The Novel Correlation of Carbonic Anhydrase II and Anion Exchanger 1 in Gills of the Spotted Green Pufferfish, *Tetraodon nigrovirids*

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ABSTRACT   A novel relationship between branchial carbonic anhydrase II (CAII) and anion exchanger 1 (AE1) was investigated in the euryhaline spotted green pufferfish (*Tetraodon nigroviridis*). The immunoblots revealed that AE1 was only detected in the membrane fraction of gills while CAII can be probed both in the membrane and cytosol fractions of gills. CAII protein abundance in the membrane fraction is salinity dependent. Immunological detection of the membrane fraction CAII protein in gills showed 3.9-fold higher in the hyposmotic (freshwater) group than the hyperosmotic (seawater;35‰) group. In contrast, there was no change in the protein level of cytosolic CAII between seawater and freshwater groups. The whole-mount immunocytochemical staining demonstrated that both AE1 and CAII were colocalized to the Na^+/K^+-ATPase-immunoreactive cells in gill epithelium of the pufferfish. The interaction between CAII and AE1 was further identified by co-immunoprecipitation because AE1 was detected in the immunoprecipitates of CAII and vice versa. Our results showed that in pufferfish gills CAII was not only expressed in the cytosol to produce the substrate for AE1 transport during Cl^- influx but also associated with the plasma membrane via AE1. Obviously, it is essential for the physiological function of AE1 to interact with CAII in the membrane of gill Na^+/K^+-ATPase-immunoreactive cells. To our knowledge, this is the first study to demonstrate the interaction of branchial CAII and AE1 in fish. The novel correlation proposed a new model of Cl^-/HCO_3^- transport in gills of the teleosts. *J. Exp. Zool. 307A:411–418, 2007.*   © 2007 Wiley-Liss, Inc.

The family of mammalian carbonic anhydrases (CA) consists of at least ten members of cytosolic forms or forms with catalytic site anchored to the extracellular surface of the cell (Geers and Gros, 2000; Kivela et al., 2000). The family of CA expressed widely and catalyzed the reversible reaction from CO_2 and water to HCO_3^- and H^+. These enzymes produce HCO_3^- for transport across membranes and consume HCO_3^- that has been transported across membranes. CA thus could be expected to have a key role in driving the transport of HCO_3^- across cells and epithelial layers (Sterling et al., 2001). CAII is one of the fastest enzymes among the CA family with a k_{cat} exceeding a million per second and appears to be almost universally expressed in certain cell types of all major mammalian tissues (Chegwidden and Carter, 2000). CAII was also found to exhibit in gill mitochondrion-rich cells (MRCs) of the tele-osts (Hirata et al., 2003). CAII was also thought to play a role of ion transport in fish gills (Hirose et al., 2003).

Plasma membrane Cl^-/HCO_3^- anion exchangers (AEs) regulate intracellular pH, chloride concentrations, and cell volume. Members of the SLC4 (solute carrier 4) mammalian gene family encode Na^+-independent Cl^-/HCO_3^- exchangers (Alper, 2006). The SLC4 family consists of ten genes, all appear to encode integral membrane proteins with very similar hydropathy plots of 10–14 transmembrane

segments and are very widely expressed across various tissues in isoforms, e.g., AE1, AE2, AE3, and AE4 (Romero et al., 2004). Plasma membrane AE proteins transport chloride and bicarbonate across most mammalian cell membranes in a one-for-one exchange reaction and act as a model for our understanding of HCO_3^- transport processes (Alper, 2006). In polarized epithelial cells, the exchangers also contributed to transepithelial secretion and reabsorption of Cl^- and acid–base equivalents (Alper, 2006). Tang and Lee (2007) recently reported that protein abundance of AE1 in membrane fractions of gills of pufferfish was salinity dependent.

The best known member among the SLC4 family is AE1 (i.e., Band 3; Romero et al., 2004). Each AE protein, including the AE1, has two large and one small domain. One large domain of human AE1 is the N-terminal cytoplasmic domain which interacted with glycolytic enzymes and cytoskeletal elements (Zhang et al., 2000). The other large domain is a transmembrane domain of about 55 kDa carrying out the anion transport function (Grinstein et al., '78). The small domain of AE1 is the C-terminal region of approximately 33 amino acids, which exhibits from the membrane to the cytosol (Lieberman and Reithmeier, '88; Fujinaga et al., '99). Furthermore, the C-terminal region of human AE1 was found to bind to CAII, the most catalytically active CA isoform in the erythrocytes (Vince and Reithmeier, '98). CAII was found to interact with AE1 through the DADD sequence of AE1 C-terminus because mutation of the most acidic amino acid sequence DADD resulted in loss of CAII binding (Vince and Reithmeier, 2000). In mammalian cells AE1 transport activity was more efficient when CAII bound to the C-terminus of AE1 (Sterling et al., 2001).

