# Ion-Deficient Environment Induces the Expression of Basolateral Chloride Channel, ClC-3-Like Protein, in Gill Mitochondrion-Rich Cells for Chloride Uptake of the Tilapia *Oreochromis mossambicus*

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## ABSTRACT

Gill mitochondrion-rich (MR) cells contain different molecules to carry out functionally distinct mechanisms. To date, the putative mechanism of $Cl^-$ uptake through the basolateral chloride channel, however, is less understood. To clarify the $Cl^-$-absorbing mechanism, this study explored the molecular and morphological alterations in branchial MR cells of tilapia acclimated to seawater (SW), freshwater (FW), and deionized water (DW). Scanning electron microscopic observations revealed that three subtypes of MR cells were exhibited in gill filament epithelia of tilapia. Furthermore, in DW-acclimated tilapia, the subtype I (ion-absorbing subtype) of MR cells predominantly occurred in gill filament as well as lamellar epithelia. Whole-mount double immunofluorescent staining revealed that branchial ClC-3-like protein and $Na^+/K^+$-ATPase (NKA), the basolateral marker of MR cells, were colocalized in tilapia. In SW-acclimated tilapia, all MR cells of gill filament epithelia exhibited faint fluorescence of ClC-3-like protein. In contrast, only some MR cells in gill filament epithelia of FW and DW tilapia expressed basolateral ClC-3-like protein; however, the fluorescence was more intense in FW and DW tilapia than in SW fish. In hyposmotic groups, the number of MR cells immunopositive for ClC-3-like protein was significantly higher in DW-exposed tilapia. Meanwhile, in gill lamellar epithelia of DW tilapia, all MR cells (subtype I) were ClC-3-like protein immunopositive. Double immunostaining of ClC-3-like protein and $Na^+/Cl^-$ cotransporter (NCC) revealed that basolateral ClC-3-like protein and apical NCC were colocalized in some MR cells in FW and DW tilapia. Moreover, both mRNA and protein amounts of branchial ClC-3-like protein were significantly higher in DW-acclimated tilapia. To identify whether the expression of branchial ClC-3-like protein responded to changes in environmental $[Cl^-]$, tilapia were acclimated to artificial waters with normal $[Na^+]/[Cl^-]$ (control), lower $[Na^+]$ (low Na), or lower $[Cl^-]$ (low Cl). Immunoblotting of crude membrane fractions for gill ClC-3-like protein showed that the protein abundance was evidently enhanced in tilapia acclimated to the low-Cl environment compared with the other groups. Our findings integrated morphological and functional classifications of ion-absorbing MR cells and indicated that ion-deficient water elevated the numbers of subtype I MR cells in both filament and lamellar epithelia of gills with positive ClC-3-like protein immunostaining and increased the expression levels of ClC-3-like protein. This study is the first to illustrate the exhibition of a basolateral chloride channel potentially responsible for $Cl^-$ absorption in the ion-absorbing subtype of gill MR cells of tilapia.

## Introduction

In animals, $Cl^-$ is the most abundant anion in intra- and extracellular environments. Chloride channels play multiple roles in diverse processes, such as regulation of transepithelial transport, cell volume regulation, cellular excitability, synaptic transmission, acidification of intracellular organelles, and blood pressure regulation (Devuyst and Guggino 2002; Jentsch et al. 2002; Nilius and Droogmans 2003). These functions are facilitated by various chloride channels that are encoded by genes belonging to several families (Jentsch et al. 2002). Moreover, chloride channels are involved in $Cl^-$ transepithelial transport in many types of cells. In the kidney, most of the filtered $Cl^-$ is reabsorbed through different mechanisms in tubular epithelial cells (Devuyst and Guggino 2002). Furthermore, basolateral chloride channels modulate net $Cl^-$ efflux from the cells participating in $Cl^-$ reabsorption in nephrons (Blaisdell and Guggino 2000; Devuyst and Guggino 2002). In general, chloride channels accomplishing the reabsorbing function are involved in cell volume regulation through swelling-activated channels when cells are exposed to a hyposmotic environment (Lang et al. 1998; Devuyst and Guggino 2002). These mechanisms are important for maintaining osmotic and ionic homeostasis.

The fish gill is the major organ responsible for both iono- and osmoregulation and is one of the most important experimental models for studying transepithelial transport physiology (Evans et al. 2005). Among the gill epithelial cells, mitochondrion-rich (MR) cells occupy only a small fraction, but they are considered to be the primary sites responsible for ion transport (Hirose et al. 2003; Evans et al. 2005; Hwang and

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Lee 2007). In addition, the primary active enzyme, NKA, is important for providing the driving force for secondary transport systems to transport ions across plasma membranes in various cell types, including branchial MR cells (Hirose et al. 2003; Evans et al. 2005; Hwang and Lee 2007). Immunocytochemical studies demonstrated that NKA is localized to the basolateral membrane of MR cells in fish (reviewed by Hwang and Lee 2007). In tilapia, all NKA immunoreactive (NKIR) cells were demonstrated to be MR cells (Uchida et al. 2000; Lee et al. 2003; Hiroi et al. 2008).

Branchial MR cells of tilapia are generally distributed in filament epithelia (Kaneko et al. 2008; Tang et al. 2008). When tilapia are exposed to ion-deficient water—that is, deionized water (DW)—however, MR cells were found in both filament and lamellar epithelia of the gills (Tang et al. 2008). Furthermore, tilapia MR cells change in size, density, ultrastructure of the apical membrane, and localization of ion transporters in response to alterations of the environment's salinity (Uchida et al. 2000; Lee et al. 2003; Inokuchi et al. 2008, 2009; Kaneko et al. 2008; Tang et al. 2008). In FW-acclimated tilapia, three subtypes of MR cells were observed and named according to their apical ultrastructures: wavy-convex (subtype I), shallow-basin (subtype II), and deep-hole (subtype III), whereas only subtype III MR cells were found in the gill epithelium of seawater (SW) tilapia (Lee et al. 1996, 2000, 2003; Hwang and Lee 2007). Moreover, different approaches were used to illustrate the major functions of subtypes I, II, and III, which might be $Na^+$ and $Cl^-$ uptake, $Na^+$and $Ca^{2+}$ uptake, and $Cl^-$ secretion, respectively (Chang et al. 2001, 2003; Hwang and Lee 2007; Inokuchi et al. 2009).

One of the major ionoregulatory functions of the MR cells in SW-acclimated teleosts is to secrete $Cl^-$, and the mechanisms for this are well known. In contrast, except for identical basolateral localization of NKA, the $Cl^-$-uptake mechanism of MR cells is controversial (Evans et al. 2005; Hwang and Lee 2007; Evans 2008). In Mozambique tilapia, Hiroi et al. (2008) and Inokuchi et al. (2008, 2009) recently demonstrated that the apical NCC was involved in $Cl^-$ uptake. In addition to the apical $Cl^-$-uptake transporter, to transport $Cl^-$ out of the cell, a basolateral chloride channel is required. The basolateral exit step for $Cl^-$ in teleosts, however, is less studied (Hirose et al. 2003; Hwang and Lee 2007; Evans 2008). Concerning the basolateral $Cl^-$ channel, members of the CLC chloride channel family, which are highly expressed in osmoregulatory organs, are thought to be the candidates (Miyazaki et al. 1999; Hirose et al. 2003).

In mammals, the CLC family has nine known members that differ in tissue distribution or subcellular localization with broad physiological functions, including transepithelial $Cl^-$ transport and cell volume regulation (Jentsch et al. 2005). Of the CLC family members, ClC-3 was demonstrated to be activated by cell swelling due to hyposmotic stimulation (Duan et al. 1997, 1999). Moreover, in diverse cell types, ClC-3 was reported to represent a major molecular entity responsible to native volume-sensitive outwardly rectifying anion channels in hyposmotic medium (Wang et al. 2000; Duan et al. 2001; Hermoso et al. 2002; Vessey et al. 2004). Importantly, Vessey et al. (2004) found that the hyposmotic activation increased ClC-3 trafficking to the plasma membrane in rabbit nonpigmented ciliary epithelial cells. In fish, cDNA encoding the ClC-3 gene have been cloned and detected in various osmoregulatory organs including the gills of Mozambique tilapia (Miyazaki et al. 1999). Recently, branchial ClC-3-like protein was verified to be expressed in the basolateral membrane of puffer fish NKIR cells, and its protein amounts in the gill membrane fraction was higher in FW than in SW (Tang and Lee 2007; Tang et al. 2010). Taken together, these findings implied the involvement of ClC-3 in $Cl^-$ regulation of tilapia gill MR cells on hyposmotic challenge. Evidence of expression and localization, however, is required.

