Isoform expression of Na^+-K^+-ATPase α-subunit in gills of the teleost *Oreochromis mossambicus*

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Lee, Tsung-Han, Jong-Chang Tsai, Mei-Jane Fang, Ming-jiun Yu, and Pung-Pung Hwang. Isoform expression of Na^+-K^+-ATPase α-subunit in gills of the teleost *Oreochromis mossambicus*. *Am. J. Physiol.* 275 (*Regulatory Integrative Comp. Physiol.* 44): R926–R932, 1998.—Three isoform-specific antibodies, 6F against the α_1-isoform of the avian sodium pump, HERED against the rat α_2-isoform, and Ax2 against the rat α_3-isoform, were used to detect the expression of Na^+-K^+-ATPase α-subunits in gills of a teleost, the tilapia (*Oreochromis mossambicus*). Tilapia gill tissue showed positive reactions to antibodies specific for α_1- and α_3-isoforms. The results of immunoblots were converted to numerical values (relative intensities) by image analysis for comparisons. Relative amounts of α_1-like isoform alone and consequently the ratio of α_1-like to α_3-like isoforms were higher in gills of seawater-adapted tilapia than in those of freshwater-adapted ones, indicating that the two isoforms respond differently to environmental salinities. In the subsequent immunocytochemical experiments, gill mitochondria-rich cells were demonstrated to immunoreact with antibodies specific for α_1- and α_3-isoforms. α_1-like and α_3-like isoforms of gill Na^+-K^+-ATPase are suggested to be involved in the ion- and osmoregulation mechanisms in tilapia. Moreover, differential expressions of two isoforms may be associated with different functions, secretion and uptake of ions and acid-base regulation, in gills of seawater- and freshwater-adapted tilapia.

chloride cell; sodium pump; ion regulation

A UNIVERSAL membrane-bound enzyme, Na^+-K^+-ATPase, actively transports Na^+ out of and K^+ into animal cells. It is not only crucial for maintaining intracellular homeostasis but also for providing a driving force for Na^+ transport in a variety of osmoregulatory epithelia, including the salt gland of the invertebrate brine shrimp; elasmobranchial rectal glands; teleostean gills; reptilian and avian nasal salt glands; and mammalian kidney tubules, bladder, and intestine. Na^+-K^+-ATPase is composed of two noncovalently linked polypeptides, a catalytic α-subunit with a molecular mass of ~110 kDa, and a smaller glycosylated β-subunit with a molecular mass of ~55 kDa (27). Sweadner (26) first showed that two electrophoretically separable forms of the α-subunit exist in mammals. Subsequently, experiments of molecular cloning and sequencing of cDNA encoding α-subunits revealed the existence of three major α-subunit isoforms, designated α_1, α_2, and α_3, in the rat and chicken (30). Tissue-specific and developmental expression of the α-subunit isoforms was demonstrated in birds and mammals and was suggested to extend to all vertebrate classes, including teleosts (24, 30). Tissue-specific expression of different isoforms was suggested to be associated with various physiological functions. The α_1-subunit functions primarily in a housekeeping capacity to maintain osmotic balance and cell volume regulation, whereas the other α-subunits fulfill more specialized requirements for cation transport necessary for differentiated cell-specific functions (12, 21).

Euryhaline teleosts inhabit environments ranging from freshwater to seawater of high salinity. Through effective mechanisms of osmoregulation, teleosts are able to retain an osmotic and ionic constancy in the internal milieus and survive in hypertonic seawater or hypotonic freshwater. Gills are the most important extrarenal organs responsible for osmoregulation in fish. Mitochondria-rich cells (MR cells, i.e., chloride cells) are the main site for active transport of ions in branchial epithelium. MR cells are suggested to have multiple functions: ion secretion in seawater-adapted fish and ion uptake and acid-base regulation in freshwater-adapted fish (5, 8, 10, 15, 16, 23), although the role of MR cells in ion uptake and acid-base regulation is still controversial (23). The biochemical mechanisms for maintenance of constant levels of ions in body fluids depend on the activity of Na^+-K^+-ATPase, and the activities of gill Na^+-K^+-ATPase in euryhaline teleosts are affected by environmental salinities and ion concentrations (4, 11, 18, 19). Moreover, biochemical and histochemical studies reveal that most of the Na^+-K^+-ATPases were found in MR cells (7, 13, 19).

When all these results were taken into account, the hypothesis that gills may express multiple isoforms of Na^+-K^+-ATPase to perform various functions was proposed (9). Previous biochemical studies provide some clues for this hypothesis. Gill Na^+-K^+-ATPase from freshwater- and seawater-adapted fish differed in enzyme kinetics (6, 22). On the other hand, in recent studies different genes encoding Na^+-K^+-ATPase α-subunits have been cloned and sequenced, and the expression of these genes in the gills and kidneys was reported in several teleosts (2, 3, 14, 25). Some other studies also examined the expression of the protein of Na^+-K^+-ATPase α-subunit in fish gills by Western blotting (9, 35). These results, however, could not confirm the possibility of the presence of more than one type of Na^+-K^+-ATPase α-subunit in gills of fish as suggested previously (9), because only the probe derived from one type of isoform was used in the same species studied. A similar situation may occur in the case of Western blots, which were performed with antibodies that were not isoform specific (9, 35).

Obviously, there are still no direct and convincing data to demonstrate the presence of multiple isoforms of Na^+-K^+-ATPase in gills of freshwater- and/or seawater-adapted teleosts. In the present study, several

antibodies were used to demonstrate tissue-specific distribution of various Na^+-K^+-ATPase α-subunit isoforms in the teleost tilapia (*Oreochromis mossambicus*). The amount of different isoforms expressed in the gills of tilapia adapted to different environments was compared, and the localization of different isoforms in gill MR cells was also investigated.

