Evidence that SLC26 anion transporters mediate branchial chloride uptake in adult zebrafish (Danio rerio)

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Submitted 19 July 2009; accepted in final form 24 July 2009

FRESHWATER (FW) fish continually face ion losses at the gill and kidney. For Cl− absorption, two different mechanisms have been proposed, one involving electroneutral Cl−/HCO3− exchange (25, 26) and another involving a Na+/Cl− cotransporter (20, 70). Although a tight linkage between Cl− uptake and HCO3− excretion in FW fish has long been recognized (see historical review by Evans, Ref. 10), the specific nature of this linkage and the participating gene products remain unknown. Members of two gene families have been implicated in Cl−/HCO3− exchange in FW fish, the SLC4 bicarbonate transporters (1, 2, 54, 57) and the SLC26 anion transporters (37, 39, 56, 62, 63). The members of the SLC4 family exhibiting anion exchange activity are characterized by their narrow substrate preference for HCO3− (or CO3−) and Cl−. The involvement of SLC4A1 (AE1) in branchial Cl−/HCO3− exchange is supported by results of in situ hybridization (65) and immunocytochemistry (72) experiments although these results should be viewed cautiously because heterologous probes and antibodies were used. In contrast to SLC4, members of the SLC26 family typically exhibit broad specificity to a variety of anions (e.g., oxalate, sulfate, iodide, and formate in addition to Cl− and HCO3−), and some may even function as anion channels (e.g., SLC26A7 and SLC26A9) (7, 24). Currently, the SLC26 family contains 11 paralogs with three of these (A3, A4, and A6) identified as Cl−/HCO3− exchangers (8). Although SLC26A1 has been implicated in renal sulfate secretion in FW fish (22, 38) and SLC26A6 is thought to play a role in the intestinal secretion of HCO3− in marine species (15, 28), there is but scant evidence for an involvement of any SLC26 family member in branchial Cl−/HCO3− exchange. Specifically, Piermarini et al. (50) demonstrated SLC26A4 (pendrin)-like immunoreactivity in gills of FW Atlantic stingray (Dasyatis sabina) using antibodies raised against human pendrin.

Using data mining techniques, we recently demonstrated that the three SLC26 genes known to exhibit Cl−/HCO3− exchange activity (za3, za4, and za6) exist in the zebrafish (Danio rerio) genome, are expressed in early development, and that their mRNA expression appears to be regulated by environmental levels of Cl− or HCO3− (3). Furthermore, it was demonstrated that targeted gene knockdown of SLC26a3, A4, or A6 resulted in a reduction of Cl− uptake in zebrafish larvae (3). These results suggest a role for SLC26 anion transporters in Cl− uptake in larval zebrafish. The present study attempts to extend these findings to adult zebrafish by testing the hypothesis that za3, za4, and za6 are expressed in the adult gill and that their expression is regulated during alterations of ambient Cl− or HCO3− in a manner consistent with their presumed role in Cl− uptake.

MATERIALS AND METHODS

Experimental animals. Adult zebrafish (D. rerio; 150–400 mg) were obtained commercially from Big Al’s Aquarium Services (Ottawa, Canada). The fish are kept in plastic aquaria supplied with recirculating, filtered fresh water. Fish were maintained at 28°C on a 14:10-h light-dark photoperiod and were fed daily using a commercial pelleted fish diet (71). Groups of 5–8 fish were acclimated in several different waters for 7 days prior to experimentation. The different waters were as follows: dechlorinated system water (hereafter referred to as control; pH = 7.30), low- and high-Cl− waters (regenerated from reverse osmosis water, with final Cl− concentrations of 0.02 and...
2 mmol/l, respectively; pH = 7.30), and base water (dechlorinated system water supplemented with either 10 or 12.5 mmol/l NaHCO₃; pH = 7.85–7.93). The ionic composition of control water was Na⁺ = 0.8 mmol/l; Cl⁻ = 0.4 mmol/l; Ca²⁺ = 0.25 mmol/l; K⁺ = 0.03 mmol/l. Low- and high-Cl⁻ media were prepared by combining appropriate quantities of NaCl and with other salts as required (CaSO₄·2H₂O; NaSO₄; K₂HPO₄) in reverse osmosis water.

