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Chloride channel ClC-3 in gills of the euryhaline teleost, *Tetraodon nigroviridis*: expression, localization and the possible role of chloride absorption

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SUMMARY

Previous studies have reported the mechanisms of ion absorption and secretion by diverse membrane transport proteins in gills of various teleostean species. To date, however, the chloride channel expressed in the basolateral membrane of mitochondrion-rich (MR) cells for Cl^- uptake in freshwater (FW) fish is still unknown. In this study, the combination of bioinformatics tools [i.e. National Center for Biotechnology Information (NCBI) database, *Tetraodon nigroviridis* (spotted green pufferfish) genome database (Genoscope), BLAT and BLASTn] were used to identify the gene of ClC-3 (*TnClC-3*), a member of the CLC chloride channel family in the *T. nigroviridis* genome. RT-PCR analysis revealed that the gene encoding for the ClC-3 protein was widely expressed in diverse tissues (i.e. gill, kidney, intestine, liver and brain) of FW- and seawater (SW)-acclimated pufferfish. In whole-mount double immunofluorescent staining, branchial ClC-3-like immunoreactive protein was localized to the basolateral membrane of Na^+/K^+-ATPase (NKA) immunoreactive cells in both the FW- and SW-acclimated pufferfish. In response to salinity, the levels of transcript of branchial *TnClC-3* were similar between FW and SW fish. Moreover, the membrane fraction of ClC-3-like protein in gills was 2.7-fold higher in FW compared with SW pufferfish. To identify whether the expression of branchial ClC-3-like protein specifically responded to lower environmental [Cl^-], the pufferfish were acclimated to artificial waters either with a normal (control) or lower Cl^- concentration (low-Cl). Immunoblotting of membrane fractions of gill ClC-3-like protein showed the expression was about 4.3-fold higher in pufferfish acclimated to the low-Cl environment than in the control group. Furthermore, branchial ClC-3-like protein was rapidly elevated in response to acute changes of environmental salinity or [Cl^-]. Taken together, pufferfish ClC-3-like protein was expressed in the basolateral membrane of gill MR cells, and the protein amounts were stimulated by hyposmotic and low-Cl environments. The enhancement of ClC-3-like protein may trigger the step of basolateral Cl^- absorption of the epithelium to carry out iono- and osmoregulatory functions of euryhaline pufferfish gills.

Key words: pufferfish, *Tetraodon nigroviridis*, euryhaline teleost, gill, mitochondrion-rich cells, basolateral chloride channel, ClC-3.

INTRODUCTION

Cl^- is the most abundant anion in animal tissue and plasma. Moreover, chloride channels play essential roles in the regulation of cellular excitability, transepithelial transport, cell volume regulation and acidification of intracellular organelles (Devuyst and Guggino, 2002; Jentsch et al., 1999; Jentsch et al., 2002). These functions are performed by a large number of different chloride channels that are encoded by genes belonging to several unrelated gene families (Jentsch et al., 2002). Chloride channels are involved in Cl^- transport in many types of epithelia. In the kidney, most of the filtered Cl^- is reabsorbed through different mechanisms operating in the apical and basolateral membranes of tubular epithelial cells (Devuyst and Guggino, 2002). Furthermore, chloride channels in the basolateral membrane mediate net Cl^- efflux from the cells that participate in Cl^- reabsorption in the nephrons (Blaisdell and Guggino, 2000; Devuyst and Guggino, 2002). These regulatory mechanisms contribute to maintaining osmotic and ionic homeostasis.

Osmoregulation and ion balance in teleosts is predominantly performed by the transportation of relevant ions (Na^+ and Cl^-) through epithelial transporter systems across the gill, the major osmoregulatory tissue (Marshall, 2002; Hirose et al., 2003; Evans et al., 2005; Hwang and Lee, 2007; Evans, 2008). In order to maintain homeostasis of plasma osmolality and ion components, marine or freshwater (FW) teleosts excrete excess salts of the blood to hyperosmotic environments or actively absorb salts from external hyposmotic environments, respectively, *via* the gill epithelial transport systems. The systems used by teleosts to adapt to seawater (SW) or FW differ not only in the direction of ion and water movements but also in the molecular components of the transporters (Marshall and Grosell, 2006).

Most teleosts are stenohaline fishes, living entirely in either SW or FW. Because euryhaline teleosts adapt to either SW or FW by efficiently switching epithelial transporter systems (Marshall and Grosell, 2006), they exhibit great ability to maintain plasma osmolality within narrow physiological ranges in different salinity environments (Marshall and Grosell, 2006; Kaneko et al., 2008). In SW-acclimated euryhaline teleosts, the secondary active Cl^- secretion in gills involves basolateral Na^+/K^+-ATPase (NKA), $Na^+/K^+/2Cl^-$ cotransporter (NKCC) and an apical chloride secretion channel, the cystic fibrosis transmembrane conductance regulator (CFTR) (Marshall, 2002; Hirose et al., 2003; Evans, 2008). By contrast, the mechanism of Cl^- uptake in gills of FW-acclimated euryhaline teleosts is more controversial and may involve different critical ion-transport proteins, such as basolateral V-type H^+-ATPase linked to apical $Cl^-/HCO3^-$ exchange (Tresguerres et al., 2006) or apical Na^+/Cl^- cotransporter (NCC) (Hiroi et al., 2008; Inokuchi et al., 2008; Inokuchi et al., 2009; Wang et al., 2009). These transport proteins are expressed in

mitochondrion-rich (MR) cells of gill epithelium. The basolateral exit step for Cl^- uptake in stenohaline or euryhaline teleosts, however, has not been reported (Hwang and Lee, 2007; Evans, 2008). Concerning the basolateral Cl^- channel involved in the Cl^- uptake pathway in branchial MR cells, Hirose et al. (Hirose et al., 2003), in their review study, have mentioned that members of the CLC chloride channel family that were highly expressed in the osmoregulatory organs might be candidates.

The CLC gene family was discovered by the cloning of ClC-0, a chloride channel expressed in the electric organ of the marine ray *Torpedo marmorata* (Jentsch et al., 1990). Since then, nine CLC genes have been identified in mammals (Jentsch et al., 2005). Generally, hyposmotic shock can result in cell swelling (Stutzin and Hoffmann, 2006). Meanwhile, ClC-3, a member of the CLC family, has been reported to be activated by cell swelling (Duan et al., 1997; Duan et al., 1999), and several studies have suggested that ClC-3 represents a major molecular entity responsible for native volume sensing in outwardly rectifying anion channels of various cell types in hyposmotic media (Wang et al., 2000; Duan et al., 2001; Hermoso et al., 2002).

