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Aquaporin-3 expressed in the basolateral membrane of gill chloride cells in Mozambique tilapia *Oreochromis mossambicus* adapted to freshwater and seawater

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Summary

We have cloned a homologue of mammalian aquaporin-3 (AQP3) from gills of Mozambique tilapia using a reverse transcription-polymerase chain reaction (RT-PCR). The deduced amino acid sequence shared 64–75% homology with other vertebrate AQP3 homologues. RT-PCR revealed that tilapia AQP3 was expressed in the brain, pituitary, kidney, spleen, intestine, skin, eye and gill in tilapia adapted to freshwater (FW) and seawater (SW). We also examined functional characteristics of tilapia AQP3 using *Xenopus* oocytes as an *in vitro* transcribed cRNA expression system. Osmotic water permeability (Pf) of *Xenopus* oocytes expressing tilapia AQP3 was about 30-fold higher than that of control oocytes, and was 80% inhibited by treatment with 0.3 mmol l^{-1} $HgCl_2$. Light-microscopic immunocytochemistry of branchial epithelia revealed that tilapia AQP3 was expressed in gill chloride cells of FW- and SW-adapted tilapia. Electron-microscopic immunocytochemistry further demonstrated that tilapia AQP3 was localized in the basolateral membrane of gill chloride cells. Basolateral localization of AQP3 in gill chloride cells suggests that AQP3 is involved in regulatory volume changes and osmoreception, which could trigger functional differentiation of chloride cells.

Key words: aquaporin, gill, osmoregulation, tilapia, *Oreochromis mossambicus.*

Introduction

For vertebrates, the control of their body fluid homeostasis is fundamental to life. Osmoregulation in teleost fishes is achieved by integrated water and ion transport in osmoregulatory organs such as the gills, kidney and intestine (Evans, 1993; Bentley, 2002). Freshwater (FW) teleosts face water load and ion loss through their body surface, and therefore excrete excess water by producing dilute urine in the kidney, and absorb ions through the gill epithelia. Conversely, to compensate for osmotic water loss across the body surface, marine teleosts drink a large amount of water, and produce isotonic urine to minimize water loss, while excess ions are actively secreted from the gills. In teleost fishes, however, little information is available on the mechanisms of water transport that are essential for maintaining plasma osmolality within physiological ranges. The branchial epithelium is one of the major sites of water exchange with external environments (Isaia, 1984), and is thus important for teleost fishes to maintain water balance of their body fluid.

Aquaporins (AQPs) are a family of integral membrane proteins that function as water-selective channels, and are importantly involved in fluid transporting mechanisms in various organs (Agre et al., 2002; King et al., 2004). More than ten AQPs have been identified in mammals; deduced amino acid sequences and the predicted six-transmembrane-spanning topology are highly conserved between species and between family members. Among AQP isoforms, AQPs 0, 1, 2, 4 and 5 are considered to be water-selective (Agre et al., 1993; Hasegawa et al., 1994; Raina et al., 1995; Yang and Verkman, 1997), whereas AQPs 3, 7, 9 and 10 are also permeable to glycerol and other small nonelectrolyte solutes, often referred to as 'aquaglyceroporins' (Ishibashi et al., 1994, 1997, 1998, 2002; Echevarria et al., 1996). AQP6 has been proposed to function in kidney endosomes as a pH-sensitive anion channel (Yasui et al., 1999a,b). In teleost fishes, several AQP homologues have been cloned to date, including AQP0 from the lens of killifish *Fundulus heteroclitus* (Virkki et al., 2001), AQP1 from the intestine of Japanese eel *Anguilla japonica* (Aoki et al., 2003), sbAQP from the kidney of sea bream, *Sparus auratus* (Santos et al., 2004), and AQP3 from the gills of European eel *Anguilla anguilla* (Cutler and Cramb, 2002), and Japanese dace *Tribolodon hakonensis* (Hirata et al., 2003). Although AQP3 may be involved in water movements and the related biological events in gills of teleosts, its physiological significance remains unclear (Cutler and Cramb, 2002; Lignot et al., 2002; Hirata et al., 2003).

In this study, we attempted to search for AQP3 in the gills

of Mozambique tilapia by PCR-based cloning in order to gain a better understanding of the mechanism and route of water movements. Here we report the cloning, tissue distribution, functional expression, and cellular and intracellular localization of tilapia AQP3. Our findings suggested the involvement of AQP3 in the mechanisms of volume regulation and osmoreception in gill chloride cells.

Materials and methods

Animals

Mozambique tilapia were maintained in tanks supplied with either recirculating FW or full-strength seawater (SW, 31–33 p.p.t.), and water temperature was maintained at 25°C. Fish were fed on commercial tilapia pellets 'Tilapia 41M' (Shikoku Kumiai Shiryo, Tokushima, Japan) once a day. Fish were anesthetized with 0.1% 2-phenoxyethanol and decapitated before removal of tissues.

Molecular cloning of tilapia AQP3 cDNA

Cloning of the partial-length cDNA

Freshwater-adapted tilapia was used for total RNA preparation. After anesthesia, the gills were dissected out and frozen in liquid nitrogen. Total RNA was extracted with RNA extraction solution (ISOGEN; Nippon Gene, Toyama, Japan) from the gills. Total RNA was treated with DNase (Promega, Madison, WI, USA) at 37°C for 1 h, and 2 μg total RNA was reverse-transcribed using Ready-To-Go™ T-Primed First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer's instructions.

Degenerate primers used for amplification were designed based on the available information in vertebrate species. Reverse transcription (RT)-PCR was carried out using sense (AQP3-df) and antisense (AQP3-dr) primers (Table 1). PCR was performed in a final volume of 20 μl containing 1× PCR buffer (TaKaRa, Shiga, Japan), 200 μmol l^{-1} of dNTPs (TaKaRa), 0.5 U *Taq* DNA polymerase (*TaKaRa r Taq*; TaKaRa), 5 μmol l^{-1} each of the primer pair, and an appropriate amount of the gill cDNA template. The PCR cycle protocol was as follows: 94°C for 3 min, 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with final incubation at 72°C for 7 min. The PCR products were analyzed on a 2% agarose gel and ligated into pBluescript SK (–) (Stratagene, La Jolla, CA, USA). The plasmid DNA was purified, and both strands of the DNA were sequenced using a DNA sequencer ABI PRISM 310 (Applied Biosystems, Foster City, CA, USA). Sequence data were analyzed with Sequencher software version 3.1.1 (Hitachi, Tokyo, Japan). All primers using for PCR-based cloning are listed in Table 1.