All experiments using live animals were performed according to institutional guidelines in accordance with the Canadian Council on Animal Care. Experimental procedures (protocol no. BL-226) were preapproved by the University of Ottawa Animal Care and Veterinary Service.

**RNA extraction and real-time RT-PCR.** Adult zebrafish were lightly anaesthetized with 0.05 mg/ml ethyl 3-aminobenzoate methanesulfonate (MS 222; Sigma) in control water until they stopped moving. The fish was then killed by removal of the head. For total RNA extraction, one complete fish was frozen in liquid nitrogen, ground on dry ice with a mortar and pestle, and stored at −80°C until sectioning. The gills were powdered on dry ice with a mortar and pestle, and stored at −80°C until needed.

Total RNA was extracted from 100 mg aliquots of powdered tissue samples (obtained from pooling tissue from 2–5 fish) using the Absolutely RNA RT-PCR Miniprep Kit (Stratagene). To remove any remaining genomic DNA, the RNA was treated on-column using RNAse-free DNase (5 µl) for 20 min at room temperature. The RNA was eluted in 50 µl of nuclease-free H₂O₂ and its quality was assessed by gel electrophoresis and spectrophotometry (Eppendorf Biophotometer). The cDNA was synthesized from 1 µg RNA by using random hexamer primers (Boehringer Mannheim) and Stratascript reverse transcriptase (Stratagene). Relative mRNA levels were assessed by multiplex quantitative PCR system. ROX (Stratagene) was used as reference dye. A Brilliant SYBR Green QPCR Master Mix Kit (Stratagene) and a Stratagene MX-4000 primers were used to amplify the inserted probes. Sense and antisense DIG-labeled RNA probes were synthesized by using 1 µg of purified PCR product in the in vitro transcription reaction by using the appropriate SP6 or T7 RNA polymerase (New England Biolabs) for 1 h at 37°C.

Fish were killed by anesthetic overdose, and the gills were removed and placed in 4% paraformaldehyde (PFA) (pH 7.4) at 4°C overnight. Samples were then placed in 15% sucrose for 2 h at room temperature following by 30% sucrose at 4°C until sectioning. The gills were embedded in OCT cryosectioning medium (VWR), incubated for 20 min, and sectioned horizontally (10-µm section) using a cryostat (Leica CM 1850). Tissue sections were placed on Superfrost Plus slide (VWR) and dried at room temperature for ~45 min prior to storage at −4°C until needed. Sections on slides were hydrated (2× 15 min) in 1× PBST (PBS with 0.1% Tween 20). Proteinase K (20 µg/ml in 1× PBST; GIBCO-BRL, Rockville, MD) was used to deproteinize samples for 20 min at room temperature. Following deproteination, samples were fixed in 4% formaldehyde (in PBS) for

**Probe design and in situ hybridization.** Primers were designed to produce probes of 748, 486, and 805 base pairs for za3, za4, and za6, respectively (see Table 1 in Ref. 3), using plasmid DNA as template. An aliquot of the PCR products was run on a 1.25% gel, and the rest was purified using a PCR purification kit (Sigma). The purified product was cloned in PCR II vector (Invitrogen), and the desired clone was purified using a plasmid miniprep purification kit (Sigma). The purified plasmid DNA was sequenced using M13 forward (−20) and M13 reverse primers to confirm identity and determine the orientation of the cloned sequence within the vector. To obtain linear DNA flanked by SP6 and T7 promoters, M13 forward (−20) and M13 reverse primers were used to amplify the inserted probes. Sense and antisense DIG-labeled RNA probes were synthesized by using 1 µg of purified PCR product in the in vitro transcription reaction by using the appropriate SP6 or T7 RNA polymerase (New England Biolabs) for 1 h at 37°C.