The primary goal of this study was to examine the morphological and functional classification of gill MR cells in tilapia to elucidate the potential mechanism for $Cl^-$ uptake in the basolateral membrane. Hence, we used different approaches to shed light on the molecular mechanism of the basolateral chloride channel ClC-3 in MR cells with special relation to alterations in their apical ultrastructures in tilapia acclimated to SW, FW, and DW. The effects of external $[Na^+]$ or $[Cl^-]$ on branchial ClC-3-like protein expression were also examined to reveal the significance of gill ClC-3-like protein in an extreme low-$[Cl^-]$ environment rather than a low-$[Na^+]$ environment. This is the first study to identify the basolateral chloride channel in a particular subtype of gill MR cells of tilapia that is potentially responsible for $Cl^-$ absorption.

## Material and Methods

### *Fish and Experimental Environments*

Tilapia (*Oreochromis mossambicus*) weighing 5–9 g were obtained from laboratory stocks. Fish were reared in a tank with 300 L of aerated local tap water (FW; $[Na^+]$ 2.6 mM, $[Cl^-]$ 0.2 mM, $[Ca^{2+}]$ 0.58 mM) at 27° ± 1°C with a daily 12-h photoperiod. SW (35‰; $[Na^+]$ 582.86 mM, $[Cl^-]$ 520.84 mM, $[Ca^{2+}]$ 15.75 mM) was prepared from local tap water with proper amounts of the synthetic sea salt Instant Ocean (Aquarium Systems, Mentor, OH). DW was prepared with a Milli-RO60 (Millipore, Billerica, MA). DW, however, was devoid of detectable levels of ions ($Na^+$, $Cl^-$, and $Ca^{2+}$). The water was continuously circulated through fabric-floss filters. Two-thirds of DW was changed every 2 d for maintenance of water quality. The parameters of the water were identical to our previous experiments (Lin et al. 2004; Tang et al. 2008). The facilities and protocols for the experimental fish were approved by the Animal Care and Utility Committee of National Chung Hsing University (approval no. 96-48).

### *Acclimation Experiments*

Before the salinity acclimation, tilapia were held in FW for 2 wk to adjust to the exposure tanks. Fish were then divided into three groups and acclimated to environments of different salinities, that is, SW, FW, and DW for 1 wk. When tilapia were

Table 1: Environmental ion concentrations

| | Nominal Value (mM) | | | Measured Value (mM) | | |
|---|---|---|---|---|---|---|
| Environment | $[Na^+]$ | $[Cl^-]$ | $[Ca^{2+}]$ | $[Na^+]$ | $[Cl^-]$ | $[Ca^{2+}]$ |
| Control | 1.0 | 1.0 | .5 | .94 ± .02 | 1.13 ± .08 | .48 ± .04 |
| Low Na | .0 | 1.0 | .5 | <.01 | .98 ± .05 | .51 ± .03 |
| Low Cl | 1.0 | .0 | .5 | 1.16 ± .04 | <.01 | .54 ± .05 |

directly transferred from FW to full-strength SW (35‰), fish died within 6 h posttransfer (Hwang 1987). To avoid the mortality in SW acclimation, tilapia were first exposed to 20‰ brackish water for 24 h and then transferred to full-strength SW (35‰). Fish used for salinity acclimation experiments were fed daily with commercial pellets except for the 2 d before the following analyses of tilapia ClC-3, including scanning electron microscopic (SEM) observation, immunoblotting, reverse transcriptase polymerase chain reaction (RT-PCR), real-time PCR, and immunofluorescent staining.

On the other hand, to determine the effects of environmental $[Cl^-]$ on branchial ClC-3-like protein expression, FW-acclimated tilapia were exposed to artificial water with different ionic concentrations (artificial-water-acclimation experiments): (1) the control group: normal $[Na^+]$/normal $[Cl^-]$; (2) the low-Na group: low $[Na^+]$/normal $[Cl^-]$; and (3) the low-Cl group: normal $[Na^+]$/low $[Cl^-]$. Fish were kept in 45-L plastic tanks containing the respective media for 1 wk at 27° ± 1°C. Half the water was changed every other day to maintain optimal water quality. Artificial waters for these three groups were prepared by dissolving appropriate amounts of NaCl, $Na_2SO_4$, $CaCl_2$, and $CaSO_4$ in DW according to the method described in previous studies with little modification (Chang et al. 2003; Chang and Hwang 2004; Inokuchi et al. 2009; Tang et al. 2010). The pH of the mediums was between 6.4 and 6.5. The nominal concentrations of $Na^+$ and $Cl^-$ in control artificial water were 1 mM $[Na^+]$ and 1 mM $[Cl^-]$, while low-Na and low-Cl groups did not contain $Na^+$ and $Cl^-$, respectively. In all three artificial waters, the $[Ca^{2+}]$ was 0.5 mM. The analysis of $Na^+$ and $Ca^{2+}$ concentrations of water samples was conducted using an atomic absorption spectrophotometer (Z-5000, Hitachi, Tokyo). The $Cl^-$ concentration of water samples was evaluated using the ferricyanide method (Franson 1985). Photometric analysis was performed using a Hitachi U-2001 spectrophotometer. Table 1 shows the actual ion concentrations of the three artificial water preparations for the control, low-Na, and low-Cl groups. In the experiment of artificial water acclimation, the fish were not fed, and the gills were sampled for analyses with the same methods described in the section of acclimation experiments.

### *SEM Observation*

After anesthetization with MS-222 (75 mg $L^{-1}$), the fish were killed, and their gills were excised. The first gill arch from each side was fixed at 4°C in 5% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.2) for 12 h. After rinsing with 0.1 M PB, specimens were postfixed with 1% osmium tetroxide in 0.2 M PB for 1 h. After rinsing with PB and dehydrating in ethanol, specimens were critical-point dried using a Hitachi HCP-2 critical-point drier (Tokyo). The gills were mounted on an aluminum specimen stub, coated with gold in an ion sputter (JFC-1600, JEOL, Tokyo), and examined with SEM (JSM-6700F, JEOL). Subtypes of MR cells were identified from SEM images according to previous studies (Table 2 in Hwang and Lee 2007).

### *Antibodies*

The primary antibodies used in this study included (1) NKA, a mouse monoclonal antibody (α5; Developmental Studies Hybridoma Bank, Iowa City, IA) raised against the α-subunit of NKA of chicken; (2) ClC-3, a rabbit polyclonal antibody (Clcn3; Alomone Labs, Jerusalem, Israel) raised against the highly conserved region, residues 592–661 of rat ClC-3, with 80% identity to residues of 591–660 of tilapia ClC-3; and (3) NCC, a mouse monoclonal antibody (T4; Developmental Studies Hybridoma Bank, Iowa City, IA) raised against the C-terminus of the human $Na^+/K^+/2Cl^-$ cotransporter (NKCC), which has been demonstrated to recognize NCC in apical membranes of MR cells of FW tilapia (Hiroi et al. 2008; Inokuchi et al. 2008,

Table 2: Primer set and annealing temperatures for real-time PCR of tilapia (*Oreochromis mossambicus*) ClC-3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

| Gene | Primer Sequence | Product Length (bp) | Annealing Temperature (°C) |
|---|---|---|---|
| ClC-3: | | | |
| Forward | 5′-CCC TGT GAT CGT GTC TAA GGA-3′ | 71 | 60 |
| Reverse | 5′-TAG CGA TTG TGA TGT CTC TGC-3′ | | |
| GADPH: | | | |
| Forward | 5′-AAC GAC AAC TTT GGC ATC GT-3′ | 71 | 60 |
| Reverse | 5′-ACC GTC TTC TGT GTG GCA GT-3′ | | |