MATERIALS AND METHODS

Animals

Tilapia, *O. mossambicus*, one of the most popular model species for research of fish osmoregulation (5, 8, 11, 13, 15, 16, 19), was used for the present study. Male or female tilapia of 6–12 g body weight were obtained from laboratory stocks. The fish were reared separately in aerated local tap water and 33% seawater at 26–29°C with a daily 12:12-h photoperiod. The water was continuously circulated through fabric-floss filters and partially refreshed every 3 days. Fish were fed with a daily diet of commercial pellets.

Preparation of Tissue Homogenates and Gill Epithelial Cells

Gills, kidneys, heart, and brain were excised and blotted dry. Gills from each individual fish yielded one sample, whereas other organs (kidneys, heart, and brain) were pooled from two or three fish to make one sample with enough protein for immnuoblottings. Gill epithelia were immediately scraped off the underlying cartilage with a scalpel, and other organs were cut into small pieces. All subsequent operations were carried out in ice. Tissues were suspended in homogenization solution (100 mM imidazole-HCl buffer, pH 7.6; 5 mM Na_2EDTA; 200 mM sucrose; and 0.1% sodium deoxycholate). Homogenization was performed in a glass Potter-Elvehjem homogenizer with a motorized Teflon pestle at 600 rpm for 20 strokes. The homogenate was then filtered through nylon gauze of 106-μm-square rectangular mesh. The residue was suspended in homogenization solution and again filtered through gauze. The filtrates were subjected to gel electrophoresis and immunoblotting.

To enrich epithelial cells (33), gill tissues were cut into small pieces in a dissociated buffer (2 mM Na_2EDTA and 1% Percoll in PBS) and stirred slowly at 4°C for 30 min. Dissociated gill cells were filtered through nylon gauze (described above) and then gently layered on a 20% Percoll discontinuous gradient. After centrifugation with the use of swing rotors at 1,000 *g* and 4°C for 10 min, the white layer of epithelial cells was separated from the red layer of blood cells. Isolated gill epithelial cells were washed with PBS and centrifuged at 1,000 *g* and 4°C for 5 min; the pellet was finally subjected to homogenization as described above. Protein concentrations of the homogenates (whole tissues or isolated epithelial cells) were determined with the reagents of Bio-Rad Protein Assay Kit using bovine serum albumin as a standard.

Na^+-K^+-ATPase Antibodies

Three primary antibodies against the catalytic subunit of Na^+-K^+-ATPase were used in the present study: *1*) mouse monoclonal antibody 6F raised against the α_1-isoform of the avian sodium pump (31), *2*) rabbit polyclonal antibody raised against the HERED sequence of the rat Na^+-K^+-ATPase α_2-isoform (24), and *3*) rabbit polyclonal antibody Ax2 raised against the rat axolemma (predominantly α_3-isoform of sodium pump) (29). Monoclonal antibody 6F was kindly provided by Dr. Douglas M. Fambrough (Dept. of Biology, Johns Hopkins Univ., Baltimore, MD), the polyclonal antibody HERED was provided by Dr. Thomas A. Pressley (Dept. of Physiology, Texas Tech Univ. Health Science Center, Lubbock, TX), and the polyclonal antibody Ax2 was provided by Dr. Kathleen J. Sweadner (Neurosurgical Research Unit, Massachusetts General Hospital, Boston, MA).

Gel Electrophoresis and Immunoblotting

Proteins within the homogenates were fractionated by electrophoresis on SDS containing 10% polyacrylamide gels (20 or 100 μg of protein/lane), except that the homogenates were heated at 37°C for 5 min rather than at higher temperatures. Rat brain microsomes (UBI, Lake Placid, NY) were used as a positive control for immunoblotting. The separated proteins were then transferred to PVDF membrane (Immobilon Transfer Membranes; Millipore, Bedford, MA) by electroblotting. Protein bands on the gel were visualized by Coomassie brilliant blue staining. The presence of transferred proteins on the blots was confirmed by staining with Amido black. After preincubation for 2 h in PBST buffer [137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.2% (vol/vol) Tween 20, pH 7.4] containing 2% (wt/vol) nonfat dried milk to minimize nonspecific binding, the blots were incubated for 1 h with primary antibody diluted in PBST (200-fold dilution), washed in PBST, and reacted for 30 min with alkaline phosphatase-conjugated secondary antibodies (goat anti-mouse IgG, Pierce, Rockford, IL; goat anti-rabbit IgG, Sigma, St. Louis, MO; both diluted 1:1,000). Blots were developed after incubation with 0.015% nitro-blue tetrazolium, 0.007% bromochloroindolyl phosphate in a reaction buffer containing 100 mM Tris, 100 mM NaCl, and 5 mM $MgCl_2$, pH 9.5. Immunoblots were scanned and imported as joint photographic experts group files into a commercial software package (Image-Pro Plus, Version 1.2, Silver Spring, MD). Stained bands in the finished images were analyzed and converted to numerical values to show the relative intensities of the immunoreactive bands.

Four repeated immunoblottings of tilapia organs (Fig. 1) were conducted using different samples. For immunoblottings of tilapia gills (Fig. 2), four individuals from each group (seawater or freshwater) were used.