Fig. 1. The relative abundance of mRNA for SLC26a3, a4, and a6 (za3, za4, and za6) in selected tissues of adult zebrafish (Danio rerio). Data are expressed relative to levels in whole body adult tissue. Data are shown are means ± 1 SE, n = 6; *statistical differences from the adult levels (assigned a value of 1) were determined using a 1-sample t-test (P < 0.05).
5 min. Fixed tissues were subsequently rinsed twice (10 min per wash) with 1× PBST and air dried at 60°C for 15 min. Hybridization buffer [100 μl of 4× SSC, 20% dextran sulfate, 50% formamide, 250 μg/ml poly(A)−, 250 μg/ml ssDNA, 0.1 mol/l DTT, 250 μg/ml tRNA, 0.5× Denhardt’s solution] was added to each probe. Each probe was then mixed well by vortexing and placed onto sections. Hybridization was performed for 16–48 h at 57°C in a humid chamber. Following hybridization, sections were washed twice (15 min per wash, 58°C) with 2× SSC and twice (15 min per wash, 58°C) with 0.2× SSC, followed by one wash in 0.1× SSC for 10 min at room temperature and two washes in 0.1× PBS (10 min per wash, room temperature). To detect hybridization, sections were incubated for 1 h at room temperature with 1% goat serum, 2 mg/ml BSA in 0.1 mol/l PBS with 0.3% Triton X-100, followed by overnight incubation at 4°C in anti-digoxigenin antibody conjugated to alkaline phosphatase (1:1,000 dilution; Roche Molecular Biochemicals, Temecula, CA). Slides were washed at room temperature in 0.1 mol/l phosphate buffer for 15 min and then briefly rinsed in water. The slides were next washed twice (5 min per wash) in coloration buffer (100 mmol/l Tris pH 9.5, 50 mmol/l MgCl2, 100 mmol/l NaCl, 0.1% Tween 20). Nitroblue tetrazolium and a single 5-bromocresyl-3-indolyl phosphate tablet (Sigma-Aldrich) were dissolved in 10 ml of H2O and layered over the sections. Color was allowed to develop in a humid chamber at room temperature for at least 4 h or until satisfactory coloration was observed. The slides were then washed twice with 0.1 mol/l PBS (15 min per wash). Coverslips were placed on the slides using 60% glycerol as mounting medium.

Once prepared, all specimens were observed and photographed using a Zeiss Axioskop microscope (Zeiss, Jena, Germany) equipped with a Hamamatsu C5985 chilled, charge-coupled device camera, using Metamorph imaging software 4.01 (Molecular Devices, Downingtown, PA).

**Antibodies and immunocytochemistry.** Gill tissues were fixed in 4% PFA, cryo-protected in sucrose, and sectioned as described previously. A polyclonal affinity-purified rabbit primary antibody was produced (Abgent) against a synthetic peptide (AEQHERINRKRKTLR) corresponding to AA’s 21–35 of zSLC26a3 (GenBank accession no. ACI05561). The specificity of this antibody against za3 was demonstrated in a previous publication (3). Sections were incubated overnight at 4°C with the za3 antibody (1:100) and α5 (1:100), a mouse monoclonal antibody against the α1-subunit of chicken Na+/K+-ATPase (University of Iowa Hybridoma Bank). For negative controls, sections were incubated with 1× PBS buffer lacking primary antibodies. Immunofluorescence was detected after incubating the sections for 1 h at room temperature with a 1:400 dilution of either Alexa-Fluor 546 coupled to goat anti-mouse IgG or Alexa-Fluor 488.
coupled to goat anti-rabbit (Fisher, Ottawa, ON, Canada). Following a 3 × 10 min wash in 0.1× PBS, sections were mounted in Vectashield mounting medium (Vector Labs) and coverslipped.