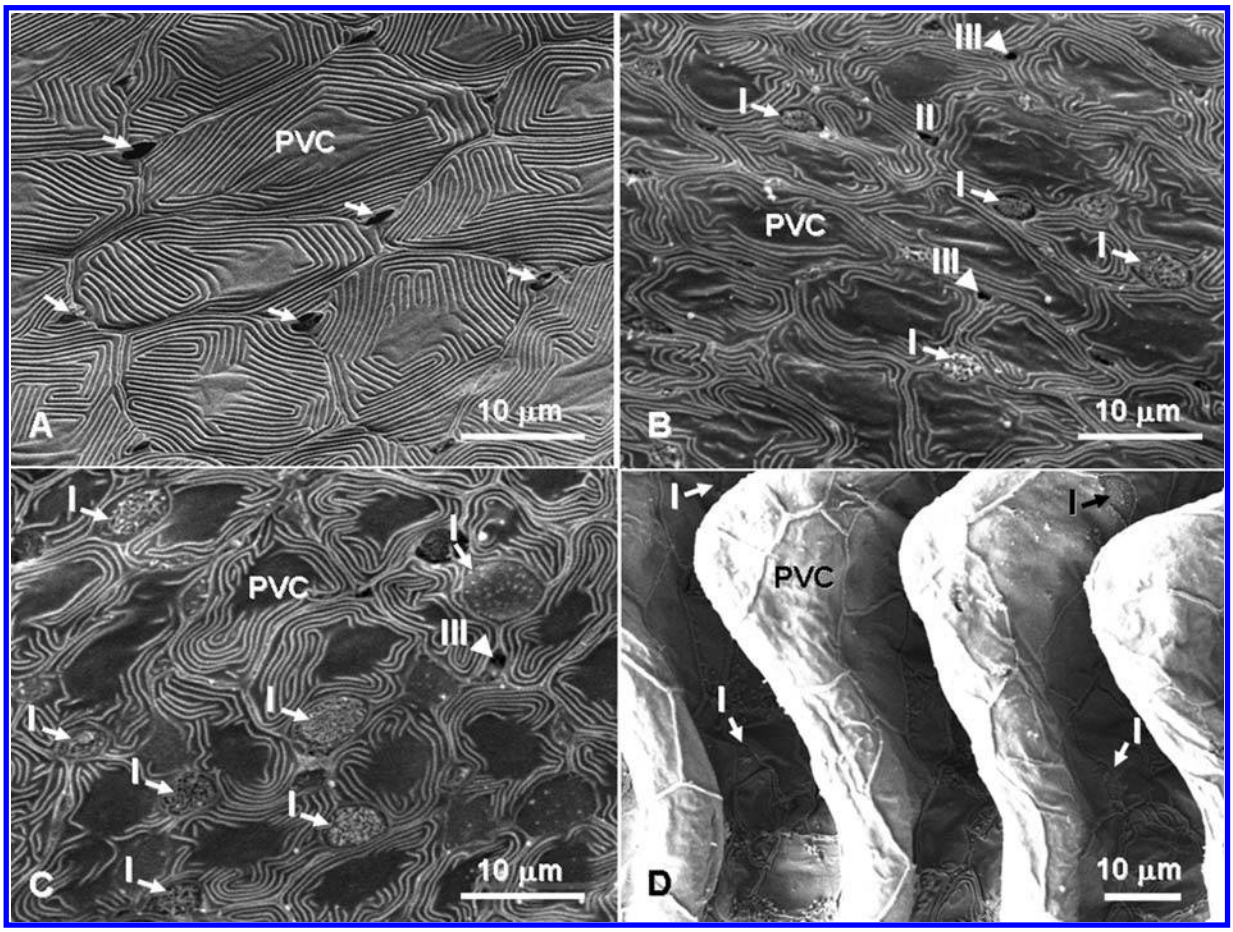


Figure 1. Scanning electron micrographs of mitochondrion-rich (MR) cells in the gill filament epithelia of tilapia acclimated to seawater (SW; *A*), freshwater (FW; *B*), and deionized water (DW; *C*). *D*, MR cells in the gill lamellar epithelia of DW-acclimated tilapia. Arrowheads in *A* indicate the apical crypts of SW MR cells (subtype III). I, II, and III in *B*, *C*, and *D* indicated subtypes of FW MR cells. Subtype I MR cells increased in number in DW-acclimated tilapia.

2009). The secondary antibody for immunoblotting was alkaline phosphatase–conjugated goat antirabbit IgG (Chemicon, Temecula, CA), and for immunofluorescent staining they were DyLight 488 or 549 conjugated goat antirabbit or antimouse IgG (Jackson Immunoresearch, West Baltimore Pike, PA).

### *Whole-Mount Fluorescent Immunocytochemistry and Confocal Microscopy*

The immunostaining procedure was performed according to Tang et al. (2010) with little modification. The first pair of gills was excised, and the gill filaments were removed from the gill arch. The gill filaments were immediately fixed in 0.5% glutaraldehyde and 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h at 4°C. After washing with 0.2% Triton X-100 in PBS (PBST), the gill filaments were postfixed and permeated with 70% ethanol for 10 min at −20°C. The gill filaments were rinsed with PBST and then incubated in PBST containing 5% bovine serum albumin for 1 h at room temperature (26°–28°C) to reduce nonspecific binding (Sigma, St. Louis, MO). The gill filaments were then incubated at room temperature for 2 h with primary polyclonal antibody Clcn3. The procedures of examination of negative controls are identical to the protocols of immunostaining except the primary antibody of anti-ClC-3. To confirm the specificity of the immunoreaction, the gill filaments were incubated with the primary antibody, which was preincubated with the antigen of ClC-3 (antigen-preabsorbed antibody). The antigen of ClC-3 with molecular weight of 35 kDa was provided by Alomone Labs. Following incubation, the gill filaments were washed several times with PBST and then labeled with DyLight 488 or 549 conjugated goat antirabbit secondary antibody (Jackson Immunoresearch) at room temperature for 2 h.

After the first staining, the gill filaments were washed several times with PBST to proceed to the second staining. The gill filaments were subsequently incubated with primary monoclonal antibodies α5 (NKA) or T4 (NCC) at 4°C for 12 h followed by labeling with Dylight 549 or 488 conjugated goat antimouse secondary antibody (Jackson Immunoresearch), respectively, at room temperature for 2 h. The samples were then washed with

