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Comparative Biochemistry and Physiology, Part A 147 (2007) 521–528
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The effect of environmental salinity on the protein expression of Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter, cystic fibrosis transmembrane conductance regulator, anion exchanger 1, and chloride channel 3 in gills of a euryhaline teleost, *Tetraodon nigroviridis*

C.H. Tang, T.H. Lee *

Department of Life Sciences, National Chung-Hsing University, Taichung 402, Taiwan

Received 12 June 2006; received in revised form 30 January 2007; accepted 30 January 2007
Available online 7 February 2007

Abstract

Chloride transport mechanisms in the gills of the estuarine spotted green pufferfish (*Tetraodon nigroviridis*) were investigated. Protein abundance of Na^+/K^+-ATPase (NKA) and the other four chloride transporters, i.e., $Na^+/K^+/2Cl^-$ cotransporter (NKCC), cystic fibrosis transmembrane conductance regulator (CFTR), Cl^-/HCO_3^- anion exchanger 1 (AE1), and chloride channel 3 (CLC-3) in gills of the seawater- (SW; 35‰) or freshwater (FW)-acclimatized fish were examined by immunoblot analysis. Appropriate negative controls were used to confirm the specificity of the antibodies to the target proteins. The relative protein abundance of NKA was higher (i.e., 2-fold) in gills of the SW group compared to the FW group. NKCC and CFTR were expressed in gills of the SW group but not in the FW group. In contrast, the levels of relative protein abundance of branchial AE1 and CLC-3 in the FW group were 23-fold and 2.7-fold higher, respectively, compared to those of the SW group. This study is first of its kind to provide direct *in vivo* evidence of the protein expression of CLC-3 in teleostean gills, as well as to examine the simultaneous protein expression of the Cl^- transporters, especially AE1 and CLC-3 of FW- and SW-acclimatized teleosts. The differential protein expression of NKA, chloride transporters in gills of the FW- and SW-acclimatized *T. nigroviridis* observed in the present study shows their close relationship to the physiological homeostasis (stable blood osmolality), as well as explains the impressive ionoregulatory ability of this euryhaline species in response to salinity challenges.

Keywords: Pufferfish; *Tetraodon nigroviridis*; Euryhaline teleost; CFTR; NKCC; Gill; Chloride transporter; Na^+/K^+-ATPase

1. Introduction

Maintaining a stable internal environment is important for vertebrate animals to survive in a variety of habitats. In response to changes of environmental conditions, the ion-transporting epithelia play the roles of modulating ion fluxes. Although ionoregulation in fish is mediated by a group of structures including the intestine and kidney, the gill is the major site for the balance of ion movement between gains and losses (Evans et al., 2005). In order to maintain the osmolality and ion balance, teleosts take up salts from fresh water (FW) through the gills and reabsorb salts in the kidney. In contrast, seawater (SW) teleosts excrete salts through the gills and absorb water in the kidney. The systems used by teleosts to adapt to FW or SW differ not only in the direction of ion and water movements but also in the molecular components of transporters. Euryhaline fish adapt to either FW or SW by switching these systems efficiently (Kato et al., 2005).

The fundamental transporters responsible for ion movement across gill epithelia have been reported in previous studies (see reviews of Perry, 1997; Hirose et al., 2003; Evans et al., 2005). Among the transporters, Na^+/K^+-ATPase (NKA) was thought to provide primary driving force for ion transport. NKA is a ubiquitous membrane-bound enzyme which is a P-type ATPase consisting of an $(\alpha\beta)_2$ protein complex. The molecular mass of

* Corresponding author. Tel.: +886 4 2285 6141; fax: +886 4 2285 1797.
E-mail address: thlee@dragon.nchu.edu.tw (T.H. Lee).

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doi:10.1016/j.cbpa.2007.01.679

the catalytic α-subunit is about 100 kDa, while the smaller glycosylated β-subunit exists with a molecular mass of approximately 55 kDa (Blanco and Mercer, 1998). Most euryhaline teleosts exhibit adaptive changes in NKA activity following salinity changes (Lin et al., 2003). In gills of euryhaline teleosts, a basolaterally located NKA creates an electrochemical gradient to transport Na^+ and Cl^- actively across epithelia in both secretory (SW) and absorptive (FW) modes (Marshall, 2002; Hirose et al., 2003; Perry et al., 2003).