Table 1. *Oligonucleotide primers used*

AQP3-df	5′-AAYCCIGCIGTNACNTTYGC-3′
AQP3-dr	5′-ACNGCRTAICCIGARTTRAANCCCAT-3′
3′AQPf-f	5′-TTCTGGCCATTGTGGATCCTTTCAAC-3′
RTG	5′-AACTGGAAGAATTCGCGGCCG-3′
5′AQPf-r	5′-GTTGAAAGGATCCACAATGGCCAGAA-3′
NUP	5′-CTAATACGACTCACTATAGGGC-3′
RT-f	5′-CTCATCCTTGTAATGTTTGGCTGTG-3′
RT-r	5′-AGAACCACAAATCCCACAGTGAAG-3′
AQP3+poly(T)	5′-$(T)_{25}$AACAAAAACCCAGAAC-3′
AQP3+kozak	5′-CCACCATGGGCAGACAAAAGGAGT-3′

3′ Rapid amplification of cDNA end (RACE)

After determination of the partial cDNA sequence, 3′ rapid amplification of cDNA end (RACE) was carried out to extend sequence information at the 3′ end. The 3′ end of tilapia AQP3 cDNA was amplified with a gene-specific primer, 3′ AQPf-f, and an adaptor primer, RTG (Table 1), in a reaction mixture (20 μl) containing 1× PCR buffer, 200 μmmol l^{-1} dNTPs, 0.5 U *Taq* DNA polymerase (*TaKaRa r Taq*; TaKaRa), 0.25 μmol l^{-1} primers, and 2 μl of tenfold diluted cDNA template. The PCR cycle protocol was as follows: 94°C for 3 min, 38 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 90 s, with final incubation at 72°C for 4 min. The clone obtained was subcloned and sequenced as described above.

5′ RACE

Total RNA was isolated as described above from the gills of FW-adapted tilapia. Total RNA (2 μg) was reverse-transcribed, and 5′ RACE-ready first-strand cDNA was synthesized using SMART™ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. For 5′ RACE, an adaptor primer, NUP, and a gene-specific primer, 5′ AQPf-r, were used (Table 1). The 5′ RACE-PCR was carried out with these primers under the same conditions as 3′ RACE-PCR. The clone obtained was subcloned and sequenced as described above.

Analysis of the amino acid sequence

Kyte-Doolittle hydropathy profile of the deduced amino acid sequence was analyzed using GENETYX-MAC version 8.0 software (Software Development, Tokyo, Japan) at a 12-residue window. The amino acid identities between tilapia AQP3 and other AQPs were analyzed by the BLAST search (Altschul et al., 1997).

Northern blot analysis

To confirm the full-length of tilapia AQP3 mRNA, total RNA was isolated from the gill of FW-adapted tilapia. Poly(A)$^+$ RNA (3 μg) was purified using Oligotex-dT30 Super (TaKaRa) and electrophoresed on a 1.2% formamide–agarose gel, and transferred to a Biodyne nylon membrane (Pall Gelman Sciences, Ann Arbor, MI, USA). The membrane was UV cross-linked (120 000 μJ cm^{-2}) using Spectrolinker XL-1500 (Spectronics Corporation, Westbury, NY, USA) and dried in a dry oven at 80°C for 15 min. The membrane was prehybridized at 42°C for 3 h in 6× SSC containing 50% formamide, 1× Denhardt's solution, 0.5% SDS and calf thymus DNA (100 ng ml^{-1}). The buffer was then replaced, and the membrane was hybridized with a [^{32}P]-labeled cDNA

probe (see below) in the same buffer at 42°C for 18 h. After hybridization, the membrane was washed in 2× SSC containing 0.1% SDS at room temperature for 5 min. The membrane was then exposed to an X-ray film RX-U (Fuji Film, Tokyo, Japan) at –80°C. A cDNA probe for tilapia AQP3 was generated with the RT-f and 5′AQPf-r (Table 1) from the tilapia AQP3 cDNA clone and radiolabeled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech) by PCR amplification.

Reverse transcription-PCR (RT-PCR) analysis

The brain, pituitary, kidney, liver, spleen, intestine, eye, gill and skin were removed from FW- and SW-adapted tilapia, and total RNA was extracted from these tissues as described above. Total RNA (2 μg) was reverse-transcribed using Superscript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA was amplified with a primer pair, RT-f and RT-r (Table 1). The PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. As a negative control, PCR was conducted in the absence of the cDNA template.

Oocyte expression

The posterior fragment of tilapia AQP3 cDNA was amplified using a primer set, RT-f and AQP3+poly(T) (Table 1), subcloned into pCR 4Blunt-TOPO (Invitrogen), excised from pCR 4Blunt-TOPO using *Nco*I and *Pst*I, and ligated into pT7Blue-2 (Novagen, Madison, WI, USA). The anterior fragment of tilapia AQP3 cDNA was amplified using a primer set, AQP3+kozak and 5′ AQPf-r (Table 1), subcloned into pBluescript SK(–) (Stratagene), excised from pBluescript SK(–) using *Nco*I, and ligated into pT7Blue-2 containing the posterior fragment of tilapia AQP3. Capped cRNA was transcribed *in vitro* using T7 mMessage mMachine kit (Ambion, Austin, TX, USA) after digestion with *Eco*RI to linearize the plasmids.

Xenopus laevis oocytes (stage V–VI) were defolliculated with collagenase (Sigma, St Louis, MO, USA) and microinjected with 10 ng of the synthesized cRNA or 50 nl of distilled water as a control, and incubated at 18°C for 48 h in modified Barth's solution (MBS; 88.0 mmol l^{-1} NaCl, 1.0 mmol l^{-1} KCl, 2.4 mmol l^{-1} $NaHCO_3$, 0.3 mmol l^{-1} $CaNO_3$ 2, 0.41 mmol l^{-1} $CaCl_2$, 0.82 mmol l^{-1} $MgSO_4$, 1000 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin).

Measurement of oocyte water permeability

After 48 h incubation, oocytes were transferred from 200 mOsm kg^{-1} (Osm_{in}) to 70 mOsm kg^{-1} (Osm_{out}) of MBS diluted with distilled water at 22°C. We acquired images of the oocyte silhouette every 15 s through a CCD camera attached to a stereomicroscope (SMZ1500, Nikon, Tokyo, Japan) up to 5 min or the time of oocyte rupture. The saved images were later analyzed with Image-Pro® Plus 4.5.1 (Media cybernetics, Silver Spring, MD, USA). The oocyte volume was calculated from the cross-sectional area of the oocyte, assuming the oocyte to be a perfect sphere.

