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Expression of mRNA and protein of Na^+-K^+-ATPase α subunit in gills of tilapia (*Oreochromis mossambicus*)

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Abstract

Using RT-PCR, partial cDNA of Na^+-K^+-ATPase α subunit of tilapia (*Oreochromis mossambicus*) was cloned and sequenced. Clone TG3, with 1685 bp encoding a protein of 565 amino acids, showed higher than 85% identity in deduced amino acid sequence with previously published animal Na^+-K^+-ATPase α-1 subunit genes. Northern blot showed that TG3 is expressed in gills, kidneys and other organs in tilapia. The amount of mRNA in gill tissue increased with the level of environmental salinity. A fragment of TG3 was constructed into a pQE30 expression vector, and the expressed fusion protein was used to raise the antibody, Ab-TG3, in rabbit. Ab-TG3 was found not only to immuno-react with a major protein of approximately 100 kDa in size in gills, kidneys, heart, and brain of tilapia, but also to recognize a similar protein in gills of mudskipper (*Periophthalmus cantonesis*) and the kidneys, heart and brain of rat. Results of immunoblotting indicate that the amount of Na^+-K^+-ATPase α submit in gills of seawater-adapted tilapia was significantly higher than that in freshwater-adapted ones. Our results indicate that the salinity-dependent stimulation of mRNA of gill Na^+-K^+-ATPase α subunit is associated with corresponding stimulation at the protein level. This provides direct evidence of enhanced transcription and translation of Na^+-K^+-ATPase α-subunit gene upon salinity challenge.

Introduction

The euryhaline teleosts inhabit environments ranging from fresh water to high-salinity seawater. Through effective mechanisms of osmoregulation, teleosts are able to maintain the osmotic constancy of internal milieu and survive in hypertonic seawater or hypotonic freshwater. Gills are the most important extra-renal organs responsible for osmoregulation in fish. Mitochondria-rich cells (MR cells, formerly chloride cells) are the main site for active transport of ions in branchial epithelium, and which secrete ions in seawater-adapted fish and absorb ions and acid-base regulation in freshwater-adapted fish, respectively (Foskett and Scheffey 1982; Hwang and Hirano 1985; Hwang 1987; Hwang 1990; Perry et al. 1992; Goss et al. 1992; Lee et al. 1996a,b). The biochemical mechanisms for maintenance of constant levels of ions in body fluids of fish depend on the activity of gill Na^+K^+-ATPase, and the enzyme activities of gills in euryhaline teleost are affected by environmental ion concentrations (DeRenzis and Bornancin 1984; Hwang et al. 1988,1989; Mayer-Gostan and Naon 1992; McCormick 1995). This enzyme is mainly located in the tubular system of the MR cells (Karnaky et al. 1976), and plays a central role in the process of ion transport in gills of freshwater- and seawater-adapted fishes (De-Renzis and Bornancin 1984; McCormick 1995).

Na^+-K^+-ATPase is composed of 2 noncovalently linked polypeptides, a catalytic α-subunit

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with a molecular weight of about 110 kDa and a smaller glycosylated β subunit with a molecular weight of approximately 55 kDa. Experiments of molecular cloning and sequencing of α-subunit cDNAs revealed three major α-subunit isoforms, designated α1, α2, and α3, in several vertebrates (Sweadner 1989; Takeyasu et al 1990; Lingrel 1992; Pressley 1992). 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. Among the 3 isoforms, α1 is the form that is predominantly expressed in the transporting epithelia in higher vertebrates (Sweadner 1989; Takeyasu et al 1990; Lingrel 1992; Pressley 1992).

Recently, a molecular biological approach was also used to study the gene expression of Na^+-K^+-ATPase in fish gills and kidneys upon salinity challenges. Genes of Na^+-K^+-ATPase α and/or β subunits have been cloned and sequenced, and the expression of these genes in the gills and kidneys was reported in several teleosts including white sucker, *Catostomus commersoni* (α subunit) (Schonrock et al. 1991), European eel, *Anguilla anguilla* (α1 and β1 subunits) (Cutler et al. 1995a,b), Atlantic salmon (partial sequence of α subunit) (*Salmo salar*) (D'Cotta et al. 1996), brown trout, *Salmo trutta* (partial sequence of α subunit) and other species (Kisen et al. 1994). The mRNA of α, α1, and β1 subunits has been found to increase after acclimation of freshwater fish to seawater (Kisen et al. 1994; Cutler et al. 1995a,b; Madsen et al. 1995) or during smoltification (D'Cotta et al. 1996). However, there is no data about the expression of the protein so far. In the present study, partial cDNA of Na^+-K^+-ATPase α subunit of tilapia (*Oreochromis mossambicus*) was cloned and sequenced, a fragment of the cloned cDNA was constructed into an expression vector, and the expressed fusion protein was used to raise an antibody. Using the DNA probe and the antibody, the expression of mRNA and protein of the Na^+-K^+-ATPase α subunit in gills of tilapia adapted to fresh water and seawater was compared.

