Molecular characterization of water-selective AQP (EbAQP4) in hagfish: insight into ancestral origin of AQP4

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1Department of Structural Pathology, Institute of Nephrology, Graduate School of Medical and Dental Sciences, Niigata University, Niigata; 2Japan Tobacco, Inc., Biohistory Research Hall, Osaka; 3Knowledge, Action, Network Research Institute, Kyoto; and 4Department of Pediatrics, School of Medicine, Fukuoka University, Fukuoka, Japan

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Nishimoto G, Sasaki G, Yaoita E, Nameta M, Li H, Furuse K, Fujinaka H, Yoshida Y, Mitsudome A, Yamamoto T. Molecular characterization of water-selective AQP (EbAQP4) in hagfish: insight into ancestral origin of AQP4. Am J Physiol Regul Integr Comp Physiol 292: R644–R651, 2007. First published September 21, 2006; doi:10.1152/ajpregu.00362.2006.—Hagfish (Eptatretus burgeri) are agnathous and are the earliest vertebrates still in existence. Pavement cells adjacent to the mitochondria-rich cells show orthogonal arrays of particles (OAPs) in the gill of hagfish, a known ultrastructural morphology of aquaporin (AQP) in mammalian freeze-replica studies, suggesting that an AQP homolog exists in pavement cells. We therefore cloned water channels from hagfish gill and examined their molecular characteristics. The cloned AQP [E. burgeri AQP4 (EbAQP4)] encodes 288 amino acids, including two NPA motifs and six transmembrane regions. The deduced amino acid sequence of EbAQP4 showed high homology to mammalian and avian AQP4 (rat, 44%; quail, 43%) and clustered with AQP4 subsets by the molecular phylogenetic tree. The osmotic water permeability of Xenopus oocytes injected with EbAQP4 cRNA increased eightfold compared with water-injected controls and was not reversibly inhibited by 0.3 mM HgCl2. EbAQP4 mRNA expression in the gill was demonstrated by the RNase protection assay; antibody raised against the COOH terminus of EbAQP4 also detected (by Western blot analysis) a major ~31-kDa band in the gill. Immunohistochemistry and immunoelectron microscopy showed EbAQP4 localized along the basolateral membranes of gill pavement cells. In freeze-replica studies, OAPs were detected on the protoplasmic face of the split membrane comprising particles 5–6 nm long on the basolateral side of the pavement cells. These observations suggest that EbAQP4 is an ancestral water channel of mammalian AQP4 and plays a role in basolateral water transport in the gill pavement cells.

mitochondria-rich cells; orthogonal arrays; pavement cell

VEGETATION LIVES IN VARIOUS habitats ranging from fresh water to sea water to dry land. The mechanisms regulating vertebrate body-fluid balance differ in diverse habitats (38), but vertebrates usually maintain a constant ionic composition and osmotic equilibrium regardless of changes in their environments (35). An exception to this constancy is seen in marine hagfish (Eptatretus burgeri), ancestral vertebrate agnathans that diverged from the main vertebrate lineage over 500 million years ago. These eel-shaped fish are the oldest living marine craniates, and they differ physiologically from other vertebrates. Hagfish are called osmoconformers because they maintain an osmotic equilibrium with the surrounding sea water, mainly by retaining extracellular concentrations of NaCl in their body fluids nearly isoosmotic to the sea level, indicating that they have neither a passive water net influx, like freshwater teleosts do, nor a net efflux, such as marine teleosts have (1, 26).

In general, the gills and kidneys play important roles in the osmotic and ionic regulation of the body fluids of aquatic vertebrates. Hagfish kidneys have a segmental arrangement of 30–40 glomeruli and two ureters, so-called archinephric ducts, that drain into the cloaca (20). Several reports demonstrate that hagfish kidneys do not concentrate urine or reabsorb NaCl (13, 31, 33); therefore, the kidneys are not major effector organs for maintenance of the NaCl concentration. On the other hand, the gills are thought to be responsible for the NaCl concentration in the plasma since Na pumps are abundantly expressed in the mitochondria-rich (MR) cells for the uptake of Na+ from the marine environment (11).