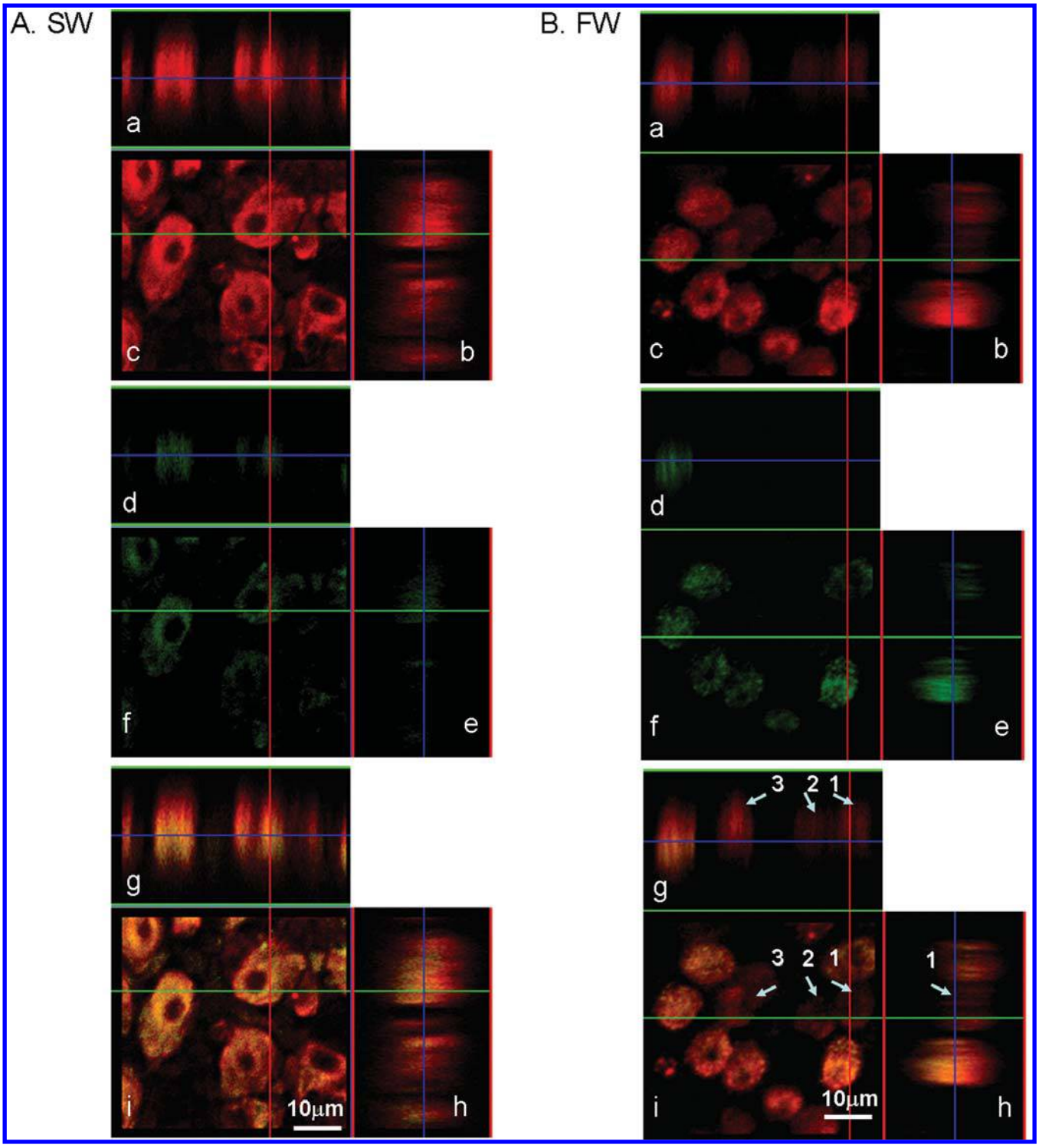


Figure 2. Confocal micrographs of immunolocalization of $Na^+/K^+$-ATPase (NKA; *red*), ClC-3-like protein (*green*), and the merged (*yellow*) 3-D images in the gill filament epithelia of tilapia acclimated to seawater (SW; *A*), freshwater (FW; *B*), and deionized water (DW; *C*). In each panel, X–Z optical sections (*a*, *d*, *g*) were cut transversely at the horizontal green lines indicated in the merged X, Y images (*c*, *f*, *i*); Y, Z optical sections (*b*, *e*, *h*) were cut at the vertical red lines indicated in the merged X, Y images (*c*, *f*, *i*); X, Y images (*c*, *f*, *i*) were cut at the blue lines indicated in the X–Z and Y, Z planes. In *A* (SW), ClC-3-like protein was colocalized with NKA. In *B* (FW) and *C* (DW), however, some NKIR cells did not exhibit ClC-3-like protein (red cells indicated by arrows with numbers).

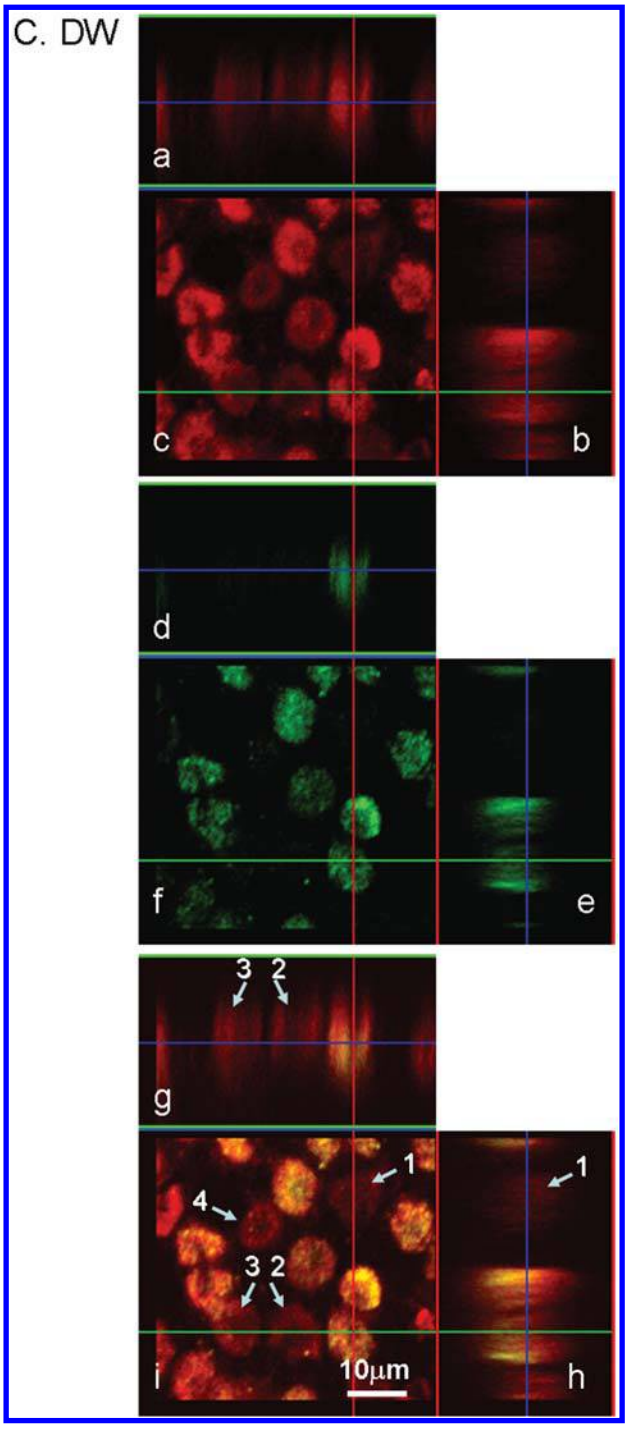


Figure 2. (*Continued*)

PBST, mounted with a coverslip, and observed with a confocal laser microscope (LSM 510, Zeiss, Hamburg, Germany). The 488-nm argon-ion laser and the 543-nm helium-neon laser were used for DyLight 488 and 549, respectively, to give the appropriate excitation wavelengths. The micrographs of immunostaining were analyzed by Zeiss LSM image software. The results of the negative control are shown in Figure A1 (available in the online edition of *Physiological and Biochemical Zoology*).

To compare the percentage of ClC-3-like protein immunopositive cells in all NKIR cells in gills of FW and DW tilapia, the areas from the afferent epithelium of the gill filaments were randomly chosen, and three gill filaments for one fish were observed. In total, 100 NKIR cells (with or without ClC-3-like protein colocalization) were randomly selected in each area, and the number of ClC-3-like protein immunopositive cells among them were counted. Five individuals ($N = 5$) were used for different groups (FW or DW).

### *RNA Extraction and Reverse Transcription*

Total RNA samples from the gills of tilapia acclimated to SW, FW, or DW were extracted using RNA-Bee (Tel-Test, Friendwood, TX) according to the manufacturer's instructions. The RNA pellet was dissolved in 30 $\mu$L DEPC-$H_2O$ and treated with the RNA cleanup protocol from the RNAspin Mini RNA isolation kit (GE Health Care, Piscataway, NJ) according to the manufacturer's instructions to eliminate genomic DNA contamination. RNA integrity was verified by 0.8% agarose gel electrophoresis. Extracted RNA samples were stored at −80°C after isolation. The concentration of extracted RNA was measured by NanoDrop 2000 (Thermo, Wilmington, DE). First-strand cDNA was synthesized by reverse transcribing 2 $\mu$g of the total RNA using a 1 $\mu$L Oligo (dT) (0.5 $\mu$g $\mu L^{-1}$) primer and a 1 $\mu$L Transcriptor Reverse Transcriptase (Transcriptor First Strand cDNA Synthesis Kit, Roche, Mannheim, Germany) according to the manufacturer's instructions.

### *Real-Time PCR*

The procedure of real-time PCR was performed according to Wang et al. (2009) using a MiniOpticon real-time PCR system (Bio-Rad Laboratories, Hercules, CA). The final volume of reaction mixture of 20 $\mu$L contained 10 $\mu$L of 2 × SYBR Green Supermix (Bio-Rad), 300 nM of the primers pairs, and 16 ng of cDNA. The standard curve of each gene was checked in a linear range with glyceraldehyde-3-phosphate dehydrogenase as an internal control. The primer sets for the real-time PCR are shown in Table 2.

### *Preparation of Crude Gill Membrane Fractions*

The procedure of preparation of crude gill membrane fractions was performed according to Tresguerres et al. (2006*b*) with little modification. The gills of the fish were excised and blotted dry immediately after the fish were killed by spinal pithing. Gill samples were immersed in liquid nitrogen and placed into ice-cold homogenization buffer (250 mM sucrose, 1 mM EDTA, 30 mM Tris, pH 7.4). Homogenization was performed in 2-mL tubes with the POLYTRON PT1200E (Lucerne, Switzerland) at maximal speed for 15 s. Debris, nuclei, and lysosomes were removed by low-speed centrifugation (13,000 *g* for 10 min, 4°C). The remaining supernatant was centrifuged at me-

