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Branchial FXYD protein expression in response to salinity change and its interaction with Na^+/K^+-ATPase of the euryhaline teleost *Tetraodon nigroviridis*

Pei-Jen Wang*, Chia-Hao Lin*,†, Hau-Hsuan Hwang and Tsung-Han Lee‡

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

*These authors contributed equally to this work

†Present address: Graduate Institute of Life Sciences, National Defense Medical Center, Taipei 100, Taiwan

‡Author for correspondence (e-mail: thlee@dragon.nchu.edu.tw)

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SUMMARY

Na^+/K^+-ATPase (NKA) is a ubiquitous membrane-bound protein crucial for teleost osmoregulation. The enzyme is composed of two essential subunits, a catalytic α subunit and a glycosylated β subunit which is responsible for membrane targeting of the enzyme. In mammals, seven FXYD members have been found. FXYD proteins have been identified as the regulatory protein of NKA in mammals and elasmobranchs, it is thus interesting to examine the expression and functions of FXYD protein in the euryhaline teleosts with salinity-dependent changes of gill NKA activity. The present study investigated the expression and distribution of the FXYD protein in gills of seawater (SW)- or freshwater (FW)-acclimated euryhaline pufferfish (*Tetraodon nigroviridis*). The full-length pufferfish FXYD gene (p*FXYD*) was confirmed by RT-PCR. p*FXYD* was found to be expressed in many organs including gills of both SW and FW pufferfish. p*FXYD* mRNA abundance in gills, determined by real-time PCR, was significantly higher in FW fish than in SW fish. An antiserum raised against a partial amino acid sequence of pFXYD was used for the immunoblots of gill homogenates and a major band at 13 kDa was detected. The relative amounts of pFXYD protein and mRNA in gills of SW and FW pufferfish were identical, but opposite to the expression levels of NKA. Immunofluorescent staining of frozen sections demonstrated that pFXYD was colocalized to NKA-immunoreactive cells in the gill filaments. In addition, interaction between pFXYD and NKA was demonstrated by co-immunoprecipitation. Taken together, salinity-dependent expression of pFXYD protein and NKA, as well as the evidence for their colocalization and interaction in pufferfish gills suggested that pFXYD regulates NKA activity in gills of euryhaline teleosts upon salinity challenge.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/211/23/3750/DC1

Key words: gill, Na^+/K^+-ATPase, pufferfish, salinity, *Tetraodon nigroviridis*, pFXYD.

INTRODUCTION

The Na^+/K^+-ATPase (NKA) is a ubiquitous membrane-bound protein that actively maintains the Na^+ and K^+ gradients between the intra- and extracellular milieu of animal cells. NKA enzyme function in humans is generated and maintained by hydrolyzing ATP which accounts for 25% of the basal metabolic rate (Cornelius and Mahmmoud, 2003). NKA is essentially involved in specialized tissue functions such as renal Na^+ reabsorption, muscle contraction and neuronal excitability.

For teleosts, NKA not only sustains homeostasis but also provides a driving force for many transporting systems, including those of gill epithelial cells. Immunocytochemical studies on gill sections as well as biochemical studies on isolated epithelial cells demonstrated that mitochondrion-rich (MR) cells had the highest level of NKA in fish gills (Dang et al., 2000; Lee et al., 2000; Sakamoto et al., 2001; Brauer et al., 2005). Most euryhaline teleosts exhibit adaptive changes in gill NKA activity following salinity challenge (Marshall, 2002; Evans et al., 2005). These have been attributed to (1) an increase in NKA α-subunit mRNA abundance (Seidelin et al., 2001; Singer et al., 2002; Scott et al., 2004), protein amounts (Lee et al., 2000; Tipsmark et al., 2002; Lin et al., 2003) or both (D'Cotta et al., 2000; Lin et al., 2004a; Lin et al., 2006); or (2) modulation of the hydrolytic rate of this enzyme as reported in gills of the Atlantic cod (*Gadus morhua*) (Crombie et al., 1996) and striped bass (*Morone saxatilis*) (Tipsmark et al., 2004). These two adaptive mechanisms are regulated by short-term (rapid) or long-term (sustained) control. Long-term regulation is found to be mediated by mineralocorticoid or thyroid hormone and leads to a significant change in the total amount of NKA, whereas short-term regulation involves protein kinases and results in modulation of NKA expression in the cell membrane (Therien and Blostein, 2000; Feraille and Doucet, 2001). In addition, a novel regulatory mechanism which revealed tissue- and isozyme-specific interaction of NKA with the members of the FXYD protein family has been elucidated in mammals and elasmobranchs (Crambert and Geering, 2003).

The FXYD proteins, so named because of their invariant extracellular motif FXYD, belonging to a family with a conserved single-span transmembrane domain (Sweadner and Rael, 2000). These proteins are characterized by a conserved FXYD motif, two identified glycine residues and a serine residue (Geering, 2005). There are seven clear members in mammals: FXYD1 (phospholemman; PLM) (Palmer et al., 1991; Crambert et al., 2002; Feschenko et al., 2003), FXYD2 (the γ subunit of NKA) (Forbush et al., 1978; Mercer et al., 1993), FXYD3 (mammary tumor marker Mat-8) (Morrison et al., 1995; Crambert et al., 2005), FXYD4 (corticosteroid hormone-induced factor, CHIF) (Attali et al., 1995; Beguin et al., 2001; Garty et al., 2002; Lindzen et al., 2003), FXYD5 (related to ion channel RIC or dysadherin) (Fu and Kamps, 1997), FXYD6 (phosphohippolin) (Yamaguchi et al., 2001) and FXYD7 (Beguin et al., 2002). In elasmobranchs, a phospholemman-like protein has been cloned (Mohmmoud et al., 2000; Mohmmoud et al., 2003) and subsequently named FXYD10 (Mohmmoud et al.,

2005). In teleosts, eight FXYD isoforms were recently cloned in Atlantic salmon (Tipsmark, 2008). Tissue-dependent expression of different FXYD isoforms and their modulation by salinity were identified by quantitative PCR. Among these isoforms, FXYD11 was predominantly expressed in gills.