Materials and methods

Animals

Tilapia (*Oreochromis mossambicus*) with 100–150 g body weight were obtained from the laboratory stock. The fish were reared in circulated and filtered fresh water or seawater (10‰ ,20‰, 30‰ or 35‰ salinity) with 12L/12D photoperiod and were kept at 26–28 °C for over 2 weeks. For the comparison in Western blot, seawater mudskipper (*Periophthalmus cantonesis*) caught from the northern coast of Taipei and 150–200 g rats provided by the National Laboratory Animal Breeding and Research Center (Taipei, ROC) were also used.

PCR amplification, cloning, and sequencing

Degenerate primers were designed to fit the amino acid sequences that are conserved among the Na^+-K^+-ATPase α subunits of vertebrates. The amino acid sequences of the 2 opposing degenerate primers are MWFDNQI (amino acids 384–390 following Lingrel et al. 1990) and QQGMKNK (amino acids 939–945). Total RNA was isolated from seawater-adapted tilapia gills by the guanidinium isothiocyanate method (Chomczynski and Sacchi 1987) using an RNAzol B RNA isolation kit (Biotech, Houston, USA). The isolated total RNA was used for the synthesis of oligo-dT-primed cDNA. The 1st-strand cDNA was used as the template for PCR. The conditions for PCR were 94 °C for 45 sec, 42 °C for 1.5 min, and 72 °C for 2 min in 35 cycles.

PCR products with the expected size were cloned into pUC19 and sequenced by the dideoxy chain-termination method (Sanger et al. 1977) using a Sequenase kit (USB, Cleveland, USA). Programs from IntelliGenetics (Mountain View, USA) were used to analyze the nucleotide sequences.

Northern blot

A 345-bp DNA fragment (encoding amino acides Ile-390 to Pro-504) from the clone TG3 was

amplified by PCR, DIG-labelled (BM, Mannheim, Germany), and then used as the probe for Northern blot. Total RNA of gills and kidneys from tilapia was isolated following the method described above, and was size-fractionated on 0.8% formaldehyde-agarose gels, then transferred to nylon membranes. Hybridization was conducted in a solution containing 5X SSC, 50% formamide, 2% blocking solution, 0.1% sodium lauryl sarcosine, 0.02% SDS, and a DIG-labeled probe at 37 °C for 16 hr. Washing conditions were 2X SSC/0.1% SDS at 50 °C for 10 min twice and 0.1X SSC/0.1% SDS at 50 °C for 15 min twice. CSPD chemiluminescent detection was conducted following the protocols from the manufacturer (BM). Membranes were exposed to X-ray films at 37 °C for 30–60 min. The films were scanned and imported as JPG files into a commercial software package (Image-Pro Plus, 1994, Media Cybernetics, Inc.), and the densities of mRNA were estimated and compared. Northern blot for each experiment was repeated 3 times using different samples.

Fusion protein and polyclonal antibody

A 375-bp BamH I-Hind III restriction fragment containing partial TG3 encoding Ile-390 to Pro-504 of TG3 was obtained from the purified plasmid DNA by cutting out with restriction enzymes, and ligated into pQE30 (QIAGEN Inc., Chatsworth, USA). DNA sequence analysis was performed to confirm the accuracy of the construction. The constructed plasmid was transformed into *Eschericha coli* strain M15. The transformants were grown overnight at 37 °C in LB broth and diluted 1:10 into fresh medium. After incubation at 37 °C for 1 h, 1 mM (final concentration) isopropyl-ß-D-thiogalactopyranoside (IPTG) was added, and incubation was continued for 3 hr. Cells were collected by centrifugation. The recombinant protein was purified by using Ni^{2+}-nitrilotriacetate-agarose (QIAGEN) according to procedures described previously (Huang et al. 1995). *E. coli* total protein and purified protein were analysed by SDS-PAGE gels as described below. The purified protein was subjected to micro sequencing to confirm the accuracy of the fusion protein.

Purified recombinant protein was dissolved in phosphate buffer saline (PBS) and mixed thoroughly with an equal volume of Freund's complete adjuvant for the first injection, and Freund's incomplete adjuvant for the second and third injections. Approximately 100 µg of recombinant protein per injection was subcutaneously injected into the back of rabbits.

