the cytoplasm of the PI (Fig. 8A). The PAS staining of serial sections of the trunk kidney (Fig. 8B) demonstrated the prominent brush border projecting into the lumen of the first segment of proximal tubules and identified the tubules as belonging to the PI. The proximal tubules are PAS positive, because the surfaces of the microvilli are coated with a particularly dense glycocalyx thought to afford physical and chemical protection to the microvilli. However, some of the PAS-positive PI lack signal for the cloned SLC26A1, suggesting heterogeneity even with the PI segments (Fig. 8, A and B). SLC26A1 mRNA localization and expression were unchanged between seawater (not shown) and freshwater fish (Fig. 8, A and B).

**Colocalization of Na\(^+/K^+\)-ATPase and V-ATPase With SLC26A1 mRNA**

Na\(^+/K^+\)-ATPase immunoreactivity was detected in the basolateral membrane of every tubule in the trunk kidney, with more intensive immunoreactivity with Na\(^+/K^+\)-ATPase found in DS compared with the proximal tubule (Fig. 8C). The ultrastructure of DS shows a more extended basolateral membrane, reaching close to the subapical region of cells. Na\(^+/K^+\)-ATPase staining in the proximal tubules was confined to the basolateral region of the cells (Fig. 6, E and F).
Figure 8, E and F, shows that the V-ATPase antibody labels the intermicrovillar membrane, where TEM showed the presence of electrondense micro dots (Fig. 6B) in the PI segment. Some of the cells also have diffuse signal throughout the microvilli. Hence, we have demonstrated that both Na\(^+\)-K\(^+\)-ATPase and V-ATPase are colocalized with SLC26A1 in the cells of PI. There were no remarkable differences in localization of these two transporter proteins between seawater (not shown) and freshwater fish (Fig. 8, C–G).

DISCUSSION

The kidney, gills, and intestine are important organs for ion regulation in teleosts. Recently, the molecular identity and specific localization of various ion transporters have been identified in the teleost gills (9, 31, 49, 52). However, the ion transport mechanisms in the kidney and intestine of teleosts are less understood. In this study, we have identified a kidney-specific SLC26A1 anion exchanger in renal tubules of rainbow trout.
trout, *O. mykiss*. The specific localization of the trout homolog of the SLC26A1 mRNA was observed in the first segment of the proximal tubule (PI), colocalized with the ion transport proteins V-ATPase and Na$^{+}$-K$^{+}$-ATPase.

Among the human homologs of SLC26 anion exchanger family, rainbow trout SLC26A1 is most closely related to the human SLC26A1 (Sat-1), according to the deduced amino acid sequence. Sat-1 is reported to act primarily as a sulfate/oxalate/bicarbonate exchanger in the proximal tubule of the mammalian kidney (19). The identity compared with the recently reported eel (*Anguilla japonica*) homolog of SLC26A1 was 75% (35). The cloned SLC26A1 has all the features of other SLC26A transporters, including the transporter signature PS01130, the sulfate transporter family conserved amino acid sequence (pfam00916), the STAS domain (pfam01740), and the anti-sigma regulatory factor SpoIIAA (COG1366). The STAS domain has disease-associated mutants in humans, and the SpoIIAA is a statistically similar site of bacterial anti-sigma factor antagonists to the COOH-terminal domain of the SLC26A anion exchangers normally found in the STAS domain (1). These findings strongly suggest that the cloned SLC26A1 represents the rainbow trout homolog of mammalian SLC26A1 (Sat-1).

The topology of SLC26A1 trout homolog was analyzed using three different programs and resulted in predictions of 10, 11, and 10 transmembrane domains, respectively. Previously predicted topologies of SLC26A anion exchangers suggest that the family members have 10–14 transmembrane helices (34). Human, mouse, rat, and eel SLC26A1 transmembrane domain predictions have been performed only on the basis of computer-based algorithms, and they suggest 12 transmembrane domains with both NH$_2$- and COOH-terminal ends located intracellularly (4, 26, 35, 41). However, it is difficult to predict the exact topologies of SLC26A anion exchangers, and different computer programs produce ambiguous results regarding the numbers of transmembrane segments for members of this family (55), as observed in the present study. Zeng et al. (55) examined the location of NH$_2$- and COOH-terminal domains of Prestin (SLC26A5) using the antibodies to the synthetic epitopes located in NH$_2$- and COOH-terminal ends and showed that both ends are cytoplasmic. In SLC26A6, it also has been suggested that both NH$_2$- and COOH-terminal ends are intracellular, by using tagged isoforms and antibodies to the tags (27). In terms of functional regulation, the STAS domain in the COOH terminus is likely an intracellular region. Although the trout homolog of the SLC26A1 may have more than 10 transmembrane domains according to hydropathy plot, the most reasonable prediction for it was derived by the PredictProtein program, which suggested a 10-transmembrane domain protein with intracellular COOH and NH$_2$ termini.