Immunocytochemistry and Confocal Microscopy

Concanavalin A and Na^+-K^+-ATPase α-subunits. Because Concanavalin A (ConA) is located at the apical surface of gill MR cells, whole mount preparations of gill filaments were made for the double labeling. Gills were excised and immediately fixed in 4% paraformaldehyde-1% glutaraldehyde in 0.1 M phosphate buffer (PB; pH 7.4) for 5 min at 4°C. The briefly

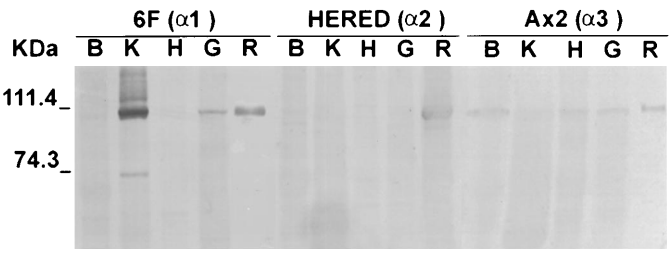

Fig. 1. Western blots of Na^+-K^+-ATPase α-subunits in various tissues from freshwater-adapted tilapia (*Oreochromis mossambicus*). Total protein (20 μg/lane) of homogenized tissues was separated on 10% SDS-PAGE, transferred to a PVDF blot, incubated with different antibodies (α_1-specific 6F, α_2-specific HERED, and α_3-specific Ax2), and visualized with alkaline phosphatase-conjugated secondary antibody, as described in MATERIALS AND METHODS. B, brain; K, kidney; H, heart; G, gill; R, positive control of rat brain microsome.

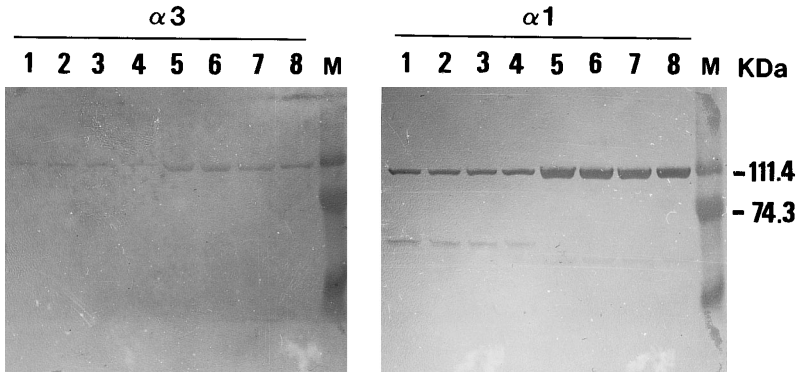

Fig. 2. Comparison of Western blots of gill Na^+-K^+-ATPase α-subunits between freshwater- and seawater-adapted tilapia (*O. mossambicus*). Total protein (100 μg/lane) of the enriched gill epithelial cells was subjected to procedures similar to those described in Fig. 1, except that the antibodies were α_1-specific 6F and α_3-specific Ax2. *Lanes 1–4*, gills from 4 individuals of freshwater group; *lanes 5–8*, gills from 4 individuals of seawater group; M, marker.

fixed gill filaments were first stained with 1% ConA (conjugated with Texas red, Sigma) for 20 min. After being permeablized with 75% ethanol for 3 min and being incubated with 10% normal goat serum (Jackson, West Grove, PA) in PBS for 30 min, the gill filaments were stained with α_1-specific monoclonal antibody (6F, diluted 1:50) or α_3-specific polyclonal antibody (Ax2, diluted 1:50) for 1 h at room temperature. Then goat anti-mouse IgG (or goat anti-rabbit IgG) conjugated with fluorescein isothiocyanate (FITC; Jackson, diluted 1:100) was added for another 1 h. The antibodies were diluted in PBS that contained 0.05% Tween 20 and 3% bovine serum albumin (Sigma). Between each step, three 10-min washes in PBS were performed.

Stained gill filaments were observed by a Bio-Rad MRC 600 confocal laser scanning microscope that was attached to a Nikon microscope and equipped with an argon laser (488 and 514 nm) for excitation. The Na^+-K^+-ATPase α_1 (or α_3) images (FITC) were taken under an A1 (BP 525–555 nm) filter set (Fig. 3*A*), whereas ConA images (Texas red) were obtained with the use of an A2 (LP 600 nm) filter set (Fig. 3*B*).

Na^+-K^+-ATPase α_1 and α_3. Gills were excised and immediately fixed in 4% paraformaldehyde-1% glutaraldehyde in 0.1 M PB (pH 7.4) for 1 h at 4°C. After rinsing briefly with PB, 10-μm-thick frozen cross sections of the gill filaments were made with a cryostat (Bright, Cambridgeshire, UK). The subsequent procedures for double staining were similar to those above, except that the two primary antibodies (α_1 and α_3) were simultaneously added for 1-h staining and then two secondary antibodies, goat anti-mouse IgG conjugated with FITC (for α_1) and goat anti-rabbit IgG conjugated with tetramethylrhodamine isothiocyanate (TRITC; for α_3), were also simultaneously added for another 1 h. Control sections were processed in parallel without primary antibody or substitution of primary antibody with nonimmune normal rabbit or mouse serum.

The slide was then observed with a Bio-Rad MRC 1000 confocal laser scanning microscope. The microscope was attached to a Zeiss microscope, and a krypton-argon laser (488 nm and 568 nm) was equipped for excitation. The Na^+-K^+-ATPase α_1 images (FITC) were taken under a 515/32 filter set, whereas Na^+-K^+-ATPase α_3 images (TRITC) were obtained with the use of a 580/32 filter set. Figures 4 and 5 demonstrate that the mutual interference of the two filter sets was excluded, because the α_1 image (FITC) could not be observed under the 580/32 filter set (Fig. 4*B*) and the α_3 image (TRITC) could not be observed under the 515/32 filter set (Fig. 5*B*).