FXYD protein members cloned from different animal tissues were thought to be involved in a variety of cellular functions. The smaller NKA γ subunit, also known as FXYD2, is the first example of a small single transmembrane protein interacting with and regulating renal NKA (Forbush et al., 1978). In mammals, significant functional effects of FXYD proteins 1–7 were demonstrated, mainly by co-immunoprecipitation and various expression systems, including their specific associations with the α/β complex of NKA, and thereby altering its kinetic properties (Therien et al., 2001; Cornelius and Mahmmoud, 2003; Crambert and Geering, 2003; Crambert et al., 2005; Garty and Karlish, 2005; Lubarski et al., 2005; Delprat et al., 2007). Elasmobranch FXYD protein (PLMS) was also found to be associated with NKA, modify its activity *in vitro* (Mahmmoud et al., 2000; Mahmmoud et al., 2003). In teleosts, however, it is not clear if FXYD proteins interact with NKA and play similar roles to those in the mammals and elasmobranchs.

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 freshwater (FW) inhabitant (Helfman et al., 1997), this pufferfish has been demonstrated to be an efficient osmoregulator in experimental conditions, as it can tolerate a direct transfer from FW to seawater (SW) or *vice versa* (Lin et al., 2004b). The great euryhalinity, wide availability and inexpensive maintenance all make the pufferfish a good experimental animal in the laboratory for studies on ionoregulation.

Salinity adaptation of euryhaline teleosts is a series of physiological responses in osmoregulatory organs, including gills, to differing ionoregulatory requirements. Lin et al. (Lin et al., 2004b) reported that the SW-acclimated pufferfish had higher protein abundance as well as activity of gill NKA than the FW-acclimated individuals. Since the estuary is an environment with changing salinities, pufferfish must have corresponding strategies for rapid ionic regulation and acclimation. Expression and functions of NKA regulatory proteins, such FXYD proteins, in the euryhaline pufferfish are thus worth investigating.

In this study, a new member of FXYD protein family, termed pufferfish FXYD protein (pFXYD) was identified. pFXYD was cloned and found to have substantial homology with the other FXYD proteins at the transmembrane domain. pFXYD was also characterized by its molecular mass, similar to the other members of the FXYD protein family, as determined by immunoblots with specific antiserum. These experiments were designed to explore the expression and distribution of pFXYD in gills of SW- and FW-acclimated euryhaline pufferfish (*Tetraodon nigroviridis*). Furthermore, the relationship between NKA and FXYD in gills was examined by immunostaining and co-immunoprecipitation to elucidate possible functions of FXYD in pufferfish.

MATERIALS AND METHODS

Experimental animals

Green spotted pufferfish (*Tetraodon nigroviridis* Marion de Procé 1822), 4–9 g body mass and 4–5 cm total length, were obtained from a local aquarium. Fish were reared in seawater (SW: $[Na^+]$ 582.86 mmol l^{-1}; $[K^+]$ 10.74 mmol l^{-1}; $[Ca^{2+}]$ 15.75 mmol l^{-1}; $[Mg^{2+}]$ 32.92 mmol l^{-1}; $[Cl^-]$ 520.84 mmol l^{-1}) and fresh water (FW: $[Na^+]$ 2.6 mmol l^{-1}; $[K^+]$ 0.04 mmol l^{-1}; $[Ca^{2+}]$ 0.58 mmol l^{-1}; $[Mg^{2+}]$ 0.16 mmol l^{-1}; $[Cl^-]$ 0.18 mmol l^{-1}) at 27±1°C with a daily 12 h:12 h L:D photoperiod for at least 4 weeks before experiments. Water was continuously circulated through fabric-floss filters and was partially refreshed every 3 days. Fish were fed a daily diet of commercial dried shrimp. The proportion of diet mass to body mass was about 1/25.

Total RNA extraction and reverse transcription

Before sampling, the fish were killed by spinal section and pithing of the brain. Total RNA was extracted from the gill epithelium using the RNeasy Mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. 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 9 μl of the total RNA (5 μg) using a 1 μl oligo(dT) primer and a 1 μl PowerScript™ reverse transcriptase (Clontech, Franklin Lakes, NJ, USA) following the manufacturer's instructions.

Primers used for PCR and real-time PCR

The pufferfish FXYD DNA sequence (p*FXYD*) was derived from the puffer genome database (http://www.genoscope.cns.fr/externe/tetranew/). The full-length p*FXYD* sequence from the database was verified by PCR and DNA sequencing experiments. To amplify the full open reading frame region (ORF) of p*FXYD*, PCR primers were designed according to the pufferfish FXYD 5′and 3′ UTR regions. p*FXYD* gene-specific primer sequences were as follows (5′ to 3′): forward – AGGTAAACCACTTGAA and reverse – CCTTC-CATTTAATCCCAGAACA. Q-PCR primers were designed using the on-line public website (https://www.genscript.com/ssl-bin/app/primer). p*FXYD* gene-specific primer sequences were as follows (5′ to 3′): forward – GCTCTGCTGCTGATGACACT and reverse – GATGCCAATGAGACAGAGGA. β-Actin primer sequences were as follows (5′ to 3′): forward – CATGTTCGA-GACCTTCAACG and reverse – GTCACACCGTCACCAGAGTC. The cDNA sequence of pufferfish *FXYD* (GenBank accession no. EF028083) and β-actin (NCBI, CAAE01015104) were aligned and compared with the sequences of other species from the NCBI database.

Polymerase chain reaction

The PCR cycle protocol was as follows: 95°C for 1 min, 30 cycles of 95°C for 1 min, 53°C for 90 s and 72°C for 2 min, with a final incubation at 72°C for 15 min. The PCR product could be stored at 4°C before running agarose gels.