The specificity of the antibody has been confirmed in preliminary experiments. Western blotting (see below) of tilapia gill tissues was run in several conditions: (1) pre-immune rabbit serum was used instead of the antiserum (2) the fusion protein of TG3 (0.2-1 µg ml^{-1}) was used to negate the antibody. The results showed no immuno-reaction in (1) and a dose-related blocking effect on the antibody in (2).

Western blot

Gills from freshwater- and seawater-adapted tilapia and seawater mudskipper, as well as brains, kidneys and hearts from freshwater-adapted tilapia and rat were homogenized in homogenization buffer (25 mM Tris-HCl, 250 mM sucrose, 20mM EDTA, and 4% sodium deoxycholate, pH 7.6) on ice. The homogenates were first centrifuged at 6000 × g for 20 min, and the supernatant was subjected to 12 000 × g for 20 min. The supernatant was centrifuged at 40 000 × g for 1 h, and the pellet was resuspended in the homogenization buffer without sodium deoxycholate for the subsequent gel electrophoresis. All above procedures were performed at 4 °C. The electrophoresed proteins on sodium dodecyl sulfate (SDS)-containing 10% polyacrylamide gels (50 µg protein per lane) were then transferred to PVDF membrane (Millipore, Bedford, USA) by electroblotting and analyzed with immunoblot using Ab-TG3 as the antibody. Immunoblots were scanned and imported as JPG files into a commercial software package (Image-Pro Plus, 1994, Media Cybernetics Inc.), and the densities of the immuno-reactions were estimated and compared by the computer. Western blot analysis for each experiment was repeated 5 times using different samples.

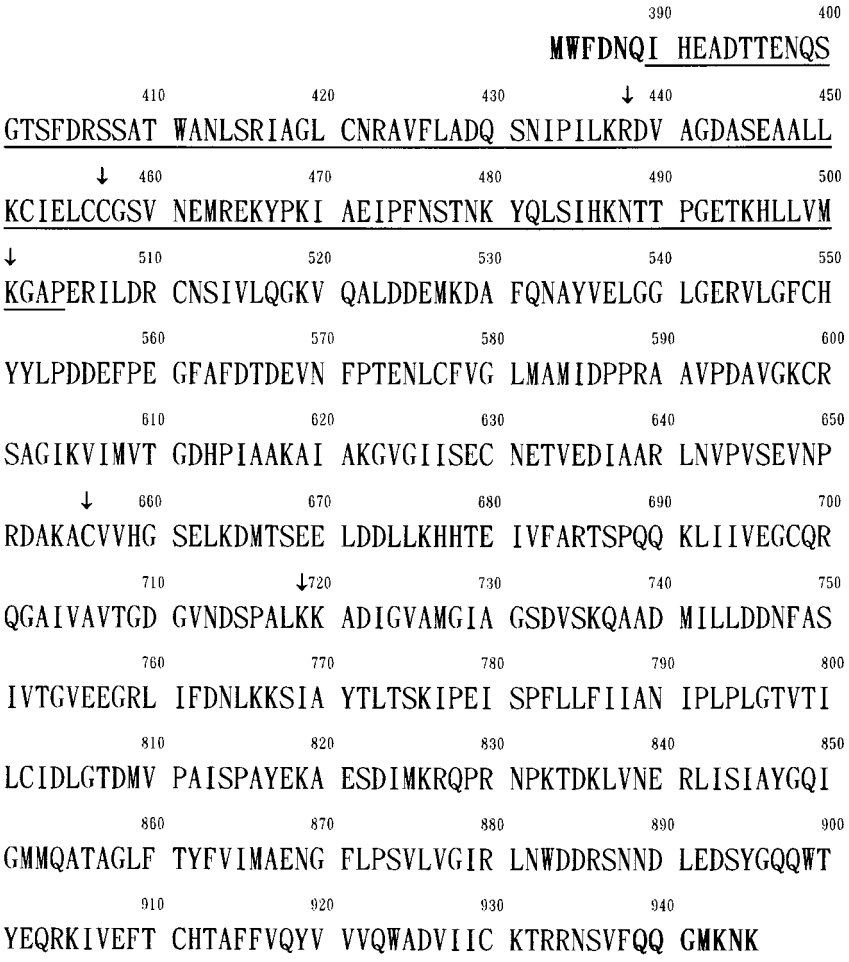

Figure 1. Deduced amino acid sequence of TG3. The numbers of amino acids follow Lingrel et al. (1990). Letters with bold face indicate the regions for the degenerate primers of PCR cloning, and the underline was designed as the probe for Northern blot. Specific amino acids highlighted with ↓ are described further in the text. The cDNA sequence of TG3 has been submitted to GenBank with the accession number U82549.

