ELSEVIER

Biochimica et Biophysica Acta 1463 (2000) 374–382

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

Molecular cloning and expression of aquapolin 1 (AQP1) in dog kidney and erythroblasts

Kazunari Higa [a], Hideharu Ochiai [a,b], Hiroshi Fujise [a,b,*]

[a] *Laboratory of Pathobiochemistry, School of Veterinary Medicine, Azabu University, Fuchinobe, Sagamihara, Kanagawa 229-8501, Japan*
[b] *High-Tech Research Center, Institute of Biosciences, Azabu University, Fuchinobe, Sagamihara, Kanagawa 229-8501, Japan*

Received 14 June 1999; received in revised form 13 September 1999; accepted 19 October 1999

Abstract

Complementary DNA of the water channel aquapolin 1 (AQP1) was cloned from dog kidney and erythroblasts. The cDNA amplified from mRNA in dog kidney was 816 bp, the same as that in bovines, but longer by 6 bp than that in humans, mice and rats. The 235-bp fragment cDNA amplified from the mRNA in dog erythroblasts, which was differentiated from peripheral blood, was completely identical to the corresponding sequence of cDNA from the dog kidney. Thus, mature red blood cells from dog may have AQP1 in their cell membranes. The amino acid sequence in dog AQP1 was 91–94% identical to that in the other species mentioned above. Dog AQP1 has six predicted transmembrane domains, two NPA motifs, one mercury-sensitive site and four consensus phosphorylation sites, the same as the other species. However, dog and bovine AQP1 have only one *N*-glycosylation site, while two glycosylation sites were found in human and rodent AQP1. *Xenopus* oocytes injected with the mRNA of the dog AQP1 exhibited high water permeability in a hyposmotic medium. Thus, dog AQP1 performs water transport the same as in the other species.

Keywords: Dog; Kidney; Erythroblast; Aquapolin; Water permeability; *Xenopus* oocyte

1. Introduction

The permeation of water across biological membranes is mediated by several water channels [1–5]. A 28-kDa protein has been isolated and defined as a water channel in red blood cells. The cDNA sequence for this water channel was then determined in human bone marrow [5], and named aquapolin 1 (AQP1) [6]. The gene product could mediate water permeation across the biological membrane when it was expressed in *Xenopus* oocytes [5]. Eight AQP and several other subgroups named aquaglyceroporins have been found to date, and together they constitute one large gene family [7]. AQP1 is present in several tissues such as kidney, lung, lactating mammary and eye, as well as in red blood cells [8].

AQP1 and the other members of the AQP family have been investigated in detail in renal tubules by several investigators [9–13]. AQP1 was found in the apical and basolateral membranes of proximal tubules and descending thin limbs [10,12] where much water reabsorption occurs. The urine of transgenic mice lacking AQP1 did not become concentrated, and the blood osmolarity increased when the water supply was restricted [14]. Thus, AQP1 was responsible for the reabsorption of water in renal tubules necessary to maintain appropriate blood osmolarity.

* Corresponding author. Fax: +81-42-769-1629;
E-mail: fujise@azabu-u.ac.jp

0005-2736/00/$ – see front matter
PII: S0005-2736(99)00218-7

AQP1 probably has a role in water permeation in other tissues where it is present.

As mentioned above, AQP1 exists in red blood cells, and it transports water when expressed in *Xenopus* oocytes. However, it is not known how and when the water channel acts in red blood cells. In dog red blood cells, there is dimorphism for ion transport and regulatory volume decrease (RVD) which are mediated by water transport; one is normal low potassium (LK) and the other is variable high potassium (HK) due to the existence of the Na,K-pump in the cell membrane [15,16]. The RVD in LK cells was mediated by a Na/Ca exchanger and in HK cells by a K-Cl cotransporter [15]. In each case, water permeation mediated by AQP1 may cooperate with each transporter for RVD. However, there is no evidence to explain the correlation between AQP1 and these ion transporters. Therefore, it is necessary to examine AQP1 in dog red blood cells to clarify the mechanism and the role of AQP1 during the volume regulation. To prepare a test model for AQP1, we cloned the cDNA of AQP1 from dog kidney and red blood cell progenitors, and expressed it in *Xenopus* oocytes.