Real-time PCR analysis

Pufferfish *FXYD* mRNA was quantified using the ABI PRISM 7000 Sequence Detection System (SYBR Green II) real-time quantitative PCR (Applied Biosystems, Foster City, CA, USA). For methods of quantifying mRNA by real-time PCR, refer to Johnson et al. (Johnson et al., 2000). PCR reactions contained 8 μl of cDNA (500× dilution), 2 μl of FXYD primer mixture (l00 nmol l^{-1}) or β-actin primer mixture (100 nmol l^{-1}), and 10 μl of SYBR Green PCR Master Mix (Applied Biosystems). Real-time PCR reactions were performed as follows: 1 cycle of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All samples were run in triplicate. Reactions for quantifying β-actin copy number were performed exactly as described above except for the use of a different probes and primers. p*FXYD* mRNA values were adjusted by the values obtained for β-actin from each DNA samples, to obtain the values reported. For each unknown sample, the corresponding p*FXYD* and β-actin values were read using linear

regression analyses from their respective standard curves (data not shown). Relative p*FXYD* expression value was obtained using the following formula: $2^{\wedge}[(Ct_{FXYD,N}–Ct_{\beta\text{-actin},N})–(Ct_{FXYD,0}–Ct_{\beta\text{-actin},0})]$, where Ct is the threshold cycle number.

Preparation of gill homogenates

Gill scrapings prepared as described above were suspended in 1 ml of homogenization solution (100 mmol l^{-1} imidazole-HCl, 5 mmol l^{-1} sodium EDTA, 200 mmol l^{-1} sucrose, 0.1% sodium deoxychlolate, pH 7.6) with 10 µl proteinase inhibitor (10 mg antipain, 5 mg leupeptin and 50 mg benzamidine dissolved in 5 ml aprotinin; 100:1). Homogenization was performed in a glass Potter-Elvehjem homogenizer with a Brinkmann polytron homogenizer (PT1200E; Kinematica, Lucerne, Switzerland) at maximal speed for 20 strokes. The homogenate was then centrifuged at 13,000 ***g*** at 4°C for 20 min. Protein concentrations of the supernatant were identified using reagents from the Protein Assay Kit (Bio-Rad, Hercules, CA, USA), using bovine serum albumin (Sigma, St Louis, MO, USA) as a standard.

Preparation of membrane fractions

The tissue scrapings were suspended in the mixture of homogenization medium and proteinase inhibitor as described previously. The membrane fraction was prepared according to the method modified from Stanwell et al. (Stanwell et al., 1994). All procedures were performed on ice. 10 µl of proteinase inhibitor was added to 1 ml of buffer A or B (1:100 each). Gill scrapings were suspended in 1 ml of buffer A (20 mmol l^{-1} Tris-base, 2 mmol l^{-1} $MgCl_2 6H_2O$, 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). Homogenization procedure was as described above. The homogenate was then centrifuged at 135,000 ***g*** for 1 h at 4°C. The pellet was suspended in 200 µl of buffer B (20 mmol l^{-1} Tris-base, 2 mmol l^{-1} $MgCl_2 6H_2O$, 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. This suspension was centrifuged again at 135,000 ***g*** for 1 h at 4°C. The supernatant, referred to as the membrane fractions, was stored at –80°C. Protein concentrations of the supernatant were determined as described above. The immunoblot of NKA, a membrane protein, was used to confirm the membrane fraction preparation (supplementary material Fig. S1).

Antiserum and antibody

The polyclonal antiserum of pFXYD was made against the specific epitope (LAAAEEHSPEDDPF) corresponding to N-terminal region of the cloned pFXYD protein. The antiserum of pFXYD was obtained from the MDBio (Taipei, Taiwan). The Na^+/K^+-ATPase (NKA) antibody is a mouse monoclonal antibody (α5) against the α subunit of the avian sodium pump (Takeyasu et al., 1988) purchased from the Developmental Studies Hybridoma Bank (The University of Iowa, Department of Biological Sciences, Iowa City, IA, USA). The secondary antibody for immunoblots was horseradish phosphatase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Pierce, Rockford, IL, USA). For immunolocalization, the secondary antibodies were Alexa-Fluor-488-conjugated goat anti-mouse and Alexa-Fluor-546-conjugated goat anti-rabbit (Molecular Probes, Eugene, OR, USA).

Immunoblots of pufferfish FXYD and NKA

Immunoblotting procedures were carried out as described by Wu et al. (Wu et al., 2003) with some modifications. For detection of pFXYD protein, protein samples were heated at 100°C for 5 min and separated by electrophoresis on sodium dodecyl sulfate (SDS)-containing 15% polyacrylamide gels (30 µg of protein/lane). The separated proteins were then transferred to PVDF membranes (Millipore, Billerica, MA, USA) by a tank transfer system (Mini Protean 3, Bio-Rad, Hercules, CA, USA). After pre-incubation for 1 h in PBST buffer containing 5% (w/v) nonfat dried milk to minimize non-specific binding, the blots were incubated for 1 h with the primary pFXYD protein antiserum diluted in 5% (w/v) nonfat dried milk sodium azide in PBST (1:500 dilution), washed in PBST, and reacted for 1 h with secondary antibody (1:15000 dilution). For detection of NKA proteins, the membrane fractions were separated by electrophoresis on SDS-containing 7.5% polyacrylamide gels. The separated proteins were then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) by electroblotting. After pre-incubation for 1 h in PBST buffer containing 5% (w/v) nonfat dried milk to minimize non-specific binding, the blots were incubated for 1 h with the primary antibody (α5) diluted in PBST (1:2500 dilution), washed in PBST, and reacted for 1 h with secondary antibody (1:5000 dilution). Blots were developed after incubation with the ECL kit (Pierce, Rockford, IL, USA). Immunoblots were photographed and imported as JPEG files into the ID image analysis software package (MCID Analysis Evaluation 7.0). Results were converted to numerical values in order to compare the relative intensities of the immunoreactive bands.