Results

Cloning and sequence

The sequence of the 1656 bp of clone TG3 obtained by RT-PCR has been submitted to GenBank with the accession number U82549. Sequence analysis of TG3 revealed that the deduced amino acid (corresponding to amino acids 392–939 in Fig. 1) shared high sequence identity with the Na^+-K^+-ATPase α subunit of vertebrates. The identical sequence has been confirmed in more than 5 distinct RT-PCR clonings. As shown in Table 1, the highest 10 proteins with amino-acid sequence homologies to TG3 are all Na^+-K^+-ATPase α subunits, and their identities are higher than 85%. Moreover, the identities with α1 subunits are higher than those with other isoforms (Table 1). Some specific amino acids, which have been found to be conserved among species (Lingrel et al. 1990), also occur at the same position of the sequence of tilapia Na^+-K^+-ATPase α subunit, i.e., Arg-438 for the tryptic site in the E_2 conformation; Leu-498 to Cys-511 for the FITC-reactive site; and Lys-501, Cys-656, Asp-710 and Lys-719 for the ATP binding pocket.

Table 1. Identities of the deduced amino acid sequences of Na^+-K^+-ATPase α subunit between tilapia and other animals

Species	Isoform	Identity (%)[a]	Reference
Chicken, *Gallus gallus*	α 1	88.8	Takeyasu et al. 1988
Eel, *Anguilla anguilla*	α 1	88.6	Cutler et al. 1995a
Rat, *Rattus norvegicus*	α 1	87.5	Shull *et al*. 1986
Horse, *Equus caballus*	α 1	87.4	Kano et al. 1989
Human, *Homo sapiens*	α 1	87.4	Kawakami et al. 1986
White sucker, *Catostomus commersoni*	α	87.4	Schonrock et al. 1991
Sheep, *Ovis aries*	α 1	87.0	Shull et al. 1985
Pig, *Sus scrofa*	α 1	87.0	Ovchinnikov et al. 1986
Pig, *Sus scrofa*	α 3	87.0	Monastyrskaya et al. 1987
Rat, *Rattus norvegicus*	α 3	85.2	Shull et al. 1986
Human, *Homo sapiens*	α 3	85.1	Ovchinnikov et al. 1988
Chicken, *Gallus gallus*	α 3	84.9	Takeyasu et al. 1990
Toad, *Bufo marinus*	α 1	84.5	Jaisser et al. 1992
Chicken, *Gallus gallus*	α 2	84.3	Takeyasu et al. 1988
Rat, *Rattus norvegicus*	α 2	84.1	Shull et al. 1986
Electric ray, *Torpedo californica*	α	82.2	Kawakami et al. 1985

[a]Percent identities of the aligned amino-acid sequences were calculated by software from IntelliGenetics (Mountain View, USA).

Expression of Na^+-K^+-ATPase α-subunit mRNA

Using the probe for TG3 (a 345-bp DNA fragment encoding amino acids Ile-390 to Pro-504), Northern blot analysis revealed that mRNA with an approximate size of 3.7 kb was detected in gills and kidneys of tilapia adapted to seawater (Fig. 2).

Moreover, the amount of mRNA of the Na^+-K^+-ATPase α subunit expressed in tilapia gills showed a positive correlation with the environmental salinity to which the fish were adapted (Fig. 3). When a similar amount (20–30 μg/lane) of total RNA from each group was loaded into the gels for comparison (Fig. 3 right), the results of Northern blots (Fig. 3 left) based on image analysis (see Materials and methods) indicated that the 10‰ , 20‰, and 30‰ groups expressed mRNA of Na^+-K^+-ATPase α subunit with amounts 2.11 ± 0.26, 2.31 ± 0.11 and 2.64 ± 0.04 (n = 3) times those in the freshwater-group, respectively.

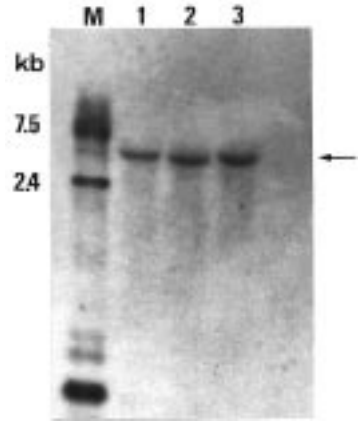

Figure 2. Expression of Na^+-K^+-ATPase α-subunit mRNA in gills and kidneys of tilapia adapted to seawater. Similar amount of total RNA were fractionated on a 0.8% formaldehyde-agarose gel as described in Materials and Methods. After blotting, the nylon membrane was hybridized with a DIG-labelled TG3 DNA probe. Lanes 1 and 2, gills of tilapia adapted to 20‰ and 30‰; lane 3, kidneys of tilapia adapted to 30‰; M, RNA ladders.