**Chloride influx measurements.** An adult zebrafish was rinsed in distilled water and then placed in 11 ml of control (normal Cl⁻) water. After a 30-min acclimation period, 0.1 µCi of Cl-36 (Amersham) was added to the water. The flux experiment was allowed to proceed for 1 h. Water samples were taken at the beginning and end of the experiment for measurements of water Cl⁻ concentrations using a microplate modification of a standard colorimetric Cl⁻ assay (73) and Cl-36 radioactivity (Beckman liquid scintillation counter). At the end of the flux period, the fish were rinsed with 1 M NaCl solution, and then anesthetized with 0.05 mg/ml MS 222. After the weights were determined, the fish were killed and individually flash frozen in liquid nitrogen. Manually pulverized zebrafish were digested overnight in 8% perchloric acid. The resulting homogenates were vortexed briefly and centrifuged prior to analysis of whole body Cl-36 activity (WBA). Cl⁻ influx (JinCl⁻; in nmol·g⁻¹·h⁻¹) was calculated according to the following formula: JinCl⁻ = WBA (CPM g⁻¹)/time (h)/water specific activity (CPM nmol⁻¹).

**Determination of whole body chloride concentrations.** Fish from control water, low Cl⁻ water, and base water groups were anesthetized with 0.05 mg/ml MS 222 in dechlorinated system water and then killed. These fish were subsequently blotted dry, wrapped in aluminum foil, and submerged in liquid nitrogen until frozen. Manually pulverized fish were transferred to prepared vials and weighed. Samples were digested overnight in 1 ml of 8% perchloric acid, and the resulting homogenates were vortexed briefly and centrifuged prior to analysis of Cl⁻ levels using a spectrophotometric method (73) modified for microplate use.

**Acid/base excretion measurements.** Adult zebrafish (200–700 mg) were acclimated for 30–45 min in constantly aerated 8 ml of control water in a plastic scintillation vial (20 ml). Water samples (1.2 ml) were removed at time 0 and 3 h later. Net acid-base flux (JNETH⁺) was determined from measurements of titratable net acid flux (JNETTA) and the change in ammonia concentration in these water samples. JNETTA was determined by titrating 1-ml water samples from the beginning and end of each flux period to pH 4.00 with 0.02 mol/l HCl and considering the difference in titrant added. Samples were continuously aerated during titration to ensure mixing and removal of CO₂ (see Ref. 34). Total ammonia in the water samples was analyzed using a micromodification of the salicylate-hypochlorite colorimetric assay of Verdouw et al. (69). JNETH⁺ was then calculated as the sum of JNETTA, and the ammonia flux (JNETNH₃) signs considered, as described by McDonald and Wood (34).

**Statistical analysis.** Data are shown as means ± 1 SE. Relative mRNA expression data were analyzed using a one-sample t-test. Comparisons between two groups were performed using unpaired t-tests, and comparisons between more than two groups were performed using one-way ANOVA followed by Bonferroni t-tests, where appropriate; the statistical level of significance was set at 5%.

**RESULTS**

SLC26 mRNA was detected in all tissues that were analyzed, although levels in some tissues (brain, liver) were uniformly low for all three genes (Fig. 1). The gill expressed relatively high levels of za6 mRNA; za3 or za4 mRNA, while present, was less abundant. Also, za4 and za6 were expressed at relatively high levels in the kidney, while za4 was highly expressed in the heart.