Together, this information suggests that ClC-3 is an important chloride channel protein involved in Cl^- regulation in hyposmotic conditions. In addition, ClC-3 mRNA was found to be expressed mainly in the osmoregulatory organs, i.e. gill, kidney and intestine, of the euryhaline teleost, Mozambique tilapia (*Oreochromis mossambicus*) (Miyazaki et al., 1999). Because the genome database of the spotted green pufferfish (*Tetraodon nigroviridis*) is available, this euryhaline species, suitable for studies on fish osmoregulation (Lin et al., 2004; Tang et al., 2007; Bagherie-Lachidan et al., 2008; Bagherie-Lachidan et al., 2009; Wang et al., 2008), was used as the experimental animal in the present study. It was hypothesized that ClC-3 would exist in this model fish exhibiting the mechanism of Cl^- absorption in gills. Expression and localization of ClC-3 were thus examined in gills of FW- and SW-acclimated pufferfish. In addition, the pufferfish have been shown to be an efficient osmoregulator in experimental conditions, because they could tolerate a direct transfer from SW to FW or *vice versa* (Lin et al., 2004). Hence, time-course experiments were performed in this study to examine the expression profiles of ClC-3 in gills of pufferfish during acute salinity challenge.

MATERIALS AND METHODS

Experimental animals

The spotted green pufferfish (*Tetraodon nigroviridis* Marion de Procé 1822) were obtained from a local aquarium and were 4–7 cm in total length and 4–8 g in body mass. SW (35‰) and brackish water (15‰; BW) used in this study were prepared from local tap water with proper amounts of synthetic sea salt (Instant Ocean, Aquarium Systems, Mentor, OH, USA). The water was continuously circulated through fabric floss filters, and the temperature was held constant at 27±1°C. Fish received a constant photoperiod cycle of 12 h:12 h light:dark cycle and were fed daily with commercial arid shrimp. Because estuaries are the natural habitats of *T. nigroviridis*, the pufferfish were reared in BW in the laboratory for two weeks. The facilities and protocols for experimental animals were approved by the Animal Care and Utility Committee of National Chung-Hsing University (approval no. 95-82).

Experimental design

Acclimation experiments

After a setting period of two weeks, fish used for salinity acclimation experiments were divided into two groups. These fish were acclimated to one of two experimental salinities, i.e. FW and SW, for two weeks. In addition, to determine the effects of environmental $[Cl^-]$ on branchial ClC-3 expression, FW-acclimated pufferfish were exposed to artificial waters with different Cl^- concentrations: (1) the control group: normal Na^+/normal Cl^- or (2) the low-Cl group: normal Na^+/low Cl^-. Fish were kept in 30 l plastic tanks containing the respective media for one week at 27±1°C. Half the water was changed every other day to maintain optimal water quality. Artificial waters for both the control and low-Cl groups were prepared by dissolving appropriate amounts of NaCl, Na_2SO_4, $CaCl_2$ and $CaSO_4$ in deionized water, according to the method described in previous studies and with little modification (Chang et al., 2003; Chang and Hwang, 2004; Inokuchi et al., 2009). The pH of the media was between 6.4 and 6.5. The nominal concentrations of Na^+ and Cl^- in two artificial water preparations were: Na^+ 1 mmol l^{-1}, Cl^- 1 mmol l^{-1} in the control group; and Na^+ 1 mmol l^{-1}, Cl^- 0.01 mmol l^{-1} in the low-Cl group. The analysis of Na^+ and Ca^{2+} concentrations of water samples was carried out using an inductively coupled plasma optic emission spectrometry (ICP-OES; Perkin Elmer Optima 2100DV, Waltham, MA, USA). The Cl^- concentration of water samples was evaluated using the Ferricyanide method (Franson, 1985). Photometric analysis was carried out using a Hitachi U-2001 spectrophotometer (Tokyo, Japan). Table 1 shows the actual ion concentrations of the two artificial water preparations for the control and low-Cl groups. Fish used for salinity-acclimation experiments were fed daily except for two days prior to the following analyses of pufferfish ClC-3, including immunoblotting, reverse transcriptase polymerase chain reaction (RT-PCR), semi-quantitative RT-PCR, immunofluorescent staining and deglycosylation. In the experiment of artificial water acclimation, fish were not fed, and then gills were sampled for analyses.

Time-course experiments

In the time-course experiments (1) the SW-acclimated pufferfish were directly transferred to FW, and (2) the pufferfish acclimated to the control artificial water were transferred to the low-Cl environment. The fish were then sampled at 0, 3, 6, 12 and 24 h to study the mechanism of short-term adjustment to Cl^- absorption. The pufferfish were not fed during the period of the time-course experiments.

ClC-3 gene identification in the *Tetraodon* genome

The method of gene identification was carried out as described by Bagherie-Lachidan et al. (Bagherie-Lachidan et al., 2008; Bagherie-Lachidan et al., 2009). Using the National Center for Biotechnology Information (NCBI), the tilapia (*O. mossambicus*) ClC-3 gene

Table 1. Environmental ion concentrations

	Nominal value (mmol l^{-1})			Measured value (mmol l^{-1})		
	$[Na^+]$	$[Cl^-]$	$[Ca^{2+}]$	$[Na^+]$	$[Cl^-]$	$[Ca^{2+}]$
Low-Cl	1.00	0.01	0.50	1.180	0.012	0.486
Control	1.00	1.00	0.50	1.120	0.980	0.513