For secretion of chloride ions in gill epithelia of SW teleosts, Silva et al. (1977) proposed a model in which a basolaterally located Na^+/Cl^- exchanger that brought chloride ions into the cell. Then pharmacological evidence proposed that basolateral Na^+/Cl^- cotransport occurred through a bumetanide-sensitive $Na^+/K^+/2Cl^-$ cotransporter (NKCC; Degnan, 1984). NKCC included two isoforms, NKCC1 and NKCC2. Because of prominent expression of NKCC1 on basolateral confinement and it has been confirmed to operate in chloride-secreting epithelia, NKCC1 was considered to be the secretory isoform. A second form, NKCC2, was only found in the kidney (Lytle et al., 1995). Instead of being diffusive, Cl^- secretion at the apical membrane may use an anion channel with characteristics resembling that of the cystic fibrosis transmembrane conductance regulator (CFTR; Marshall et al., 1995). CFTR was reported to exist in gills of teleosts by electrophysiological, molecular biological, pharmacological, biochemical, and immunocytochemical studies (Marshall and Singer, 2002). On the other hand, for absorption of chloride ions in gills of FW teleosts, the model was not conclusive (Hirose et al., 2003). Chloride influx, however, was shown to be stimulated by infusion of bicarbonate with a good correlation between the rates of Cl^- absorption and base secretion (Kerstetter and Kirschner, 1972; de Renzis and Maetz, 1973). An epithelial Cl^-/HCO_3^- exchanger in fish gills was reported in a kinetic, pharmacological and correlative morphological study (Goss et al., 1995; Tresguerres et al., 2006). The studies of Wilson et al. (2000a, 2002) proved the distribution of Cl^-/HCO_3^- exchanger 1 (AE1) in fish gills. In addition, the chloride channel to absorb chloride from cell to blood in gills of FW teleosts is not clear (Evans et al., 2005; Tresguerres et al., 2006). A member of the CLC family was suggested to be the chloride channel in gills of FW teleosts (Hirose et al., 2003). Transporters of the CLC family, such as CLC-3, are important for transepithelial Cl^- transport in different organs of mammals (Kawasaki et al., 1994; Sasaki et al., 1994; Weylandt et al., 2001). In teleosts, the mRNA of CLC-3 was demonstrated to be abundant in the gills (Miyazaki et al., 1999).

The spotted green pufferfish (*Tetraodon nigroviridis*) is an advanced tetraodontid teleost whose native range covers the rivers and estuaries of Southeast Asia (Rainboth, 1996). Being a peripheral FW inhabitant (Helfman et al., 1997), this pufferfish was demonstrated to be an efficient osmoregulator in experimental conditions, as it can tolerate a direct transfer from FW to SW or vice versa (Lin et al., 2004; Lin and Lee, 2005). The present set of experiments was designed to identify the adaptive responses of protein expression of Cl^- transporters in gills of the spotted green pufferfish acclimated to salinities of a hyperosmotic environment (i.e., SW) or a hyposmotic environment (i.e., FW). This study, to our knowledge, is the first to provide direct *in vivo* evidence of CLC-3 protein expression in teleostean gills, as well as the first to compare simultaneous protein expression of the four Cl^- transporters of SW- and FW-acclimatized euryhaline teleost.

2. Materials and methods

2.1. Fish and experimental environments

The spotted green pufferfish (*T. nigroviridis*) were obtained from a local aquarium with the body weight of 5.6±0.4 g and the total length of 5.6±0.5 cm. Seawater (35‰; SW) used in this study was prepared from local tap water with proper amounts of synthetic sea salt (Instant Ocean, Aquarium Systems, Mentor, OH, USA). The spotted green pufferfish were reared in either SW or FW at 27±1 °C with a daily 12 h photoperiod for at least 2 weeks before sampling (Lin et al., 2004). The water was continuously circulated through fabric-floss filters. The fish were fed with commercial arid shrimp daily.

2.2. Preparation of membrane fractions for NKA, AE1, CLC-3, NKCC and gill lysates for CFTR

Gill arches of the fish were excised and blotted dry. The gill epithelia were immediately scraped off from the underlying cartilage with a scalpel. All procedures were performed on ice. Gill scrapings were suspended in 300 μL of (a) buffer A (20 mmol L^{-1} Tris–base, 2 mmol L^{-1} $MgCl_2 \cdot 6H_2O$, 2 mmol L^{-1} EDTA, 0.5 mmol L^{-1} EGTA, 1 mmol L^{-1} DTT, 250 mmol L^{-1} sucrose, proteinase inhibitor, pH 7.4) for NKA, AE1, and CLC-3, or (b) ice-cold SEI buffer (300 mmol L^{-1} sucrose, 20 mmol L^{-1} EDTA, 100 mmol L^{-1} imidazole, pH 7.4) for NKCC and CFTR, respectively. Homogenization was performed in 2 mL tubes with the Polytron PT1200E (Lucerne, Switzerland) at maximal speed for 25 strokes. Membrane fractions of NKA, AE1, and CLC-3 were prepared according to the method modified from Stanwell et al. (1994). After homogenization, the homogenates for NKA, AE1, and CLC-3 were then centrifuged at 135,000 *g* for 1 h at 4 °C. Supernatants were the mixture of cytosol protein. The pellets were resuspended in 300 μL buffer B (20 mmol L^{-1} Tris–base, 2 mmol L^{-1} $MgCl_2 \cdot 6H_2O$, 5 mmol L^{-1} EDTA, 0.5 mmol L^{-1} EGTA, 1 mmol L^{-1} DTT, 5 mmol L^{-1} NaF, 0.1% Triton X-100, proteinase inhibitor, pH 7.5) and vortexed every 10 min during a 1 h incubation period at 4 °C. Dissolved pellets were centrifuged again at 135,000 *g* for 1 h at 4 °C. Supernatants were the mixture of membrane fractions for NKA, AE1, and CLC-3. According to Tipsmark et al. (2004), the homogenates for NKCC was centrifuged at 1000 *g* for 20 min at 4 °C. The membrane fraction of NKCC was the pellet isolated from the supernatant by a second centrifugation at 50,000 *g* for 30 min at 4 °C. The homogenates for CFTR were carried out as described by Marshall et al. (2002b) with little modification. After centrifugation at 2000 *g* at 4 °C for 6 min, the pellet was