Within the gill, cells enriched with SLC26 mRNA were located predominantly on the filament epithelium especially in the interlamellar regions (Figs. 2 and 3) and less frequently on the lamellar epithelium at the bases of lamellae (Fig. 2). This cellular distribution of za3 was confirmed using immunocytochemistry (Fig. 2D). Interestingly, however, only a relatively small proportion of the za3 positive cells coexpressed Na⁺/K⁺-ATPase (NKA; Fig. 3D). In situ staining or immunofluorescence was not observed when sections were treated with sense probes (Figs. 2C and 3C) or when primary antibody was omitted, respectively.
Maintaining fish in water containing low levels of Cl\(^{−}\) for 10 days resulted in marked increases in the expression of SLC26 mRNA levels (Fig. 4A). The za3 mRNA was increased 40−80 times between 5 and 10 days, za4 was increased 20−30 times at 5 and 7 days, and za6 was increased about 30 times at 7 and 10 days. Exposure of fish to water containing high ambient Cl\(^{−}\) levels was without effect (Fig. 4B). Fish kept in water with elevated NaHCO\(_3\) also exhibited time-dependent increases in SLC26 mRNA expression between 3 and 10 days of exposure (Fig. 5A) that was associated with increased rates of Cl\(^{−}\) uptake (Fig. 5B) when measured in control (normal Cl\(^{−}\)) water. Net acid flux, when assessed in control water, was significantly reduced (equivalent to an increase in net base excretion) in fish exposed for 10 days to elevated NaHCO\(_3\) (Fig. 6C) owing to reduced (albeit not statistically significant) net titratable alkalinity flux (Fig. 6C), a result that is consistent with increased rates of Cl\(^{−}/\)HCO\(_3\) exchange. Whole body Cl\(^{−}\) and high HCO\(_3\)\(^{−}\)-exposed fish, respectively.

DISCUSSION

The results of this study, demonstrating the presence of SLC26 anion transporter mRNA and protein (SLC26a3 only) in the gill and their apparent regulation by ambient Cl\(^{−}\) levels, provide indirect evidence for the involvement of this gene family in Cl\(^{−}\) uptake in adult zebrafish (D. rerio). On the basis of their restricted localization to cells on the filament and at the bases of lamellae as well as the correspondence between transporter mRNA levels, Cl\(^{−}\) uptake rates, and net acid excretion, we suggest that three paralogs of the SLC26 family (za3, za4, and za6) are expressed in mitochondrion-rich cells where they function as Cl\(^{−}/\)HCO\(_3\)\(^{−}\) exchangers. To our knowledge, this is only the second study to implicate SLC26 anion transporters in Cl\(^{−}\) uptake in FW fish. Previously, Piermarini et al. (50) provided immunocytochemical evidence for the participation of SLC26a4 (pendrin) in Cl\(^{−}\) uptake in FW Atlantic stingray (Dasyatis sabina).

Mitochondrion-rich cells express Cl\(^{−}/\)HCO\(_3\)\(^{−}\) exchangers in zebrafish gills. In addition to recent compelling and direct evidence obtained using isolated gill cells of rainbow trout (40), there is considerable correlative evidence implicating MRCs as sites of Cl\(^{−}/\)HCO\(_3\)\(^{−}\) exchange in FW fish. First, the rate of Cl\(^{−}\) uptake in FW teleosts is positively correlated with the apical surface area of mitochondrion-rich cells (MRC) apical membranes (30, 31, 44, 46). Second, treatments known to modify rates of Cl\(^{−}\) uptake (e.g., inhibition using thiocyanate or stimulation by elevated ambient Cl\(^{−}\) levels) were shown to specifically and appropriately alter the intracellular concentration of Cl\(^{−}\) in MRCs (35, 36). Third, the surface area of exposed MRC apical membranes is increased in brown bullhead (Ictalurus nebulosus) (13) or rainbow trout (14, 43) during metabolic alkalosis, a response presumably aimed at increasing rates of Cl\(^{−}\) uptake and net base excretion during metabolic compensation of the alkalosis. Thus, the conclusion of the present study that SLC26 anion transporters are restricted to the MRCs of zebrafish is entirely consistent with previous observations.