The osmotic water permeability (Pf) was calculated using the following equation (Preston et al., 1992):

$$Pf = V_0 \times d(V/V_0)dt / [S \times Vw \times (Osm_{in}–Osm_{out})] ,$$

where $d(V/V_0)dt$ is the initial rate of increase in relative oocyte volume; V_0 is the initial volume of the oocyte (V_0=9×10^{-4} cm^3); S is the initial oocyte surface area (S=0.045 cm^2); Vw is the molar volume of water (Vw=18 cm^3 mol^{-1}).

Effects of mercuric chloride were examined by incubating oocytes in MBS containing 0.3 mmol l^{-1} $HgCl_2$ for 10 min prior to Pf measurements. To examine the recovery of the $HgCl_2$-induced inhibition by a reducing agent, oocytes were incubated for 10 min in MBS containing 5 mmol l^{-1} β-mercaptoethanol following 10 min incubation in $HgCl_2$. Significant differences in Pf values at P<0.01 were determined by one-way analysis of variance (ANOVA), followed by Fisher's PLSD, using StatView 5.0 software (Hulinks, Tokyo, Japan).

Antibody

A polyclonal antibody was raised in a rabbit against a synthetic peptide corresponding to part of the C-terminal region of tilapia AQP3 molecules (amino acid residues 267–279 in tilapia AQP3). The antigen conjugated with keyhole limpet hemocyanin (KLH) was emulsified with complete Freund's adjuvant, and immunization was performed in a New Zealand white rabbit. The antiserum was obtained after several booster injections, and the specific antibody was affinity-purified from the antiserum with the antigen peptide (QIAGEN, Hilden, Germany).

Western blot analysis

The specificity of the antibody raised against the synthetic peptide was confirmed by western blot analysis. Prior to western blotting, the sample was subjected to immunoprecipitation. The gill was isolated from FW-adapted tilapia, and the gill filaments were scraped in 1 ml of lysis buffer consisting of IP buffer (pH 7.4; 140 mmol l^{-1} NaCl, 2 mmol l^{-1} KCl, 10 mmol l^{-1} Hepes, 5 mmol l^{-1} EDTA), inhibitors (10 mmol l^{-1} benzamidine, 1 μg ml^{-1} Pepstatin A and 2 mmol l^{-1} phenyl methyl sulfonyl fluoride) and 1% Triton X-100, and left on ice for 20 min to lyse the cells. The lysate was centrifuged at 5000 ***g*** for 5 min at 4°C, and the supernatant was incubated with 2 μl of the tilapia AQP3 antibody at 4°C for 16 h. Slurry (20 μl) containing 50% protein A sepharose beads (Amersham Pharmacia Biotech) blocked overnight with 1% bovine serum albumin (BSA) was added to the sample, and the mixture was incubated for 1 h at 4°C. After washing five times with IP buffer containing inhibitors and centrifugation at 10000 ***g*** for 30 s, 30 μl of hot Laemmli buffer (Laemmli, 1970) containing 5% β-mercaptoethanol was added to beads binding to the antibody, and the mixture was incubated for 15 min at 65°C. The sample was centrifuged at 10 000 ***g*** for 2 min. The supernatant was separated by SDS–polyacrylamide gel electrophoresis using PAG Mini 'DAIICHI' 15/25 (Daiichi

pure chemicals, Tokyo, Japan). After electrophoresis, the protein was transferred from the gel to a polyvinylidene fluoride microporous membrane (Immobilon-P Transfer Membrane; Millipore, Billerica, MA, USA). After blocking with a blocking solution (Block Ace; Dainippon Pharmaceutical, Osaka, Japan) for 1 h at room temperature, the membrane was incubated with anti-tilapia AQP3 diluted 1:100 with 20 mmol l^{-1} Tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 1.5 h at room temperature. After rinsing in TBS-T, the membrane was stained by the avidin–biotin–peroxidase complex (ABC) method (Hsu et al., 1981), using commercial reagents (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA). Briefly, the membrane was incubated with biotinylated anti-rabbit IgG for 30 min, and then with ABC for 30 min at room temperature. The membrane was finally incubated with 0.02% 3,3′-diaminobenzidine tetrahydrochloride (DAB) containing 0.005% H_2O_2 for 3 min to visualize the immunoreactive bands.

Fluorescence microscopy

The gills were dissected out and fixed in 4% paraformaldehyde (PFA) in 0.1 mol l^{-1} phosphate buffer (PB, pH 7.4) for 16 h at 4°C. After fixation, the gills were dehydrated in ethanol, and embedded in Paraplast. Sections (4 μm) were cut and mounted on MAS-coated slides (Matsunami, Osaka, Japan). For the detection of chloride cells in the sections, we used an antibody specific for Na^+/K^+-ATPase. The antiserum (NAK121) was raised in a rabbit against a synthetic peptide corresponding to part of the highly conserved region of the α-subunit (Uchida et al., 2000). The sections were incubated with anti-tilapia AQP3 diluted 1:200 with PBS containing 2% normal goat serum (NGS), 0.1% BSA, 0.02% KLH and 0.01% sodium azide (NB-PBS) overnight at 4°C, and then with goat anti-rabbit IgG labeled with Alexa Fluor 488 (Molecular probes, Eugene, OR, USA) diluted 1:1000 with NB-PBS for 3 h at room temperature. After rinsing in PBS, the sections were incubated with Alexa Fluor 546-labeled anti-Na^+,K^+-ATPase (Katoh and Kaneko, 2003) diluted 1:2000 with NB-PBS at 4°C for 8 h. The sections were observed under a fluorescence microscope (Nikon E800) with blue (excitation, 450–490 nm; emission, 520–560 nm) and green (excitation, 510–560 nm; emission, >590 nm) excitation filter blocks for Alexa Fluor 488 and Alexa Fluor 546, respectively. To confirm the specificity of the immunoreaction for tilapia AQP3, the sections were incubated with the pre-immune serum instead of anti-tilapia AQP3.