RESULTS

Immunoblotting of Na^+-K^+-ATPase α_1 and α_3

Due to the high sequence homology of Na^+-K^+-ATPase α-subunits among vertebrates (9), we were able to detect the distribution of three rat or chicken isoform-specific antibodies of Na^+-K^+-ATPase α-subunit in different tissues, e.g., brain, kidneys, heart, and gills of

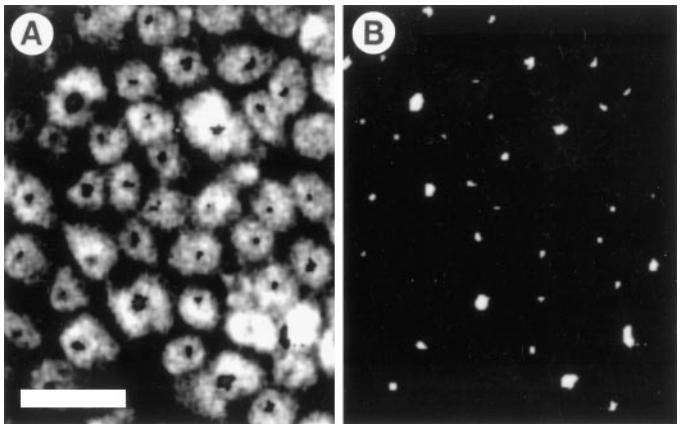

Fig. 3. Confocal images of double staining of concanavalin A (ConA) and Na^+-K^+-ATPase α_1-subunit in gill filaments from freshwater-adapted tilapia (*O. mossambicus*). Same gill filament was doubly stained with ConA and α_1-specific antibody (6F) as described in MATERIALS AND METHODS, and images were obtained from a surface view of the gill filament. Magnification ×510 (scale = 30 μm). *A*: image of Na^+-K^+-ATPase α_1-subunit. *B*: image of ConA.

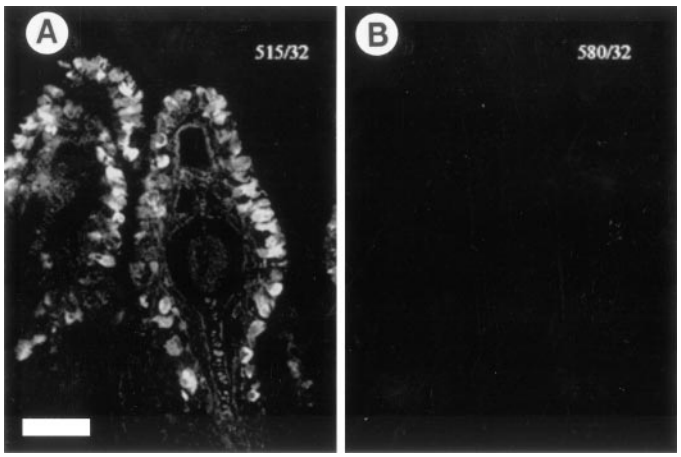

Fig. 4. Confocal images of the immunocytochemistry of Na^+-K^+-ATPase α-subunits in frozen cross sections of gill filaments from freshwater-adapted tilapia (*O. mossambicus*). Same frozen section was doubly stained with antibodies (α_1-specific 6F and α_3-specific Ax2) and was observed under different filter sets as described in MATERIALS AND METHODS. α_1 images were obtained under 515/32 (*A*) and 580/32 (*B*) filter set, respectively. Magnification ×200 (scale = 50 μm).

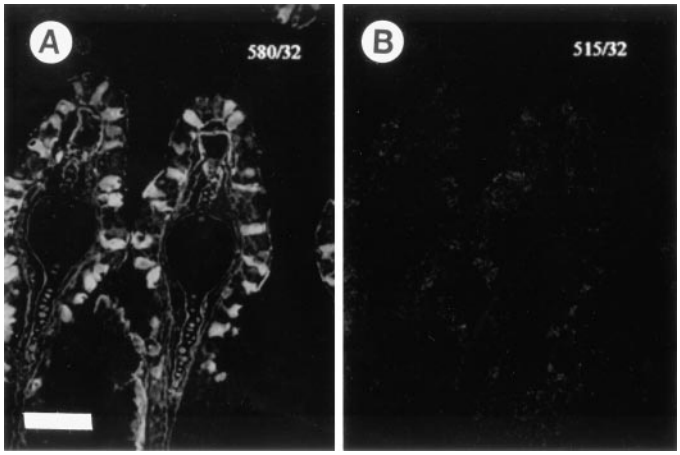

Fig. 5. Confocal images of the immunocytochemistry of Na^+-K^+-ATPase α-subunits in frozen cross sections of gill filaments from freshwater-adapted tilapia (*O. mossambicus*). Same frozen section was doubly stained with antibodies (α_1-specific 6F and α_3-specific Ax2) and was observed under different filter sets as described in MATERIALS AND METHODS. α_3 images were obtained under 580/32 (*A*) and 515/32 (*B*) filter set, respectively. Magnification ×200 (scale = 50 μm).

freshwater-adapted tilapia (Fig. 1), and the results revealed a tissue-specific distribution of the three isoforms. We detected a major immunoreactive band of the α_1-specific antibody with a relative molecular mass of ~100 kDa in the homogenates from kidneys and gills. We failed to detect any major bands of the α_2-specific antibody HERED. We detected a major protein band of the α_3-specific antibody Ax2 in brain, heart, and gills, but not in kidneys, of freshwater-adapted tilapia.

To reconfirm the expression of α_1-like and α_3-like isoforms of Na^+-K^+-ATPase in tilapia gills, enriched gill epithelial cells, instead of whole gill tissues, were used in the subsequent immunoblotting. The results demonstrate the coexistence of the protein of α_1-like and α_3-like subunits of Na^+-K^+-ATPase in gills of both freshwater- and seawater-adapted tilapia (Fig. 2). Moreover, the two isoforms appeared to respond differently to environmental salinities. The results of Western blots (Fig. 2) based on image analysis (see MATERIALS AND METHODS) indicate that the amount of Na^+-K^+-ATPase α_1-like subunit in the seawater group was significantly higher than that in the freshwater group, but no significant difference in the amount of α_3-like subunit was found between the two groups (Table 1).