Immunolocalization

The first left and right gill arches with filaments were excised and fixed immediately in a mixture of methanol and DMSO (4:1 v/v) at –20°C for 3 h (Chen et al., 2004). After washing with phosphate-buffered saline (PBS; 137.00 mmol l^{-1} NaCl, 2.68 mmol l^{-1} KCl, 10.14 mmol l^{-1} Na_2HPO_4, 1.76 mmol l^{-1} KH_2PO_4, pH 7.4), the arch and one row of the filaments of the gills were removed. The remaining filaments were perfused with 30% sucrose in PBS for 1 h at room temperature. Gill tissue was then mounted in OCT (optimal cutting temperature) compound (Tissue-Tek, Sakura, Torrance, CA, USA) for cryosectioning. Sections of gills were cut at 5–7 µm thick using a Cryostat Microtome (Microm HM 505E, Walldorf, Germany) at –25°C. The sections were placed on 0.01% poly-L-lysine (Sigma, St Louis, MO, USA)-coated slides, and kept in slide boxes at –20°C before staining. Samples were rinsed with PBS three times and then incubated in 5% bovine serum albumin (Sigma) and 2% Tween 20 (Merck, Hohenbrunn, Germany) in PBS for 0.5 h at room temperature. Cryosections were then incubated at room temperature for 1 h with 300× diluted pFXYD polyclonal antiserum. Following incubation, the sections were washed several times with PBS, and then labeled with 500× diluted Alexa-Fluor-546-conjugated goat anti-rabbit secondary antibody at room temperature for 2 h. After the first staining, the cryosections were washed several times with PBS to continue the second staining. The sections were subsequently incubated with 100× diluted NKA monoclonal antibody α5 for 3 h at room temperature followed by labeling with Alexa-Fluor-488-conjugated goat anti-mouse secondary antibody at room temperature for 1 h. The samples were then washed with PBS, mounted using coverslips with Clearmount™ mounting solution (Zymed, South San Francisco, CA, USA), and observed with a confocal laser scanning microscope (LSM 510, Zeiss, Hamburg, Germany) to determine immunolocalization. The micrographs of immunofluorescence staining were controlled by the Zeiss LSM image software.

Immunoprecipitation

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

Statistical analyses

Values are expressed as means ± s.e.m. Results were analyzed using Student's *t*-test and $P<0.05$ was set as the level of significance.

RESULTS

Characterization of pufferfish FXYD expressed in the gills

A 267 bp full-length pufferfish FXYD (p*FXYD*) cDNA was cloned, which encoded an 84 amino acid residue protein. The full-length cDNA contained 16 bp of 5′ untranslated region (UTR) and 216 bp of 3′ untranslated region (UTR) except for its poly(A)$^+$ tail. Fig. 1A shows the deduced amino acid sequence from the cloned full-length cDNA of p*FXYD*. Seventeen FXYD proteins from seven vertebrate species were aligned and compared. The phylogenetic tree of FXYD proteins showed a close relationship among fish FXYDs and human FXYD3 and FXYD4 (Fig. 1B). According to the hydropathy analysis, pFXYD contained one transmembrane domain (35–55 residue of the pFXYD peptide sequence; gray background in Fig. 1B), which was highly conserved with the other FXYD proteins (40–70% identity). The first 18 amino acids of pFXYD peptide sequence were predicted as the signal peptide (underlined) and threonine71 (circled) as the possible site for phosphorylation by PKA. Based on the alignment, pFXYD protein is a small protein containing the highly similar FXYD motif and two glycine residues (G39 and G50) at the conserved transmembrane domain in frame (Fig. 1A).