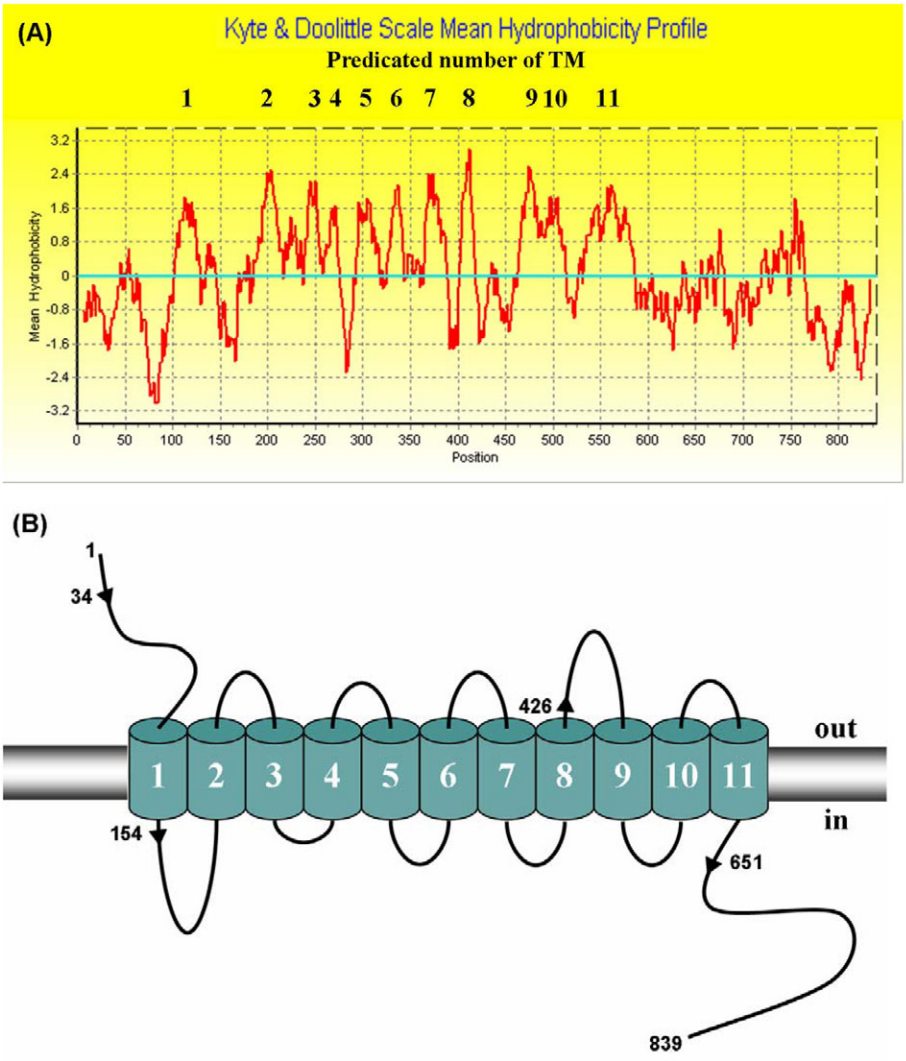

Fig. 1. Hydropathy plot and schematic representation of the predicted transmembrane (TM) region of identified ClC-3 protein in *Tetraodon nigroviridis* (TnCLC-3). (A) Kyte–Doolittle hydropathy plot (Kyte and Doolittle, 1982) of identified TnClC-3 protein. (B) Potential membrane-spanning regions were numbered 1–11 (white). Potential *N*-glycosylation sites were indicated by solid triangles.

(*OmClC-3*) sequence (accession no. AF182215) (Miyazaki et al., 1999) was retrieved. To identify homologous ClC-3 in pufferfish, *T. nigroviridis*, the *OmClC-3* sequence was subjected to the *Tetraodon* genome using the BLAT program in Genoscope (http://www.genoscope.cns.fr/externe/tetranew/). The sequence with the highest BLAT score and the highest degree of sequence identity was chosen to submit to a BLAST search against the non-redundant (nr) nucleotide database to cross-reference and confirm sequence identity with ClC-3 orthologs in NCBI. A putative ClC-3 gene of *T. nigroviridis* (*TnClC-3*) was confirmed by BLASTn. Subsequently, the coding sequence (CDS) of *TnClC-3* with high homology to the *OmCLC-3* CDS was used as a template for primer design. The deduced amino acid sequence of the identified TnClC-3 protein was analyzed by computer program (BioEdit, version 7.0.5.3) using the Kyte–Doolittle method (Kyte and Doolittle, 1982) to predict the membrane topology (Fig. 1).

RNA extraction and reverse transcription

Each total RNA sample from various tissues (i.e. gill, kidney, intestine, liver and brain) was extracted from pufferfish by using the RNA-Bee^{TM} (Tel-Test, Friendwood, TX, USA), following the manufacturer's instructions. The RNA pellet was dissolved in 30 µl of DEPC-H_2O and treated with the RNA clean-up protocol from the RNAspin Mini RNA isolation kit (GE Health Care, Piscataway, NJ, USA), following 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. First-strand cDNA was synthesized by reverse transcribing 2 µg of the total RNA using a 1 µl Oligo(dT) (0.5 µg µl^{-1}) primer and 1 µl of SuperScript^{TM} Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions.

RT-PCR analysis

For PCR amplification, 2 µl of first-strand cDNA was used as a template in a 50 µl final reaction volume containing 0.2 mmol l^{-1} dNTP, 1.25 units of Ex Taq polymerase (Takara, Otsu, Shiga, Japan) and 0.2 µmol l^{-1} of each primer. The specific primers used for *TnClC-3* and *glyceraldehyde-3-phosphate dehydrogenase* (*TnGAPDH*) (acting as an internal control) are shown in Table 2. The reaction of PCR amplification of *TnClC-3* and *TnGAPDH* was performed under the following conditions: 5 min denaturation at 95°C for one cycle, followed by 40 cycles of denaturation at 95°C (1 min), annealing at 58°C (1 min), extension at 72°C (25 s) and a final single extension at 72°C for 5 min.

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was carried out as described by Marone et al. (Marone et al., 2001) with little modification. The linear portion

Table 2. Primer sets and annealing temperatures for spotted green pufferfish (*Tetraodon nigroviridis*) *ClC-3* (*TnClC-3*) and *glyceraldehyde-3-phosphate dehydrogenase* (*TnGAPDH*)

Gene	Primer sequence	Product length (bp)	Annealing temperature (°C)
TnClC-3			
Forward	5′-CAA GTA CAG CAA GAA CGA GGC-3′	294	58
Reverse	5′-AAC ACG CCC AGG AGG ATG AA-3′		
TnGAPDH			
Forward	5′-CTG GTG CTA AGC GTG TGG TTG-3′	375	58
Reverse	5′-CCA CTG GCA CCC TGA AAG C-3′		

of the amplification curves for *TnClC-3* and *TnGAPDH* were simultaneously determined by different cycles of PCR amplification with first-strand cDNA from both FW and SW groups. The optimal number of cycles used for *TnClC-3* and *TnGAPDH* reactions was 25 cycles. The PCR products were analyzed by 1.5% agarose gels containing the Safeview DNA stain (GeneMark, Taipei, Taiwan) to confirm the expected molecular weight of the amplification product, and the band intensities were quantified by MCID software version 7.0, rev. 1.0 (Imaging Research Inc., Ontario, Canada).

Antibodies

The primary antibodies used in this study included (1) ClC-3: a rabbit polyclonal antibody (Clcn3; Alomone labs, Jerusalem, Israel) against the residues 592–661 of rat ClC-3, and (2) NKA: a mouse monoclonal antiserum (α5; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) raised against the α-subunit of avian NKA. The secondary antibody for immunoblotting was alkaline phosphatase conjugated goat anti-rabbit IgG (Chemicon, Temecula, CA, USA). For immunofluorescent staining, the secondary antibodies were Alexa-Fluor 488-conjugated goat anti-rabbit IgG or Alexa-Fluor 546-conjugated goat anti-mouse IgG (Molecular Probe, Eugene, OR, USA).

Whole-mount fluorescent immunocytochemistry and confocal microscopy

The gill filaments were removed from gill samples fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). After washing in PBS, the gill filaments were postfixed and permeated with 70% ethanol for 10 min at –20°C. The gill filaments were rinsed with PBS and then incubated in 5% BSA (Sigma, St Louis, MO, USA) for blocking. The gill filaments were then incubated at room temperature for 3 h with primary polyclonal antibody Clcn3. Following incubation, the gill filaments were washed several times with PBS, and they were then labeled with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (Invitrogen) at room temperature (25–28°C) for 2 h. After the first stain, the gill filaments were washed several times with PBS before proceeding to the second staining step. The gill filaments were subsequently incubated with primary monoclonal antibody α5 for 3 h at room temperature, followed by labeling with Alexa Fluor 546-conjugated goat anti-mouse secondary antibody (Invitrogen) at room temperature (25–28°C) for 2 h. The samples were then washed with PBS, mounted with a coverslip and observed with a confocal laser scanning microscope (Leica Lasertechnik, Heidelberg, Germany). The 488 nm argon–ion laser and the 543 nm helium–neon laser were used for observing the fluorescent staining of Alexa Fluor 488 and Alexa Fluor 546, respectively, to give the appropriate excitation wavelengths.