Transmission electron microscopy

The gills were fixed in 2% PFA, 0.2% glutaraldehyde (GA) in 0.1 mol l^{-1} PB for 8 h at 4°C. After washing in PBS for 1 h, the gills were immersed in 30% sucrose in PBS for 1 h, and embedded in Tissue-Tek OCT compound (Sakura Finetek, Tokyo, Japan) at –20°C. Cryosections (20 μm) were cut on a cryostat (CM 1100, Leica, Wetzlar, Germany) at –20°C, and mounted on MAS-coated slides (Matsunami). The sections were immunocytochemically stained by the ABC method, using commercial reagents (Vectastain ABC kit). In brief, the sections were incubated sequentially with: (1) 0.6% H_2O_2 for 30 min, (2) 2% NGS for 30 min, (3) anti-tilapia AQP3 diluted 1:100 at 4°C overnight (for controls, the pre-immune serum instead of anti-tilapia AQP3), (4) biotinylated anti-rabbit IgG for 1.5 h, (5) ABC for 1.5 h, and (6) 0.02% DAB containing 0.005% H_2O_2 for 7 min. The sections were then post-fixed in 1% osmium tetroxide in 0.1 mol l^{-1} PB for 10 min. After dehydration in ethanol, the sections were embedded in Spurr's resin (Polysciences, Warrington, PA, USA). Ultrathin sections were cut using a diamond knife and mounted on grids. The specimens were observed using a transmission electron microscope (JEOL-1010, JEOL, Tokyo, Japan).

Results

Characterization of AQP3 expressed in the tilapia gills

We identified 1808 bp of full-length tilapia AQP3 cDNA containing 87 bp of a 5′ untranslated region (UTR), 909 bp of an open reading frame, and 812 bp of a 3′ UTR except for its poly(A)$^+$ tail (DDBJ/EMBL/GenBank accession number AB 126941). This cDNA encodes a protein with 303 amino acid residues. Fig. 1A shows alignment of deduced amino acid sequences of AQP3 from Mozambique tilapia, European eel, Japanese dace, *Xenopus* and human. DDBJ/EMBL/GenBank accession numbers for sequences are as follows: European eel (AJ319533), Japanese dace (AB055465), *Xenopus* (AJ131847) and human (NM_004925). Tilapia AQP3 shared high identities with those from European eel (75%), Japanese dace (72%), *Xenopus* (66%) and human (64%), whereas the identities to the other AQPs (AQP0–2, 4–12) were less than 30%.

According to the hydropathy analysis (Fig. 1B), tilapia AQP3 contained six putative transmembrane domains, five connecting loops, and cytoplasmic N- and C-terminal domains, all of which are conserved among AQP molecules. The second and fifth loops contained consensus NPA motifs, which is also characteristic of AQP molecules.

Northern blot analysis showed that the hybridized signal was detected as a single band of approximately 1.8 kb in the gill of FW tilapia (Fig. 2). The size of mRNA was in agreement with that of the cDNA for tilapia AQP3 obtained in this study.

Tissue distribution of tilapia AQP3 mRNA

RT-PCR analysis followed by electrophoresis and staining with ethidium bromide characterized the tissue-specific expression pattern of tilapia AQP3 in fish adapted to FW and SW. A representative result is shown in Fig. 3. The PCR amplification yielded a band of the predicted size (500 bp) in the brain, pituitary, kidney, spleen, intestine, eye, gill and skin of tilapia adapted to both FW and SW. No positive band was detected in the liver. In addition, those PCR products were confirmed to be tilapia AQP3 cDNA fragments by subcloning and sequencing.

Measurement of oocyte water permeability

To determine the characteristics of tilapia AQP3, osmotic volume changes were measured in control *Xenopus* oocytes

A

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Tilapia          1 MGRQKEYLDKLSRFFQIRNLLLRQALAECLGTLILVMFGCGAVAQRVLSGGSHGLFLTVNFAFGFAAMLGILVCGQVSGGHLNPAVTFALCLLGRERWRKFPMYFLFQTIGAFFGSAIIF 120
European eel     1 MGKQKFYLDKLARTFQIRNRLLRQGLAECLGTLILVMFGCGAVAQVVLSAGSHGLFLTVNFAFGFGATLGILVCGQVSGGHLNPTVTFAQCLLGREPWIKFPVYFLFQTLGAFLGSGVIF 120
Japanese dace    1 MGWQKAMLDKLAQTFRIRNKLLRQGLAECLGTLILVMFGCGSVAQMVLSGGSHGRFLTINLAFGFAATLGILVCGQVSGGHLNPAVTFALCLLGRAKWRKFPVYFLFQTIGSFLGAAIIF 120
Xenopus          1 MGSQKDFVNKCNQMLRLRNKLLRQALSECLGTLILVMFGCGSVAQVVLSKGSHGQFLTVNLAFGFAVMLGILISGQVSGGHLNPAVTFALCILAREPWIKFPVYSIAQTLGAFLGAGIVY 120
Human            1 MGRQKELVSRCGEMLHIRYRLLRQALAECLGTLILVMFGCGSVAQVVLSRGTHGGFLTINLAFGFAVTLGILIAGQVSGAHLNPAVTFAMCFLAREPWIKLPIYTLAQTLGAFLGAGIVF 120
                   ** **           *  **** * ************** *** *** * ** *** * ****   ****  ***** **** **** * * *  * * * *   ** * * *

Tilapia        121 GMYYDALLLRPGSFNLTSTNN---TAGIFATYPARHLTLVNGFFDQIIGTTALIVCVLAIVDPFNNPIPQGLEAFTVGFVVLVIGLSMGFNSGYAVNPARDFGPRLFTSMSGWGGAVFTA 237
European eel   121 GLYYDAMWDFG--KNDLIVVGEKATAGIFATYPSNHLTLLNGFFDQLIGTAALIVCILAIVDPYNNPIPRGLEAFTVGFVVLVIGLSMGFNSGYAVNPARDFGPRLFTALAGWGREVFTA 238
Japanese dace  121 AEYHDAMYDFAGETNQLLVSGPKATAGIFATYPNPHLTILNGFFDQVIGTASLIVCVLAIVDPYNNPVPPGLEAFTVGFSILLIGLSMGFNSGYAVNPARDFGPRLFTAIAGWGSEVFTT 240
Xenopus        121 GLYYDAIWFFAN--DQLYVTGENGTAGIFTTFPSDHLTLMNGFFDQFIGTAALIVCVLAIVDPNNNPIPRGLEAFTVGFVVLVIGTSMGFNSGYAVNPARDFGPRLFTSLAGWGTEVFWA 238
Human          121 GLYYDAIWHFAD--NQLFVSGPNGTAGIFATYPSGHLDMINGFFDQFIGTASLIVCVLAIVDPYNNPVPRGLEAFTVGLVVLVIGTSMGFNSGYAVNPARDFGPRLFTALAGWGSAVFTT 238
                     * **                 ***** * *  **   ****** **      * ****** *** * ********   * ** **********************   ***  **