Fish kidneys lack of a great deal of similarity to mammalian kidneys (12, 54). In regard to basic structure and morphology, the fish kidney consists of only head and trunk kidney. Embryologically, head kidney derived from pro-nephros, and trunk kidney from mesonephros. The head kidney is the anterior portion of the kidney and consists primarily of lymphoid tissue, whereas the trunk kidney is composed of many nephrons and interstitial lymphoid tissue. However, nephrons of teleosts are devoid of the loop of Henle found in higher vertebrates. In the present study, light microscopy showed distinct features of each tubule type in rainbow trout trunk kidney, and TEM observations revealed both unity and differences in ultrastructure between segments of nephron. Although every cell in the renal tubules has a rich population of mitochondria, differences in the mitochondrial fine structure and its distribution were observed. This is consistent with observations in English sole, where the mitochondria are well-developed and extended throughout the cytoplasm in PII (15). Our observations showed equally developed mitochondria in both PI and PII with larger numbers of smaller mitochondria appearing to be present in PII of rainbow trout. The extensive brush border and large lysosomes in PI tubules observed in this study also have been shown in English sole. Moreover, lysosomes were PAS positive (15), as also observed in this study. In addition, similar structural features of proximal tubules, such as brush border and lysosomes, were reported in mammalian kidney (29), suggesting functional similarity of this segment to vertebrate kidney tubules. The DS was differentiated from proximal tubules essentially by the prominent basolateral infoldings and fewer microvilli on the lumen in our observations, in agreement with the previous observation in southern flounder (15), whereas this segment of the mammalian kidney shows fewer mitochondria (29).

In our Western blot analysis, the antibodies recognized one major specific protein band of ~100 kDa for Na$^{+}$-K$^{+}$-ATPase and two major bands for V-ATPase at ~70 kDa. These are close to the predicted sizes for these transport proteins and suggest good cross-reactivity for these heterogeneous antibodies. It is possible that there exist two V-ATPase A-subunits with different but close molecular masses in rainbow trout, as is the case in humans (17) and chickens (13). In the immunocytochemistry using the antibody to Na$^{+}$-K$^{+}$-ATPase, a difference in the distribution of immunoreactivity was observed between the proximal and distal tubules. The difference may reflect the intensity and localization of the tubular system (basolateral membrane) of the cells, which can be observed with TEM, and supports the suggestion that Na$^{+}$-K$^{+}$-ATPase is located in the basolateral membrane of every cell in both proximal and distal tubules.

There have been 11 SLC26 genes identified, and 6 of them (A1, A2, A4, A6, A7, and A11) have been reported to be expressed in the mammalian kidney (34). Specifically, immunolocalization of SLC26A1 and A6 has been observed in the proximal tubules of the rat and mouse nephron (19, 23, 40). The expression of SLC26A1 anion exchanger mRNA was shown by Northern blot analysis to be solely expressed in trunk kidney in seawater-adapted rainbow trout. In situ hybridization showed specific localization of SLC26A1 anion exchanger mRNA in the PI segment of the nephron in both seawater and freshwater fish. This result is consistent with the previous reports in mammals as mentioned above.