To date, it has become understanding that the gill epithelium was the primary site of transport processes that countered not only the influences of osmotic and ionic gradients but also pH regulation and nitrogenous waste excretion. The gill epithelium was mainly composed of pavement cells, MRCs, and mucous cells. Compared with pavement cells, MRCs occupied a much smaller fraction of branchial epithelial surface area, but were considered to be the major sites for active physiological processes in gills (Evans et al., 2005). In addition, the Na^+/K^+-ATPase (NKA) highly expressed in the basolateral membrane of osmoregulatory epithelial cells was thought to be a marker for MRCs (Hirose et al., 2003). Although

AE1 protein was found to exhibit in MRCs of fish gills (Wilson et al., 2000a, 2002), the correlation and interaction between CAII and AE1 in fish gills, to our knowledge, has never been reported. To confirm the functional interaction of CAII and AE1 in fish gill, this study reports the exhibition of CAII protein in both the cytosol and the membrane fractions and illustrates the correlation, interaction, and localization of CAII and AE1 in NKA immunoreactive MRCs of the spotted green pufferfish, *Tetraodon nigroviridis*.

MATERIALS AND METHODS

Experiment animals and environments

The spotted green pufferfish, *T. nigroviridis* were obtained from a local aquarium with the body weight of 7.7 ± 0.4 g and the total length of 6.4 ± 0.6 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, Ohio, USA). The spotted green pufferfish were reared in either SW or freshwater (FW) at $27 \pm 1°C$ with a daily 12 hr photoperiod for at least 2 weeks before sampling. The water was continuously circulated through fabric-floss filters. The fish were fed with commercial arid shrimp daily.

Preparation of gill cytosol and membrane fractions

Immediately after fish were killed by spinal pithing, the 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. Ten microliter 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 or B in the following process. Gill scrapings were suspended in 300 μl of buffer A (20 mmol L^{-1} Tris-base, 2 mmol L^{-1} MgCl$_2 \cdot$6H$_2$O, 2 mmol L^{-1} ethylene diamine tetra acetate, 0.5 mmol L^{-1} ethylene glycol bis(b-aminoethyl ether)-N, N-tetraacetic acid, 1 mmol L^{-1} dithiothreitol, 250 mmol L^{-1} sucrose, proteinase inhibitor, pH 7.4). Homogenization was performed in 2 ml tubes with the Brinkmann polytron homogenizer (PT1200E, Kinematica, Lucerne, Switzerland) at maximal speed for 25 strokes. Membrane fractions were prepared according to Tang and Lee (2007). After homogenization, the homogenates were then centrifuged at 135,000 g for 1 hr 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$6H$_2$O, 5 mmol L^{-1} ethylene diamine tetra acetate, 0.5 mmol L^{-1} ethylene glycol bis (b-aminoethyl ether)-N, N- tetraacetic acid, 1 mmol L^{-1} dithiothreitol, 5 mmol L^{-1} NaF, 0.1% Triton X-100, proteinase inhibitor, pH 7.5) and vortexed every 10 min during a 1 hr incubation period at 4°C. Dissolved pellets were centrifuged again at 135,000 g for 1 hr at 4°C. Supernatants were the mixture of membrane fractions. Protein concentrations were identified by reagents from the Protein Assay Dye (Bio-Rad, Hercules, CA, USA) using bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) as a standard. The samples were stored at −80°C before immunoblotting.

Antibodies

The primary antibodies used in this study included (1) 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; (2) Carbonic anhydrase II (CAII): a rabbit polyclonal antibody (ab6621; Abcam, Cambridge, UK) raised against the CAII of Human; (3) NKA: a mouse monoclonal antiserum (α5; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) raised against the α-subunit of avian NKA. The secondary antibodies for immunoblotting were alkaline phosphatase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Chemicon, Temecula, CA, USA). For immunocytochemistry 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).