Tilapia        238 RDCWFLVPIFAPFLGSILGVLIYQLMVGFHTEGEARDKKQGTVQENLQLANVASSNNSKEATKEIY 303
European eel   239 HSYWFFVPIFAPFLGTVVGVLVYQLTVGFHVEGEARDKMEATEDERIKLSNVATKDAA-------- 296
Japanese dace  241 RQCWFLVPFFAPFLGSIIGVIVYQLMVGWYLEAEARDKTNKDMEESLKLNDVTGKN---------- 296
Xenopus        239 GNQWWWVPIVSPLLGSFAGVLVYQLMIGCHIEPAP----QSTQQENIKLADVKNKDRL-------- 292
Human          239 GQHWWWVPIVSPLLGSIAGVFVYQLMIGCHLEQPP----PSNEEENVKLAHVKHKEQI-------- 292
                     *  **   * **   **  ***  *   *           *   *
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B

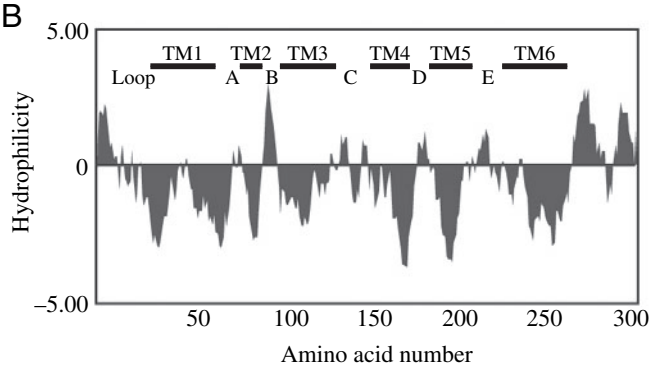

Fig. 1. Sequencing analysis. (A) Alignment of deduced amino acid sequences of AQP3 from Mozambique tilapia, European eel, Japanese dace, *Xenopus* and human. Asterisks and hyphens indicate identical residues and gaps introduced for alignment, respectively. Two NPA motifs are shaded. The antibody was raised against a synthetic peptide corresponding to the underlined sequence. (B) Hydropathy analysis of the deduced amino acid sequence of tilapia AQP3. Characteristic features of AQP are conserved in tilapia AQP3: putative six transmembrane domains (TM1–6) and five connecting loops (A–E). Consensus NPA motifs are present in loops B and E.

and those injected with tilapia AQP3 cRNA. The volume increased more rapidly in oocytes expressing tilapia AQP3 than in controls after transfer to the hypoosmotic solution (Fig. 4A). The Pf values were about 30-fold higher in AQP3 expressing oocytes than in control oocytes (Fig. 4B). The increase in Pf mediated by AQP3 expression was inhibited by pre-treatment with 0.3 mmol l^{-1} $HgCl_2$, and this inhibition was fully reversed by 5 mmol l^{-1} β-mercaptoethanol treatment following the incubation in $HgCl_2$.

Western blot analysis

In Western blot analysis, the antibody raised against the synthetic peptide corresponding to part of the C-terminal region of tilapia AQP3 recognized two protein bands with molecular mass of 26 and 28 kDa (Fig. 5). The 28 kDa band corresponding to the mass predicted from the open reading frame of the cloned cDNA was the major one, while the smaller band of 26 kDa was somewhat faint.

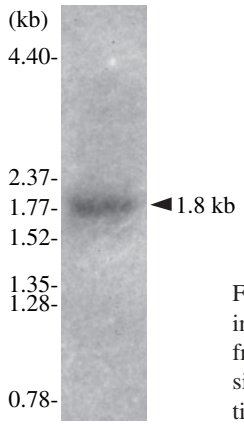

Fig. 2. Northern blot analysis of AQP3 in the gill of tilapia adapted to freshwater. An arrowhead indicates the signal detected with the [^{32}P]-labeled tilapia AQP3 cDNA specific probe.

Cellular and intracellular localization of tilapia AQP3 in the gills

Na^+/K^+-ATPase-immunoreactive chloride cells observed by light-microscopic immunocytochemistry were mostly located in the afferent vascular half of the gill filaments, whereas they were rarely observed in the efferent side of the filaments and on the lamellar epithelia (Fig. 6C,D). Chloride cells were apparently larger and more intensively stained with anti-Na^+/K^+-ATPase in SW-adapted fish than in FW-adapted fish. The intense immunoreaction for AQP3 was detected in chloride cells in both FW- and SW-adapted tilapia (Fig. 6A,B).

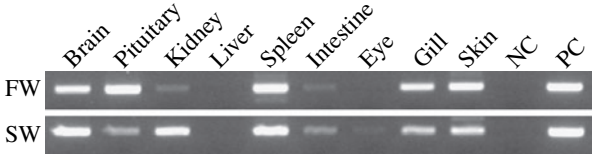

Fig. 3. Tissue distribution of AQP3 mRNA, detected by RT-PCR, in freshwater (FW)- and seawater (SW)-adapted tilapia. Total RNAs were reverse-transcribed and amplified by PCR to produce 500 bp bands. NC, negative control; PC, positive control.