Expression of Na^+-K^+-ATPase α-subunit protein

To study the Na^+-K^+-ATPase α subunit protein expression, a TG3 fusion protein was overexpressed and used as an antigen for the production of an antibody as described in Materials and methods. Fig. 4 indicates that a fusion protein of 15–16 kDa in size was overexpressed in the transformed *E. coli* with TG3 insert (lane 1) but not in wild-type cells which lacked any insert (lane 2). The fusion protein was purified, and its size and amino-acid sequence were confirmed (data not shown).

Ab-TG3 was raised against the TG3 fusion protein. The polyclonal antibody, Ab-TG3, was

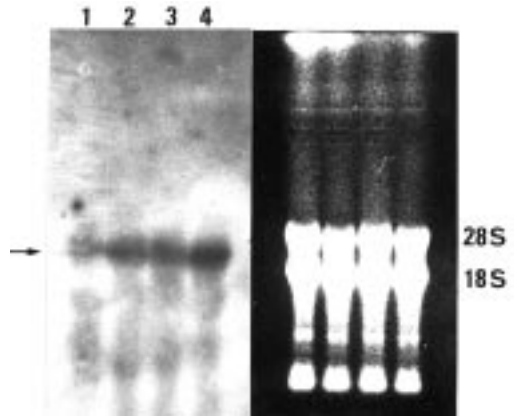

Figure 3. Expression of Na^+-K^+-ATPase α-subunit mRNA in gills of tilapia adapted to different salinities. Materials and methods were similar to those of Figure 2. Left, Northern blot; Right, ethidium bromide staining of the same gel. Locations of 18S and 28S rRNA are indicated. Lane 1, fresh water; lane 2, 10‰; lane 3, 20‰; lane 4, 30‰.

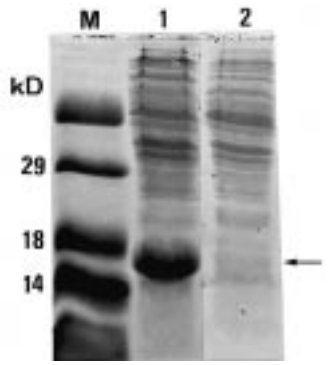

Figure 4. Overexpression of the fusion protein of the fragment containing partial TG3 encoding Ile-390 to Pro-504 of TG3. Proteins were separated on 15% SDS-PAGE gels and stained with coomassie blue as described in Materials and Methods. A, total protein of the transformant with TG3 insert (lane 1) and the wild-type cells without insert (lane 2) are shown. M, protein markers.

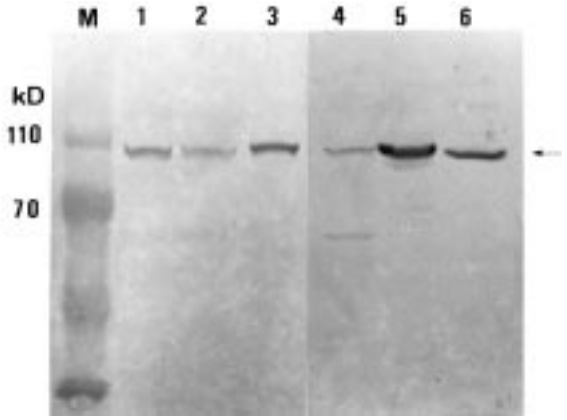

Figure 5. Expression of Na^+-K^+-ATPase α-subunit protein in different organs of tilapia and mudskipper. Similar amounts of membrane protein were electrophoresed on 10% SDS-PAGE gels, and the separated proteins were transferred onto a PVDF membrane and probed with the antiserum, Ab-TG3, as described in Materials and Methods. Lanes 1 and 2, gills of seawater-and freshwater-adapted tilapia; lane 3, gills of mudskipper; lanes 4, 5, and 6, heart, brain, and kidneys of freshwater-adapted tilapia; M, protein markers.