resuspended in SEI buffer and centrifuged again at 2000 *g* for 6 min. The obtained pellets of CFTR and NKCC as described above were resuspended in SEI buffer. 10 μL of proteinase inhibitor (10 mg antipain, 5 mg leupeptin, and 50 mg benzamidine dissolved in 5 mL aprotinin) was added to 1 mL of buffer A, B, or SEI. Protein concentrations were identified by reagents from the Protein Assay Dye (Bio-Rad, Hercules, CA, USA), using bovine serum albumin (Sigma, St. Louis, MO, USA) as a standard. All of the membrane fractions and gill lysates were stored at −80 °C before immunoblotting.

2.3. Preparation of erythrocyte fractionation

The blood of the spotted green pufferfish was collected from the caudal veins. Then the blood was centrifuged at 600 *g* for 10 min to bring down erythrocytes. The low speed works were performed because the red blood cells were heavily packed with hemoglobin. After removing the supernatant, the pellet was suspended in isotonic buffer (0.9% NaCl in 5 mmol L^{-1} sodium phosphate, pH 8), and then centrifuged again. Finally, the pellet was re-suspended in hypotonic buffer (5 mmol L^{-1} sodium phosphate, pH 8, without NaCl). Protein concentrations were quantified with the Protein Assay Dye (Bio-Rad, Hercules, CA, USA), using bovine serum albumin (Sigma, St. Louis, MO, USA) as a standard. All erythrocyte fractionations were stored at −80 °C before immunoblotting.

2.4. Antibodies/antisera

The primary antibodies/antisera used in the present study included (1) NKA: a mouse monoclonal antiserum (α5; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) raised against the α-subunit of avian NKA; (2) NKCC: a mouse monoclonal antiserum (T4; Developmental Studies Hybridoma Bank) raised against the c-terminus of human NKCC; (3) CFTR: a mouse monoclonal antibody (M3A7; NeoMarkers, Fremont, CA, USA) raised against the NBD2 domain of human CFTR; (4) AE1: a rabbit polyclonal antiserum (tAE1) was kindly provided by Prof. P. P. Hwang (Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan) raised against the AE1 of tilapia; (5) CLC-3: a rabbit polyclonal antibody (Clcn3; Alomone labs, Jerusalem, Israel) against the residues 592–661 of rat CLC-3. The secondary antibodies were alkaline phosphatase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Jackson Immuno Research, West Grove, PA, USA).

2.5. Immunoblots of NKA, AE1, CLC-3, NKCC, and CFTR

Immunoblotting procedures were carried out according to Lee et al. (2000) with little modification. Proteins of the membrane fractions of NKA, AE1, or CLC-3, were heated together with the sample buffer at 37 °C for 30 min. For NKCC, membrane fractions were heated at 65 °C for 10 min. The gill lysates for CFTR were heated at 60 °C for 15 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 (25 μg of protein/lane). The separated proteins were then transferred to PVDF membranes (Millipore, Bedford, MA, USA) by electroblotting. After preincubation for 3 h in PBST buffer containing 5% (wt/vol) nonfat dried milk to minimize nonspecific binding, the blots were incubated at 4 °C for overnight with primary antibodies diluted in 1% BSA and 0.05% sodium azide in PBST, washed in PBST, and incubated at room temperature for 1.5 h with secondary antibodies. Blots were developed after incubation with BCIP/NBT kit (Zymed, South San Francisco, CA, USA). Immunoblots were photographed and imported as TIF files into the commercial image analysis software package (Kodak digital Science, 1995). Results were converted to numerical values in order to compare the relative intensities of the immunoreactive bands.

2.6. Negative controls

The samples used for negative controls of different transporters were identical to those for immunoblots. For NKA, NKCC, and AE1, pre-immune sera of mouse (NKA, NKCC) or rabbit (AE1) were used to substitute those primary antisera,