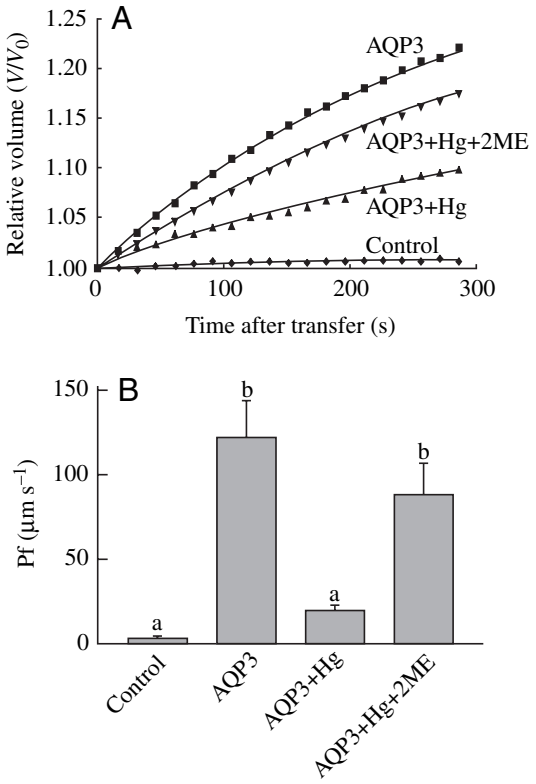

Fig. 4. Functional expression of tilapia AQP3 in *Xenopus* oocytes. (A) Representative data of osmotic oocyte volume *V* changes in control oocytes (control), tilapia AQP3 cRNA-injected oocytes (AQP3), those with 0.3 mmol l^{-1} $HgCl_2$ treatment (AQP3+Hg) and those with $HgCl_2$ treatment followed by 5 mmol l^{-1} β-mercaptoethanol (AQP3+Hg+2ME). (B) Osmotic water permeability (Pf) in different experimental groups. Values are means ± S.E.M. Values marked with different letters are significantly different from one another at $P<0.01$.

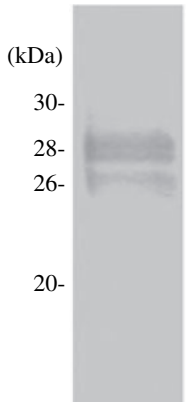

Fig. 5. Western blot analysis with an antibody raised against a synthetic peptide corresponding to part of the C-terminal region of tilapia AQP3. After immunoprecipitation with anti-tilapia AQP3, the gill protein sample was separated by SDS-polyacrylamide gel electrophoresis and transferred to a membrane. Immunoreactive bands were detected by the ABC method. Positions of molecular mass markers (kDa) are shown on the left.

The specific immunoreaction was not observed in the other part of the gills. The immunoreactions for AQP3 and Na^+/K^+-ATPase coincided completely with each other (Fig. 6E,F). There was no significant difference in AQP3 immunoreactivity in chloride cells between FW and SW gills. In the controls, where sections were incubated with the pre-immune serum, the immunoreaction was not detected in either FW or SW fish (Fig. 6G,H).

Electron-microscopic immunocytochemistry further revealed that AQP3 was localized in the basolateral membrane, but not in the apical membrane, of gill chloride cells in FW- and SW-adapted tilapia (Fig. 7A,C). The specificity of the immunoreaction was confirmed by replacement of the specific antibody with the pre-immune serum: the immunoreaction in the basolateral membrane was extinguished, although mitochondria showed some non-specific reaction (Fig. 7B,D).

Discussion

In the present study, we succeeded in cloning a cDNA encoding AQP from tilapia gills. This AQP was identified as an AQP3 homologue because it shows high identity (more than 64%) with AQP3 from other vertebrate species, and is distinct from other AQP isoforms, sharing less than 30% identity. The deduced amino acid sequence shows that tilapia AQP3 shares the characteristic features of AQP molecules: six transmembrane domains, five connective loops, cytoplasmic N- and C-terminal domains, and consensus NPA motifs in the second and fifth loops. These findings suggest that tilapia AQP3 functions as a water channel molecule.

To further evaluate the water-transporting function of tilapia AQP3, the synthesized cRNA encoding tilapia AQP3 was injected into *Xenopus* oocytes to allow its expression, and osmotic swelling was measured in the oocytes following transfer to the hypoosmotic solution. The AQP3-expressed oocytes swelled in the hypoosmotic medium, absorbing water according to the osmotic gradient created between internal and external media, while the swelling was not evident in the control oocytes. The Pf values calculated from the time-course changes in oocyte swelling were about 30 times higher in the cRNA-injected oocytes than in the control. The increase in the Pf value of cRNA-injected oocytes was about 80% inhibited by pre-treatment with Hg^{2+}, a potent inhibitor of AQP that combines 'mercury-sensitive' cysteine residues of AQP to block its aqueous pore (Kuwahara et al., 1997). This Hg^{2+}-induced inhibition was restored by β-mercaptoethanol, the reducing agent that may dissociate Hg^{2+} from AQP. Thus, the inhibition by Hg^{2+} was not due to non-specific toxicity of mercury compounds, but was a reversible phenomenon. These results indicate that AQP3 expressed in the *Xenopus* oocytes and incorporated into the vitellin membrane increased water permeability, providing functional evidence of tilapia AQP3 as a water channel. Because of high homology with mammalian AQP3, tilapia AQP3 is also likely to function as an aquaglyceroporin, although we did not address its glycerol-transporting ability in this study.

In the present study, AQP3 was extensively expressed in various tissues examined, except for the liver, in tilapia adapted to both FW and SW. It should be noted that these AQP3-expressing tissues include major osmoregulatory organs (gills, kidney and intestine), suggestive of its involvement in osmoregulatory processes. In mammalian species, AQP3 is