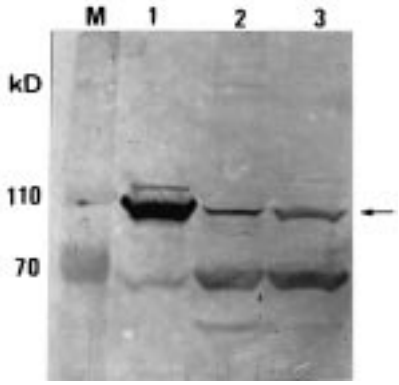

Figure 6. Expression of Na^+-K^+-ATPase α-subunit protein in organs of rat. Materials and methods were similar to those of Figure 5. Lane 1, brain; lane 2, heart; lane 3, kidneys; M, protein markers.

used in Western blot analysis, and the results reveal that the antibody recognizes a major protein with an approximate size of 100 kDa in gills, kidneys, brain, and heart of tilapia (Fig. 5). Moreover, Ab-TG3 was also found to recognize a major band of a similar size in gills of mudskipper and in heart, brain, and kidneys of rat (Fig. 5, 6). Although, some protein bands with sizes smaller than 70 kDa were observed, they may have been degraded proteins or other non-specifically recognized proteins.

The amount of Na^+-K^+-ATPase α subunit protein expressed in tilapia gills, similar to that of mRNA, showed a positive correlation with the environmental salinities to which the fish were adapted. When a similar amount of membrane proteins from each group was loaded into the gels for comparison (Fig. 7, lanes 1 and 2), the results

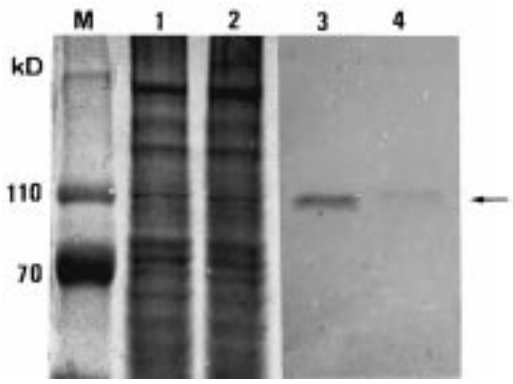

Figure 7. Comparison of the expression of Na^+-K^+-ATPase α-subunit protein in gills of seawater- and freshwater-adapted tilapia. Materials and methods were similar to those of Figure 5. One gel was stained with coomassie blue (left), and another gel was transferred onto a PVDF membrane and probed with the antiserum, Ab-TG3 (right). Lanes 1 and 3, seawater; lanes 2 and 4, fresh water; M, protein markers.

of Western blots (Fig. 7, lanes 3 and 4) based on image analysis (see Materials and Methods) indicate that the amount of Na^+-K^+-ATPase α subunit in the seawater group is 5.4 ± 1.1 (n = 5) times that of the freshwater group.

Discussion

In this study, we cloned and sequenced a cDNA fragment of Na^+-K^+-ATPase α subunit, and over-expressed the fusion protein of the recombinant partial Na^+-K^+-ATPase α subunit to raise a polyclonal antibody. By using these DNA probe and antibody, we demonstrated that a stimulation of the expression of Na^+-K^+-ATPase α-subunit mRNA is associated with expression of the protein in tilapia gills upon salinity challenges.

The TG3 clone was confirmed to be partial cDNA of Na^+-K^+-ATPase α subunit based on the following results: (1) higher than 85% identities with the published Na^+-K^+-ATPase α subunit in the deduced amino-acid sequence; (2) the presence of some conserved specific amino acids for the tryptic site in the E_2 conformation, FITC-reactive site and ATP binding pocket (Lingrel et al. 1990); and (3) a single 3.7-kb transcript in gills, kidneys, and other organs. Based on the comparisons of the 565 amino acid sequences from different species, TG3 reveals higher identities with

Tilapia Na^+-K^+-ATPase α subunit										
TG3	487									497
	K	N	T	T	P	G	E	T	K	H	L

Vertebrate Na^+-K^+-ATPase consensus										
α1	K	N	A	N	A	G	E	P	R	H	L
			P		P	S		S	Q	Y	
					T				k		
α2	E	R	E	D	D	P	Q	–	G	H	I
				E	S				S		V
α3	E	T	E	D	P	N	D	N	R	Y	L

Figure 8. Comparison of amino acid sequence 487-497 of tilapia TG3 with that of a consensus of Na^+-K^+-ATPase a-subunit sequences. The cosensuses follow Cutler et al. (1995a), which were derived from the a isoform subunit sequences of vertebrates so far published. "—" represents a deletion in the sequence.