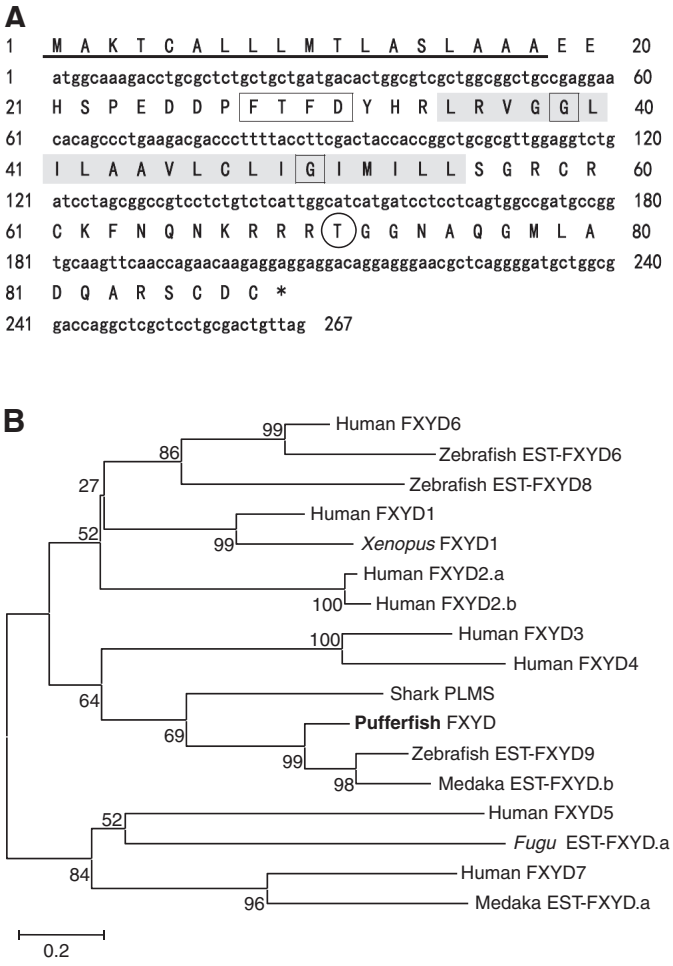

Fig. 1. (A) Pufferfish FXYD (pFXYD) nucleotide sequence and deduced amino acid sequence. The transmembrane domain is shaded gray; the predicted signal peptide is underlined; the circle indicates the site for phosphorylation; the boxes indicate the highly similar FXYD motif and two glycine residues (G39 and G50). (B) Phylogenetic tree of pFXYD with known human FXYD members and related EST sequences from various teleost fish. Accession numbers are: shark PLMS, CAD88978; human F*X*YD1, NP_005022; human F*X*YD2.a, NP_001671; human F*X*YD2.b, NP_067614; human F*X*YD3, NP_005962; human F*X*YD4, NP_775183; human F*X*YD5, NP_054883; human F*X*YD6, NP_071286; human F*X*YD7, NP_071289; zebrafish EST-F*X*YD6, AW153757; zebrafish EST-F*X*YD8, AI958251; zebrafish EST-F*X*YD9, AW455046; medaka EST-F*X*YD.a, AU169681; medaka EST-F*X*YD.b, AU169966; *Fugu* EST-F*X*YDa, CA332188; *Xenopus* F*X*YD1, NP_001011320.

Tissue distribution of pufferfish *FXYD* gene

RT-PCR analysis followed by electrophoresis and ethidium bromide staining characterized the tissue-specific expression pattern of FW and SW pufferfish *FXYD*. A representative result is shown in Fig. 2. The PCR amplification yielded a band of the predicted size (138 bp) from the gill, kidney, gut, liver, eye, brain, muscle and heart of both FW- and SW-acclimated pufferfish. Those PCR products were confirmed to be pufferfish FXYD cDNA fragments by subcloning and sequencing (data not shown). β-Actin was cloned as the internal control to confirm the cDNA quality.

p*FXYD* mRNA abundance detected by real-time PCR

For quantification of p*FXYD* mRNA abundance, the real-time PCR primer was checked by RT-PCR and a 138 bp major band could be detected (Fig. 3A). FW pufferfish had significantly higher levels of p*FXYD* mRNA than the SW individuals (Fig. 3B). The results indicated that hyperosmotic shock reduced the expression of p*FXYD* mRNA.

Immunoblotting of the pufferfish FXYD

Immunoblots of total gill lysates of both FW- and SW-acclimated pufferfish revealed single immunoreactive bands of pFXYD of approximately 13 kDa molecular mass. The specificity of this antiserum to pufferfish FXYD was confirmed by the negative control experiment using rabbit pre-immune serum to replace the pFXYD antiserum (Fig. 4A). Further comparisons of the relative abundance of pFXYD protein in the membrane fractions from gills of FW and SW pufferfish was conducted (Fig. 4C), using actin as the loading control (Fig. 4B, upper panel). The immunoblots showed a single pFXYD-immunoreactive band at approximately 13 kDa in the membrane (Fig. 4B) fractions of both FW- and SW-acclimated pufferfish gills. Based on image analysis, the FW fish had about 5.4-fold more pFXYD protein than the SW group (346.0±68.8 *vs* 63.7±11.2 in arbitrary unit; N=6; Fig. 4C).

Immunolocalization of pFXYD and Na^+/K^+-ATPase (NKA)

Fig. 5 shows the confocal images of frozen longitudinal sections of gill filaments of FW- and SW-acclimated pufferfish double immunostained with antibody specific to the NKA α subunit and antiserum to pFXYD. Confocal micrographs reveal that pFXYD (Fig. 5A,D, red cells) and NKA-immunoreactive cells (Fig. 5B,E,

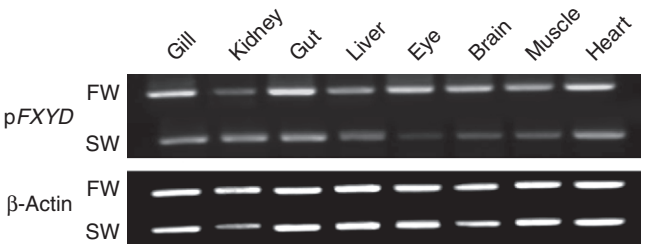

Fig. 2. RT-PCR analysis showing expression of p*FXYD* in various tissues of fresh water (FW)- and seawater (SW)-acclimated pufferfish. The p*FXYD* gene was found in the gill, kidney, gut, liver, eye, brain, muscle and heart of the pufferfish acclimated to fresh water (FW) or seawater (SW). β-Actin was used as an internal control.

green cells) colocalized (Fig. 5C,F yellow cells) in gill filaments of both FW and SW pufferfish.

Co-immunoprecipitation of pFXYD and NKA

Immunoblotting was used to examine the interaction between pFXYD and NKA. The results showed that when pFXYD and NKA were precipitated, band were found at 100 kDa (Fig. 6A, lane 1) and 13 kDa (Fig. 6B, lane 1) corresponding to the molecular masses of pufferfish NKA and FXYD protein, respectively. Lane 2 was the negative control in which no antibody was used in immunoprecipitates. Lane 3 was the positive control, which demonstrated the immunoprecipitation efficiency. The present data demonstrated that pFXYD interacted with NKA in gills of pufferfish.

DISCUSSION

The present study is the first to provide evidence of a novel FXYD protein that associates with NKA in gills of euryhaline teleosts. In mammals, the FXYD family has seven known members (FXYD1–7) that share a conserved signature sequence encompassing the transmembrane and adjacent regions (Sweadner and Rael, 2000). In fish, three gene products have be found in the zebrafish, EST-FXYD6, EST-FXYD8 and EST-FXYD9, which are homologues of the FXYD proteins according to the provisional terminology suggested by Sweadner and Rael (Sweadner and Rael, 2000). In addition, a phospholemman-like protein cloned from shark has been named FXYD10 (Mahmmoud et al., 2005). Pufferfish FXYD (p*FXYD*) was cloned in this study and identified as an FXYD homologue because it showed high identity at the transmembrane domain with the other FXYD proteins from different vertebrate species. According to the alignment with other vertebrate FXYD proteins, the deduced amino acid sequence showed that pFXYD shared the characteristic features of FXYD molecules: one transmembrane domain with an extracellular N-terminal and a cytoplasmic C-terminal and a highly similar FXYD motif at the N terminus. The phenylalanine (F) and aspartic acid (D) of the FXYD motif and two glycine residues (G39 and G50) at the conserved transmembrane domain of pFXYD were identical to those of the other 17 FXYD proteins from seven vertebrate species compared in this study (Fig. 1A). The FXYD motif is required for structural interaction with NKA (Beguin et al., 2001). Interestingly, the FXYD motif was present as FxFD in pFXYD protein. The tyrosine was replaced by phenylalanine (Y to F substitution), which is also found in FXYD proteins of other teleost species, such as zebrafish FXYD9 (EST) and medaka FXYDb (EST). Recently, FXYD9 of Atlantic