Recently, the aquaporin (AQP) water channel has been identified, and its physiological relevance to fluid homeostasis has been demonstrated (7, 29, 43). Among AQPs, AQP4 has the most potential for high water permeability, and it has orthogonal arrays of particles (OAPs) in the lipid bilayer (15, 44). OAPs are plasma membrane specializations visualized by means of freeze-fracture electron microscopy. Several years prior to the molecular identification of AQPs, OAPs were hypothesized as representing a morphological signature of water-transporting units. The localization of OAPs has been described in several cell types, including the basolateral plasma membranes of the kidney collecting duct principal cells (21), gastric parietal cells (6), astroglial cells (19), and skeletal muscle fibers (28), and lens of eye (40) in mammals. The localization of these OAPs is concordant with that of AQP4 in these tissues (43) except that of AQP0 in the lens of eye, suggesting that the OAPs mostly consist of AQP4. Furthermore, OAPs were demonstrated to consist of AQP4 by the disappearance of OAPs in AQP4-knockout mice (41).

Bartels (2) found OAPs in the basolateral plasma membrane of pavement cells in hagfish gills. Hence, we hypothesized that the OAPs are constructed by an AQP4 homolog even in very primitive ancestral vertebrates. Although OAPs have been observed in several vertebrates in freeze-fracture studies (5), the molecule(s) forming OAPs have not been fully elucidated, and little is known about the relationship between AQPs and OAPs in vertebrates. The present report demonstrates that the AQP homolog EbAQP4 has high water permeability and is of

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relevance for gill and pavement cell-specific basolateral membrane water transport in hagfish. The clustering into the AQP4 subsets by phylogenetic tree analysis, the evolutionarily preserved intramolecular OAPs signature by sequence alignments and appearance of OAPs, functional mercury-insensitive water permeability, and basolateral membrane localization with regard to EbAQP4 have provided insight that hagfish EbAQP4 may be the prototype of the mammalian AQP4 in the vertebrate lineage.

MATERIALS AND METHODS

Animals. Hagfish (E. burgeri) were obtained from Misaki Marine Biological Station, University of Tokyo. They were maintained unfed in artificial aerated sea water (~1,050 mOsm) at 10°C for up to 3 mo. The hagfish were then anesthetized in MS-222 (Sigma) and killed by decapitation; the relevant tissues were immediately dissected out with reference to Romer’s atlas of hagfish anatomy (32). The procedures for this present study were approved by the Animal Committee at Niigata University School of Medicine, and all animals were treated according to the guidelines for animal experimentation of Niigata University.

RT-PCR. Total RNA was extracted from the tissues using Isogen reagent (Nippon Gene). For RT-PCR, we made degenerate primers corresponding to the conserved NPA motifs in the mammalian AQP0-AQP5 water channels family: sense strand, 5′-CA(C/T)AC(A/T)TIT-A(A/C/T)CCICG(A/G/C/T)G/T-3′; anti-sense strand, 5′-CC(A/G/C/T)TA(A/C/T)CCA(A/G)TA(A/G/C/T)A/C/TCCA-3′. One microgram of total RNA was reverse-transcribed by SuperScript II reverse transcriptase (Invitrogen) at 42°C for 60 min and then heated at 72°C for 10 min. The synthesized cDNA was used for subsequent PCR with the degenerate primers as follows: 94°C for 30 s, 45°C for 30 s, 72°C for 2 min, 35 cycles. After ethanol precipitation, the PCR products were ligated into the T-easy vector (Promega) and then sequenced. Both 5′ and 3′ RACE-PCR were performed using total RNA with the gene-specific primers.

Phylogenetic tree inference. Multiple alignment of amino acid sequences was carried out by XCED (23). Phylogenetic trees were inferred by the neighbor-joining method using the distance matrix estimated by the maximum likelihood method based on the Jones-Taylor-Thornton model (34). Heterogeneity of evolutionary rates among sites was included by a discrete gamma distribution with an estimated shape parameter of 1.25 (45). Amino acid sites where gaps exist in the alignment were excluded from the calculation. The bootstrap analysis was carried out by the methods of Felsenstein (14).