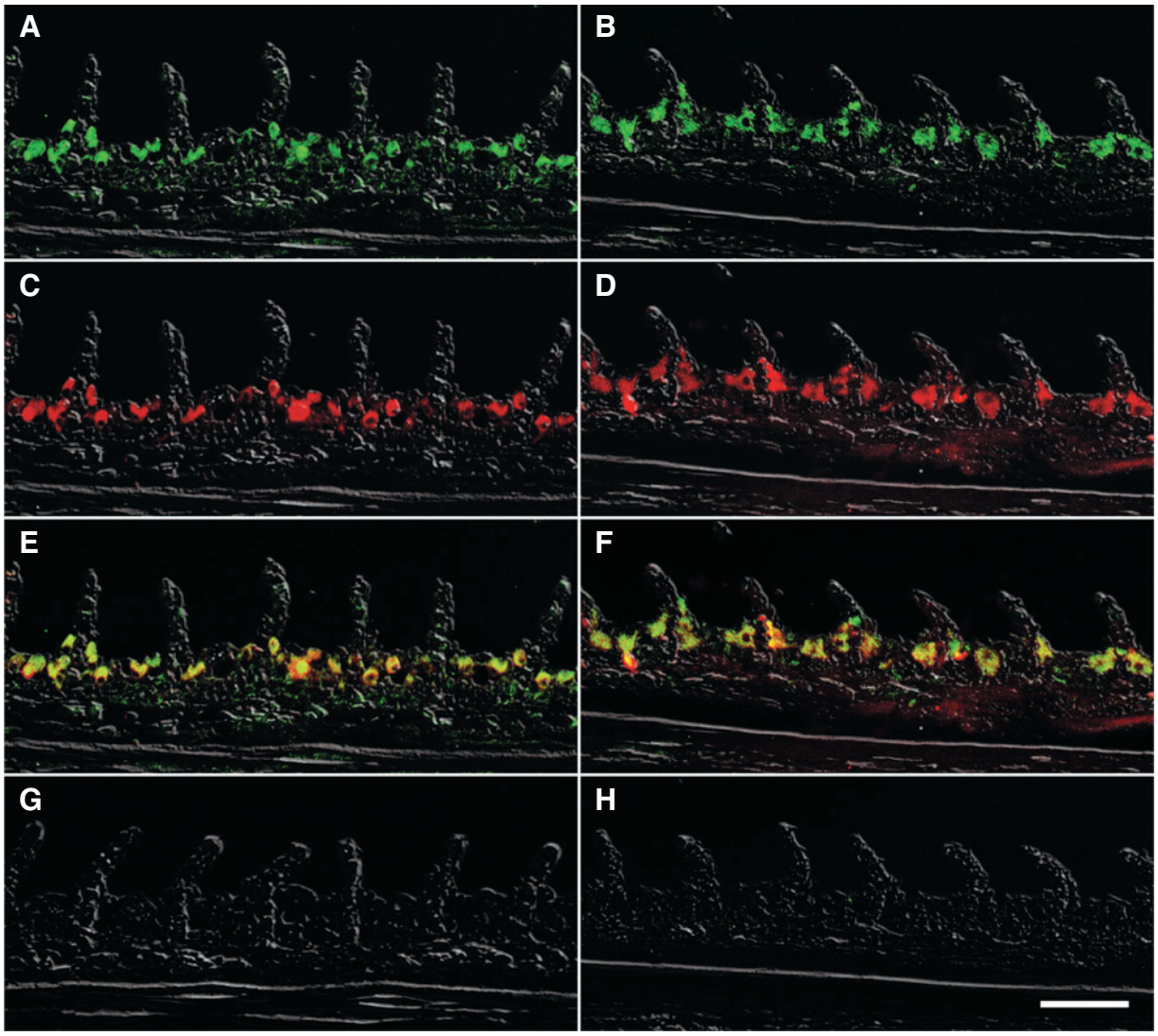

Fig. 6. Double immunofluorescence microscopy of gills in tilapia adapted to freshwater (FW; A,C,E,G) and seawater (SW; B,D,F,H), stained with anti-tilapia AQP3 (A,B) and anti-Na^+/K^+-ATPase as a marker of chloride cells (C,D). E and F are merged images of A,C and B,D, respectively. AQP3-immunoreactive cells are observed in both FW and SW gills (A,B), and immunoreactions for AQP3 and Na^+/K^+-ATPase coincided completely with each other (E,F). No immunoreaction is observed in control sections (G,H). Bar, 50 μm.

ubiquitously expressed in the epithelia of the urinary tract, digestive tract and respiratory tract and epidermis of the skin. It has been suggested that mammalian AQP3 is involved in osmoregulation and osmoprotective systems against dehydration in terrestrial life (Ma et al., 2000; Matsuzaki et al., 1999; Kreda et al., 2001; Hara and Verkman, 2003).

A specific antibody was raised against tilapia AQP3. In western blot analysis, the antibody recognized one major band of approximately 28 kDa and another faint band of 26 kDa. The 28 kDa band corresponds well to the predicted mass of tilapia AQP3, whereas the minor 26 kDa band may represent a degenerated product of the native AQP3. In spite of the unexpected immunoreactive band of smaller molecular mass, the result suggests high specificity of the antibody and its availability for immunocytochemical detection of AQP3.

The Na^+/K^+-ATPase is a key enzyme in the ion-transporting functions of chloride cells in both FW- and SW-acclimated fish (McCormick, 1995), and the antiserum specific for this enzyme serves as a specific marker for their immunocytochemical detection (Ura et al., 1996). As Na^+/K^+-ATPase is located in the tubular system, which is continuous with the basolateral membrane (Karnaky et al., 1976; Hootman and Philpott, 1979; Hirose et al., 2003), the widespread distribution of the tubular system in the cytoplasm results in cell labeling with the nucleus remaining unstained. In the present light-microscopic immunocytochemistry, the AQP3 immunoreaction coincided completely with the Na^+/K^+-ATPase immunoreaction, suggesting the colocalization of both molecules in the basolateral membrane of gill chloride cells. The basolateral localization of AQP3 was further confirmed by the electron-

microscopic immunocytochemistry, in which intensive immunoreactive signals were detected along the membrane of the tubular system. It is notable that the observed immunoreaction for AQP3 in gill chloride cells at light- and electron-microscopic levels showed no clear distributional difference between FW and SW tilapia. This suggests that this molecule is not directly related to water transport for FW and SW adaptation, but is involved in more fundamental mechanisms common to chloride cell functions in FW and SW.

Chloride cells are typically classified into two types on the basis of their ion-transporting functions; that is, FW-type cells that absorb NaCl in fish adapted to hypoosmotic environments, and SW-type cells that excrete excess NaCl in hyperosmotic environments (McCormick, 1995). In Mozambique tilapia, morphological and functional differences between FW and SW types have been well described in chloride cells of the gills (Uchida et al., 2000) and embryonic yolk-sac membrane (Shiraishi et al., 1997; Kaneko et al., 2002). In general, SW-type chloride cells are larger than the FW type and form multicellular complexes together with adjacent accessory cells, whereas FW-type cells exist individually without forming cellular complexes. In the present study, gill chloride cells detected with anti-Na^+/K^+-ATPase were larger in SW-adapted tilapia than in FW fish, indicating that FW- and SW-type cells are the predominant cell types in tilapia adapted to respective environments. Recent studies have shown that those two types of chloride cells with different ion-transporting functions can be transformed from one type to another. According to *in vivo* sequential observations on chloride cells in the yolk-sac membrane of tilapia embryos, single FW-type cells are transformed into multicellular SW-type cells in response to transfer from FW to SW (Hiroi et al., 1999). Similarly, in killifish, SW-type gill chloride cells are transformed into FW-type cells as a short-term response after transfer from SW to FW (Katoh and Kaneko, 2003). These findings indicate plasticity in the ion-transporting functions of chloride cells.