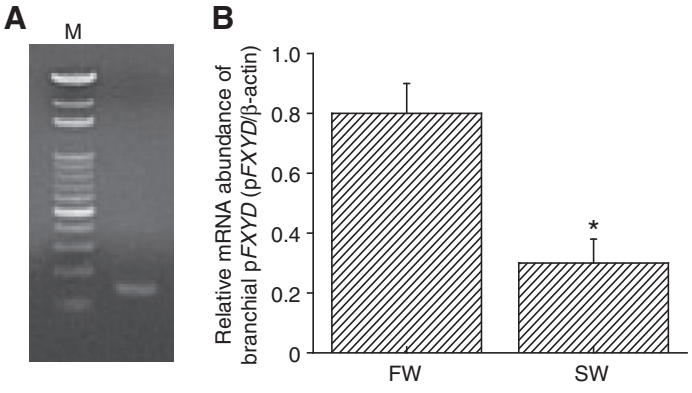

Fig. 3. Quantification of relative mRNA abundance of branchial p*FXYD*. (A) RT-PCR analysis confirmed the primer specificity. M, markers. (B) Comparison between branchial mRNA abundance of freshwater (FW)- and seawater (SW)-acclimated pufferfish, as quantified by real-time PCR. β-Actin was used as an internal control. Values are means ± s.e.m. (*N*=6). The asterisk indicates a significant difference between the FW and SW groups (*P*<0.05, Student's *t*-test).

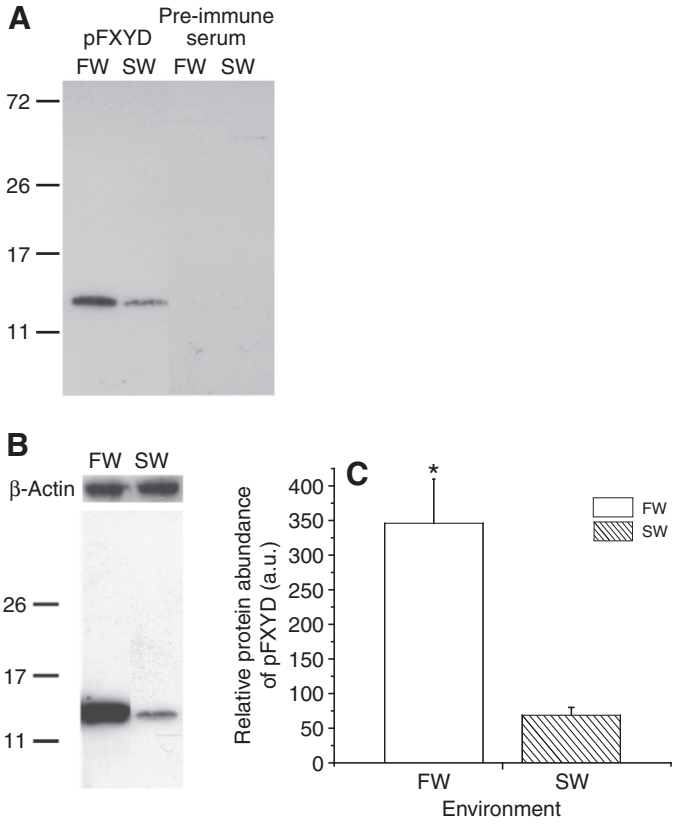

Fig. 4. (A) Specificity analysis of the pFXYD antiserum raised against a synthetic peptide corresponding to the N-terminal region of pufferfish FXYD. Total gill lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane. A single immunoreactive band was detected in both freshwater and seawater gill samples by the HRP detection system. The pre-immune serum was used as the negative control, which revealed no immunoreactive band. (B) Membrane fractions had an immunoreactive band at 13 kDa. β-Actin was used as the loading control. (C) The relative abundance of the pFXYD protein expressed in gills of pufferfish acclimated to fresh water (FW) was significantly higher than in the SW group (*N*=6). a.u., arbitrary units. The asterisk indicates a significant difference (*P*<0.05, Student's *t*-test). Values are means ± s.e.m.