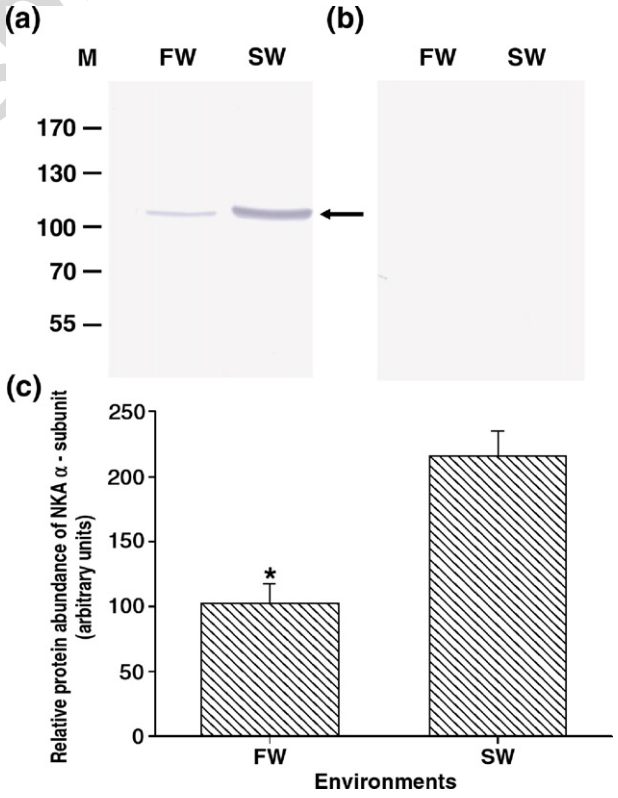

Fig. 1. Representative immunoblot and negative control of the pufferfish (*Tetraodon nigroviridis*) gills probed with (a) a monoclonal antiserum α5 to NKA α-subunit and (b) the pre-immune serum of mouse, respectively. The immunoreactive bands had molecular masses centered at 105 kDa (arrow). The immunoreactive bands of SW-acclimatized fish were more intensive than FW-acclimatized individuals. (c) Relative abundance of immunoreactive bands of NKA α-subunit in gills of different salinity groups ($n=4$). Expression of NKA α-subunit was 2-fold higher in the SW group than the FW group. The asterisk indicated a significant difference ($P<0.05$) by unpaired *t*-test. Values are means ± S.E.M. M, marker; FW, fresh water; SW, seawater.

respectively. CFTR or CLC-3 was confirmed by incubating the lysate or membrane fraction with the antigen pre-absorbed antibody. The antigen of CFTR was synthesized on the basis of the recognized sequence of the monoclonal antibody, M3A7, by MDBio, Inc, Taipei, Taiwan. The antigen of CLC-3 with molecular weight of 35 kDa was provided by the manufacturer, Alomone labs.

2.7. Statistical analysis

The significance of the difference between treatments was assessed by unpaired *t*-test ($P<0.05$). Values were expressed as means±S.E.M.

3. Results

3.1. Branchial Na^+/K^+-ATPase (NKA) expression

Immunoblotting of membrane fractions of gill tissues from pufferfish acclimatized to fresh water (FW) or seawater (SW) resulted in a single immunoreactive band of about 105 kDa (Fig. 1a). The immunoreactive band of negative control was absent by replacing the primary antibody (α5) with the pre-immune serum of mouse (Fig. 1b). Quantification of immunoreactive bands between the two environmental groups revealed significant differences. Gill NKA α-subunit protein amounts of the SW-acclimatized fish were about 2-fold higher than those of the FW group (Fig. 1c).

3.2. Branchial $Na^+/K^+/2Cl^-$ cotransporter (NKCC) expression

Immunoblotting of membrane fractions of gill tissues from pufferfish acclimatized to FW or SW resulted in a single immunoreactive band of about 170 kDa (Fig. 2a). The immunoreactive band of negative control was absent by replacing the primary antibody (T4) with the pre-immune serum of mouse (Fig. 2b). The result showed that NKCC expressed only in SW-pufferfish rather than FW individuals.

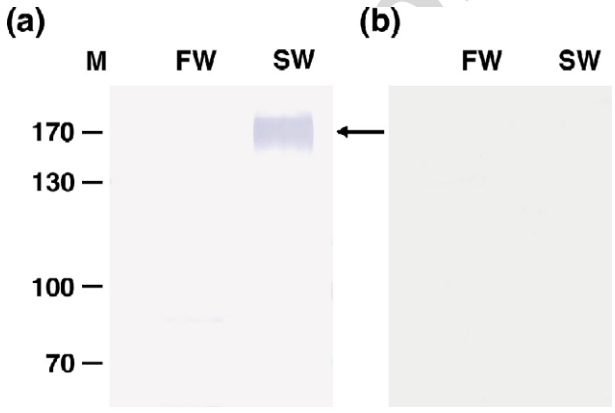

Fig. 2. Representative immunoblot and negative control of the pufferfish (*Tetraodon nigroviridis*) gills probed with (a) a monoclonal antiserum (T4) to NKCC and (b) pre-immune serum of mouse, respectively. The immunoreactive bands only detected only in SW fish ($n=8$) had molecular masses centered at 170 kDa (arrow). M, marker; FW, fresh water; SW, seawater.

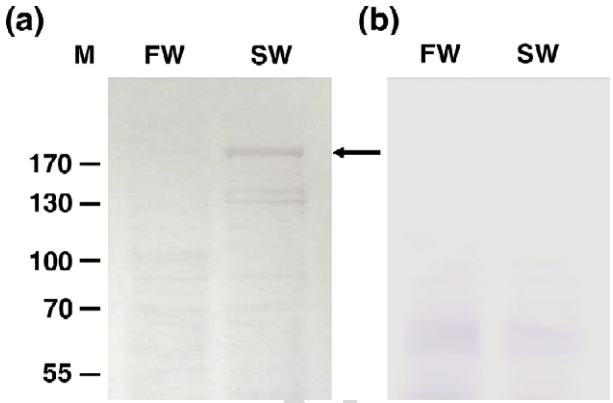

Fig. 3. Representative immunoblot and negative control of the pufferfish (*Tetraodon nigroviridis*) gills probed with (a) a monoclonal antibody (M3A7) to CFTR and (b) the primary antibody pre-incubated with the antigen, respectively. The immunoreactive bands detected only in SW-acclimatized fish ($n=8$) had molecular masses centered at 172 kDa (arrow). M, marker; FW, fresh water; SW, seawater.