It has also been reported that chloride cells are equipped with an autonomous mechanisms of functional differentiation from the FW to SW type that are independent of endocrine and nerve systems (Shiraishi et al., 2001). Chloride cells in the 'yolk ball', a yolk-sac preparation separated from the body of FW-adapted tilapia embryos, have been shown to form SW-type multicellular complexes after SW transfer. Considering the functional plasticity of chloride cells, it is most probable that, in response to transfer from FW to SW, FW-type cells detect changes in the environmental salinity in a direct or indirect manner, and this triggers transformation into the SW type. One possible way is to detect a fluctuation in the external salinity *via* the apical membrane of chloride cells facing the external medium. It is more likely, however, that increased environmental salinity results in a slight increase in internal osmolality, which could be detected by the basolateral membrane of chloride cells. In fact, changes in osmolality on the basolateral side have been shown to affect the rate of Cl^- secretion by opercular epithelia of killifish (Zadunaisky et al., 1995; Marshall et al., 2000).

Based on our observations that AQP3 is intensively located in the basolateral membrane of chloride cells, we propose a hypothesis that basolateral AQP3 is involved in osmoreception by chloride cells. It is expected that the AQP3-rich basolateral membrane is more permeable to water than the other membranes. This is supported by our finding that AQP3-expressed *Xenopus* oocytes exhibited higher water permeability than control oocytes, resulting in oocyte swelling in the hypo-osmotic medium. Since the surface area of the basolateral membrane is enlarged because of intensive

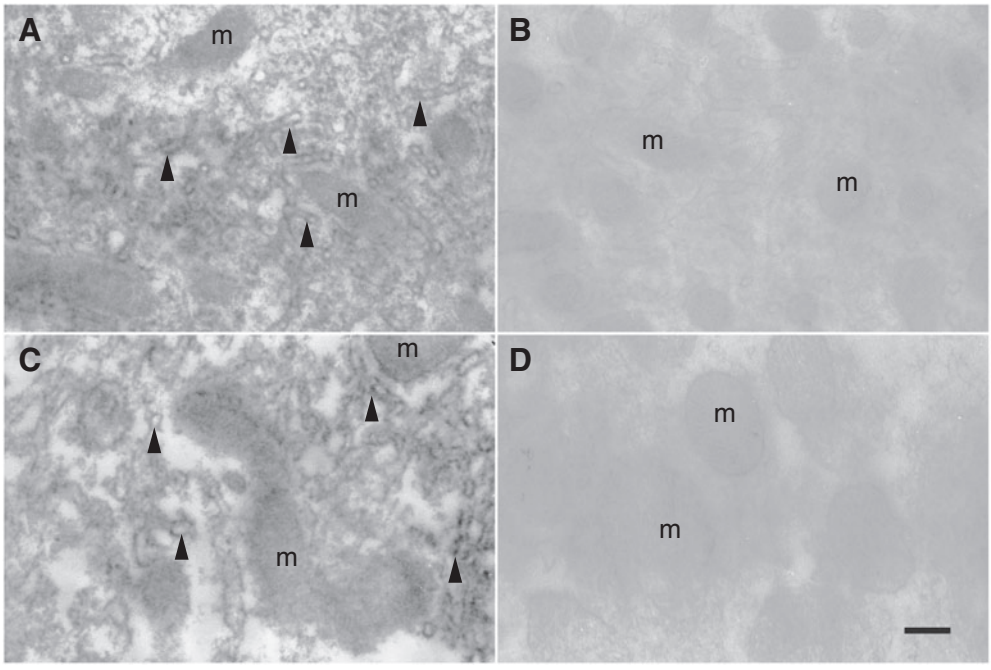

Fig. 7. Electron-microscopic immunocytochemistry of gill chloride cells in tilapia adapted to freshwater (FW; A,B) and seawater (SW; C,D), incubated with anti-tilapia AQP3 (A,C) and with pre-immune serum as controls (B,D). Intense immunoreaction for AQP3 is observed along the membrane of the tubular system (arrowheads) continuous with the basolateral membrane in chloride cells of FW- and SW-adapted tilapia (A,C), but not in controls (B,D). m, mitochondrion. Bar, 200 nm.

infoldings of the tubular system, the ratio of the surface area to the cell volume is thought to be much higher in chloride cells than in any other cell type in the gills. Such structural characteristics may further enhance the possible osmosensitivity of chloride cells. Transfer of fish from SW to FW, for example, may induce a slight decrease in blood osmolality, which creates an osmotic gradient between the intracellular fluid and blood. Subsequently, water moves into chloride cells through basolateral AQP3 according to the osmotic gradient, resulting in cell swelling. Conversely, transfer from FW to SW may lead to shrinkage of chloride cells. In mammalian renal cells, incubation in a hyperosmotic medium decreases cell volume, which leads to alterations in intracellular ion concentrations and an increase in Na^+/K^+-ATPase activity (Bowen, 1992; Yordy and Bowen, 1993).

It is likely that cell volume changes, presumably facilitated by AQP molecules located in the plasma membrane, may also occur in the other osmoregulatory organs, the kidney and intestine, in which the gene expression of AQP3 was confirmed in the present study. In Mozambique tilapia and Japanese eel, it has been reported that prolactin (PRL)-producing cells in the pituitary are sensitive to changes in osmotic pressure of the extracellular fluid. The PRL secretion from organ-cultured pituitaries increased when the medium osmolality was slightly reduced (Grau et al., 1981; Suzuki et al., 1991), and this osmosensitive PRL release was linked with volume changes in PRL cells (Weber et al., 2004). This process might also be mediated by AQP3, since we detected substantial expression of AQP3 in the tilapia pituitary.

List of abbreviations

ABC	avidin–biotin–peroxidase complex
AQP	aquaporin
ANOVA	analysis of variance
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
cRNA	complementary RNA
DAB	3,3′-diaminobenzidine tetrahydrochloride
FW	freshwater
GA	glutaraldehyde
KLH	keyhole limpet hemocyanin
NGS	normal goat serum
Pf	water permeability
PFA	paraformaldehyde
PLSD	protected least significant difference
PRL	prolactin
RACE	rapid amplification of cDNA ends
RT-PCR	reverse transcriptase-polymerase chain reaction
S	initial surface area
SW	seawater
UTR	untranslated region
V_0	initial volume
Vw	molar volume of water

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