Table 1. *Comparison of isoform expression of Na^+-K^+-ATPase α-subunit in gills of freshwater- and seawater-adapted tilapia (O. mossambicus)*

Isoform	Seawater	Freshwater	Seawater/Freshwater
α_1-Like	72.14 ± 19.80*†	23.13 ± 6.92†	3.44 ± 1.68
α_3-Like	4.68 ± 1.42	3.76 ± 1.60	1.61 ± 1.35
α_1-Like/α_3-like	15.62 ± 2.07*	6.65 ± 2.08	2.50 ± 0.78

Means ± SD are indicated. Values are presented as arbitrary units based on data from image analysis described in MATERIALS AND METHODS. *Seawater group is significantly different ($P < 0.05$, *t*-test) from the freshwater group in α_1-like and in α_1-like/α_3-like; †α_1-like is significantly different ($P < 0.05$, *t*-test) from α_3-like in the seawater group and in the freshwater group.

Consequently, the ratio of α_1-like to α_3-like subunits in the seawater group was also higher than that in the freshwater group (Table 1).

Double Staining of Na^+-K^+-ATPase α_1 and α_3 in MR Cells

ConA, which can be used to identify carbohydrate residues distributed on the apical cell surface of gill MR cells, is one of the indicators for MR cells (17). Figure 3 shows the images of the surface view of a whole-mounted gill filament that was doubly stained with ConA and anti-Na^+-K^+-ATPase α_1-antibody. All cells that were stained with α_1-specific antibody (Fig. 3*A*) were also labeled with ConA (Fig. 3*B*). Similar results were obtained in the case of double staining of ConA and α_3-specific antibody (data not shown). These results confirm that Na^+-K^+-ATPase α_1-like and α_3-like subunits are stained exclusively in MR cells.

Figure 6 shows the confocal images of a frozen cross section of gill filament that was doubly stained with both antibodies specific for Na^+-K^+-ATPase α_1 and α_3. The staining patterns of these two isoforms of enzyme are not identical in the same gill sections (compare Fig. 6*A* with 6*B*). Some Na^+-K^+-ATPase α_3-positive MR cells were weakly stained with the α_1-specific antibody (Fig. 6*B*), whereas several Na^+-K^+-ATPase α_1-positive MR cells were weakly or negatively stained with the α_3-specific antibody (Fig. 6*A*). However, it should be noted there were a few MR cells strongly stained with both these two isoforms of the enzyme (Fig. 6, *A* and *B*).

Negative control experiments, in which normal goat serum was used instead of the isoform-specific antibodies, have been conducted (data not shown) to confirm the above positive results.

DISCUSSION

The present study demonstrates for the first time that two isoforms, α_1- and α_3-like, of Na^+-K^+-ATPase are expressed in gill MR cells of the euryhaline teleost tilapia (*O. mossambicus*) and that the expressions of the two isoforms in the gills vary depending on environmental salinities to which the fish are adapted. These results suggest that these two isoforms of Na^+-K^+-ATPase α-subunit are involved in the mechanisms of ion regulation in freshwater- and seawater-adapted tilapia.

Tissue-specific and developmental expression of the α-subunit isoforms has been reported in birds and mammals and was also suggested to extend to all vertebrate classes, including teleosts (24, 27). However, direct proof of teleostean α-subunit expression has not been available until now. With recent advances in molecular cloning, the genes of Na^+-K^+-ATPase α-subunits have been isolated from several teleosts, including white sucker (*Catostomus commersoni*; α-subunit (26), European eel (*Anguilla anguilla*; α_1 subunit) (2), Atlantic salmon (*Salmo salar*; partial sequence of α-subunit) (3), brown trout (*Salmo trutta*; partial sequence of α-subunit), and other species (14). The mRNA of α- or α_1-subunit has been found to increase after acclimation of freshwater fish to seawater (2, 14, 17) or during

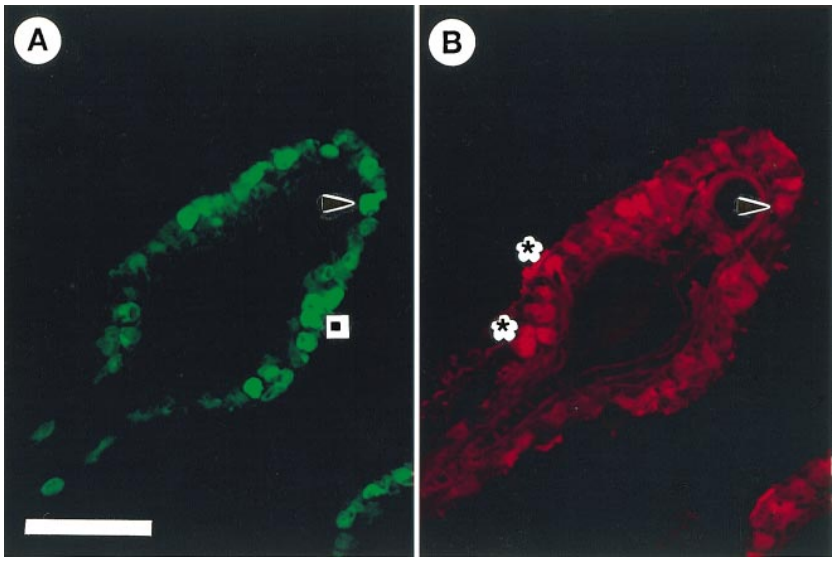

Fig. 6. Confocal images of the immunocytochemistry of Na^+-K^+-ATPase α-subunits in frozen cross sections of gill filaments from freshwater-adapted tilapia (*O. mossambicus*). Same frozen section was doubly stained with antibodies (α_1-specific 6F and α_3-specific Ax2) and was observed under different filter sets as described in MATERIALS AND METHODS. α_1 image was obtained under a 515/32 filter set (*A*), and α_3 was image obtained under a 580/32 filter set (*B*). *α_3-positive cells weakly stained with the antibody specific for α_1. Square, α_1-positive cells weakly or negatively stained with the antibody specific for α_3. Arrowhead, cells strongly stained with both antibodies. Magnification ×330 (scale = 50 μm).