Immunoprecipitation

Immunoprecipitation (IP) with primary antibody of either CAII or AE1 was carried out with the Catch and Release reversible IP system (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's manual. After elution with non-denaturing elution buffer, the samples were stored at −80°C before immunoblotting.

Immunoblots of the cytosol, membrane fractions, and IP samples

Proteins of those samples were heated together with the sample buffer at 37°C for 30 min. The pre-stain protein molecular weight marker was purchased from Fermentas (SM0671; Hanover, MD, USA). All samples were divided by electrophoresis on sodium dodecyl sulfate-containing 7.5 or 10% polyacrylamide gels (25 µg of protein/lane) dependent on target protein. The separated proteins were then transferred to PVDF membranes (Millipore, Bedford, MA, USA) by electroblotting. After preincubation for 3 hr in Phosphate buffer saline containing 0.1% Tween 20 (PBST) buffer containing 5% (wt/vol) non-fat dried milk to minimize non-specific binding, the blots were incubated overnight at 4°C with primary antibodies diluted in 1% BSA and 0.05% sodium azide in PBST, then washed in PBST, and incubated at room temperature for 1.5 hr 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. Immunoreactive bands were analysis using MCID software version 7.0, rev. 1.0 (Imaging Research Inc., Ontario, Canada). Results were converted to numerical values to compare the relative protein abundance of the immunoreactive bands.

Whole-mount fluorescent immunocytochemistry and confocal microscopy

The gill filaments were removed from gill samples fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After washing in phosphate-buffered saline (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). The gill filaments were then incubated at room temperature for 2 hr with primary polyclonal antibodies tAE1 or ab6621, respectively. Following incubation, the gill filaments were washed several times with PBS, and then labeled with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (Molecular Probes) at room temperature for 2 hr. After the first staining, the gill filaments were washed several times with PBS to proceed the second staining. The gill filaments were subsequently incubated with primary monoclonal antiserum α5 for 1 hr at room temperature followed by labeling with Alexa Fluor 546-conjugated goat anti-mouse secondary antibody (Molecular Probes) at room temperature for 2 hr. The samples were then washed with PBS, mounted with a coverslip, and observed with a confocal laser scanning microscope (LSM 510, Zeiss, Hamburg, Germany). The 488 nm argon–ion laser and the 543 nm helium–neon laser were used

for Alexa Fluor 488 and Alexa Fluor 546, respectively, to give the appropriate excitation wavelengths. The micrographs of immunocytochemistry were controlled by the Zeiss LSM image software.

Statistical analysis

Statistical significance was determined by unpaired t-test ($P < 0.05$) for group data analysis. Values were expressed as means $\pm$ SEM.

RESULTS

Immunoblots of anion exchanger 1 (AE1) and carbonic anhydrase II (CAII) in cytosol and membrane fractions

Immunoblots of AE1 in gills of FW-acclimatized spotted green pufferfish revealed a single immunoreactive band at approximately 118 kDa in membrane fractions rather than cytosol fractions ($n = 6$; Fig. 1). In contrast, CAII immunoreactive bands at approximately 30 kDa were detected by immunoblots in both cytosol and membrane fractions of gills of pufferfish acclimatized to FW or SW ($n = 6$; Figs. 2a and b). Quantification of immunoreactive bands of gill CAII between two environmental groups showed that the protein abundance of cytosol fractions were similar (Fig. 2c), whereas the average protein level of membrane fractions of FW pufferfish was significantly (about 3.9-fold) higher than that of the SW individuals (Fig. 2d).
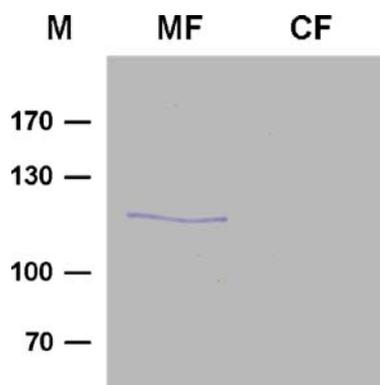

Fig. 1. Representative immunoblot of pufferfish (*Tetraodon nigroviridis*) gills probed with a polyclonal antiserum (tAE1) to anion exchanger 1 (AE1). AE1 immunoreactive bands had molecular masses centered at 118 kDa. AE1 were only detectable in membrane fractions ($n = 4$). M, marker; MF, membrane fractions; CF, cytosol fractions.