α-1 isoforms than with other α isoforms, but the differences in identities are less than 5% (Table 1). Comparing the whole amino acid sequences among different isoforms of the Na^+-K^+-ATPase α subunit from various species, Takeyasu et al. (1990) found that amino-acid sequence differences between α-isoforms within a single species are approximately twice that between corresponding α-isoforms of 2 species. Moreover, as many as 77 positions were found to be isoform-specific residue positions (Takeyasu et al. 1990). One of these isoform-specific positions, amino-acid 487–497 region which is located just at the NH_2-terminal of the FITC-reactive site, has been used as targets for site-directed isoform-specific antibodies (Pressley 1992), and consensus of this region for each isoform has been derived for identifying the type of isoform for the cloned sequence (Cutler et al. 1995a). Following these studies, comparisons of amino acid sequence 487–497 of TG3 with those of the consensuses further indicated that TG3 may be partial cDNA of α-1 subunit of Na^+-K^+-ATPase, since TG3 shares 8/11 amino acids with α-1 but only 1/11 with α-2, and 3/11 with α-3, respectively (Fig. 8). According to the same method, the Na^+-K^+-ATPase α subunit of white sucker cloned by Schonrock et al. (1991)

likely belongs to α-1, while that of electric ray, *Torpedo californica*, (Kawakami et al. 1985) is an α-3-like isoform (comparison data not shown). Other types of α-subunit isoforms, however, remain to be cloned to confirm the presence of multiple α-isoforms of Na^+-K^+-ATPase in fish.

The Northern blot analysis results of tilapia gills and kidneys demonstrate the existence of a major mRNA of Na^+-K^+-ATPase α-subunit approximately 3.7 kb in size, being in agreement with the transcript size found in the different organs of several fishes (Cutler et al. 1995a) and higher vertebrates (Sweadner 1989). However, a larger size with about 4.0–4.15 kb of mRNA was reported in electric ray (Kawakami et al. 1985) and white sucker (Schonrock et al.1991), and a smaller transcript of 3.5 kb in size was found in eel (Cutler et al. 1995a). On the other hand, 2 distinct α-subunits, 4.15 and 3.8 kb in length, were detected in the Northern blot of brain of white sucker (Schonrock et al. 1991), and a major 3.7-kb and a minor 1.8-kb transcripts were reported in gills of Atlantic salmon (*Salmo salar*) (D'Cotta et al. 1996). Whether these smaller minor transcripts are another isoform or a nonfunctional truncated form, as the authors suggested, remains to be certified.

The present study establishes for the first time a polyclonal antibody which was raised against the fusion protein derived from a partial Na^+-K^+-ATPase α-subunit cDNA. This antibody was demonstrated to recognize Na^+-K^+-ATPase α subunits not only from different tissues of teleost but also those tissues from other animals, suggesting a general antibody for different isoforms and for different species. Ura et al. (1996) raised a polyclonal antibody against a synthetic oligopeptide which was based on the sequences of Na^+-K^+-ATPase α subunits from different vertebrates and invertebrates. In gills and kidneys of masu salmon (*Oncorhynchus masou*), Ura's antibody recognized the intact form of Na^+-K^+-ATPase (αβ-complex, 150 kDa) and some proteolytic fragments of α subunits (60–65 kDa) but never reacted with the intact form of the α subunit. The antibody from the present study, similar to the antibodies established in the previous study (Pressley 1992), showed a specific reaction with an approximately 100-kDa protein corresponding to the intact Na^+-K^+-ATPase α subunit.

Na^+-K^+-ATPase is mainly located in the tubular system of MR cells (Karnaky et al. 1976), and plays a central role in the transport mechanisms of different ions in gills of freshwater- and seawater-adapted fishes (McCormick 1995). On the other hand, transepithelial ion permeabilities, and thus ionic fluxes, in the gills of seawater-adapted fish are much higher than those in the gills of freshwater-adapted fish (Payan et al. 1984). It has been well documented that the activities of branchial Na^+-K^+-ATPase in euryhaline fish (with some exceptions) increase with elevated environmental salinities, and that this is mediated by hormones (DeRenzis and Bornancin 1984: McCormick 1995). Evident differences in the branchial Na^+-K^+-ATPase between seawater- and freshwater-adapted fish suggest that the capacities (i.e., activities) of the enzyme may be required differentially not only to perform the transport functions of different ions as described above but also to balance the different rates of ionic fluxes in gill epithelia between seawater- and freshwater-adapted fish. However, whether synthesis of new protein of Na^+-K^+-ATPase was involved in this salinity-dependent increase of activity is not clear until recently. Northern blot analysis demonstrated that expression of Na^+-K^+-ATPase α-subunit mRNA in gills of seawater-adapted fish is higher than that in freshwater-adapted ones (Kisen et al. 1994; Cutler et al. 1995a). D'Cotta et al. (1996) found that enhanced transcript levels of the gill Na^+-K^+-ATPase α subunit are concurrent with the elevated enzyme activity in Atlantic salmon during smoltification. Based on the data of Nothern blot and enzyme activity, Madsen et al. (1995) demonstrated that an increased abundance of Na^+-K^+-ATPase α-subunit mRNA is part of the molecular mechanism behind the increased enzyme activity induced by seawater transfer, cortisol, growth hormone, and IGF-I. The present study provides direct data for the first time of the expressed protein level of gill Na^+-K^+-ATPase to link those reported previously (as described above), and the transcription and function (activity and/or ion fluxes) levels of the enzyme. The present study demonstrates that the augmented mRNA expression of Na^+-K^+-ATPase α subunit is associated with the increase in the α-subunit protein in fish gills upon seawater adaptation.