In zebrafish (19) and other species including rainbow trout (11, 12), killifish (Fundulus heteroclitus) (29), and tilapia (Oreochromis mossambicus) (16, 51), a variety of MRC subtypes have been identified on the basis of their ultrastructure or...
the composition and distribution of ion transporters (52; see also Table 1 in Ref. 19). At least three subtypes exist on the yolk sac of larval zebrafish (18) and presumably on the gills of adults; MRCs enriched with NKA (termed NaR cells), MRCs expressing Na\(^+\)-Cl\(^-\) cotransporters (termed NCC cells), and those enriched with V-type H\(^+\)-ATPase (termed HR cells). Because activity of V-type H\(^+\)-ATPase is likely to play an integral role in Cl\(^-\)/HCO\(_3^-\) exchange (see below), it is possible that the SLC26 anion transporters are restricted to the HR MRCs. In rainbow trout, MRCs have been subdivided into two groups according to their abilities to bind peanut lectin agglutinin (PNA). MRCs negative for PNA (termed PNA\(^-\) cells) are thought to be Na\(^+\)-absorbing acid-secreting cells (55) possessing apically bound V-type H\(^+\)-ATPase (termed HR cells). By analogy, we expect that the NaR cells may be one of the MRC subtypes expressing SLC26 anion transporters in the zebrafish gill. However, because SLC26a3 protein expression was observed in NKA-enriched and NKA-poor cells (Fig. 2), it is likely that the SLC26 transporters are localized to more than one MRC subtype, potentially in both NaR and HR MRCs. In rainbow trout, it was recently proposed that the PNA\(^+\) MRCs contain an apical membrane Cl\(^-\)/HCO\(_3^-\) exchanger, while the PNA\(^-\) MRCs house Cl\(^-\)/HCO\(_3^-\) exchangers on the basolateral membranes. The cellular distribution of SLC26 transporters in zebrafish gill will need to be verified through future research utilizing zebrafish-specific V-type H\(^+\)-ATPase antibodies. Interestingly, and in contrast to existing models, Ivanis et al. (21) concluded that Na\(^+\)/H\(^+\) exchanger isoform 3 resides within PNA\(^-\) cells rather than the PNA\(^+\) cells that are presumed to be the sites of Na\(^+\) uptake in rainbow trout.

The ability of an electroneutral Cl\(^-\)/HCO\(_3^-\) exchanger to function on the apical membrane of MRCs in FW fish is thermodynamically constrained by an unfavorable Cl\(^-\) gradient that is unlikely to be overcome (at least at the macroscopic level) by the modest prevailing HCO\(_3^-\) gradient (41, 68). In this regard, the involvement of V-type H\(^+\)-ATPase to energize Cl\(^-\) uptake can be envisaged in two different ways. First, as discussed by Tresguerres et al. (68), cytosolic carbonic anhydrase (32) working in concert with a V-type H\(^+\)-ATPase (to prevent acid accumulation) may serve to create microenvironments enriched with HCO\(_3^-\) as CO\(_2\) is hydrated in close proximity to the apical membrane of MRCs.
proximity to apical membrane Cl⁻/HCO₃⁻ exchangers. For this scheme to work with a basolateral localization of the V-type H⁺-ATPase, it is essential that the basolateral membranes and apical membranes are near each other, which indeed is a likely scenario in the NaR MRCs, because the basolateral membranes are highly infolded and extend throughout the cytoplasm (23). The resultant high levels of HCO₃⁻ formed from CO₂ hydration could serve to drive electroneutral Cl⁻/HCO₃⁻ exchange, while the H⁺ could be removed from the cell by basolateral H⁺ secretion via the V-type H⁺-ATPase. A second potential mechanism linking V-type H⁺-ATPase and Cl⁻/HCO₃⁻ exchange at the gill is the pumping of H⁺ across the apical membrane into the water, which could serve to lower HCO₃⁻ levels in an external microenvironment to potentially enlarge the outwardly directed HCO₃⁻ gradient (33). Similarly, Boisen et al. (4)

suggested that an apically positioned V-type H⁺-ATPase could serve to increase intracellular HCO₃⁻ levels that would also serve to enhance the HCO₃⁻ gradient. Of these various possibilities, the former seems more likely, especially considering that analogous cell types in mammals (e.g., base-secreting intercalated cells of the cortical collecting duct) are known to express apical membrane SLC26A4 and basolateral V-type H⁺-ATPase (58, 64).