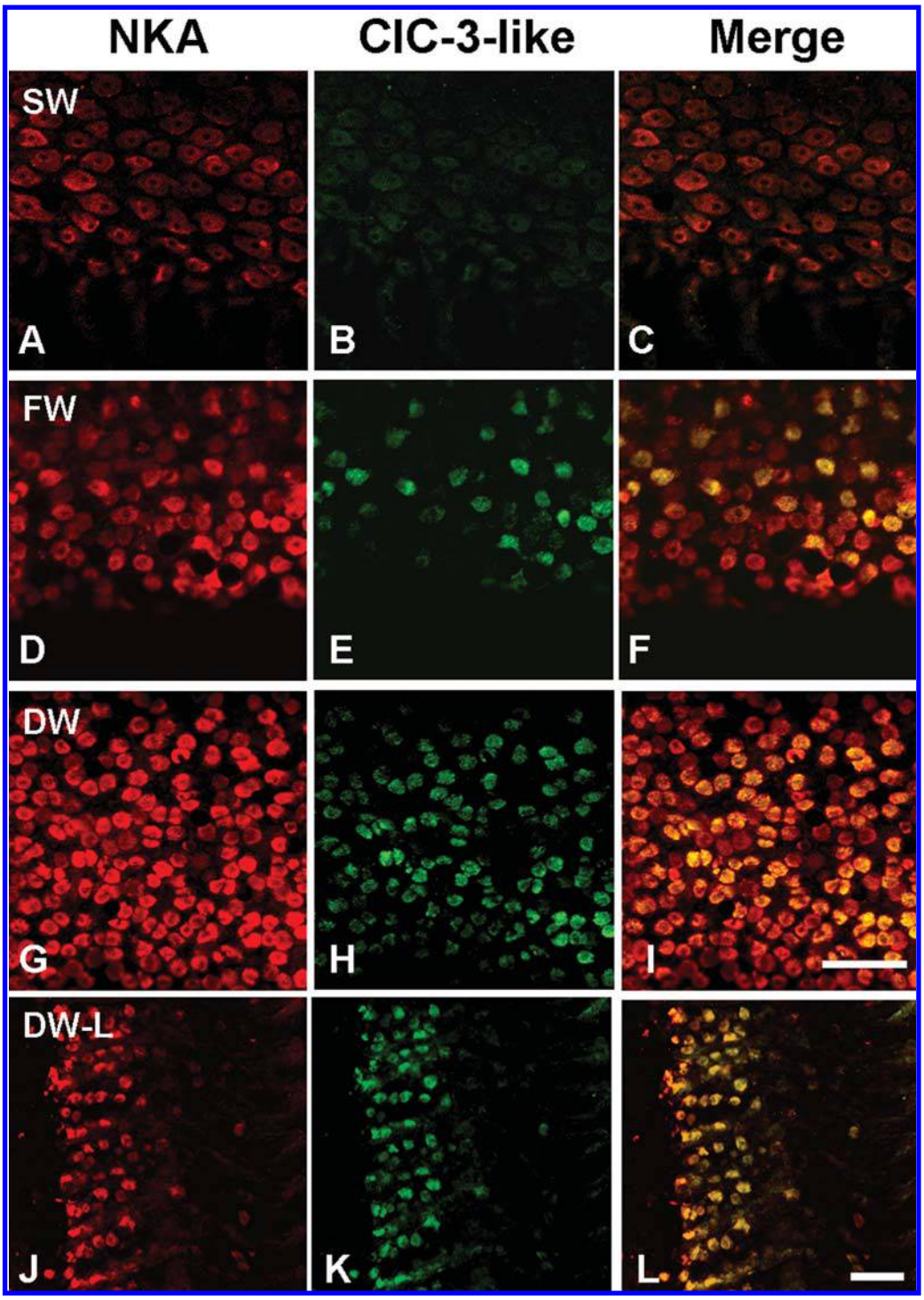


Figure 3. Double immunofluorescent staining with anti-$Na^+/K^+$-ATPase (NKA; *A*, *D*, *G*, *J*; *red*) and anti-ClC-3 (*B*, *E*, *H*, *K*; *green*) in the gill filament epithelia of tilapia acclimated to seawater (SW; *A*, *B*, *C*), freshwater (FW; *D*, *E*, *F*), and deionized water (DW; *G*, *H*, *I*) and in the gill lamellar epithelia of DW-acclimated tilapia (*J*, *K*, *L*). *C*, *F*, *I*, *L* = merged images. Scale bars = 40 $\mu$m.

dium speed (20,800 *g* for 1 h, 4°C). The resulting pellet was resuspended in homogenization buffer and stored at −80°C. The pelleted fraction should contain large fragments of the plasma membrane along with membranes from the Golgi and the endoplasmic reticulum. This fraction is therefore referred to as the crude membrane fraction. Aliquots of crude cell-membrane fractions were saved for protein determination analysis. Protein concentrations were determined with the reagents

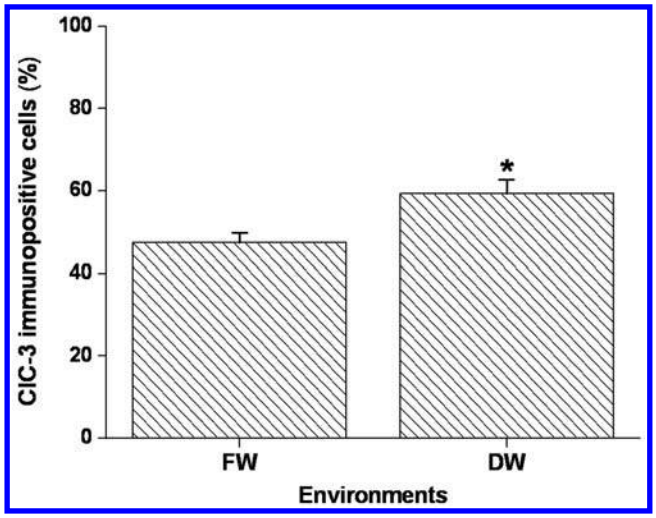


Figure 4. Comparisons of relative percentages of ClC-3-like protein immunoreactive cells in all NKIR cells in gill filament epithelia of tilapia acclimated to freshwater (*FW*) and deionized water (*DW*). The asterisk indicates a significant difference ($P < 0.05$, $N = 5$) by unpaired *t*-test.

of a BCA Protein Assay Kit (Pierce, Hercules, CA, ) using bovine serum albumin (Pierce) as a standard. The crude membrane fractions were stored at −80°C until immunoblotting.

### *Immunoblotting*

Immunoblotting procedures were modified from Tang et al. (2009). Proteins of the membrane fraction were heated together with the sample buffer at 37°C for 30 min. The prestained protein molecular weight marker was purchased from Fermentas (SM0671; Hanover, MD). All samples were separated by electrophoresis on sodium dodecyl sulfate–containing 7.5% polyacrylamide gels (20 $\mu$g of protein/lane). The separated proteins were then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA) by electroblotting. After preincubation for 3 h in PBST buffer containing 5% (w/v) nonfat dried milk to minimize nonspecific binding, the blots were incubated overnight at 4°C with primary antibody (Clcn3) diluted in 1% BSA and 0.05% sodium azide in PBST, washed in PBST, and incubated at room temperature (26°–28°C) for 2 h with secondary antibodies. Blots were developed after incubation with a BCIP/NBT kit (Zymed, South San Francisco, CA). To correct for the differences in loading, protein amounts of each lane were quantified after staining with Coomassie blue. Coomassie blue–stained gels and developed immunoblots were photographed and imported as TIFF files. The lanes of the gel stained with Coomassie blue and immunoreactive bands were analyzed using MCID software (ver. 7.0, rev. 1.0; Imaging Research, St. Catharines, Ontario). The results were converted to numerical values to compare the relative protein abundance of the immunoreactive bands. The relative protein abundance of branchial ClC-3-like protein in each sample was defined as the ratio of antibody/Coomassie blue. For negative controls, the procedures are identical to the protocols of immunoblot except the primary antibody. To confirm the specificity of the immunoreaction, the antigen-preabsorbed antibody was used to replace the primary antibody. The antigen of ClC-3 with molecular weight of 35 kDa was provided by Alomone Labs.

### *Statistical Analysis*

To compare the density of MR cells of ClC-3-like protein immunopositive in two hyposmotic environments (DW and FW), the significance of the difference between treatments was assessed by unpaired *t*-test ($P < 0.05$). Values were compared using a one-way ANOVA with Tukey's pairwise comparisons, and $P < 0.05$ was set as the significance level in the other experiments. Values were expressed as the means ± SEM unless stated otherwise.

## Results

### *Different Subtypes of MR Cells*

Different subtypes of MR cells were observed in the gills of tilapia by SEM, and the compositions of MR cell subtypes were found to vary with the acclimated environments (Fig. 1). In SW, all MR cells were located in the epithelium of the gill filament and exhibited apical crypts (subtype III, deep-hole; Fig. 1*A*). In FW, three subtypes (I, wavy-convex; II, shallow-basin; III, deep-hole) of MR cells were found in the epithelium of the gill filaments (Fig. 1*B*). Furthermore, when tilapia were exposed to DW, most MR cells in the filament epithelia were subtype I (Fig. 1*C*). In addition, MR cells were found to exist in the epithelium of gill lamellae only in DW-exposed tilapia, and the lamellar MR cells were subtype I (Fig. 1*D*). The other subtypes of MR cells were never found in the lamellar epithelia.