Whole-mount immunocytochemistry for AE1, CAII, and Na$^+$/K$^+$-ATPase (NKA)

Distributions of branchial AE1 and CAII were determined by the whole-mount immunofluorescent staining and the samples were double stained with NKA. In gills of FW pufferfish, both AE1 (green cells in Fig. 3A) and CAII (green cells in Fig. 3D) were colocalized to NKA immunoreactive cells (red cells in Fig. 3B and E) and thus revealed yellow color of the merged images (Fig. 3C and F).

Immunoprecipitation of CAII and AE1

To examine the interaction of CAII and AE1, immunoprecipitated CAII was probed in the complexes of IP for AE1 and vice versa using immunoblotting analyses. The results showed predominant bands at 30 (Fig. 4a) and 118 kDa (Fig. 4b) corresponding with the molecular weights of CAII and AE1, respectively. Indeed, CAII was detected in immunoprecipitates of AE1 and CAII (Fig. 4a) and AE1 was detected in immunoprecipitates of CAII and AE1 (Fig. 4b). The data demonstrated that AE1 interacted with CAII in gills of pufferfish.

DISCUSSION

The branchial epithelium of fishes is a tissue of multiple functions that plays a central role in a suite of physiological responses including osmoregulation, ionoregulation, and pH regulation to environmental and internal changes. This study is the first of its kind to compare the effect of environmental salinity on the protein expression of carbonic anhydrase (CAII) in teleostean gills, as well as to examine interaction of CAII and anion exchanger 1 (AE1) in fish gills.

Heterologous antibodies were used in this study because homologous antibodies of Na$^+$/K$^+$-ATPase (NKA), AE1, and CAII to pufferfish were not found. The antibody to NKA (α5) was generally used in fish samples and the specificity of α5 to fish was commonly accepted (Lee et al., '98; Piermarini and Evans, 2001; Tipsmark et al., 2002; Choe et al., 2004; Lin et al., 2004; 2006; Brauer et al., 2005). Meanwhile, Tang and Lee (2007) revealed that using a non-homologous antiserum (tAE1) raised against tilapia AE1 the molecular weight of immunoreactive bands in the immunoblots of pufferfish gills at 118 kDa (Fig. 1) was identical to the glycosylated form of AE1 protein (Borgese et al., 2004). Similarly, Wilson et al. (2000a) used the non-homologous antibody raised against rainbow trout
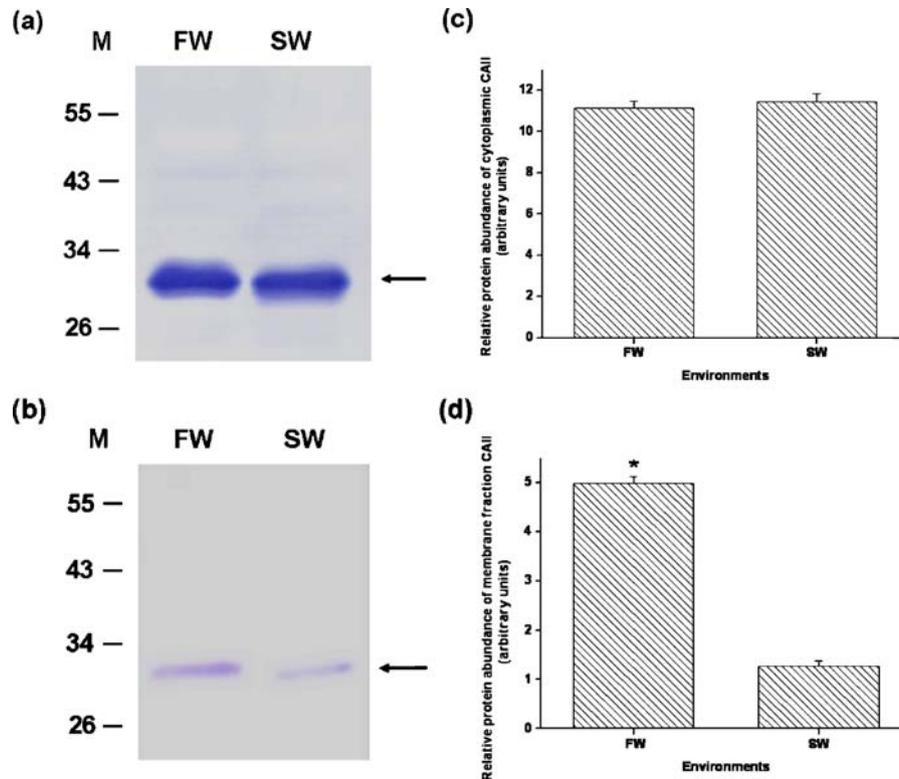
Fig. 2. Representative immunoblots of FW- and SW-acclimatized pufferfish gills detected by a polyclonal antibody (ab6621) to carbonic anhydrase II (CAII) with immunoreactive bands at 30 kDa in the cytosol (**a**) and the membrane fractions (**b**) of gills. Relative intensities of CAII immunoreactive bands ($n = 6$) revealed that no significant difference of cytopalsmic CAII was found between the FW and SW groups (**c**) while significant increase of CAII protein abundance in membrane fraction was observed in pufferfish exposed to FW (**d**). The asterisk indicted a significant difference ($P < 0.05$) by unpaired t-test. Values were mean $\pm$ SEM. M, marker; FW, freshwater; SW, seawater.