smoltification (3). So far, two or more genes of Na^+-K^+-ATPase α-subunits have never been found in the same fish species studied. On the other hand, Ura et al. (35) raised a polyclonal antibody against a synthetic oligopeptide that was based on the sequences of Na^+-K^+-ATPase α-subunits from different vertebrates and invertebrates, and Hwang et al. (9) established a polyclonal antibody that was raised against the fusion protein derived from a partial Na^+-K^+-ATPase α_1 cDNA of tilapia (*O. mossambicus*). Neither antibody showed isoform-specific specificity. Because of the high identities of cDNA sequences of different isoforms, Northern blots using one isoform as the probe cannot discriminate between the mRNAs of two isoforms of similar size. A similar possibility may occur in the case of Western blots with antibodies that are general for all isoforms.

With the use of three isoform-specific antibodies, the present study provides direct evidence for the presence of multiple isoforms of Na^+-K^+-ATPase α-subunit in different organs of a teleost.

In accordance with Takeyasu et al. (30) and Pressley (24), sequences of NH_2-terminal and of 11 amino acids (487–497) in the central portion of the Na^+-K^+-ATPase α-subunit are markedly different between isoforms. Cutler et al. (2) reported that Na^+-K^+-ATPase α_1 of European eel shared 8 of 11 amino acids (487–497) with the consensus α_1 sequence compared with 3 of 11 or 1 of 11 with the α_2 or α_3 sequences, respectively; and the NH_2-terminal region of eel α_1 is also more similar to that of α_1 than to those of the other isoforms. The amino acid 384–504 of Na^+-K^+-ATPase α_1 of tilapia was found to show 83.5–84.3, 73.3, and 76.0–76.9% homologies with those of rat and chicken α_1, α_2, and α_3, respectively (9). In accordance with Hwang et al. (9), the Na^+-K^+-ATPase α-subunit of white sucker (*C. commersoni*) and electric ray (*Torpedo californica*) shows the highest homology with α_1 and α_3 of rat and chicken, respectively. On the other hand, mouse monoclonal antibody 6F, which was raised against the α_1-isoform of the avian sodium pump, has been demonstrated to be α_1 specific and to show broader species specificity (1). The rabbit polyclonal antibody, which was raised against the HERED sequence of the rat Na^+-K^+-ATPase α_2-isoform, shows species specificity for mammals only (24), suggesting that the possibility for the presence of α_2 in fish still cannot be excluded. Rabbit polyclonal antibody Ax2 raised against rat axolemma (predominately α_3 of the sodium pump) has been found to have good reactivity with α_3 but poor reactivity with α_2 (20). On the basis of this information, the present results indicate that at least two isoforms of Na^+-K^+-ATPase α-subunit, α_1-like and α_3-like, are expressed in tilapia tissues, including gills. However, we must note that the relative expression of these isoforms in tissues of tilapia appears to be different from what has been reported in mammals. Indeed, Cutler et al. (2) also found that the mRNA expression pattern of α_1 in various organs of the European eel was different from that of mammals. With regard to this discrepancy, two possibilities are proposed. *1*) The functions, and thus the relative expression, of these isoforms in fish may be different from those in mammals. *2*) Because the antibodies used in the present study are heterogeneous for tilapia, there may be some problem of crossreaction. The abundance of isoforms in some organs of tilapia may be too low to be detected by these heterogeneous antibodies. It is necessary to raise antibodies specific to fish isoforms to answer the above questions.

By using four site-specific-directed polyclonal antibodies, Pressley (24) found that both catfish brain and gill show positive reactions to the LEAVE-directed anti-

body (general for all Na^+-K^+-ATPase α-subunits), whereas only catfish brain was found to positively react with TED-directed antibody (specific for Na^+-K^+-ATPase α_3). Pressley (24) indicated the possibility of the presence of multiple isoforms in teleosts and argued that the gill must express an isoform other than α_3. The inconsistency between Pressley's results and the present data on gills should not merely be ascribed to the different species studied. Rather, differences in the antibodies (as described above, TED vs. Ax2) and the experimental methods used in Pressley's study and the present studies should be considered. Pressley used the homogenates of gill tissue, whereas in the present study we used homogenates of enriched gill epithelial cells. The enrichment procedure apparently enhances the reaction of the epithelial cells to the α_3-specific antibody (Ax2).