Ribonuclease protection assay. 32P-labeled anti-sense cRNA probes for EbAQP4 mRNA were synthesized by in vitro transcription by using linearized plasmids with EbAQP4 cDNA inserted as described (43). Total RNA samples (10 μg) were hybridized with the mixture of cRNA probes (1×106 cpm each) overnight at 45°C, and nonhybridized probes were digested with ribonuclease A and ribonuclease T1. The samples were electrophoresed on 6% polyacrylamide slab gels containing SDS. The separated bands were blotted onto nitrocellulose membranes. The membranes were incubated with 5% milk in PBS-T (80 mM Na2HPO4, 20 mM NaH2PO4, 100 mM NaCl, 0.1% Tween-20, pH 7.4) for 1 h and then incubated with affinity-purified rabbit anti-EbAQP4 antibodies (dilution 1:200) at 4°C overnight. The membranes were washed three times for 30 min each in PBS-T and incubated with horseradish peroxidase-labeled second antibody (1:1,000 EnVision; DAKO) at room temperature for 60 min. The immunoreactivity was visualized by a chemiluminescence detection system (ECL Plus; Amersham Pharmacia Biotech). The visualized membranes were stained by Coomassie blue to confirm equal loading of the samples.
Immunohistochemistry. Tissues were fixed in methyl-Carnoy fixative, dehydrated in ethanol, and embedded in paraffin. They were sectioned at a thickness of 4 μm, and the sections were dewaxed and rehydrated. They were incubated with rabbit anti-EbAQP4 antibodies (dilution 1:20) for 60 min and then reacted with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:5 EnVision; Amersham Pharmacia Biotech) for 2 h, gently washed in PBS several times, postfixed with glutaraldehyde, counterstained with 2% aqueous uranyl acetate and lead citrate for 5 min, and then examined using an electron microscope (Hitachi).

Immunogold electron microscopy. Ultrathin sections of glycol methacrylate-embedded tissues were collected on nickel grid meshes. They were then incubated with 5% nonfat milk for 1 h and subsequently with affinity-purified anti-EbAQP4 antibody (dilution 1:20) for 16 h. After several washes with PBS, the sections were incubated with gold (10 nm)-labeled anti-rabbit IgG (1:20 AuroProbe EM; Amersham Pharmacia Biotech) for 2 h, gently washed in PBS several times, postfixed with glutaraldehyde, counterstained with 2% aqueous uranyl acetate and lead citrate for 5 min, and then examined using an electron microscope (Hitachi).

Expression in Xenopus oocytes. EbAQP4 cDNA was digested by BamHI and EcoRI and ligated into the high-level expression vector pCS2, which contains the 5'-untranslated region of the Xenopus β-globin cDNA (a gift from Dr. M. Maeno) (37). Capped cRNA was synthesized in vitro by SP6 RNA polymerase after linearization with NotI as described previously (24). As a positive control, capped cRNA was synthesized in vitro from rat AQP4 cDNA ligated into the pXG-ev vector, also containing the 5'-untranslated region of the Xenopus β-globin cDNA, by T3 RNA polymerase after linearization with NotI (a gift from Drs. M. Yasui and P. Agre) (22). Xenopus laevis oocytes (stage V-VI) were defolliculated with collagenase (Yakulto) and microinjected with 10 ng in 50 nl of the synthesized cRNA or 50 nl of water as a control. After incubation in 200 mOsm, modified Barth’s buffer at 22°C for 3 days, the oocytes were transferred to 70 mOsm modified Barth’s buffer at 22°C, and their swelling was monitored by video microscopy. The coefficient of osmotic water permeability (Pf) was assessed by a real-time quantitative imaging method.