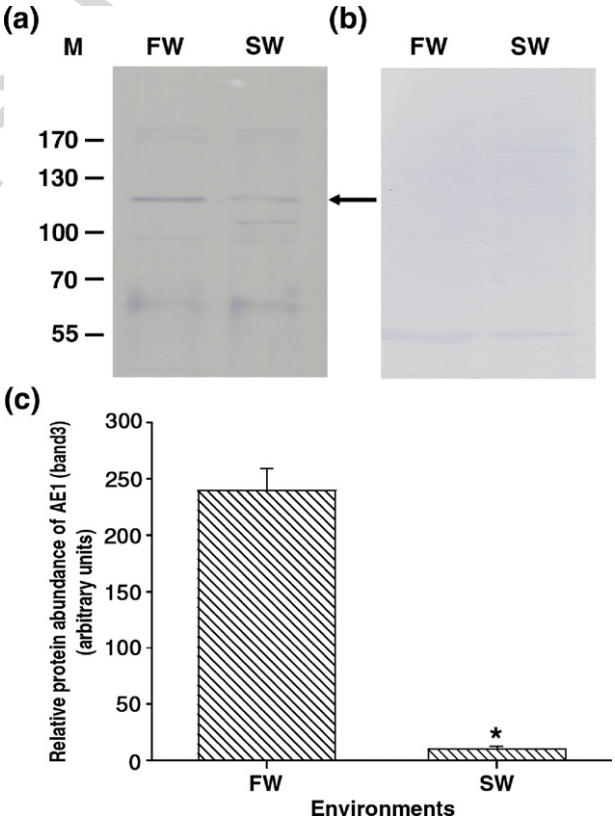

Fig. 4. Representative immunoblot and negative control of the pufferfish (*Tetraodon nigroviridis*) gills probed with (a) a polyclonal antiserum (tAE1) to AE1 and (b) the pre-immune serum of rabbit, respectively. The immunoreactive bands had molecular masses centered at 118 kDa (arrow). The immunoreactive bands of FW-acclimatized fish were more intensive than SW-acclimatized individuals. (c) Relative abundance of immunoreactive bands of AE1 in gills of different salinity groups ($n=4$). Expression of AE1 in the FW group was 23-fold higher than that in the SW group. The asterisk indicted a significant difference ($P<0.05$) by unpaired *t*-test. Values are means±S.E.M. M, marker; FW, fresh water; SW, seawater.

3.3. Branchial cystic fibrosis transmembrane conductance regulator (CFTR) expression

Immunoblotting of gill lysates from pufferfish acclimatized to FW or SW revealed a major immunoreactive band of about 172 kDa (Fig. 3a). The immunoreactive band of negative control was absent by preincubation of the primary antibody (M3A7) with the antigen (Fig. 3b). The result showed that CFTR expressed only in SW fish rather than FW individuals.

3.4. Cl^-/HCO_3^- anion exchanger 1 (AE1) expression in gills and erythrocytes

Immunoblotting of membrane fractions of gill tissues from pufferfish acclimatized to FW or SW resulted in a single immunoreactive band of about 118 kDa (Fig. 4a). The immunoreactive band of negative control was absent by replacing the primary antibody (tAE1) with pre-immune serum of rabbit (Fig. 4b). Quantification of immunoreactive bands between the two environmental groups showed significant differences. Gill AE1 protein abundance of the FW pufferfish was about 23-fold higher than that of the SW individuals (Fig. 4c).