Preparation of gill membrane fractions

Immediately after the fish were killed by spinal pithing, the gills were extracted and gill arches were removed and blotted dry. Gill samples were immersed in liquid nitrogen and placed into ice-cold homogenization buffer (250 mmol l^{-1} sucrose, 1 mmol l^{-1} EDTA, 30 mmol l^{-1} Tris). 40 µl of a proteinase inhibitor cocktail (Roche, Mannheim, Germany) were added to 1 ml of homogenization buffer. Homogenization was performed in 2 ml tubes with the Polytron PT1200E (Lucerne, Switzerland) at maximal speed for 20 strokes. Debris, nuclei and lysosomes were removed by low speed centrifugation (13,000 ***g*** for 10 min, 4°C). The remaining supernatant was centrifuged at medium 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 plasma membrane (both apical and basolateral) along with membranes from the Golgi body and the endoplasmic reticulum but no small cytoplasmic vesicles as they typically do not pellet down unless greater forces (100,000 ***g*** for >1 h) are applied (Tresguerres et al., 2007a; Tresguerres et al., 2007b). This fraction is therefore referred to as the cell membrane fraction. Aliquots of cell membrane fractions were saved for protein determination analysis. Protein concentrations were determined with the reagents of a BCA Protein Assay Kit (Pierce, Hercules, CA, USA) using bovine serum albumin (Pierce) as a standard.

Immunoblotting

Immunoblotting procedures were carried out according to Tang et al. (Tang et al., 2009) with little modification. Proteins of the membrane fractions 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, USA). All samples were divided by electrophoresis on sodium dodecyl sulfate (SDS)-containing 7.5% polyacrylamide gels (15 µg of protein per lane). The separated proteins were then transferred to PVDF membranes (Millipore, Bedford, MA, USA) by electroblotting. After preincubation for 3 h in PBST (phosphate-buffered saline containing 0.1% Tween 20) buffer containing 5% (wt/vol.) non-fat dried milk to minimize non-specific binding, the blots were incubated at room temperature for 3 h with primary antibody diluted in 1% BSA and 0.05% sodium azide in PBST, washed in PBST and incubated at room temperature for 2 h with secondary antibody. Blots were developed after incubation with BCIP/NBT kit (Zymed, South San Francisco, CA, USA). To correct for 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 gels stained with Coomassie Blue and immunoreactive bands were analyzed using a software package (MCID software; Imaging Research, Inc.). 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 given by the ratio of antibody/Coomassie Blue.

Table 3. Overview of the information of *ClC-3* gene in spotted green pufferfish (*Tetraodon nigroviridis*) (*TnClC-3*)

Gene	Sequence identity (%)	Chromosome				Genomic length (bp)
		Chromosome number	Start location (bp)	End location (bp)	Total size (bp)	
TnClC-3	88.9	chrUn_random	21730410	21738958	171761319	8549

Sequence identity denotes sequence identity to *Oreochromis mossambicus ClC-3* (*OmClC-3*).
Genomic length denotes total length of *TnClC-3* gene.

Negative controls

The samples used for negative control of ClC-3 were identical to those for immunoblots. The specificity of anti-ClC-3 antibody (Clcn3) was confirmed by incubating the membrane fraction with the antigen pre-absorbed antibody. The antigen of ClC-3 with a molecular weight of 35 kDa was provided by the manufacturer, Alomone labs.

Deglycosylation

30 µg protein samples of the branchial membrane fractions of FW pufferfish were treated with *N*-glycosidase F (Sigma), according to the instructions of the manufacturers.

Statistical analysis

In the acclimation and artificial water experiments, statistical significance was determined using a Student's *t*-test ($P<0.05$) for group data analysis. In the time-course experiments, the effect of FW- or low-[Cl^-] transfer at different time points were assessed by comparison with the 0 h values, using Dunnett's test in which salinity effects were detected by one-way analysis of variance (ANOVA) ($P<0.05$). Measured time-course variables of SW-transfer or control-transfer groups were assessed using the same method to elucidate the stress of handling fish upon transfer. Values were expressed as means ± s.e.m.

RESULTS

Gene identification

According to Bagherie-Lachidan et al. (Bagherie-Lachidan et al., 2008; Bagherie-Lachidan et al., 2009), the combination of bioinformatics tools, i.e. the *Tetraodon* genome database (Genoscope), the National Center for Biotechnology Information (NCBI) database, BLAT and BLASTn were used, and the *chloride channel 3* (*ClC-3*) gene was identified in the genome of *T. nigroviridis*. *Tetraodon nigroviridis* ClC-3 was designated *TnClC-3* (accession no. FJ534534). *TnClC-3* displayed 88.9% sequence identity to *O. mossambicus ClC-3* (*OmClC-3*) (accession no. AF182215). The information of the *TnClC-3* gene is described in Table 3. The partial region of the identified *TnClC-3* gene was amplified by RT-PCR using the specific primers to yield PCR products of expected size (Table 2). The PCR products were also sequenced and aligned to confirm that the identified *TnClC-3* was identical to the sequence initially identified in the database. The membrane topology of deduced amino acid sequences of identified TnClC-3 was analyzed by a computer program, and the hydropathy plot (Fig. 1A) indicated that the identified TnClC-3 consisted of 11 transmembrane regions. The schematic representation is shown in Fig. 1B.

Distribution of the identified *TnClC-3*

RT-PCR with first-strand cDNA from gill, kidney, intestine, brain and liver of FW- and SW-acclimated pufferfish was conducted to determine the distribution of identified *TnClC-3* mRNA, and *glyceraldehyde-3-phosphate dehydrogenase* (*TnGAPDH*) mRNA was used as an internal control. After 40 cycles of PCR reaction with the specific primers of identified *TnClC-3*, the expected 294-bp products were found in each tested tissue from either FW or SW pufferfish (Fig. 2). Furthermore, the semi-quantitative RT-PCR analysis revealed that the mRNA abundance of gill *TnClC-3* was similar between FW- and SW-acclimated groups (Fig. 3).

Localization of ClC-3-like protein in gills of pufferfish

Localization of branchial ClC-3-like protein was determined by the whole-mount immunofluorescent staining method, and the samples were counter-stained with NKA, the basolateral marker of MR cells. In the whole-mount immunofluorescent staining, most NKA-immunoreactive cells were distributed in the afferent vascular region of the gill filaments, and no MR cells were found in gill lamellae in either experimental group. Immunoreaction of branchial ClC-3-like protein (green cells in Fig. 4B,E) were colocalized to NKA (red cells in Fig. 4A,D) and thus resulted in a yellow color of the merged images (Fig. 4C and F) to indicate branchial ClC-3-like protein expressed in the basolateral region of MR cells in FW (Fig. 4A–C) and SW (Fig. 4D–F) fish.