Fig. 2. The hydrophobicity profile of EbAQP4 calculated by the Kyte-Doolittle method. A: six putative transmembrane domains are indicated by I–VI. B: three-dimensional structure of EbAQP4 by GTOP homology modeling using rat AQP4 (2d57A) in Protein Data Bank databases. Six helices and two small helices are visualized by MOLMOL. Residues Gly 143, Trp 217, and Leu 225 are symbolized by tetrahedrons; residues His 96 and Tyr 236 are symbolized by half cones.

Fig. 3. Comparison of the primary sequences of EbAQP4 with those of hAQP4, rAQP4, qAQP4, and hAQP1. A serine preceding the 2nd NPA known as a mercury-insensitive site is boxed. + Known feature of the sequence for hydrophobic residues Gly 143, Trp 217, Leu 225 is boxed. Specific residues His 96 and Tyr 236, important for the interface between neighboring AQP4 tetramers. h = Human, r = rat, q = quail.
Freeze-fracture electron microscopy. Samples were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 24 h at 4°C; they were then washed with cacodylate buffer, immersed in 30% glycerol in cacodylate buffer for 2 h, and then frozen in liquid nitrogen. Frozen samples were fractured at −110°C and platinum-shadowed unidirectionally at an angle of 45° in Balzers Freeze Etching System (BAF060; BAL-TEC). Samples were then immersed in household bleach, and the replicas floating off the samples were picked up on formvar-filmed grids and examined with a Hitachi H-7500 electron microscope at an acceleration voltage of 100 kV (16).

RESULTS

Nucleotide sequence of the clone and structure of the deduced protein. A PCR product with the expected length (~460 bp) was obtained by RT-PCR. The isolated clone was regarded as a member of the AQP family since the deduced protein contained the two NPA motifs preserved in other members of the AQP family. The 5' and 3' RACE PCR were used to obtain the entire sequence of the clone, including the start and stop codons (GenBank accession no. AB258403). The coding region of the clone consisted of 864 nucleotides, resulting in a 288-amino acid sequence with a molecular mass of ~31 kDa. (Fig. 1).

A computer analysis of the deduced protein obtained using the algorithm of Kyte and Doolittle (24a) demonstrated six hydrophobic regions that highly correspond to the bilayer-spanning domains (Fig. 2A). The three-dimensional (3-D) structure prediction of the deduced protein showed six helices and two small helices with 49% similarity to rat AQP4 (Protein Data Bank entry name: 2d57A) by gene-to-protein (GTOP) homology modeling (Fig. 2B).

The presumed amino acid sequence was relatively highly homologous to human AQP4 (43%), rat AQP4 (44%), and quail AQP4 (43%). The hydrophilic residues Gly 143, Trp 217, and Leu 225, responsible for AQP tetramers, were conserved (Fig. 3). Moreover, His 96 and Tyr 236, critical amino acids for the cytoplasmic interface between neighboring AQP4 tetramers in mammals were also found (Fig. 3) as visualized in Fig. 2B. To establish a possible evolutionary trace of the clone, a phylogenetic tree was inferred by the neighbor-joining method. A sequence comparison with other members of the AQP family showed that the clone was clustered into the AQP4 subset (Fig. 4). Hence, this clone was designated as E. burgeri AQP4 (EbAQP4).

Expression of EbAQP4 and immunolocalization. Tissue expression of EbAQP4 was examined by the ribonuclease protection assay using total RNA isolated from various organs of hagfish. Strong expression was detected in the hagfish gill (Fig. 5), whereas no detectable signal was demonstrated in other hagfish tissues.
Membrane fractions prepared from hagfish gills were analyzed by Western blot with the EbAQP4 antibody. The antibody recognized a major protein band with an approximate size of ~31 kDa in the hagfish gill (Fig. 6). The immunoreactive band was not identified in other hagfish tissues, however.

In the gill, immunoreactivity for EbAQP4 was selectively observed in the gill epithelia (Fig. 7A). At a higher magnification, two types of cells were distinguished in the epithelium: pavement cells and MR cells. Immunoreactivity was observed in the basolateral membrane of pavement cells, which are typically small and resemble squamous cells. No immunolabeling was demonstrated in the adjacent MR cells, which are spherical, relatively larger than pavement cells, and singly intercalated between the pavement cells (Fig. 7B). No immunostaining was demonstrated in the gill using the preimmune serum (Fig. 7C).