It has been well documented that tissue-specific, cell-specific, and developmental stage-specific distribution of Na^+-K^+-ATPase α-isoforms are of physiological significance. Previous studies on the correlation of isoform distribution and enzyme kinetics in mammals indicate that α_1-, α_2-, and α_3-isoforms differ in the affinities to cardiac glycoside, Na^+ and K^+ ions, etc. (12, 27). Recent studies further demonstrate that not only isoform-specific but also tissue-specific differences in the expression of Na^+-K^+-ATPase α-subunits are related to the apparent affinities for both Na^+ and K^+ ions (21, 32). Jewell and Lingrel (12) and Munzer et al. (21) have previously hypothesized that α_1 represents a "housekeeping" form of Na^+-K^+-ATPase that is capable of responding to typical physiological demands and that, in the case of neurons or other excitable tissues, the larger influxes of Na^+, such as those likely to occur during repeated action potentials, overwhelm the capacity of housekeeping pumps and require the involvement of other types of isoforms that can deal with an excess of intracellular Na^+. According to the present results, two isoforms of Na^+-K^+-ATPase α-subunits are differentially expressed in the gills of sweater- and freshwater-adapted tilapia and appear to be of physiological significance. Considering the dramatic differences in ionic compositions of seawater and freshwater environments, the intracellular and extracellular conditions in the gill cells of fish adapted to the two environments may be subtly different. Different combinations of two or more isoforms with different enzymatic characteristics, rather than only one type of isoform, may be necessary to carry out excretion and uptake of various ions. Indeed, previous studies indicated that branchial Na^+-K^+-ATPase shows differences in the enzyme kinetics and optimal activity conditions between freshwater- and seawater-adapted coho salmon, *Oncorhynchus kisutch* (6), or rainbow trout (22). The present results provide some clues for this inference; however, further studies are needed to confirm it.

Most tissues express more than one isoform because of the multicellular composition of these tissues and because different isoforms are expressed in a cell-specific manner (28). For example, in the central nervous system of rat, neural cell bodies have been found to contain predominantly one of three α-subunits; however, mixtures of any two isoforms or all three have also been found. Glia were observed to express either α_1 or α_2 or both. In the case of myelinated tracts, some had predominantly α_3 in the axons, others had α_1 and α_2 (20, 28). In the present study we found that some gill MR cells express both α_1-like and α_3-like subunits of Na^+-K^+-ATPase, whereas others predominantly express either α_1-like or α_3-like. It is not surprising to find heterogeneity at the level of enzymatic expression in animals other than mammals. However, this provides some clues for our previous hypothesis concerning the structures and functions of gill MR cells. Recent studies report that several types of gill MR cells with different structures are found in freshwater- and seawater-adapted fish (8, 10, 13, 15, 34). Moreover, the composition of these different types of MR cells was found to correlate with the ionic composition in environments, suggesting that these MR cells are responsible for the transport of different ions in fish adapted to diverse environments (15, 34). To test this hypothesis, further studies are needed to examine whether these types of MR cells show subtle differences in the expression of α_1-like, α_3-like, or both subunits of Na^+-K^+-ATPase.

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REFERENCES

1. **Arystarkhova, E., and K. J. Sweadner.** Isoform-specific monoclonal antibodies to Na,K-ATPase α subunits. *J. Biol. Chem*. 271: 23407–23417, 1996.
2. **Cutler, C. P., I. L. Sanders, N. Hazon, and G. Cramb.** Primary sequence, tissue specificity and expression of Na^+,K^+-ATPase α_1 subunit in the European eel (*Anguilla anguilla*) *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 111B: 567–573, 1995.
3. **D'Cotta, H. C., C. Gallais, B. Saulier, and P. Prunet.** Comparison between parr and smolt Atlantic salmon (*Salmo salar*) α subunit gene expression of Na^+/K^+ATPase in gill tissue. *Fish Physiol. Biochem*. 15: 29–39, 1996.
4. **De Renzis, G., and M. Bornancin.** Ion transport and gill ATPases. In: *Fish Physiology*, edited by W. S. Hoar and D. J. Randall. New York: Academic, 1984, vol. XB, p. 65–129.
5. **Foskett, J. K., and C. Scheffey.** The chloride cell: definitive identification as the salt-secretory cell in teleosts. *Science* 215: 164–166, 1982.
6. **Giles, M. A., and W. E. Vanstone.** Changes in ouabain-sensitive adenosine triphosphatase activity in gills of coho salmon (*Oncorhynchus kisutch*) during parr-smolt transformation. *J. Fish. Res. Board Can*. 33: 54–62, 1976.
7. **Hootman, S. R., and C. W. Philpott.** Ultracytochemical localization of Na^+,K^+-activated ATPase in chloride cells from the gills of a euryhaline teleost. *Anat. Rec*. 193: 99–130, 1979.
8. **Hwang, P. P.** Tolerance and ultrastructural response of branchial chloride cells on salinity changes in euryhaline teleost, *Oreochromis mossambicus*. *Mar. Biol. (Berl.)* 94: 643–649, 1987.
9. **Hwang, P. P., M. J. Fang, J. C. Tsai, C. J. Huang, and S. T. Chen.** Expression of mRNA and protein of Na^+-K^+-ATPase α subunit in gills of tilapia (*Oreochromis mossambicus*). *Fish Physiol. Biochem*. In press.