Protein expression of branchial ClC-3-like protein

Immunoblotting of membrane fractions of gill tissues from fish acclimated to FW or SW showed a single immunoreactive band of about 102 kDa (Fig. 5A). The immunoreactive band of the negative control was absent by preincubation of the primary antibody (Clcn3) with the antigen (Fig. 5B). Quantification of immunoreactive bands between the two environmental groups revealed significant differences ($N=6$). Branchial ClC-3-like

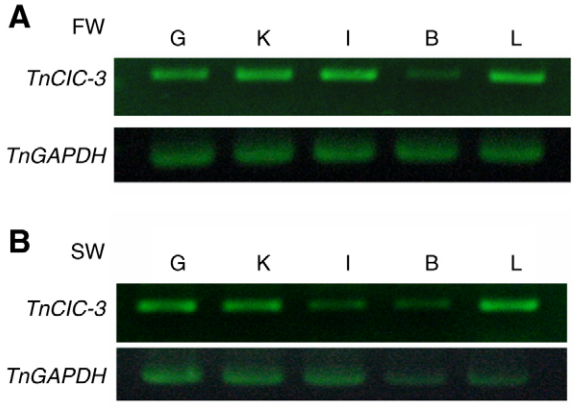

Fig. 2. Distribution of *ClC-3* genes in freshwater (FW) (A) and seawater (SW) (B) *Tetraodon nigroviridis* (*TnClC-3*) tissues by RT-PCR analysis. *Tetraodon nigroviridis glyceraldehyde-3-phosphate dehydrogenase* (*TnGAPDH*) was used as an internal control for all cDNAs used in the analysis. *TnClC-3* and *TnGAPDH* amplicons were 294 and 341 bp, respectively. 294 bp products were found in each tested tissue of FW and SW fish. G, gill; K, kidney; I, intestine; B, brain; L, liver.

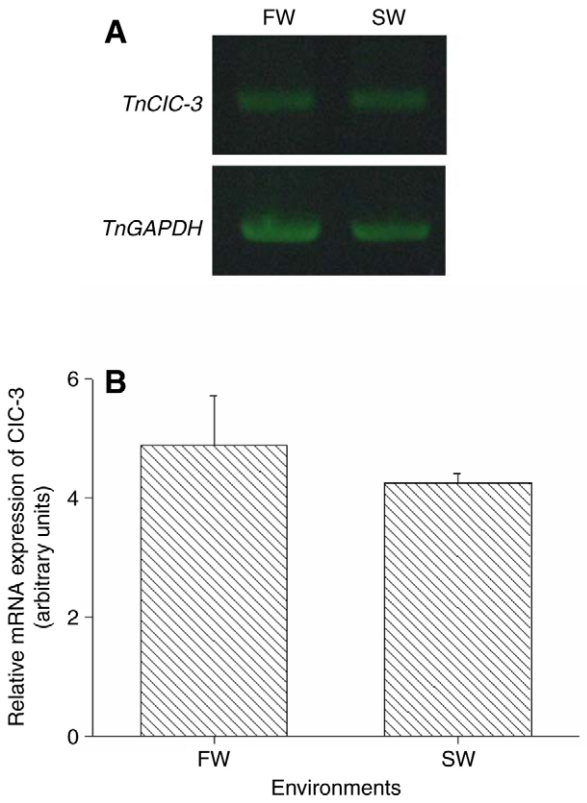

Fig. 3. Semi-quantitative RT-PCR analysis. (A) Branchial *TnClC-3* mRNA levels in freshwater (FW)- and seawater (SW)-acclimated pufferfish were determined by semi-quantitative RT-PCR. (B) Histogram of semi-quantitative RT-PCR results. Band densities representing *TnClC-3* mRNA levels in gills were normalized to *TnGAPDH*. No significant different was found between the FW and SW groups. Values are means ± s.e.m. (*N*=4).

protein amounts were 2.7-fold higher in the FW-acclimated pufferfish than in the SW group (Fig. 5C). To further illustrate the response of branchial ClC-3-like protein to changes in environmental Cl^- concentration, pufferfish were acclimated to artificial waters (control and low-Cl) as described above. A 102 kDa single immunoreactive band was also detected in the gill membrane fraction of pufferfish acclimated to two artificial waters. The immunoreactive band of low-Cl-acclimated pufferfish was substantially denser than that of the control group (Fig. 6A). Quantification of immunoreactive bands between the two environmental groups showed that the abundance of branchial ClC-3-like protein in low-Cl-acclimated pufferfish was 4.3-fold higher than that of the control group (Fig. 6B).

Time-course transfer experiments

To determine the short-term changes in expression of gill ClC-3-like protein in response to salinity or [Cl^-] changes, we performed two sets of time-course experiments. Transfer of SW-acclimated pufferfish to FW induced a 1.9-fold increase in protein abundance of gill ClC-3-like protein after 6 h post-transfer and reached a maximum level from 6 to 24 h post-transfer (Fig. 7A,B). However, the expression of gill ClC-3-like protein was elevated in response to the low-Cl environment at 3 h post-transfer, and the significantly higher protein amounts were sustained at 24 h after transfer (Fig. 7C,D).

Deglycosylation of gill CLC-3-like protein

The molecular weight of immunodetected ClC-3-like protein (102 kDa) was not in the range (93 kDa) that was calculated by bioinformatics analysis (Gasteiger et al., 2005) (www.expasy.org/tools/pi_tool.html). In order to detect whether branchial ClC-3-like protein was an *N*-glycosylated glycoprotein, the membrane fraction samples were treated with *N*-glycosidase F and caused the 102 kDa immunoreactive band to collapse to about 90 kDa (Fig. 8). Therefore, pufferfish ClC-3-like protein of 102 kDa was *N*-glycosylated membrane protein synthesized on ribosomes and transported to the Golgi complex where the glycosyl transferases-assembled complex oligosaccharide chains were located.

DISCUSSION

Anion channels are expressed in all biological membranes. Because chloride is the most abundant inorganic anion in the intracellular media and in body fluid, anion channels are commonly referred to as chloride channels (Schmieder et al., 2002). Chloride channels are involved in a broad range of physiological functions, including the transepithelial transport, cell volume regulation, stabilization of membrane potential, synaptic inhibition, extracellular and vesicular acidification, and endocytotic trafficking that may be tissue-, cell type-specific or housekeeping distribution in various tissues (Schmieder et al., 2002; Jentsch et al., 2005). In mammals, chloride channels are important for transepithelial chloride transport in osmoregulatory organs (Devuyst and Guggino, 2002). Excellent osmoregulatory ability is crucial for euryhaline teleosts living in hyper- and hyposmotic environments. The apical chloride channel, CFTR and basolateral NKCC responsible for chloride secretion has been illustrated in review studies (Marshall, 2002; Hirose et al., 2003; Evans, 2008). In FW fish, the chloride absorption mechanism is composed of the apical Cl^-/HCO_3^- exchange (i.e. pendrin) (Piermarini et al., 2002) or NCC (Hiroi et al., 2008; Inokuchi et al., 2008; Inokuchi et al., 2009). However, the basolateral chloride channel to absorb chloride from cell to blood in gills of FW-acclimated teleosts is yet not clear (Evans, 2008; Tresguerres et al., 2006). In the present investigation, we therefore used different experimental designs to assess the gill expression of ClC-3, a member of the CLC chloride channel family, in gills of a euryhaline teleost, the spotted green pufferfish (*T. nigroviridis*).