AE1 to demonstrate that AE1 protein was expressed in gills of freshwater (FW) tilapia (*Oreochromis mossambicus*) with a molecular weight of 116 kDa. In contrast, the anti-human CAII antibody (ab6621) used in this study detected a single band at 30 kDa in gills of pufferfish (Fig. 2), similar to the molecular weight of CAII found in carp (30 kDa; Rahim et al., '88), spiny dogfish (33 kDa; Wilson et al., 2000b), and lampreys (32 kDa; Choe et al., 2004).

Because AE1 was a membrane-bound protein (Alper, '91), this study used the method modified from Stanwell et al. ('94) to separate the membrane and cytosol fractions of gills. Immunoblots confirmed that the membrane and cytosol proteins were successfully separated because AE1 expressed only in the membrane fractions (Fig. 1). Tang and Lee (2007) reported that the protein abundance of AE1 in gill membrane fractions of FW pufferfish was 23-fold higher than seawater (SW) individuals but in erythrocytes the abundance was similar between FW and SW. In

contrast, this study demonstrated for the first time that the CAII proteins were detected not only in cytosol but also in membrane fractions of fish gills (Fig. 2). Higher mRNA levels of branchial CAII were induced by environmental acidification in the Osorezan dace (*Tribolodon hakonensis*; Hirata et al., 2003) and FW-transfer in the killifish (*Fundulus heteroclitus*; Scott et al., 2005). Boisen et al. (2003) described that in fish active Cl^- uptake was strongly dependent on branchial carbonic anhydrase (CA) regardless of external media. In this study, more protein amounts (3.9-fold) of membrane fractions CAII were found in FW pufferfish rather than SW individuals (Fig. 2). The protein amounts of cytosol fractions CAII between FW- and SW-acclimatized individuals, however, were highly expressed but not significantly different (Fig. 2).