An additional consideration with respect to SLC26A6 is that the teleost fish paralogs characterized so far, appear to be electrogenically operating in an nHCO₃⁻/Cl⁻ mode (14, 26). This transport stoichiometry would be favorable for Cl⁻/HCO₃⁻ exchange across the apical membrane, because the inside negative membrane potential would fuel the activity of nHCO₃⁻/Cl⁻ exchange by SLC26a6. The activity of a V-type H⁺-ATPase would act to hyperpolarize the gill cell membrane and thus anion exchange via SLC26a6, an effect that would be most pronounced with an apical localization of the V-type H⁺-ATPase (14). However, while the presence of an electronegic SLC26a6 and V-type H⁺-ATPase in the apical membrane would provide a very powerful system for Cl⁻ uptake from low ambient Cl⁻ concentrations, it would effectively short-circuit the capacity of these cells to participate in acid-base regulation, because it would not be possible to transfer net acid (or net base) to the external environment (68).

SLC26 transporter expression during exposure to low ambient Cl⁻ or elevated HCO₃⁻. Zebrafish exhibit a remarkable capacity to tolerate ion-poor water owing to their high affinity and high capacity (JMAX) ion uptake mechanisms (4). Indeed, the affinity constant (Kₘ) for Cl⁻ uptake in zebrafish acclimated to soft water (~40 μM NaCl) was reported to be only 8 μM, which is the lowest Kₘ ever reported for a fish (4). The results of the present study suggest that the low Kₘ and high JMAX for Cl⁻ uptake in zebrafish acclimated to low Cl⁻ water may reflect increased branchial expression of SLC26 anion transporters. However, given that SLC26 transporters normally exhibit Km8 for Cl⁻/HCO₃⁻ exchange in the low micromolar per liter range (e.g., Ref. 59), it is unclear as to how they would function at low environmental Cl⁻ levels. One possibility [see also Grosell et al. (15)] is that one or more of the zebrafish SLC26 Cl⁻ transporters exhibit unusually low Km8 compared with orthologous mammalian genes. Interestingly, base excretion was not elevated in fish after their return from low Cl⁻ water to normal water, despite increased rates of Cl⁻ uptake (4). It is conceivable that increased rates of Cl⁻/HCO₃⁻ exchange were being matched by similar increases in the rate of Na⁺/H⁺ exchange (or some other Na⁺ uptake mechanism linked to H⁺ excretion) so as to prevent changes in blood acid-base status. Clearly, this is an area that merits further research.

Zebrafish were exposed to elevated external HCO₃⁻ based on results obtained using rainbow trout in which similar treatment was used to inhibit Cl⁻ uptake and induce metabolic alkalosis (45). The expectation was that fish experiencing an extended period of compromised Cl⁻ uptake (owing to a diminished outwardly directed HCO₃⁻ gradient), would compensate and thus exhibit increased rates of Cl⁻ uptake and base excretion upon return to normal water. Indeed, upon the return of fish to normal water after 10 days exposure to elevated HCO₃⁻, JinCl⁻ and JNETTA were increased and decreased (i.e., base excretion was increased), respectively, although it is notable that the
increase in $J_{\text{net TA}}$ was substantially less than the change in $J_{\text{NET TA}}$, a difference that is difficult to reconcile. Notwithstanding the discrepancy in the magnitude of the changes in $J_{\text{inCl}}$ and $J_{\text{NET TA}}$, the effects of high- $\text{HCO}_3^-$ exposure are consistent with the concomitant increased expression of mRNA for SLC26 transporters. Taken together, the results of these experiments suggest that transcriptional regulation of SLC26 transporters may be an important mechanism for modulating rates of Cl$^-$/ $\text{HCO}_3^-$ exchange during environmental perturbation.