### *Localization of ClC-3-Like Protein in Gill Epithelial Cells*

The localization of ClC-3-like protein in tilapia gill epithelium was examined by double immunofluorescent staining with NKA, the basolateral marker of MR cells, and observed with a confocal laser microscope (Figs. 2, 3). Three-dimensional (3-D) images of confocal micrographs (Fig. 2*A*) indicated that all examined NKIR cells (i.e., MR cells) exhibited basolateral ClC-3-like protein (i.e., colocalization of NKA and ClC-3-like protein in all observed cells) in SW tilapia (Fig. 2*A*), although the immunoreaction of ClC-3 is weak (Fig. 3*B*). Moreover, 3-D images clearly showed that in FW- and DW-acclimated tilapia, only a proportion of NKIR cells displayed the basolateral immunoreaction of ClC-3-like protein (Fig. 2*B*, 2*C*). Further examination of ClC-3-like protein localization on randomly chosen areas of gill filament epithelia of FW and DW tilapia also revealed that only some NKIR cells exhibited basolateral ClC-3-like protein (Fig. 3*D*–3*I*). Interestingly, in DW-acclimated tilapia, all lamellar NKIR cells displayed basolateral ClC-3-like protein (Fig. 3*J*–3*L*).

The percentage of ClC-3-like protein immunoreactive cells in all examined MR cells of gill filament epithelia was signifi-

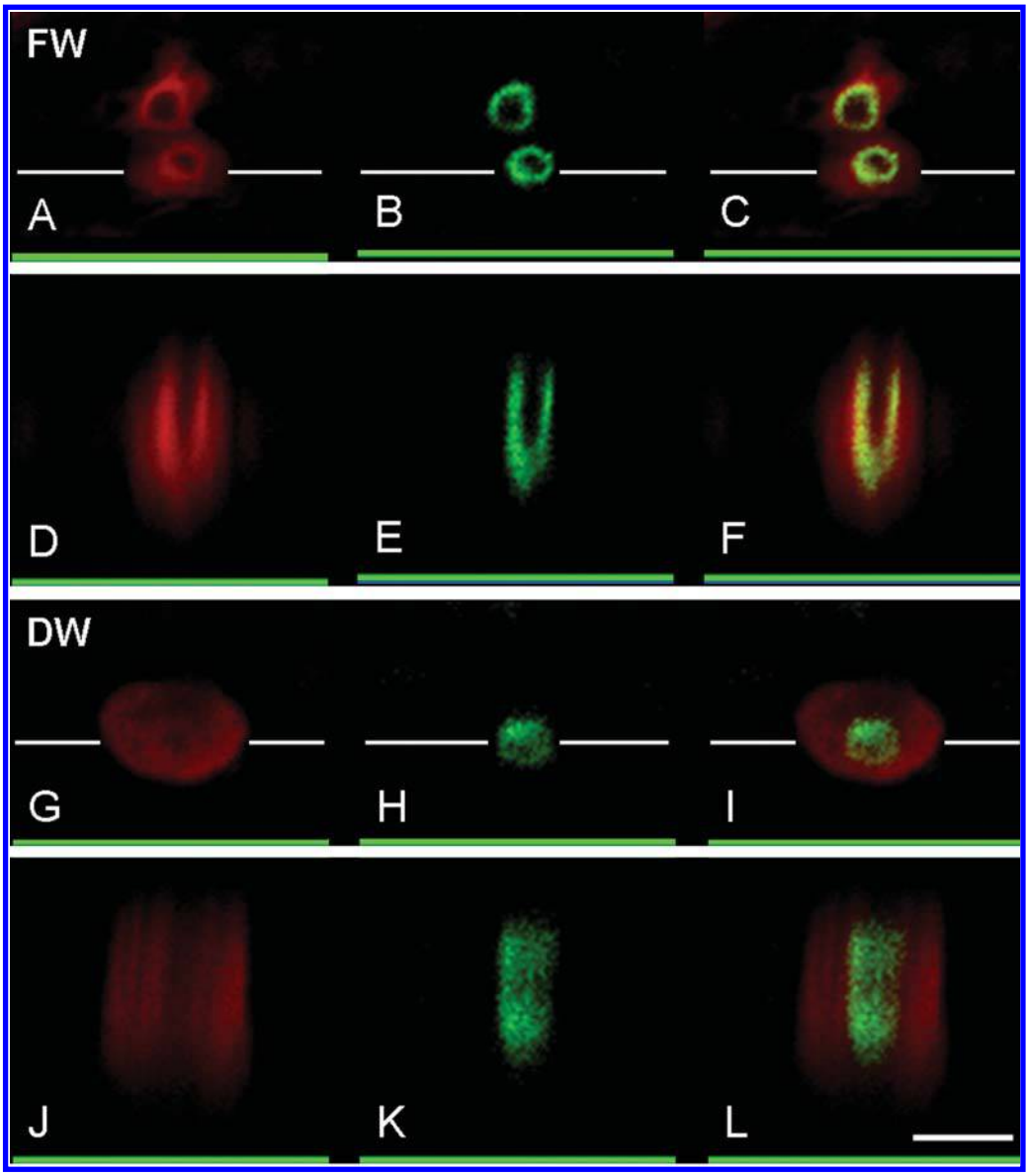


Figure 5. Immunolocalization of ClC-3-like protein (*red*) and $Na^+/Cl^-$ cotransporter (*green*) in the gill filaments of tilapia acclimated to freshwater (*FW*; *A–F*) and deionized water (*DW*; *G–L*). X–Z optical sections were cut horizontally at the transverse white lines indicated in the merged X–Y images. *D–F*, *J–L*, X–Z optical sections. Scale bar = 10 $\mu$m.

cantly higher in the DW group (about 60%) compared with that in the FW group (about 50%; Fig. 4). Furthermore, in our observation, the total number of ClC-3-like immunoreactive cells was also increased in DW-acclimated tilapia.

*Double Immunofluorescent Staining of ClC-3 and NCC*

In the gills of both the FW- and DW-acclimated tilapia, double immunofluorescent staining of ClC-3-like protein (red) and NCC (green) was present in the 3-D confocal micrographs (Fig. 5). In either FW or DW, ClC-3-like protein (Fig. 5*A*, 5*D*, 5*G*, 5*J*) immunoreactive cells were found to exhibit apically stained NCC (Fig. 5*B*, 5*E*, 5*H*, 5*K*).

*Expression of Branchial ClC-3 mRNA and ClC-3-Like Protein*

Branchial ClC-3 mRNA abundance of SW-, FW-, and DW-acclimated tilapia was determined by a real-time PCR (Fig. 6*A*) and semiquantitative PCR (Fig. A2, available in the online edition). The results revealed that branchial ClC-3 mRNA in the DW group was about 2.4-fold and 2.8-fold higher than in the SW- and FW-acclimated tilapia, respectively.

Immunoblotting of crude membrane fractions of gill tissues from tilapia acclimated to SW, FW, or DW resulted in a single immunoreactive band of about 105 kDa. The immunoreactive band in the DW group was more intensive than in the other groups (Fig. 7*A*). The immunoreactive band of the negative

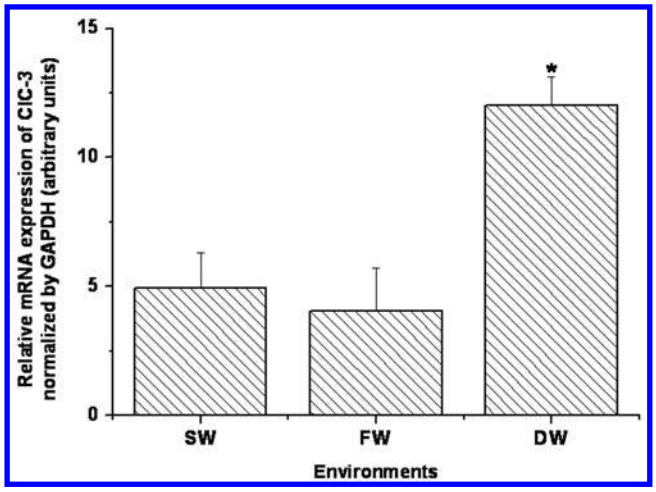


Figure 6. Levels of ClC-3 transcript in the gills of tilapia exposed to different environments analyzed by real-time polymerase chain reaction ($N = 5$). The expression of gill ClC-3 mRNA was significantly higher in the deionized water (*DW*) group than in the seawater (*SW*) and freshwater (*FW*) groups. The asterisk indicates a significant difference ($P < 0.05$) using Tukey's multiple comparison test following a one-way ANOVA. Values are means ± SEM.

control was absent following incubation of the PVDF membrane of crude gill membrane fraction with the antigen-preabsorbed antibody (Fig. 7*B*). Quantification of immunoreactive bands of different groups showed that the amounts of gill ClC-3-like protein of the DW tilapia were higher than those of FW (about 2.3-fold) and SW (about 1.9-fold) fish (Fig. 7*C*).