Mammalian cells with the highest AE1 expression were the type A (or α) intercalated cells of renal collecting ducts. AE1 was present in the basolateral membrane of those cells. Type A (or α)
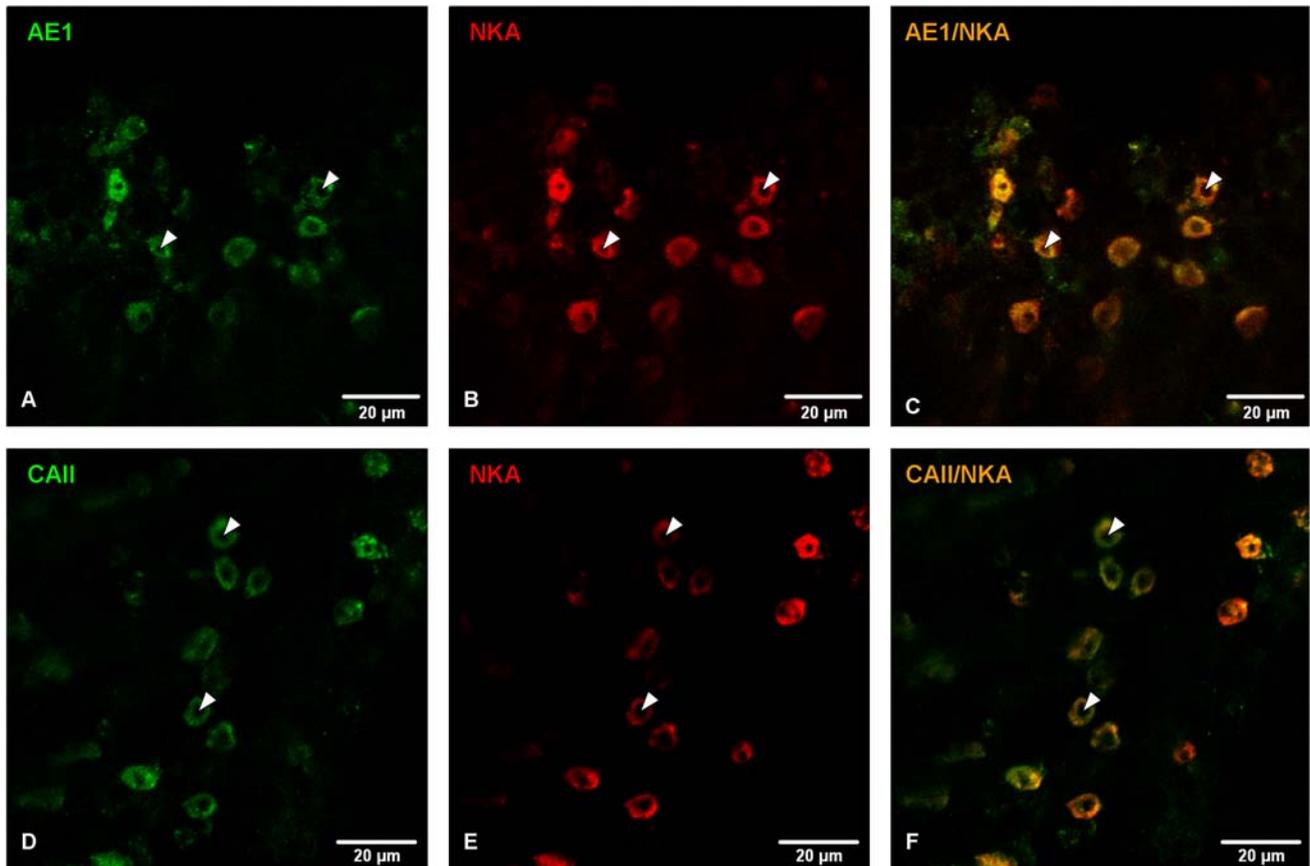
Fig. 3. Confocal laser scanning micrographs of whole-mount preparations of the gill filaments in freshwater pufferfish. Gill filaments were double stained with anti-anion exchanger 1 (AE1; green; **A**) or anti-carbonic anhydrase II (CAII; green; **D**) and anti-Na^+/K^+-ATPase (NKA; red; **B** and **E**). The merged images (**C** and **F**) of double-stained gill filaments showed that AE1 and CAII were colocalized to the same cells, the NKA-immunoreactive cells (i.e., mitocondrion-rich cells; MRCs). Scale bar, 20 μm. Arrowheads indicate the nuclei of immunoreactive cells.

intercalated cells were also rich in mitochondria and their abundant cytoplasmic CA guaranteed rapid generation of H^+ and HCO_3^- (van Adelsberg et al., '93). In fish gills, there were several reviews (Claiborne et al., 2002; Hirose et al., 2003; Perry and Gilmour, 2006) as well as original paper (Hirata et al., 2003) described that CA (or CAII) was exhibited in mitochondrion-rich cells MRCs. Meanwhile, AE1 was also found to appear in MRCs of fish gill epithelium (Wilson et al., 2000a, 2002) In this study, whole-mount immunocyto-chemical staining revealed that AE1 and CAII were colocalized in the NKA immunoreactive cells (i.e., MRCs; Lin et al., 2004) of pufferfish gills (Fig. 3). Similar localization of AE1 and CAII in fish gill MRCs might be corresponding to the type A (or α) intercalated cells of mammalian kidneys with functions of disposing of the intracellular HCO_3^- as well as regulating intracellular pH, $[Cl^-]$, and cell volume and extracellular compart-mental or systemic pH and $[Cl^-]$ (Alper, '91).