The SLC26 anion exchanger family is known to transport a large number of monovalent and divalent anions. The previously cloned rat SLC26A1 expressed in *Xenopus* oocyte and SF9 cells demonstrated Na$^{+}$-independent sulfate and oxalate transport (4, 19). Although the sulfate concentration in seawater is ~25 mM, it has been reported that
plasma sulfate concentration is \(-0.2\) mM, and that in urine is \(~70\) mM, in the marine teleosts, the long-horned sculpin and southern flounder \((14, 30)\). Many previous studies have suggested that the proximal tubule excretes sulfate in marine teleosts \((2, 3, 5, 7, 32, 36, 38, 42–45)\); however, molecular mechanisms of sulfate excretion in teleosts have not been revealed. In the membrane vesicles of kidney tubules of southern flounder, Renfro and Pritchard \((44)\) showed that sulfate transport from interstitium to cell can be trans-stimulated by a basolateral membrane pH gradient and that it was an electroneutral, Hg- and DIDS-inhibitable process. In isolated renal tubules, sulfate secretion is Na\(^+\) gradient dependent, although there is no effect of a Na\(^+\) gradient in the basolateral membrane vesicles. The study in brush border vesicles also suggested the presence of an anion exchanger that secretes sulfate from the cell to the lumen, because the transport stimulated the counter ion gradient \((45)\). Pelis and Renfro \((38)\) reviewed the previous findings on teleost sulfate transport and proposed a hypothetical model of sulfate excretion in proximal tubules. They suggested that different sulfate/anion exchangers (sulfate and 2HCO\(_3\)-2/2OH\(-2/2Cl\(-/oxalate\) exist in the apical and basolateral membranes of the proximal tubules and that Na\(^+\)/H\(^+\) exchanger on either apical or basolateral membranes is required to prevent the tendency for intracellular acidification. In their model, Na\(^+\)-K\(^+\)-ATPase located on the basolateral membrane generates the Na\(^+\) gradient for Na\(^+\)/H\(^+\) exchange by Na\(^+\) transport into the interstitium. We also identified Na\(^+\)-K\(^+\)-ATPase in the basolateral membrane in the cells of proximal tubules, whereas we also detected the specific localization of V-ATPase in the brush-border membrane and subapical region. The Na\(^+\)-K\(^+\)-ATPase localization agrees with the proposed model in the review by Pelis and Renfro \((38)\). Similarly, the V-ATPase can transport protons from cytoplasm to lumen and is consistent with the proposed model in terms of function, that is, the excretion of protons to prevent the intracellular acidification and/or membrane depolarization (Fig. 9).

In this study, an acute increase in cloned SLC26A1 mRNA expression was detected at 3 h after freshwater rainbow trout were injected with excess sulfate. At the same time, it was observed that plasma sulfate concentration of injected rainbow trout reached at least six times higher than the normal plasma level \((0.45\) mM), and the excess plasma sulfate rapidly decreased to the initial level. These results strongly suggest the hypothesis that kidney-specific rainbow trout SLC26A1 is involved in sulfate excretion. In both seawater and freshwater, in situ hybridization showed the quite similar localization and expression of rainbow trout SLC26A1 in the present study. In the eel, the SLC26A1 has been cloned from kidney and localized to proximal tubules of the kidney \((35)\). Interestingly, in that study, the mRNA expression was greater in freshwater-than in seawater-adapted eels, and the authors suggested that the eel SLC26A1 is involved in sulfate reabsorption across the basolateral membrane, possibly contributing to osmoregulation in freshwater. However, it should be noted that SO\(_4\)\(^{2-}\) in plasma of freshwater eels is \(~37.5\) meq \((35)\), whereas that of freshwater rainbow trout was \(~0.36\) mM in this study.

In the mammalian kidney, reabsorption of filtered sulfate occurs primarily in the proximal tubules and is required for sulfate homeostasis in mammals \((16)\). Although the sulfate homeostasis in freshwater fish has been less demonstrated than that in seawater fish, the kidney of freshwater teleosts should be a site of net sulfate reabsorption because of the low levels of sulfate and limitation of drinking in freshwater environments \((38)\). Hence, the SLC26A1 anion exchanger expressed in the PI in freshwater rainbow trout might be involved in sulfate reabsorption. Interestingly, in some tubules identified as PI, no SLC26A1 signal was observed. This raises the interesting possibility that subtypes of cells exist within the segment, possibly transporting a variety of ions such as Na\(^+\), Cl\(^-\), Mg\(^{2+}\), SO\(_4\)\(^{2-}\), Ca\(^{2+}\), or HCO\(_3\) \((15)\). This is significant in that this is the first time it has been shown that cells in the PI may differentially express certain transporters.

**Perspectives**

SLC26 A1, sulfate/anion exchanger, was cloned from seawater-adapted rainbow trout, and excretion of excess plasma sulfate corresponded to the increase in the SLC26A1 expression. The exchanger was distributed specifically in the first segment of proximal tubules and colocalized with both Na\(^+\)-K\(^+\)-ATPase in basolateral membrane and V-ATPase in apical membrane. However, some PI segments without the signals also were observed. This finding suggests the existence of more than one type of mitochondria-rich cell in the first segment of proximal tubules in terms of function. These findings provide an important step to further our understanding of sulfate transport mechanism in fish renal tubules at the molecular level, the physical roles SLC26 anion exchangers in teleosts, and relatively unexplored kidney physiology in teleosts.

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