Other candidates for Cl$^-$/ $\text{HCO}_3^-$ exchange in FW fish. The presence of SLC26 anion transporters in the gill of zebrafish does not exclude the possible involvement of other genes in Cl$^-$ uptake. Indeed, prior to the demonstration of pendrin (SLC26a4)-like immunoreactivity on the gill of Atlantic stingray (50), it was generally held that branchial Cl$^-$/ $\text{HCO}_3^-$ exchange was accomplished by one or more members of the SLC4 gene family. This conclusion was supported by the results of studies utilizing immunocytochemistry (67, 72) or Western blot analysis (66), in situ hybridization (65), and data obtained from pharmacological studies employing the Cl$^-$ blocker (66, 69, 45, 47, 53). However, upon reinterpretation, the conclusions from some of these studies are questionable. For example, the 24-mer in situ probe used by Sullivan et al. (65), while homologous with rat AE1 [SLC4A1; (27)] shares 63–71% sequence identity with zA3, zA4, and zA6 and thus may have hybridized to multiple mRNA targets, including members of the SLC26 gene family. In addition, disulfonic stilbene derivatives, such as SITS and DIDS, are not specific blockers of SLC4 anion exchangers but also inhibit members of the SLC26 gene family (64). Finally, the antibody used by Wilson et al. (72) was generated against denatured rainbow trout red blood cell SLC4A1 protein (5), and thus its specificity for SLC4A1 vs. other SLC4 and SLC26 proteins is uncertain. Although AE1 was recently identified in gill MRCs of pufferfish (Tetraodon nigroviridis) using an antibody generated against tilapia (67), its localization to the basolateral membrane suggests a role for HCO$_3^-$ reabsorption rather than transepithelial Cl$^-$ uptake. Another member of the SLC4 family, SLC4A2 (AE2), although present in zebrafish kidney (60, 61) has not been identified in the gills of any fish species. A candidate for Cl$^-$ uptake in FW fish is the Na$^+$/Cl$^-$ cotransporter, which was recently shown by elegant immunocytochemistry to reside on the apical membrane of type-II (FW type) MRCs in tilapia (Oreochromis mossambicus) (17) and a novel type of MRC in zebrafish larvae (70).

**Perspectives**

Although more than 70 years have passed since the seminal studies of Krogh (25, 26) gave rise to the concept of Cl$^-$/ $\text{HCO}_3^-$ exchange in fish gills, a great deal of uncertainty still surrounds the underlying molecular mechanisms. The results of the present and previous studies indirectly implicating SLC4 and SLC26 transporters in Cl$^-$/ $\text{HCO}_3^-$ exchange now need to be confirmed using more direct approaches. However, until functional gene knockout models become more routinely available for use in fish, this will remain a challenging proposition. Additional experiments are also required to elucidate the role of V-type H$^+$/ATPase in facilitating Cl$^-$/ $\text{HCO}_3^-$ exchange.

In addition to the potential involvement of SLC4 and SLC26 gene family members, Na$^+$/Cl$^-$ cotransporters (members of the SLC12 gene family) are also implicated in Cl$^-$ uptake in FW fish (17, 70). The apparent redundancy of Cl$^-$ uptake mechanisms is not only testimony to the importance of ionic homeostasis but also suggests an evolutionary divergence of Cl$^-$ uptake mechanisms with only some being linked to acid-base regulation. With such redundancy of mechanisms, is it conceivable that fish could adjust the rates of Cl$^-$/ $\text{HCO}_3^-$ exchange to compensate an acid-base disturbance without compromising overall Cl$^-$ uptake. For example, a decrease in the rate of Cl$^-$/ $\text{HCO}_3^-$ exchange to correct metabolic acidosis could be accompanied by an equivalent increase in the rate of Cl$^-$ uptake via Na$^+$/Cl$^-$ cotransport. The relative roles of these various putative Cl$^-$ uptake pathways in the FW fish gill should be a focus of future research.

**ACKNOWLEDGMENTS**

We are grateful to Vishaal Saxena for excellent technical assistance.

**GRANTS**

This work was financially supported by National Sciences and Engineering Research Council Discovery and Research Tools and Infrastructure Grants (to S. F. Perry) and National Science Foundation Grant I0B-0743903 (to M. Grosell).

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