The CLC family plays multiple roles in biological membranes, and ClC-3 is a member that is ubiquitously expressed in diverse tissues of mammals (Jentsch et al., 1999; Jentsch et al., 2002; Jentsch et al., 2005). In fish, ClC-3 was cloned from cDNA libraries of the euryhaline tilapia (*O. mossambicus*), and it was found that *OmClC-3* mRNA was broadly expressed in different tissues in FW- and SW-acclimated tilapia (Miyazaki et al., 1999). In the present study, the hydropathy plot was analyzed by a computer program to show that the amino acid sequence of identified TnClC-3 protein was comprised of 11 transmembrane regions (Fig. 1), which coincided with the common structural features of the CLC family with 10 to 12 transmembrane regions (Jentsch et al., 1999; Waldegger and Jentsch, 2000). The RT-PCR analysis showed that the *TnClC-3* gene was expressed in various tissues of pufferfish, including osmoregulatory tissues (i.e. gill, kidney and intestine) (Fig. 2), and this might indicate that *TnClC-3* plays diverse functions in different tissues.

Extra- or intracellular osmotic disturbances alter the cell volume and trigger a multitude of intracellular signaling events, including various secondary message cascades, phosphorylation or dephosphorylation of target proteins, as well as altered gene or

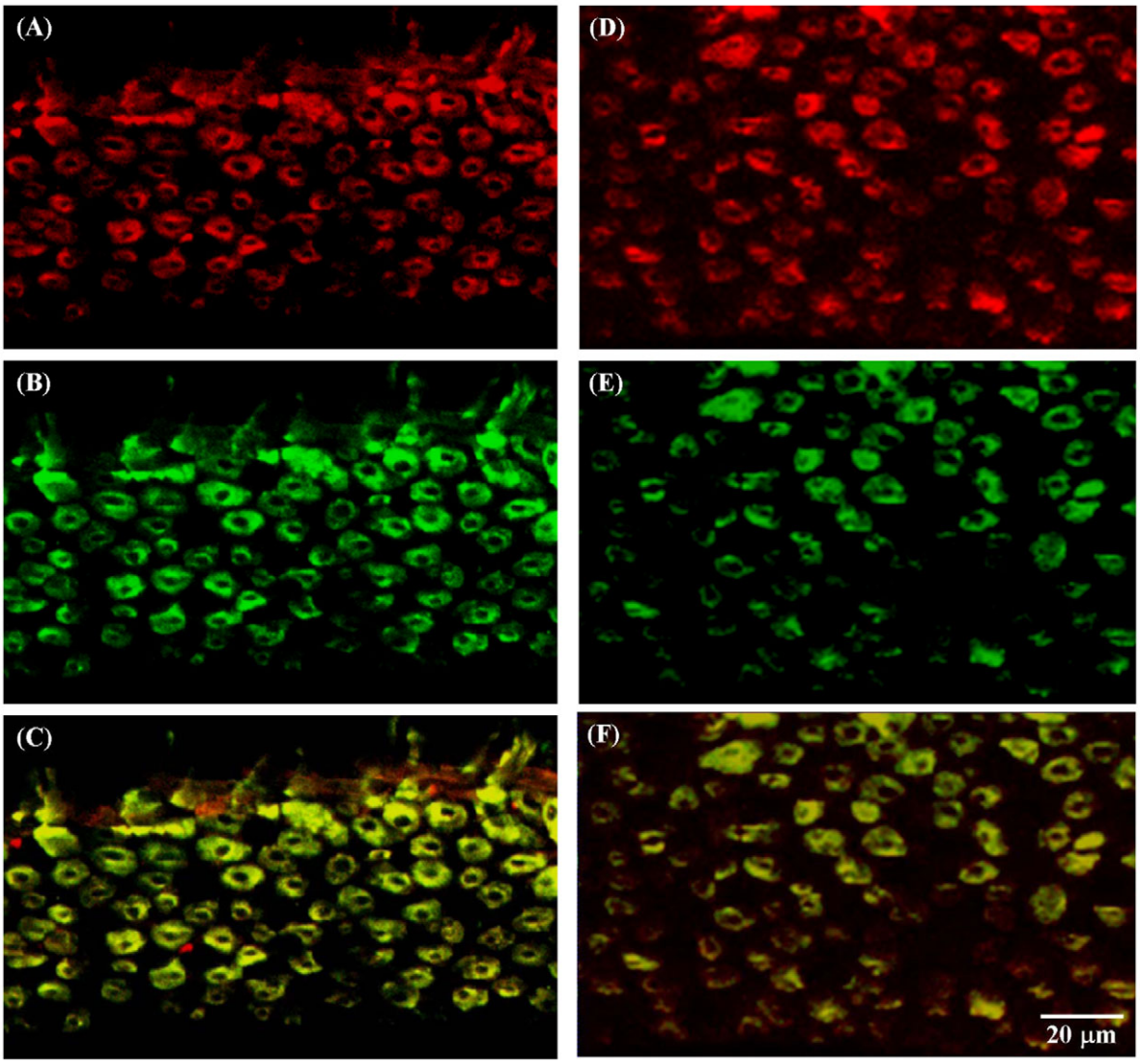

Fig. 4. Confocal laser scanning micrographs of whole-mount immunofluorescent staining of the gill filaments in freshwater (FW)- and seawater (SW)-acclimated pufferfish. Gill filaments were double stained with anti-Na^+/K^+-ATPase (NKA) (red; A and D) and anti-ClC-3 (green; B and E). The merged images (C and F) of double-stained gill filaments showed that ClC-3-like protein was colocalized to NKA-immunoreactive cells (i.e. mitochondrion-rich cells). Scale bar, 20 μm.

protein expression (Waldegger et al., 1997a; Waldegger et al., 1997b; Lang et al., 1998; Duan et al., 1999). ClC-3 expression in mammalian cells is strongly inhibited by hyperosmotic cell shrinkage whereas it is activated by hyposmotic cell swelling (Duan et al., 1997). Furthermore, several studies demonstrated that ClC-3 represents a major molecular entity responsible for native volume sensing of outwardly rectifying anion channels in hyposmotic medium in cardiac cells (Duan et al., 1997; Duan et al., 2001; Wang et al., 2003), smooth muscle cells (Duan et al., 2001; Wang et al., 2003), bovine epithelial cells (Wang et al., 2000), HeLa cells and ClC-3-expressed *Xenopus* oocytes (Kawasaki et al., 1994; Hermoso et al., 2002) as well as non-pigmented ciliary epithelial cells (Vessey et al., 2004). Because the gill is in direct contact with the external environment and is crucial to osmoregulation in fish (Evans et al., 2005), environmental salinity changes would cause alteration of the levels of related genes or proteins to compensate for osmotic homeostasis. In pufferfish gills, the semi-quantitative RT-PCR revealed that mRNA abundance of branchial *TnClC-3* was similar between FW and SW groups (Fig. 3) whereas the amounts of ClC-3-like protein were significantly elevated by the hyposmotic environment (Fig. 5). The lack of correlation between mRNA and protein was also found in the other ion transport proteins, for example, branchial CFTR of killifish (*Fundulus heteroclitus*) (Scott et al., 2004). The constant mRNA expression in different environments might be attributed to the regulatory mechanism of translation rather than transcription being activated in pufferfish upon hyposmotic challenge. In addition, increased ClC-3-like protein expression in gills of FW-acclimated pufferfish was in accordance with the hypothesis that ClC-3 is a hyposmotically activated chloride channel (Duan et al., 1997). In contrast to ClC-3, NKCC protein was inhibited by hyposmotically induced cell swelling (Klein et al., 1993; Haas et al., 1995; Lytle, 1998). Similarly, decreased protein expression of branchial NKCC was also found in gills of FW-acclimated euryhaline teleosts (Scott et al., 2004; Tipsmark et al., 2004; Tang and Lee, 2007; Tse et al., 2007; Wilson et al., 2007).