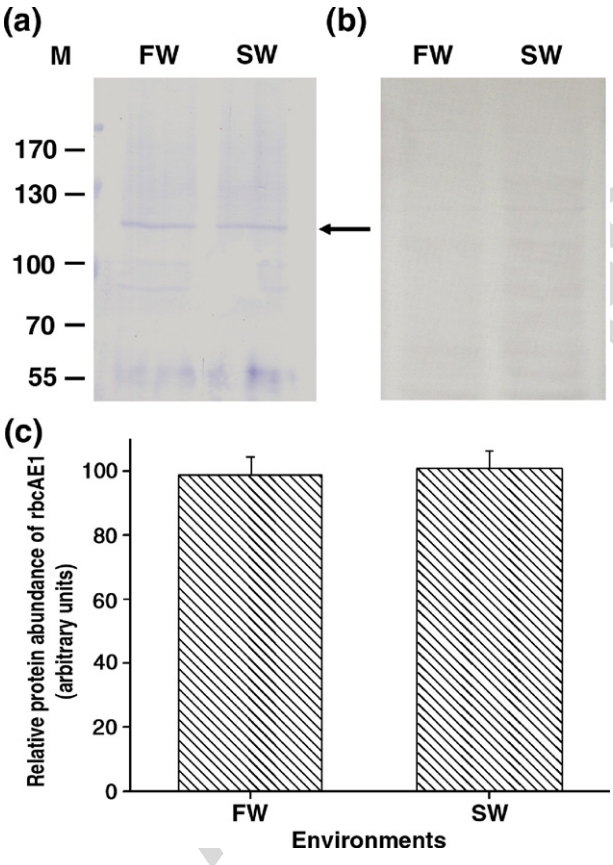

Fig. 5. Representative immunoblot and negative control of the pufferfish (*Tetraodon nigroviridis*) erythrocytes probed with (a) a polyclonal antiserum (tAE1) to AE1 and (b) the pre-immune serum of rabbit, respectively. The immunoreactive bands had molecular masses centered at 118 kDa (arrow). The intensities of the immunoreactive bands in FW- and SW-acclimatized individuals were similar. (c) Relative abundance of immunoreactive bands of AE1 in erythrocytes of different salinity groups ($n=4$). No significant difference of expression of erythrocytes AE1 was found between the FW and SW groups. Values are means±S.E.M. M, marker; FW, fresh water; SW, seawater.

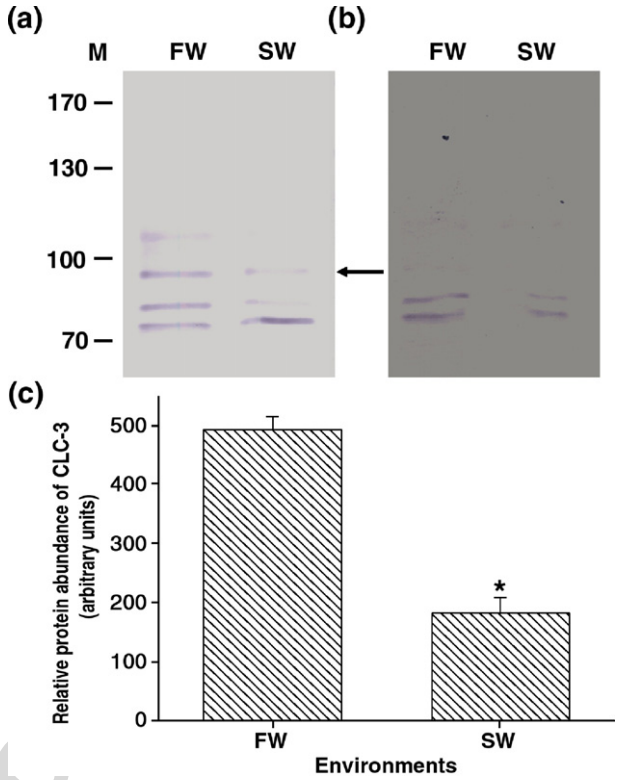

Fig. 6. Representative immunoblot and negative control of the pufferfish (*Tetraodon nigroviridis*) gills probed with (a) a primary antibody to CLC-3 (Clcn3) and (b) the primary antibody pre-incubated with the antigen. Compared to the negative control, one specific protein band with molecular weight of 96 kDa was obtained (arrow). The immunoreactive bands of FW-acclimatized fish were more intensive than SW-acclimated individuals. (c) Relative abundance of immunoreactive bands of CLC-3 in gills of different salinity groups ($n=4$). Expression of CLC-3 was 2.7-fold higher in the FW group than the SW group. The asterisk indicated a significant difference ($P<0.05$) by unpaired *t*-test. Values are means±S.E.M. M, marker; FW, fresh water; SW, seawater.

Immunoblotting of erythrocytes from pufferfish acclimatized to FW or SW revealed a single immunoreactive band of about 118 kDa (Fig. 5a). The immunoreactive band of negative control was absent by replacing the primary antibody (tAE1) with pre-immune serum of rabbit (Fig. 5b). No significant difference was found between quantification of immunoreactive bands of FW and SW pufferfish (Fig. 5c).

3.5. Branchial chloride channel (CLC-3) expression

Immunoblotting of membrane fractions of gill tissues from fish acclimatized to FW or SW showed a immunoreactive band of about 96 kDa (Fig. 6a). The immunoreactive band of negative control was absent by preincubation of the primary antibody (Clcn3) with the antigen (Fig. 6b). Quantification of immunoreactive bands between the two environmental groups revealed significant differences. Gill CLC-3 protein amounts of the FW pufferfish were about 2.7-fold higher than those of the SW individuals (Fig. 6c).

4. Discussion

Teleostean species are about 95% stenohaline, living entirely in either FW or SW. The remaining 5% are euryhaline, having the capacity to resist dramatic changes in environmental salinities, a feature that is found among teleost lineages and has apparently evolved many times (Evans, 1984). This capacity to evolve euryhalinity may be one of the reasons that teleosts can be found in almost all aquatic habitats. Spotted green pufferfish, *T. nigroviridis*, is one species of euryhaline teleosts living in estuaries (Lin et al., 2004). Most euryhaline teleosts including the pufferfish exhibit adaptive changes in gill NKA activity following salinity changes. Previous studies reported that some euryhaline fish responded with higher gill NKA expression (mRNA, protein, or activity) in fresh water (FW), e.g., milkfish, *Chanos chanos* (Lin et al., 2003), killifish, *Fundulus heteroclitus* (Scott et al., 2004), and striped bass, *Morone saxatilis* (Tipsmark et al., 2004). The other euryhaline teleosts, however, responded with higher gill NKA expression in seawater (SW), e.g., tilapia, *Oreochromis mossambicus* (Lee et al., 2000, 2003), Atlantic salmon, *Salmo salar* (D'Cotta et al., 2000), and the spotted green pufferfish (Lin et al., 2004). Immunoblots of gill samples prepared by membrane protein purification in this study confirmed our previous findings (Lin et al., 2004) by immunoblots of total lysates of gills demonstrating that the protein abundance of branchial NKA of SW pufferfish is about 2-fold higher than that of FW individuals (Fig. 1). Differential expression of gill NKA upon salinity challenge may be attributed to different affinities of NKA for Na^+ and K^+ in various euryhaline species (Lin and Lee, 2005).