Activity of pumps, transporters, channels, and exchangers may be regulated in a number of ways. One important aspect of regulation of transport proteins was interaction with other proteins. The C-terminal tail of AE1 was shown to bind to CAII, the most catalytically active CA isoform of human erythrocytes (Vince and Reithmeier, '98). Follow-ing studies with GST fusion proteins of segments of AE1 C-terminus indicated that interaction of AE1 with CAII was ionic and mediated by the most acidic sequence (DADD) of the AE1 C-terminus (Vince and Reithmeier, 2000). In addition, Sterling et al. (2001) reported that in mammalian cells AE1 transport activity was more efficient when CAII bound to the C-terminus of AE1. In fish gills, Choe et al. (2004) suggested that CAII might bind to the Cl^-/HCO_3^- exchanger in MRCs of FW lamprey. The interaction of CAII and AE1 was further demonstrated in this study by co-IP because AE1 was detected in immunopreci-pitates of CAII and vice versa (Fig. 4). Since DADD
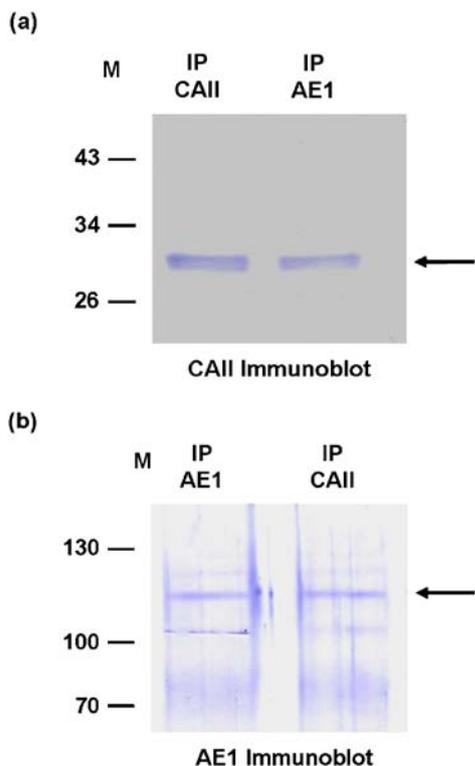
(a)

(b)

Fig. 4. Co-immunoprecipitation of carbonic anhydrase II (CAII) with anion exchanger 1 (AE1; **a**) or AE1 with CAII (**b**). CAII and AE1 were immunoprecipitated from gill total lysates by primary antibodies, and then immune complexes were analyzed by immunoblots for CAII and AE1, respectively. In either immunoprecipitated CAII or AE1, the immunoblotting analyses for CAII and AE1 revealed immunoreactive bands at 30 (**a**) and 118 kDa (**b**), respectively.
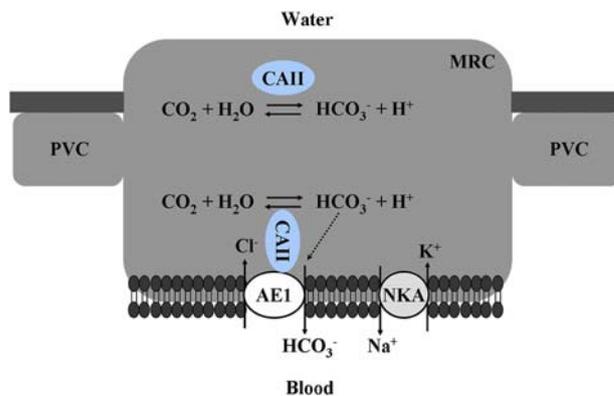

Fig. 5. A proposed model for interaction of carbonic anhydrase II (CAII) with anion exchanger 1 (AE1) in the mitochondrion-rich cell. CAII catalyses the reversible conversion of CO_2 and water to HCO_3^- and H^+, whereas AE1 transports HCO_3^- in exchange for Cl^- reversibly across the plasma membrane. The present model depicts CAII in the MRC as either membrane form associated with the plasma membrane via AE1 or free form in the cytosol. MRC, mitochondrion-rich cell; PVC, pavement cell.

was also found in the predicted AE1 C-terminal sequence of pufferfish genomic database (Genoscope; http://www.genoscope.cns.fr/); it is thus possible that the efficiency of AE1 transport activity in fish gills was affected by CAII binding.

Taken together, the results of this study illustrated a model of AE1 regulation and function in MRCs (Fig. 5) and indicated that expression of CAII and AE1 in membrane fractions of gills of *T. nigroviridis* responded to salinity changes for maintenance of cellular and physiological homeostasis.

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