Another interesting finding is the 2.6-fold increase of gill Na^+-K^+-ATPase α-subunit mRNA upon seawater adaptation compared to a 5.4-fold increase of protein. This was also reported in the expression of rat myocardial Na^+-K^+-ATPase α-2 subunit; a 5-fold increase in the mRNA on day 2 and 14-fold increase in protein on day 4 were stimulated by cutaneous administrations of T_3 (triiodothyronine) (Hensley et al. 1992). On the contrary, α-1 mRNA increased to 3 fold by day 1, and the protein 2 fold by day 4 in hyperthyroid rat (Hensley et al. 1992). The inconsistency in either the augmentation level or timing of steady state between mRNA and protein of Na^+-K^+-ATPase α (and/or β) subunit has been reported in different tissues or cells under various physiological conditions (Nici et al. 1991; Azuma et al. 1993; Ohara et al. 1993; Lee et al. 1995). This phenomenon may indicate that changes in translation or postranslational kinetics are involved as Hensley et al. (1992) suggested, and these changes may be dependent on the isoform, cell, species, or physiological conditions. Further studies are needed to determine the time course of changes in the expression of gill Na^+-K^+-ATPase α-subunit mRNA and protein following salinity challenges.

It has been well known that the different isoforms of Na^+-K^+-ATPase α and β subunits exhibit tissue- and cell-specific and developmental patterns of expression (Lingrel 1992; Takeyasu et al. 1990). The previous results on various rodent cell lines suggest that the α1 gene functions primarily in a housekeeping capacity to maintain osmotic balance and cell-volume regulation, while the other α-subunit genes fulfill more specialized requirements for cation transport necessary for differentiated cell-specific function (review of Lingrel et al. 1990). Many tissues express more than 1 isoform because of the multicellular composition of these tissues. Jewell et al. (1992) demonstrated that α isoforms have distinct Na^+ affinity to developed enzymes with different properties for specific cellular conditions. Previous studies indicate that branchial Na^+-K^+-ATPase shows differences in the optimal activity condition between freshwater- and seawater-adapted coho salmo, *Oncorhynchus kisutch* (Giles and Vanstone 1976) or rainbow trout, *Oncorhynchus mykiss* (Pagliarani et al. 1991). Several studies also report that actinomycin D reveals different effects on the branchial activities in freshwater- and seawater-adapted fish (Motais 1969; Gallis et al. 1979; Eib and Hossner 1985; Beckman and Zaugg 1990). On the other hand, Bell and Sargent (1979) did not find different isoforms of Na^+-K^+-ATPase in gills of eel based on SDS-page analysis of the partial purified enzyme. In the study by Pressley (1992), an antibody designed for all α isoforms recognized the gill tissues of freshwater catfish (*Ictalurus punctatus*), however those antibodies which were designed specifically for α 1, 2, and 3 isoforms, respectively, did not react with the same tissues. The previous studies (Kisen et al. 1994; Cutler et al. 1995a; Madsen et al. 1995; D'Cotta et al. 1996) and the present results on the expression of Na^+-K^+-ATPase α subunit suggest the existence of only 1 isoform of mRNA and/or protein in the gills of either seawater- or freshwater-adapted fish. These results, however, could not exclude the possibility of the presence of more than 1 type of Na^+-K^+-ATPase α subunit in gills of fish. So far, only the probe derived from one type of isoform is available in the same species studied. Because of the high identities of cDNA sequences of different isoforms, Northern blots with only the probe of 1 isoform may not discriminate the mRNAs of 2 isoforms of similar size. A similar possibility may occur in the case of Western blots with Ab-TG3 which is general for different isoforms as suggested above. Our preliminary experiments (Lee, Yu, Tsai, Fang and Hwang, unpublished data) show that gill tissues from either seawater- or freshwater-adapted tilapia react with monoclonal and polyclonal antibodies which were raised against avian α-1 subunit and rat α-3 subunit, respectively, but not with another anti-rat-α-2 polyclonal antibody. It will be of much interest and remains to be investigated whether various isoforms of Na^+-K^+-ATPase α subunit are expressed differentially in gills of fish adapted to different environments.

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