According to the current model of salt excretion in gills of SW teleosts, the driving force for Cl^- secretion is the Na^+ electrochemical gradient established by NKA (Marshall, 2002). In order to secret excess Cl^-, mRNA expression of branchial NKCC increased after transfer to SW in the European eel, *Anguilla anguilla* (Cutler and Cramb, 2002), killifish (Scott et al., 2004), and striped bass (Tipsmark et al., 2004). Moreover, protein abundance of NKCC detected also by the monoclonal antibody T4 was upregulated after SW acclimation in tilapia (Wu et al., 2003), brown trout, *Salmo trutta* (Tipsmark et al., 2002), striped bass (Tipsmark et al., 2004), and killifish (Scott et al., 2004). Lytle et al. (1995) reported that the monoclonal antibody T4 identified immunoreactive proteins ranging in mass from 146 to 205 kDa. The molecular weights of NKCC immunoreactive bands detected by T4 in the killifish, striped bass, shark rectal gland (Scott et al., 2004; Tipsmark et al., 2004; Lytle et al., 1995) and the pufferfish (Fig. 2a) fell into this range. Brown trout and tilapia, however, had both lower (i.e. 109 and 105 kDa) and higher (i.e. 223 and 282 kDa) molecular weights. The lower bands may be resulted from protein degradation and the higher bands may be due to different levels of glycosylation (Tipsmark et al., 2002; Wu et al., 2003). In this study, fresh gill samples were used for immunoblots and a single band of glycosylated form of NKCC at 170 kDa was found only in the SW-acclimatized pufferfish rather than the FW group (Fig. 2a). The immunoreactive band was further confirmed to be NKCC by the experiment of negative control (Fig. 2b). NKCC expressed in gills of SW pufferfish conforms to the current model of Cl^- excretion (Hirose et al., 2003). Although the monoclonal antibody T4 used in this study has been demonstrated to recognize both secretory and absorptive isoforms of NKCC in a variety of animal tissues (Lytle et al., 1995) as well as the thiazide-sensitive Na^+/Cl^- cotransporter (NCC) in FW teleosts (Dr. Junya Hiroi, personal communication), NCC mRNA sequence of *T. nigroviridis* can not be found in the database (Tetraodon Genome browser of Genoscope). Thus, compared to the other FW-acclimatized euryhaline teleosts, discrepancy of gill NKCC expression in the FW pufferfish might be due to the lack of NCC expression.

Similar to NKCC, CFTR protein is expressed only in gills of SW-acclimatized pufferfish (Fig. 3a) to secrete excess Cl^- for homeostasis. In SW-acclimatized killifish and mudskipper, *Periophthalmodon schlosseri*, gill CFTR had a molecular weight of about 150 kDa (killifish, Marshall et al., 2002b; Katoh and Kaneko, 2003; mudskipper, Wilson et al., 2000b), 160 kDa (killifish, Scott et al., 2004), or 175 kDa (killifish; Marshall et al., 2002a), respectively. In this study, the negative control (Fig. 3b) performed by pre-incubation of the antibody with the antigen (synthetic peptide) together with prediction of the CFTR molecular weight (about 170 kDa) according to the amino acid sequence of *T. nigroviridis* from NCBI (National Center for Biotechnology Information, accession number AAR16330) database confirmed that the major band (172 kDa) of the immunoblot (Fig. 3a) was the target protein. Different from expression in the pufferfish, CFTR protein abundance in gills of killifish did not change significantly after SW transfer, although mRNA expression was elevated for a prolonged period (Scott et al., 2004). In gills of the Atlantic salmon, CFTR mRNA levels were elevated after transfer from FW to SW (Singer et al., 1998, 2002). Following SW exposure, upregulation of the Cl^- channel CFTR mRNA in gills of killifish and Atlantic salmon suggested that gene played a role of ionoregulation during SW acclimation. In killifish, dissimilar changes in CFTR mRNA and protein expression suggested post-transcriptional regulation of protein abundance. Elevated Cl^- conductance in SW might therefore be modulated primarily by post-translational mechanisms (e.g., intracellular trafficking) and might not involve changes in CFTR protein abundance (Scott et al., 2004). Recently, Marshall et al. (2005) proposed a model in which CFTR in killifish was activated by phosphorylation of protein kinase A (PKA), which in turn was stimulated by cAMP, similar to the mechanism in mammalian tissues (Tabcharani et al., 1990; Steagall et al., 1998). Since phosphorylation is necessary for activation and sustained operation of the channel, it is intriguing to clarify the regulatory mechanisms of CFTR in gills of pufferfish upon salinity challenge.