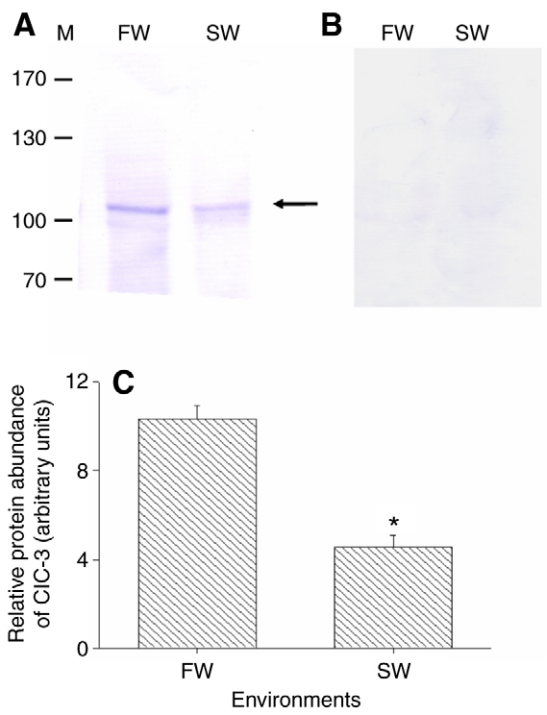

Fig. 5. The representative immunoblot and negative control of the freshwater (FW)- and seawater (SW)-acclimated pufferfish (*Tetraodon nigroviridis*) gills probed with (A) a primary antibody to ClC-3 (Clcn3) and (B) the primary antibody pre-incubated with the antigen. Compared with the negative control, a single immunoreactive band with molecular weight of about 102 kDa was obtained (arrow). The immunoreactive bands of FW-acclimated pufferfish were more intensive than SW-acclimated individuals. (C) Relative abundance of immunoreactive bands of ClC-3-like protein in gills of different salinity groups (N=6). Expression of ClC-3-like protein was 2.7-fold higher in the FW group than the SW group. The asterisk indicated a significant difference (P<0.05) by Student's t-test. Values are means ± s.e.m. M, marker.

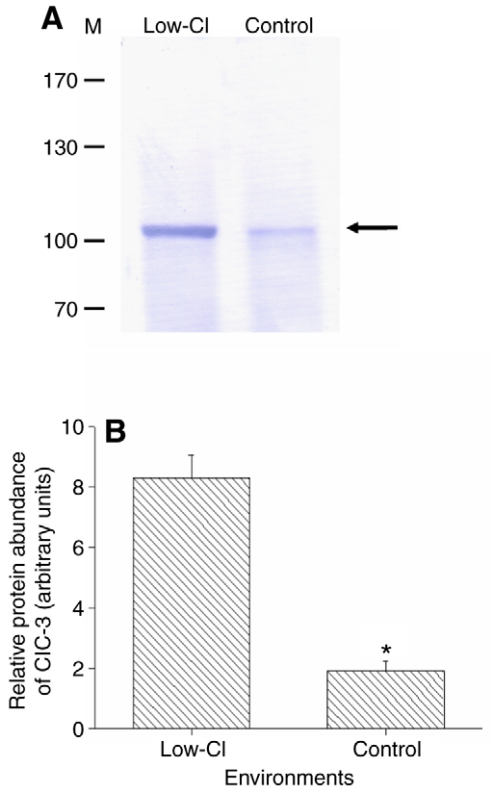

Fig. 6. (A) The representative immunoblot of artificial-water-acclimated pufferfish (*Tetraodon nigroviridis*) gills probed with a primary antibody to ClC-3 (Clcn3). A single immunoreactive band with molecular weight of about 102 kDa is indicated by an arrow. The immunoreactive bands of low-Cl-acclimated pufferfish were more intensive than control-acclimated individuals. (B) Relative abundance of immunoreactive bands of ClC-3-like protein in gills of different salinity groups (N=6). Expression of ClC-3-like protein was 4.3-fold higher in the low-Cl group than the control group. The asterisk indicated a significant difference (P<0.05) by Student's t-test. Values are means ± s.e.m. M, marker; low-Cl, normal Na^+/low Cl^- artificial water; control, normal Na^+/normal Cl^- artificial water. The measured ion concentrations of these two artificial waters were shown in Table 1.

Chloride channels expressed in plasma membranes play an important role in transepithelial transport. Results of previous studies, however, were inconsistent in whether the ClC-3 protein is an intracellular channel or a plasma membrane channel (Nilius and Droogmans, 2003), even though the ClC-3 chloride channel, activated by hyposmotically induced cell swelling, has been verified. Weylandt et al. provided the evidence to show that a proportion of the ClC-3 protein is present on the plasma membrane (Weylandt et al., 2001). In rabbit non-pigmented ciliary epithelial cells, an enhanced shift in ClC-3 immunofluoresence from intracellular locations to the plasma membrane is clearly evident in cells exposed to hyposmotic solutions as compared with isosmotic solutions (Vessey et al., 2004). Moreover, Huang et al. demonstrated that the Cl^- current was significantly increased in the *ClC-3* gene-transfected mammalian epithelial cell line, tsA, followed by calcium/calmodulin-dependent protein kinase II (CaMKII) activation (Huang et al., 2001). Subsequently, increases in the level of intracellular Ca^{2+} induced a translocation of ClC-3 from the cytoplasmic compartment to the plasma membrane in tsA and the human colonic tumor cell line T84. In addition, the preparation of membrane fractions was successfully used to detect ClC-3 expression in plasma membranes (Shimada et al., 2000). According to Tresguerres et al. (Tresguerres et al., 2007a; Tresguerres et al., 2007b), the membrane fraction used in the present study to detect the ClC-3-like protein in gills of pufferfish should contain large fragments of plasma membrane along with membranes from the Golgi and the endoplasmic reticulum. Therefore, branchial ClC-3-like protein should be predominantly expressed in the plasma membrane in pufferfish gills.

The localization of membrane transporter proteins is related to their function in epithelia. In a previous model, CFTR was proposed as the basolateral chloride channel in the pavement and MR cells in opercular epithelium of FW killifish (Marshall, 2002). However, branchial CFTR was expressed in SW-acclimated rather than FW-acclimated pufferfish, as shown both by immunoblotting (Tang et al., 2007) and immunofluorescent staining (C.H.T. and T.H.L., unpublished data). Because the present study has demonstrated that ClC-3-like protein was colocalized to NKA in the basolateral membrane of MR cells (Fig. 4), the chloride channel of the working model of the basolateral Cl^- exit step in gills of pufferfish would be ClC-3-like protein rather than CFTR.