### *Artificial Water Experiments*

To further clarify the response of branchial ClC-3-like protein to changes in environmental $Cl^-$ concentration, tilapia were acclimated to three artificial waters (i.e., control, low-Na, and low-Cl), as described above. The results of immunoblotting showed that a 105-kDa single immunoreactive band was also detected in the branchial membrane fraction of tilapia acclimated to artificial waters. The immunoreactive band of low-Cl-exposed tilapia was denser than that of the control and low-Na groups (Fig. 8*A*). Quantification of immunoreactive bands among the different groups showed that the abundance of branchial ClC-3-like protein in low-Cl-acclimated tilapia was about 2.8-fold and 2.4-fold higher than in the control and low-Na-acclimated groups, respectively (Fig. 8*B*).

## Discussion

Because $Cl^-$, the predominant inorganic anion, is cotransported with other ions to maintain charge, cell volume, and intracellular pH and [$Cl^-$], the chloride channels display multiple functions in a broad range of physiological regulation (Jentsch et al. 2002, 2005). One of the important functions of chloride channels in epithelial cells of vertebrates is to modulate $Cl^-$ movement for osmoregulation (Devuyst and Guggino 2002; Miyazaki et al. 2002; Schmieder et al. 2002; Evans et al. 2005). The $Cl^-$ secretory mechanisms of the gill, the major osmoregulatory organ of fish, are well established (Hirose et al. 2003; Evans et al. 2005; Evans 2008). The $Cl^-$-uptake mechanisms in gill MR cells of FW teleosts, however, have received less attention. In previous review articles, the apical $Cl^-$ uptake was supposed to occur via the $Cl^-/HCO3^-$ exchanger or the NCC in FW teleosts (Tresguerres et al. 2006*a*; Evans 2008; Hwang 2009). Meanwhile, at the basolateral membrane, the cellular mechanisms for branchial $Cl^-$ uptake in fish are not clear yet. To propose a basolateral chloride channel for $Cl^-$ uptake, CLC family members highly expressed in the osmoregulatory organs were considered (Miyazaki et al. 1999; Hirose et al. 2003). However, only a few studies (Miyazaki et al. 1999, 2002; Tang and Lee 2007; Tang et al. 2010) investigated the CLC family in fish. Therefore, this study assessed the relationship between morphological changes of apical membranes of MR cells and the expression and localization of branchial ClC-3-like protein as well as salinity/artificial water (with different [$Cl^-$]) acclimation in euryhaline Mozambique tilapia to reveal the potential $Cl^-$ absorption function of basolateral ClC-3-like protein in the ion-absorbing subtype of MR cells.

When vertebrate cells are exposed to hyposmotic solutions, the regulatory volume decrease (RVD) process is activated to reduce cell volume during hyposmotically induced cell swelling (Hoffmann and Dunham 1995). ClC-3, a member of the CLC family, is an important molecule underlying volume-sensitive osmolyte/anion channels and the RVD process in HeLa cells and *Xenopus laevis* oocytes (Hermoso et al. 2002). Furthermore, ClC-3 is activated by hyposmotic shock while strongly inhibited by hyperosmotic conditions (Duan et al. 1997). Using different approaches, expression of endo- or exogenous ClC-3 was demonstrated to generate outwardly rectifying currents in hyposmotic medium in various cell types (Kawasaki et al. 1994; Duan et al. 1997, 2001; Wang et al. 2000, 2003; Hermoso et al. 2002; Vessey et al. 2004). Based on these functional studies of ClC-3, this chloride channel was thought to be our investigative target to propose a potential role of the basolateral exit step for $Cl^-$ uptake in gills.

The branchial MR cell is the dominant site to modulate ion movement and is in direct contact with external environments via the apical membrane (Evans et al. 2005; Kaneko et al. 2008). Changes of environmental salinity would therefore lead to alteration of the apical ultrastructure, which is closely related to ion-transporting functions of MR cells (Kaneko et al. 2008). To integrate the functional morphology and the molecular mechanisms of $Cl^-$ uptake in gill MR cells, tilapia were acclimated to environments of different salinities, that is, SW, FW, and DW in this study. The ultrastructure of apical membranes of branchial MR cells typically formed a crypt (deep-hole subtype; Fig. 1*A*) in SW-acclimated tilapia, and this subtype of MR cell is equipped with basolateral NKCC and apical cystic fibrosis transmembrane conductance regulator to play a role in $Cl^-$ secretion (Hiroi et al. 2008; Kaneko et al. 2008). In FW-acclimated tilapia, however, three subtypes of MR cells with different apical surface ultrastructures were identified (Fig. 1*B*; Lee et al.

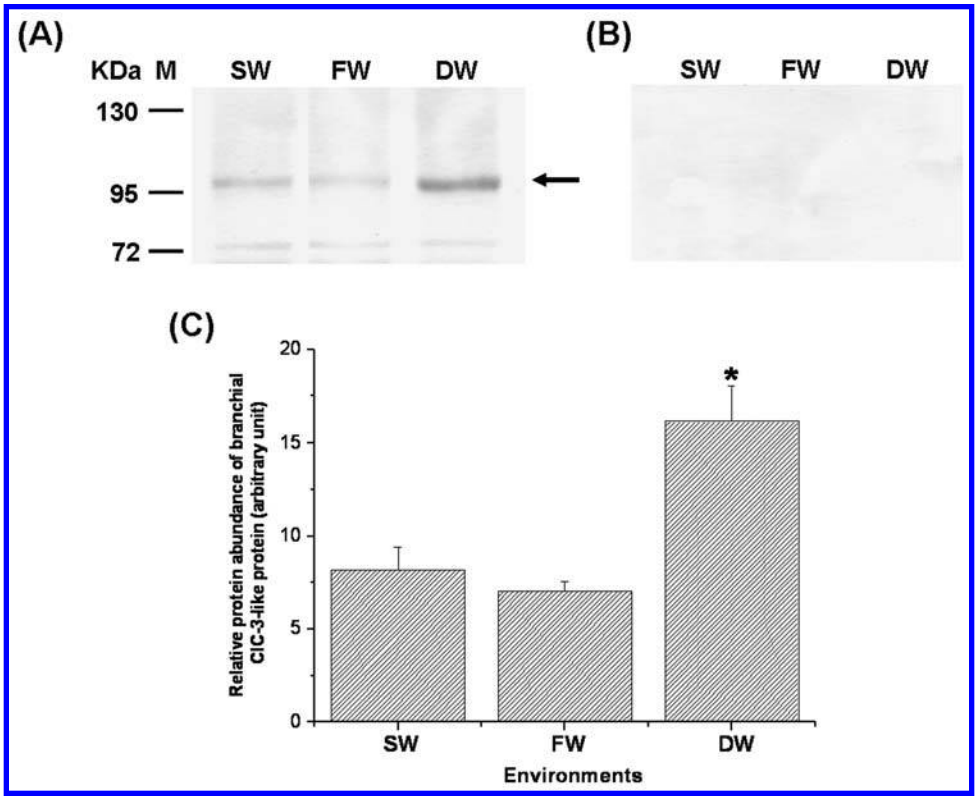


Figure 7. Representative immunoblot of crude membrane fractions of tilapia gills probed with a polyclonal antibody to ClC-3 (*A*) and the primary antibody preincubated with the antigen (*B*). Compared with the negative control, a single immunoreactive band with a molecular weight of about 105 kDa was obtained (*arrow*). The immunoreactive bands of fish acclimated to deionized water (*DW*) were more intense than those of fish acclimated to seawater (*SW*) and freshwater (*FW*). *C*, Relative abundance of immunoreactive bands of ClC-3-like protein in the gills of different salinity groups ($N = 6$). Expression of ClC-3-like protein was higher in the DW group than in the SW and FW groups. The asterisk indicates a significant difference ($P < 0.05$) using Tukey's multiple comparison test following a one-way ANOVA. Values are means $\pm$ SEM. $M$ = marker.