FW teleosts absorb Cl^- actively from the hypotonic external milieus to counterbalance for those passively lost Cl^-. To modulate Cl^- absorption, the plasma membrane anion exchanger (AE) protein facilitates the one-to-one electroneutral exchange of Cl^- for HCO_3^- and thereby contributes to pH regulation, CO_2 metabolism, cell volume regulation and

maintenance of intracellular Cl^- and HCO_3^- levels (Romero et al., 2004). The best known member of the AE protein family in the teleost is the electroneutral, SITS-sensitive Cl^-/HCO_3^- AE1 protein (Hirose et al., 2003), similar to the erythrocyte AE1 (Band 3, Alper, 1991). Using a non-homologous antibody raised against rainbow trout erythroid band 3 protein (AE1), Wilson et al. (2000a) demonstrated that AE1 protein was expressed in gills of FW tilapia, *O. mossambicus*, with a molecular weight of 116 kDa. In this study, fresh gill samples of FW and SW pufferfish were used for immunoblots. Pufferfish AE1 protein was detected by a non-homologous antiserum (tAE1) raised against tilapia AE1 and the negative control of the pre-immune serum substituting for tAE1 (Fig. 4b) confirmed that the immunoreactive band at 118 kDa (Fig. 4a) was the glycosylated form of AE1 protein in gills (Borgese et al., 2004). Comparison of the erythrocytes AE1 protein abundance between FW and SW pufferfish further confirmed that upregulation of the AE1 protein abundance was mainly attributed to gills, not erythrocytes (Fig. 5). Elevated levels of gill AE1 indicated a possible role of absorbing Cl^- in FW-acclimatized pufferfish, similar to that in FW tilapia and coho salmon (Wilson et al., 2000a, 2002).

To date, the channel responsible for taking Cl^- from epithelial cells to blood in gills of teleosts is not clear (Hirose et al., 2003; Evans et al., 2005; Tresguerres et al., 2006). Miyazaki et al. (1999) have cloned OmCLC-3, a member of the CLC chloride channel family, from cDNA libraries of the euryhaline tilapia and found that OmCLC-3 mRNA exhibited in various FW- and SW-acclimatized tilapia tissues, including the gill. The present study reported that for the first time to our knowledge, CLC-3 protein was exhibited in gills of the teleost and the abundance was compared between FW- and SW-acclimatized individuals. 2.7-fold abundance of branchial CLC-3 was expressed in FW pufferfish rather than SW individuals (Fig. 6). CLC-3 is also a candidate for volume-sensitive osmolyte and anion channels (VSOACs; Duan et al., 1997; Wang et al., 2000). Most vertebrate cells responded to hyposmotically induced cell swelling by actively decreasing cell volume, a process known as regulatory volume decrease (RVD) (Hoffmann and Dunham, 1995). Since VSOACs are activated on cell swelling in most vertebrate cells, native VSOACs are considered to be a major pathway of RVD through transport of Cl^- and organic osmolytes. CLC-3 is an important molecular component underlying VSOACs and the RVD process in HeLa cells and *Xenopus laevis* oocytes (Hermoso et al., 2002). Using the opercular epithelium sharing functional aspects with gill epithelium, Marshall et al. (2005) demonstrated that in hyposmotic (FW) shock, the ionoregulatory cells of the killifish swelled and protein phosphatase, but not PKC, was involved in the recovery process (RVD). In cardiac cells of guinea pig or NIH/3T3 cells, CLC-3 channels were opened due to cell swelling or endogenous PKC inhibition while closed by PKC activation, protein phosphatase inhibition, or elevation of intracellular $[Ca^{2+}]$ (Duan et al., 1997). Taken together, increasing levels of CLC-3 protein in gills of the pufferfish after exposure to the hyposmotic environments (FW) may be attributed to RVD response of ionoregulatory cells, activation of protein phosphatase, or inhibition of PKC.

In contrast to differential expression in gills of pufferfish upon salinity challenge, the protein abundance of NKA and Cl^- transporters in the brain, a non-osmoregulatory organ, was not salinity-dependent (data not shown). In summary, this is the first report to illustrate simultaneous protein expression of four Cl^- transporters as well as NKA in gills of teleosts. SW-acclimatized *T. nigroviridis* expressed NKCC and CFTR in gills to secrete excess Cl^- while higher protein abundance of AE1 and CLC-3 was found in gills of FW-acclimatized pufferfish to absorb Cl^- for blood homeostasis.

Acknowledgments

The anti-AE1 antibody was kindly provided by Prof. Pung-Pung Hwang in the Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan. The monoclonal antibody of Na^+/K^+-ATPase α-subunit (α5) and $Na^+/K^+/2Cl^-$ cotransporter (T4) were purchased from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, John Hopkins University School of Medicine, Baltimore, MD 21205, and the Department of Biological Sciences, University of Iowa, Iowa City, IA 52242, under Contract N01-HD-6-2915, NICHD, USA. This study was supported by a grant from the National Science Council of Taiwan to T.H.L. (NSC 93-2321-B-005-010).

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