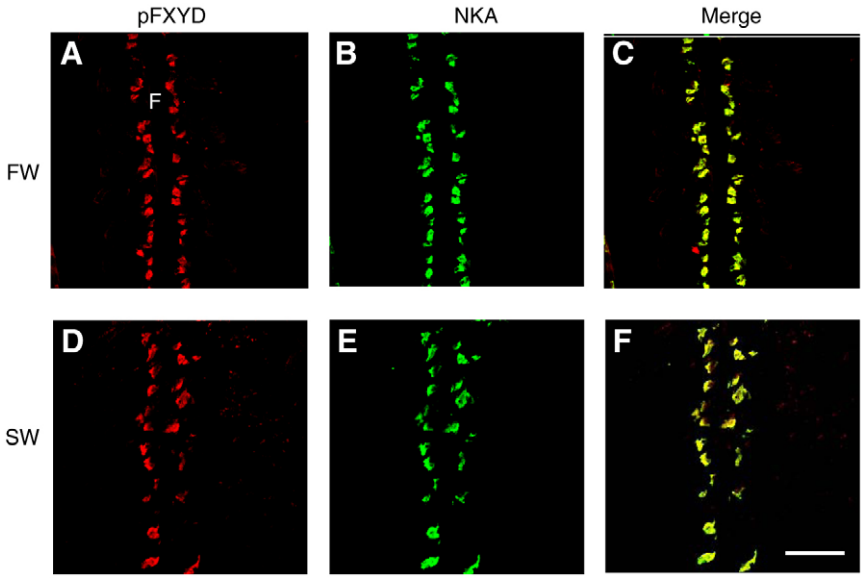

Fig. 5. Immunolocalization of pFXYD protein (red; A and D) and Na^+/K^+-ATPase (NKA; green; B and E) in frozen longitudinal sections of gills of pufferfish acclimated to fresh water (FW) or seawater (SW). After double staining of the same sections, merged confocal microscope images revealed that NKA and pFXYD are colocalized (yellow in C and F) in the basolateral membrane of epithelial cells in gill filaments. F, filament. Scale bar, 50 μm.

salmon was also shown to have the FxFD motif in its protein sequence (Tipsmark, 2008). The phylogenetic tree (Fig. 1B) showed the mostly close relationship among the above proteins and revealed the evolutionary character of FXYD protein. All FXYD proteins in mammals and shark were reported to have the function of being regulators of the NKA (Garty and Karlish, 2006; Delprat et al., 2007). PLM (FXYD1) and PLMS were found to inhibit NKA activity (Feschenko et al., 2003; Mahmmoud et al., 2003; Silverman et al., 2005). In addition, FXYD3 (Mat-8) decreased the apparent affinities for both Na^+ and K^+ ion of NKA when expressed in *Xenopus* oocytes (Crambert et al., 2005). FXYD4 (CHIF), however, decreased the affinity for extracellular K^+ and increased the affinity for intracellular Na^+ but with no change in maximal pump current (Beguin et al., 2001). Although the phylogenetic tree of pFXYD and the other FXYD proteins showed close relationship with human FXYD3 and FXYD4 (Fig. 1B), from alignment analysis human FXYD3 has the highest similarity with pFXYD, suggesting the possibility of similar functions.

Our results indicated that the p*FXYD* gene is expressed in several organs of the pufferfish including gills (Fig. 2). In mammals, tissue-specific distribution of different FXYD members was found, e.g. PLM (FXYD1) was detected mainly in the brain, heart and skeletal muscle (Feschenko et al., 2003; Wetzel and Sweadner, 2003; Zhang et al., 2003); the Na^+/K^+-ATPase (NKA) γ subunit (FXYD2) was detected only in the kidney (Pu et al., 2001; Wetzel and Sweadner, 2001); CHIF (FXYD4) was detected in kidney and colon (Shi et al., 2001; Garty et al., 2002); and FXYD7 was brain-specific (Beguin et al., 2002). In addition, the elasmobranch PLMS (FXYD10) is expressed in the rectal gland, the osmoregulatory organ of shark (Mahmmoud et al., 2003). Our results showed the osmoregulatory organs: gill, kidney and gut all expressed the pFXYD protein (data not shown). In addition, the levels of p*FXYD* mRNA in gills of FW-acclimated pufferfish were higher than in the SW-acclimated group (Fig. 3B). For pufferfish acclimating to different salinity, pFXYD might play the important role for adjusting ion regulation.

The significance of the role of teleostean branchial NKA in ion transport has been confirmed in a range of species (reviewed by Hwang and Lee, 2007) since the first studies of Epstein et al. (Epstein et al., 1967) on killifish, *Fundulus heteroclitus*, and Kamiya and Utida (Kamiya and Utida, 1968) on eels, *Anguilla japonica*. Significantly higher branchial NKA activity as well as α-subunit protein abundance were found in SW- than FW-acclimated pufferfish (Lin et al., 2004b). Since the elevation of pufferfish gill NKA activity and α-subunit protein abundance occurred within 3 h post-transfer from FW to SW (C.-H.L. and T.-H.L., unpublished data), it was postulated that pufferfish NKA expression was rapidly modified by FXYD protein upon salinity challenge. In this study, expression of p*FXYD* mRNA was found, by real-time PCR, as well as protein levels, determined by immunoblot using pufferfish FXYD antiserum. The specificity of the antiserum was confirmed by the 13 kDa major band and the negative control (Fig. 4A). The higher p*FXYD* mRNA and protein levels in gills of FW-acclimated pufferfish (Figs 3 and 4) is opposite to the trend of the NKA protein abundance and activity. The phylogenetic tree revealed a close relationship between pFXYD and the shark FXYD10 and human FXYD3 and FXYD4 (Fig. 1B). Since these FXYD proteins have been demonstrated to associate specifically with NKA and affect the pump function (Beguin et al., 2001; Feschenko et al., 2003; Mahmmoud et al., 2003; Crambert et al., 2005; Silverman et al., 2005), it is suggested that pFXYD also functions as a NKA regulator through their inhibition of NKA activity when pufferfish are exposed to FW.