1996). By manipulating a single ambient ion level together with the ion-influx data, these experiments have demonstrated that one subtype of MR cell corresponded to regulation of one major ion in tilapia (Chang et al. 2001, 2003; Chang and Hwang 2004).

The subtype I wavy-convex MR cell characterized by a wide apical membrane and rough surface was thought to be the $Cl^-$ absorption subtype (Chang et al. 2001, 2003; Inokuchi et al. 2009), and the density of subtype I MR cells in epithelia of gill filaments increased when tilapia were acclimated to DW, the extreme hyposmotic environment (Fig. 1*C*; Inokuchi et al. 2008; Tang et al. 2008). Furthermore, the Z-plane images of confocal micrographs revealed that branchial ClC-3-like protein in tilapia was colocalized with NKA, the basolateral marker of MR cells, in all examined environments (Fig. 2). In SW-acclimated tilapia, although all MR cells exhibited immunoreaction of ClC-3-like protein, the fluorescent intensity of a single ClC-3-like protein immunopositive cell was quite weaker than in the FW and DW groups (Fig. 3*A*–3*I*). This result implied branchial ClC-3-like protein might play a significant role when tilapia were exposed to hyposmotic environments. In addition, ClC-3-like protein might also play a role in regulation of intracellular $[Cl^-]$ or acid-base homeostasis in SW tilapia. Similar to the proportion of subtype I MR cells, the density of ClC-3-like protein immunopositive MR cells of filament epithelia was higher in DW-exposed tilapia compared with FW individuals (Fig. 4). Interestingly, MR cells appearing in the gill lamellae were only found in DW-acclimated tilapia (Tang et al. 2008), and this study is the first to demonstrate that all lamellar MR cells are the wavy-convex subtype I MR cell (Fig. 1*D*) with positive ClC-3-like protein immunostaining (Fig. 3*J*–3*L*). Hence, in tilapia gills, gill ClC-3-like protein was proposed to be a basolaterally localized chloride channel in the subtype I MR cells. Recently, Inokuchi et al. (2009) demonstrated that NCC was localized in the apical membrane of $Cl^-$-absorbing MR cells (subtype I) of tilapia to transport $Cl^-$ from external environments into MR cells. Meanwhile, double immunofluorescent staining in this study showed that apical NCC was expressed in most basolateral ClC-3-like protein–expressing MR cells in FW- and DW-acclimated tilapia (Fig. 5). It is thus presumed that environmental $Cl^-$ was transported into blood through apical NCC and basolateral ClC-3-like protein in the subtype I MR cells of the gill epithelium when tilapia were exposed to hyposmotic media.

The abundance of osmoregulation-related genes or proteins

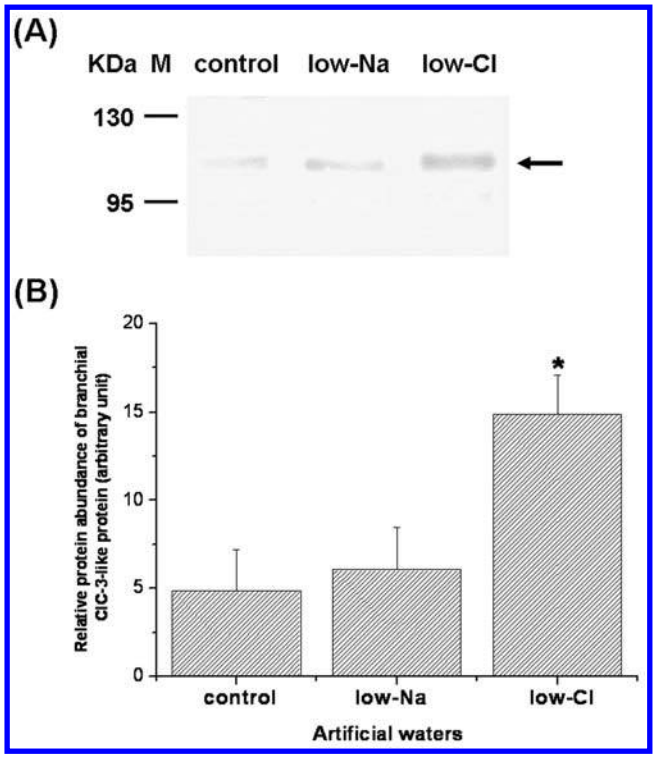


Figure 8. *A*, Representative immunoblot of gills from fish acclimated to artificial water probed with a primary antibody to ClC-3 (Clcn3). A single immunoreactive band with molecular weight of about 105 kDa is indicated by an arrow. The immunoreactive bands of low-Cl-acclimated tilapia were more intense than control and low-Na-acclimated individuals. *B*, Relative abundance of immunoreactive bands of ClC-3-like protein in the gills of different artificial water groups ($N = 5$). Expression of ClC-3-like protein was about 2.8-fold and 2.4-fold higher than in the control and low-Na-acclimated groups, respectively. The asterisk indicates a significant difference ($P < 0.05$) using Tukey's multiple comparison test following a one-way ANOVA. Values are means ± SEM. *M* = marker; *control* = normal $[Na^+]$/normal $[Cl^-]$ artificial water; *low-Na* = low $[Na^+]$/normal $[Cl^-]$ artificial water; *low-Cl* = normal $[Na^+]$/low $[Cl^-]$ artificial water. The measured ionic concentrations of these three artificial waters are shown in Table 1.

of branchial MR cells are regulated to maintain osmotic homeostasis when euryhaline teleosts are exposed to environments of different salinities. The levels of ClC-3 mRNA as well as ClC-3-like protein in crude membrane fractions of tilapia gills were significantly increased in an ion-deficient environment (DW; Figs. 6, 7), which coincided with the characteristic of hyposmotic activation of ClC-3 in mammals. It was assumed that branchial ClC-3-like protein was more essential in DW-acclimated tilapia than in FW-acclimated individuals. Moreover, Chang et al. (2003) demonstrated that $Cl^-$ influx was enhanced in tilapia acclimated to artificial water with an extremely low $Cl^-$ concentration. Our study also verified that the abundance of branchial ClC-3-like protein increased in response to an environment with extremely low $[Cl^-]$ (Fig. 8). When compared with SW tilapia, however, the abundance of ClC-3 mRNA and ClC-3-like protein was not elevated in FW fish. Taken together, these findings suggested the existence of an alternative mechanism of basolateral $Cl^-$ absorption specific for FW tilapia. Meanwhile, ClC-3-like protein basolaterally localized in subtype I MR cells might play a crucial role in $Cl^-$ uptake for physiological homeostasis when tilapia were exposed to a chloride-poor environment.

While ClC-3 has been confirmed to mediate outward $Cl^-$ currents in hyposmotic solutions in different cell types (Wang et al. 2000; Duan et al. 2001; Hermoso et al. 2002), whether the subcellular localization of ClC-3 protein is to an organelle or the plasma membrane is still debatable (Nilius and Droogmans 2003). Vessey et al. (2004) reported that when rabbit nonpigmented ciliary epithelial cells were exposed to a hyposmotic solution, an enhanced shift in ClC-3 protein from an intracellular location to the plasma membrane was observed. Furthermore, the preparation of membrane fractions was successfully used to detect ClC-3 expression in plasma membranes (Shimada et al. 2000; Huang et al. 2001). Taken together with our data of tilapia ClC-3-like protein from the crude gill membrane fractions containing large fragments of the plasma membrane along with the membrane from the Golgi and the endoplasmic reticulum (Tresguerres et al. 2006*b*), ClC-3-like protein should be predominantly expressed in the plasma membrane of branchial cells of tilapia.

Integrating the data of the apical ultrastructures of gill MR cells (both filament and lamellar epithelia) and immunolocalization of ClC-3-like protein in this study, we demonstrated that the ion-absorbing subtype I MR cells of tilapia contained apically located NCC as well as basolaterally located ClC-3-like protein and NKA. In addition, combined with the functional characteristics of mammalian ClC-3 and the results of enhanced abundance of gill ClC-3-like protein in DW-exposed tilapia, increased amounts of basolateral ClC-3-like protein in the gills of tilapia acclimated to low-$[Cl^-]$ environments was thought to be responsible for $Cl^-$ uptake. Our study provided diverse evidence for the $Cl^-$-absorbing mechanism in the basolateral membrane of a particular subtype of branchial MR cells as well as integrated morphological and functional classifications of $Cl^-$-absorbing MR cells to illustrate a potential model for transporting $Cl^-$ through the gill epithelium of DW-exposed tilapia. ClC-3-like protein reported here may be one of the mechanisms of basolateral $Cl^-$ uptake, and the alternative mechanisms will be examined in our future studies.

## Acknowledgments

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