10. **Hwang, P. P., and R. Hirano.** Effects of environmental salinity on intercellular organization and junctional structure of chloride cells in early stages of teleost development. *J. Exp. Zool*. 236: 115–126, 1985.
11. **Hwang, P. P., C. M. Sun, and S. M. Wu.** Changes of plasma osmolality, chloride concentration and gill Na-K-ATPase activity in tilapia *Oreochromis mossambicus* during seawater acclimation. *Mar. Biol. (Berl.)* 100: 295–299, 1989.
12. **Jewell, E. A., and J. B. Lingrel.** Comparison of the substrate dependence properties of the rat Na,K-ATPase α_1, α_2, and α_3 isoforms expressed in Hela cells. *J. Biol. Chem*. 266: 16925–16930, 1991.
13. **Karnaky, K. J., Jr.** Structure and function of the chloride cell of *Fundulus heteroclitus* and other teleosts. *Am. Zool*. 26: 209–224, 1986.
14. **Kisen, G., C. Gallais, B. Auperin, H. Klungland, O. Sandra, P. Patrick, and O. Andersen.** Northern blot analysis of the Na^+,K^+-ATPase α subunit in salmonids. *Comp. Biochem. Physiol. B Biochem. Mol. Biol*. 107B: 255–259, 1994.
15. **Lee, T. H., P. P. Hwang, H. C. Lin, and F. L. Huang.** Mitochondria-rich cells in the branchial epithelium of the teloest, *Oreochromis mossambicus*, acclimated to various hypotonic environments. *Fish Physiol. Biochem*. 15: 513–523, 1996.
16. **Li, J., J. Eygensteyn, R. A. C. Lock, P. M. Verbost, A. J. H. van der Heijden, S. E. Wendelaar Bonga, and G. Flik.** Branchial chloride cells in larvae and juveniles of freshwater tilapia *Oreochromis mossambicus*. *J. Exp. Biol*. 198: 2177–2184, 1995.
17. **Madsen, S. S., M. K. Jensen, J. Nohr, and K. Kristiansen.** Expression of Na^+-K^+-ATPase in the brown trout, *Salmo trutta*: in vivo modulation by hormones and seawater. *Am. J. Physiol*. 269 (*Regulatory Integrative Comp. Physiol*. 38): R1339–R1345, 1995.
18. **Mayer-Gostan, N., and R. Naon.** Effects of ambient ion concentrations on gill ATPase in fresh water eel, *Anguilla anguilla*. *Fish Physiol. Biochem*. 10: 75–89, 1992.
19. **McCormick, S. D.** Hormonal control of gill Na^+,K^+-ATPase, and chloride cell function. In: *Fish Physiology*, edited by C. M. Wood and T. J. Shuttleworth. New York: Academic, 1995, vol. XIV, p. 285–315.
20. **McGrail, K. M., J. M. Phillips, and K. J. Sweadner.** Immunofluorescent localization of three Na,K-ATPase isozymes in the rat central nervous system: both neurons and glia can express more than one Na,K-ATPase. *J. Neurosci*. 11: 381–391, 1991.
21. **Munzer, J. S., S. E. Daly, E. A. Jewell-Motz, J. B. Lingrel, and R. Blostein.** Tissue- and isoform-specific kinetic behavior of the Na,K-ATPase. *J. Biol. Chem*. 269: 16668–16676, 1994.
22. **Pagliarani, A., V. Ventrella, R. Ballestrazzi, F. Trombetti, M. Pirimi, and G. Trigari.** Salinity-dependence of the properties of gill Na^+, K^+-ATPase in rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol. B Biochem. Mol. Biol*. 100B: 229–236, 1991.
23. **Perry, S. F.** The chloride cells: structure and function in the gills of freshwater fishes. *Annu. Rev. Physiol*. 59: 325–347, 1997.
24. **Pressley, T. A.** Phylogenetic conservation of isoform-specific regions within α-subunit of Na,K-ATPase. *Am. J. Physiol*. 262 (*Cell Physiol*. 31): C743–C751, 1992.
25. **Schonrock, C., S. D. Morley, Y. Okawasa, K. Lederis, and D. Richter.** Sodium and potassium-ATPase of the teleost fish *Catostomus commersoni* sequence, protein, structure and evolutionary conservation of the alpha subunit. *Biol. Chem*. 372: 279–286, 1991.
26. **Sweadner, K. J.** Anomalies in the electrophoretic resolution of Na^+/K^+-ATPase catalytic subunit isoforms reveal unusual protein-detergent interactions. *Biochim. Biophys. Acta* 1029: 13–23, 1979.
27. **Sweadner, K. J.** Isozymes of the Na^+, K^+-ATPase. *Biochim. Biophys. Acta* 988: 185–220, 1989.
28. **Sweadner, K. J.** Overlapping and diverse distribution of Na-K-ATPase isozymes in neurons and glia. *Can. J. Physiol. Pharmacol*. 70: S255–S259, 1992.
29. **Sweadner, K. J., and R. C. Gilkerson.** Two isozymes of the Na,K-ATPase have distinct antigenic determinants. *J. Biol. Chem*. 260: 9016–9022, 1985.
30. **Takeyasu, K., V. Lemas, and D. M. Fambrough.** Stability of Na^+-K^+-ATPase α-subunit isoforms in evolution. *Am. J. Physiol*. 259 (*Cell Physiol*. 28): C619–C630, 1990.
31. **Takeyasu, K., M. M. Tamkun, K. L. Renaud, and D. M. Fambrough.** Ouabain-sensitive (Na^++K^+)-ATPase activity expressed in mouse L cells by transfection with DNA encoding the α-subunit of an avian sodium pump. *J. Biol. Chem*. 263: 4347–4354, 1988.
32. **Therien, A. G., N. B. Nestor, W. J. Ball, and R. Blostein.** Tissue-specific versus isoform-specific differences in cation activation kinetics of the rat Na,K-ATPase. *J. Biol. Chem*. 271: 7104–7112, 1996.
33. **Tsai, J. C.** Characterization of the pattern of cytokeratin proteins in the epidermal cells of loach, *Misgurnus anguillicaudatus* (Cypriniformes). *Fish Physiol. Biochem*. 15: 307–316, 1996.
34. **Tsai, J. C., and P. P. Hwang.** The wheat germ agglutinin (WGA) binding sites and development of the mitochondria-rich cells in gills of tilapia (*Oreochromis mossambicus*). *Fish Physiol. Biochem*. In press.
35. **Ura, K., K. Soyano, N. Omoto, S. Adachi, and K. Yamauchi.** Localization of Na-K-ATPase in tissues of rabbit and teleosts using an antiserum directed against a partial sequence of the α-subunit. *Zool. Sci*. 13: 219–227, 1996.