The relative abundance of the pufferfish FXYD protein in the membranes of the gills was analyzed in the present study (Fig. 4B,C). The membrane proteins of gill homogenates was assayed by ultracentrifugation (Stanwell et al., 1994) and the NKA α subunit (a membrane protein) was found to be present in one band only in the membrane fraction (supplementary material Fig. S1). Using this protocol to separate membrane protein, our results showed a major 13 kDa band in immunoblots of membrane fractions from pufferfish gills. The pFXYD protein was significantly more abundant in the membrane fraction of the FW-acclimated group (Fig. 4C). This suggests that the abundance of pFXYD protein in branchial cells is correlated with the environmental salinity. The epitope sequence of our pFXYD antiserum covered the predicted N-terminal signal peptide. It was thus reasonable that the antiserum recognized both cytosol and membrane pFXYD. However, it has been shown that mammalian FXYD1 and FXYD4, as well as elasmobranch PLMS (FXYD10), determined their orientation in the membrane and become mature proteins after cleavage of the 20-amino acid N-

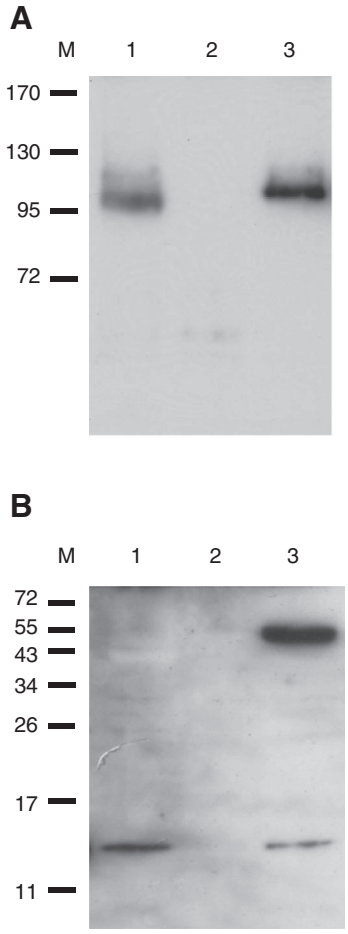

Fig. 6. Co-immunoprecipitation of Na^+/K^+-ATPase (NKA) with pFXYD protein (A), and pFXYD with NKA (B). pFXYD and NKA were immunoprecipitated from gill total lysates of freshwater pufferfish by primary antibodies, then the immune complexes were analyzed by SDS-PAGE and subsequent immunoblotting for NKA and pFXYD protein, respectively. Immunoreactive bands of NKA and FXYD were detected at 100 kDa (A) and 13 kDa (B), respectively. In B, the 55 kDa band in lane 3 is the IgG heavy chain of pFXYD antibody. M, markers; lane 1, western blot detection of the opposite antibody (experimental group); lane 2, negative control; lane 3, positive control of immunoblot using the same antibody with immunoprecipitation.

terminal signal peptide (Palmer et al., 1991; Beguin et al., 2001; Mahmmoud et al., 2003). In this study, the N-terminal 18-amino acid sequence was predicted to be the signal peptide of the pFXYD protein (Fig. 1A). Since the pFXYD sequence was very similar to the FXYD4 and FXYD10 sequences (Fig. 1B), the immature pFXYD protein in organelles of the cytosol might also be matured and transported to membrane through cleavage of its signal peptide. Furthermore, the threonine71 of pFXYD peptide sequence were predicted to be the site of phosphorylation by PKA (Fig. 1A). Because phosphorylation at a specific residue of FXYD1 (PLM) was found to result in transportation of PLM from an intracellular compartment to the plasma membrane (Lansbery et al., 2006), phosphorylation may also play an important role in transport of pFXYD in gill epithelial cells of pufferfish. The intracellular transport mechanisms of pFXYD protein should be investigated in future studies.

In mammals and elasmobranchs, most FXYD proteins were colocalized to NKA in different organs (Wetzel and Sweadner, 2001; Feschenko et al., 2003; Mahmmoud et al., 2003; Crambert et al., 2005; Lubarski et al., 2005; Delprat et al., 2007). The present study revealed that pFXYD protein was colocalized to NKA immunoreactive (NKIR) cells in gill filaments of FW- or SW-acclimated pufferfish (Fig. 5C,F, yellow cells). In gills of the euryhaline teleosts, epithelial NKIR cells are mitochondrion-rich (MR) cells responsible for ionoregulation, as NKA was detected in their basolateral membrane (Hwang and Lee, 2007). Although in some FW-acclimated euryhaline fish NKIR cells are distributed in epithelia of both gill filaments and lamellae (Sakamoto et al., 2001; Lin et al., 2006), the pufferfish NKIR cells were normally observed in filament epithelia of both FW- or SW-acclimated individuals (Fig. 5B,E) (Lin et al., 2004b), similar to the situation in tilapia (Lee et al., 1996; Lee et al., 2003; Uchida et al., 2000). Hence, the colocalization of pFXYD and NKA suggests that pFXYD may interact with NKA at the basolateral membrane of MR cells in gill filaments of pufferfish.

In addition to colocalization, all mammalian FXYD proteins have been demonstrated to interact with NKA and alter its kinetic properties (reviewed by Garty and Karlish, 2006). Shark FXYD protein (PLMS) also associate with NKA and modify its activity (Mahmmoud et al., 2000; Mahmmoud et al., 2003). Interaction between NKA α subunit and FXYD proteins, including FXYD1, 2, 3, 4, 7 and 10 was demonstrated mainly by co-immunoprecipitation (Therien et al., 2001; Cornelius and Mahmmoud, 2003; Crambert and Geering, 2003; Garty and Karlish, 2005; Crambert et al., 2005). Interaction between pFXYD and NKA protein of the teleost, the pufferfish, was also proved by co-immunoprecipitation in this study (Fig. 6). Since pFXYD protein was found to interact with, as well as colocalize to, NKA α subunit in gills (Fig. 5), pFXYD was suggested to regulate NKA activity *via* interaction with NKA α subunit when pufferfish experience salinity challenge.

Taken together, salinity-dependent expression of pFXYD protein and its interaction with NKA in gills of the euryhaline teleost was first reported in this study. Pufferfish exposed to SW experienced osmotic stress because the osmolality of plasma was hypotonic to the external environment, and the mRNA and protein levels of pFXYD were reduced to elevate NKA activity through their interaction in epithelial NKIR cells of gill filaments. By contrast, FW-acclimated pufferfish had increased pFXYD mRNA and protein levels to inhibit NKA activity. The pFXYD protein regulation of NKA appears to exist in all vertebrates from human to fish. More detailed investigation of the interaction of FXYD and NKA in euryhaline teleosts will be intriguing and provide insights into the understanding of teleost ionoregulation.

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