Ultrastructural observations revealed the localization of EbAQP4 on the basolateral membrane of pavement cells (Fig. 8). Immunogold staining for EbAQP4, however, was not detected in other cell types of the gill epithelia, such as MR cells.

Water permeability of EbAQP4. The osmotic water permeability coefficient (Pf) of Xenopus oocytes injected with EbAQP4 cRNA was eight times higher than that of water.

Fig. 7. A: immunolocalization of EbAQP4 in gill of hagfish. EbAQP4 immunoreactive cells are observed in the luminal side of the gill. B: at a high magnification, the lateral membrane of pavement cells (arrows) is labeled, whereas adjacent mitochondria-rich cells (MR) are not stained. C: no staining is observed with preimmune serum. Scale bars: 100 μm in both A and C, 10 μm in B.

Fig. 8. Immunoelectron micrographs of a pavement cell (PC) and a MR cell stained with anti-EbAQP4 antibody in the gill of hagfish. Immunogold particles are found on the boundary of basolateral membrane (arrows) between a pavement cell and a MR cell (A and B). The arrow head points to an occluding junction between a pavement cell and a MR cell. m = mitochondria. Scale bars: 1 μm in both A and B.
injected control oocytes (Fig. 9). The water permeability of *Xenopus* oocytes injected with rat AQP4 cRNA was 13 times higher than that of water-injected oocytes. The Pf values of oocytes injected with either EbAQP4 or rat AQP4 cRNA were not affected by treatment with 0.3 mM HgCl₂.

*Freeze-fracture replica study.* To observe the OAPs in the gill, we analyzed the split membrane by freeze-fracture study. We identified these cells by the occluding junctions, which are a known feature of the junction located between pavement cells and MR cells (Fig. 10A). OAPs were found exclusively on the basolateral plasma membrane of the pavement cells. On the protoplasmic-face images (protoplasmic leaflets) of the split membrane, the arrays were composed of 5- to 6-nm particles (Fig. 10B). The number of particles forming an array ranged from 4 to 12. Complementary arrays of pits were difficult to identify on the extraplasmic leaflets (E-faces) because of their small size. The density of the arrays of particles varied considerably between individual pavement cells.

**DISCUSSION**

The kidneys and gills are important organs for osmoregulation in marine teleosts. The kidney is not a critical organ for the NaCl regulation in hagfish because the ratios between the plasma and the urine concentration for Na⁺ and Cl⁻ are close to one, indicating that there is no net active Na⁺ and Cl⁻ reabsorption or secretion in the kidney (1). EbAQP4 is exclusively expressed in the gill, as shown by the ribonuclease protection assay, supporting the idea that an osmotic gradient facilitating water transport exists in the gill.

The hagfish gill epithelium consists of four cell types. The epithelial surface consists of pavement cells and MR cells, whereas the deep layers of the epithelium contain basal cells and intermediate cells. The mucus-secreting pavement cells cover most of the gill surface and are mainly present in the multilayered columnar epithelium of the lateral part of the gill, sending thin cytoplasmic processes toward the basal lamina (4). MR cells are characterized by the presence of carbonic anhydrase at the apical plasma membrane and Na⁺-K⁺-ATPase at the basolateral membrane. Interestingly, there are some unique similarities between mammalian kidney collecting duct principal cells and hagfish gill pavement cells with regard to the presence of OAPs. In addition, these cells have peculiar adjacent neighbors: intercalated cells in the mammalian kidney and MR cells in the gill, respectively. OAPs have been demonstrated in both the principal cells in the mammalian kidney and the pavement cells in the gill, and AQP4 has been localized in the basolateral membrane of the principal cells. In contrast, OAPs are absent in both the intercalated cells in the mammalian kidney and the MR cells in the gill, and those cells have an H⁺-secreting property characterized by rod-shaped particles in the apical membrane (4, 8, 9). Moreover, intercalated cells and MR cells are always separated from one another at the epithelial surface by principal cells and pavement cells, respectively. Although the extracellular acid-base composition and/or osmotic environments of mammals and hagfish are extremely different, this cellular organization that they have in common might be importantly preserved in regard to acid-base balance and water homeostasis in vertebrate evolution.