To further clarify whether ClC-3-like protein expression in gills of pufferfish was specifically affected by changes in environmental Cl^- concentration, the pufferfish were subjected to artificial water environments. Artificial water experiments have been used to

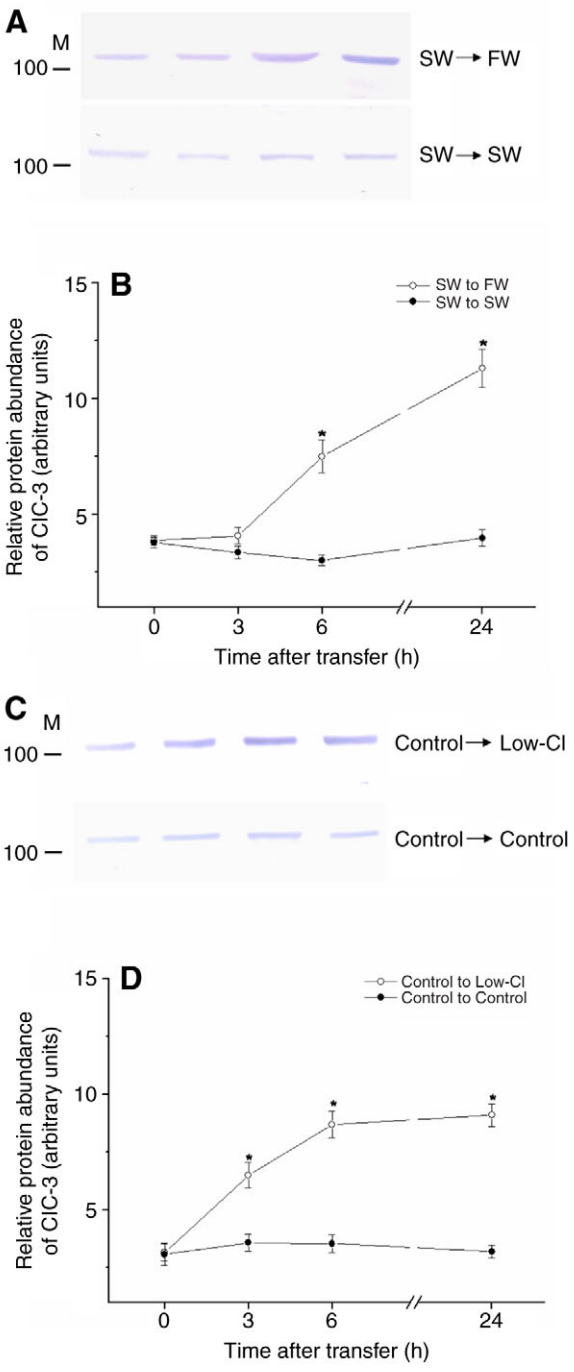

Fig. 7. Changes of relative protein abundance of ClC-3 like protein in pufferfish gills following direct transfer from seawater (SW) to freshwater (FW) (A and B) and from control artificial water (control) to low [Cl^-] artificial water (low-Cl) (C and D). The SW and Control fish were transferred simultaneously to SW and control artificial water, respectively, as the sham groups of the time-course experiments. (A and C) Representative immunoblots of ClC-3-like protein probed with a primary antibody Clcn3 revealed a single immunoreactive band at approximately 102 kDa. Relative protein abundance of ClC-3-like protein increased significantly during the first 6 h post-transfer and elevated 3.3-fold at 24 h after transfer compared with the 0 h when transferred from SW to FW (B). Relative protein abundance of ClC-3-like protein following transfer from control to low-Cl elevated significantly during the first 3 h post-transfer and increased 2.7-fold at 6 h and 24 h after transfer compared with 0 h (D). The asterisks indicate significant differences (P<0.05). Values are means ± s.e.m. (N=5). M, marker.

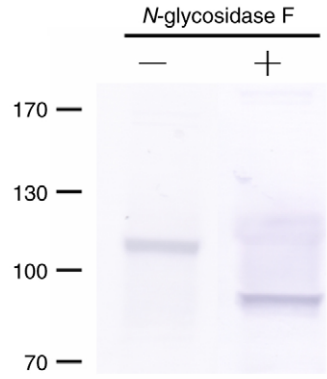

Fig. 8. *N*-Glycosidase F digestion of ClC-3-like protein in gills of freshwater (FW) pufferfish. Gill membrane fractions were prepared from FW-acclimated pufferfish and treated without (–) or with (+) *N*-Glycosidase F. The samples were detected by immunoblotting with anti-ClC-3 antibody (Clcn3). *N*-glycosidase F caused the 102 kDa band to collapse to a band about 95 kDa (arrow) and demonstrated that the single band at 102 kDa of the immunoblots detected in this study using fresh pufferfish gill samples was the glycosylated form of ClC-3-like protein.

examine iono- and osmoregulatory mechanisms of single ions in several studies (Chang et al., 2003; Chang and Hwang, 2004; Yan et al., 2007; Inokuchi et al., 2009). Moreover, Chang et al. verified that Cl^- influx was enhanced in tilapia acclimated to a medium of low Cl^- concentration (Chang et al., 2003). This study provided the direct *in vivo* evidence that ClC-3-like protein abundance specifically increased in response to environments of low Cl^- concentration (Fig. 6). Taken together, the results of the low [Cl^-] experiments implied the possible role for Cl^- uptake of the basolateral chloride channel, ClC-3.

The pufferfish is a peripheral FW teleost (Helfman et al., 1997), and this species displayed efficient osmoregulatory ability in experimental conditions, because it can tolerate a direct transfer from SW to FW or *vice versa* (Lin et al., 2004). The time-course experiments were conducted to show that ClC-3-like protein was significantly elevated at 6 h post-transfer from SW to FW. In addition, to survive in the extreme environment of low Cl^- concentration, branchial ClC-3-like protein was rapidly modulated within 3 h after transfer. This might indicate that the osmotic imbalance of pufferfish was initially adjusted at a short time scale (3 to 6 h) and achieved osmotic homeostasis within 24 h, which was similar to striped bass (*Morone saxatilis*) (Tipsmark et al., 2004). Meanwhile, the results implied that the euryhalinity of pufferfish was well developed to promptly activate hyper-osmoregulatory mechanisms in pufferfish upon hyposmotic challenge.

The molecular weight of immunodetected ClC-3-like protein (102 kDa) was not in the range (93 kDa) that was calculated by bioinformatics analysis (Gasteiger et al., 2005) (www.expasy.org/tools/pi_tool.html). ClC-3 was reported as the *N*-glycosylated glycoprotein in mammals (Schmieder et al., 2001; Gentzsch et al., 2003). Hence, enzymatic digestion of N-linked oligosaccharide residues was performed on gill membrane fractions. The immunoreactive band shifted to about 90 kDa after treatment with *N*-glycosidase F (Fig. 8). Therefore, the single band at 102 kDa of the immunoblots detected in this study using fresh pufferfish gill samples was the glycosylated form of ClC-3-like protein.

In conclusion, a potential mechanism of Cl^- uptake in fish gill epithelium was illustrated in this study. The hyposmotic or low Cl^-

concentration environment induced ClC-3 protein expression in basolateral membranes of branchial MR cells, which might contribute to the basolateral exit step for Cl^- absorption. The regulatory mechanisms and pathways of ClC-3 trafficking to the plasma membrane have already been identified in mammals (Duan et al., 1999; Ogura et al., 2002; Zhao et al., 2007). To realize more detailed characteristics of ClC-3 expression in fish, the regulatory mechanism will be examined in future studies.

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