The phylogenetic tree constructed by analysis of comparative sequences in water-selective AQPs shows that EbAQP4 is clustered in the AQP4 subset, suggesting that EbAQP4 is an orthologous gene of AQP4. The primary amino acids sequence of EbAQP4 has 44% similarity to that of rat AQP4 (22);
however, the 3-D structure similarity between EbAQP4 and rat AQP4, as shown by GTOP homology modeling, is 49%, much higher than the primary amino acid sequence similarity. This is a good indication that the 3-D structure similarity is more often conserved than the primary amino acid sequence similarity. In addition, the unique amino acid feature and placement with regard to stabilization of OAPs was preserved in both rats and hagfish (47). Furthermore, immunostaining of EbAQP4 in the basolateral membrane of the pavement cells is in agreement with the finding that OAPs are localized in the basolateral membrane of pavement cells (2). Hence, it is highly probable that EbAQP4 forms OAPs in the pavement cells.

OAPs are present in the plasma membranes of numerous cell types in various animals, and AQP4 is likely to be the main OAPs forming AQP in nonocular tissues (39). The number and size of OAPs in cell membranes have been found to change under various conditions (5, 36): acute dehydration increases the number of OAPs in the principal cells of the kidney (27), and histamine treatment causes the rearrangement of OAPs in gastric cells (10). These results suggest that the formation of OAPs in association with AQP4 expression is regulated physiologically. AQP4 is localized in the basolateral membrane of principal cells of the renal collecting ducts (7, 29, 43). AQP2 is also expressed in the principal cells of renal collecting ducts at the apical membrane and is regulated by two processes: 1) short-term redistribution of AQP2 to the apical plasma membrane from endosomal vesicles, increasing water permeability via protein kinase A phosphorylation of serine 256 (17, 30), and 2) long-term biosynthesis via CREB phosphorylation (25, 46). AQP4, in contrast, is regulated via protein kinase C phosphorylation (18, 48). Reversible phosphorylation is one of the most common ways by which the function of proteins can be physiologically regulated (12). In addition, the activity of many channels has also been shown to be regulated in this manner (42). Since EbAQP4 contains two potential protein kinase C sites, phosphorylation of EbAQP4 might be associated with the Pk of EbAQP4, either through direct gating or in association with conformational changes in OAPs evoked by other multimeric protein interactions.

Functional roles of AQP4 are of particular interest. One of the characteristic features of the pavement cells are mucus synthesis and secretion (4), but not of MR cells. This finding was investigated by ultrastructural study. For this reason, a possible role of EbAQP4 in the pavement cells may be mucus secretion as roles of AQP3 and/or AQP4 have been shown in some acinar cells in mammalian secretory glands (7). In this regard, EbAQP4 may permit water entry through the base.

Other intriguing role for AQP4 is relation with cell adhesion (47). It has been suggested (15) that small OAPs (2–12 tightly packed square particles), constructed by a low AQP4M1/AQP4M23 expression ratio, provide weak adhesion between rough junctions of the mitochondria-rich cell of amphibian epidermis. In this manner, EbAQP4 may permit water entry through the base. It is possible because a residue of Arg9 in the AQP4M1 NH2 terminus interferes with the organization of OAPs constructed by coherence between the neighboring AQP4 tetramers (47). Since EbAQP4 also contains Arg7 in the NH2 terminus, the formation of OAPs by EbAQP4 may be interfered with by this residue, resulting in a small assembly of OAPs. Some pavement cells adjoin each other and between-pavement cells are tightly connected at occluding junctions that have high trans-epithelial resistance (3). It may be acceptable to presume that EBAAQP4 in the junctions are involved in regard to cell-cell contact across the adjoining membranes (47). Further studies are necessary to elucidate between the molecular characteristics and physiological roles with regard to evolutionarily conserved AQP4 orthologs.

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