2. Materials and methods

2.1. Animals

The kidney for RNA sampling was obtained from a normal LK dog of the Japanese Shiba breed [17]. The peripheral blood for the preparation of the red blood cell progenitor was also obtained from the Japanese Shiba dog. All experiments were performed in accordance with the guidelines of The Laboratory Animal Care Committee of Azabu University and The Japanese Animal Welfare Guide.

2.2. Progenitors of red blood cells

The progenitors of red blood cells, mostly erythroblasts, were prepared basically according to the method of Wada et al. [18]. Peripheral blood was obtained from the dog, and the mononuclear cells were separated by centrifugation with Ficoll-Conray (Pharmacia). The cells were first incubated in Iscove's modified Dulbecco's medium (IMDM; Gibco-BRL) with 10% FCS and phytohemagglutinin-stimulated leukocyte conditioned medium for 7 days (first phase). After removal of phagocytic cells by the carbonyl iron method [19], the cells were again incubated in IMDM medium with 30% FCS, 1% deionized bovine serum albumin (Sigma), 10 mM 2-ME, 300 μg/ml iron-saturated transferrin (Sigma, MO) and 2 U/ml recombinant erythropoietin (Snow Brand Milk Product, Japan) (second phase). On day 3 of the second phase, the differentiated cells were harvested. The cells were examined with benzidine and determined to be erythroid progenitors, mostly erythroblasts.

2.3. RNA isolation and RT-PCR

Total RNA was extracted from both kidney cells and the erythroblasts, according to the acid guanidinium thiocyanate–phenol–chloroform method with a kit reagent (Isogen, Wako). The resulting RNA was dried and resuspended in diethylpyrocarbonate-treated water. The mRNA was then purified using an oligo-dT cellulose column. First-strand cDNA was synthesized using avian myoblastosis reverse transcriptase and a random hexanucleotide-primer. The cDNA served as a template to generate fragments or the full-length sequence for AQP1.

The PCR primers used are indicated in Fig. 1. Sense and antisense oligonucleotides (se1 and an1) were first designed for PCR primers between the transmembrane region (TM) 4 and COOH-terminal whose sequences were conserved among the species reported [13,20,22]. The PCR was performed using Tag DNA polymerase for 35 cycles of 94°C for 15 s/50°C for 30 s/72°C for 60 s, and the products were examined by electrophoresis. After successful amplification of the fragment cDNA, several oligonucleotide primers complementing various regions of the NH_2-terminal and COOH-terminal ends, including non-coding regions, were also designed as shown in Fig. 1, and several PCR were performed.

2.4. Sequencing of cDNA

The fraction of the gel containing the amplified cDNA was excised and purified by phenol extraction. Then, the cDNA was ligated into pCR2.1 plasmid and the plasmid was transformed into competent

Escherichia coli cells (TA Cloning Kit, Invitrogen). The PCR products from several positive clones were examined. The sense and antisense strands of cDNA were sequenced by the dideoxynucleotide termination method with a DNA polymerase kit (Amplitag DNA polymerase, ABI). The sequencing reactions were run on an automated DNA sequencer (ABI 373, Applied Biosystems). The cDNA was examined by assessment of nucleotide sequences of mammals stored in the GenBank with the assistance of a computer program (Genetyx-Mac). Hydropathy analyses of the deduced amino acid sequence were also examined using this computer program according to Kyte and Doolittle [23].

2.5. Expression of AQP1 in Xenopus oocytes

The cDNA of the AQP1 in the pCR2.1 vector was digested at *Kpn*I–*Not*I sites and then subcloned into pCI expression vector at *Kpn*I–*Not*I sites. The cDNA was cut to linearize with *Sca*I and was transcribed in vitro with T7 RNA polymerase using a Capping Kit (Stratagene). The cRNA was then purified by phenol–chloroform extraction. The cRNA concentration was determined by optical density, and its quality was assessed by gel electrophoresis. For the expression marker, an Xpress tag was inserted into the 5′-end of the AQP1 cDNA with pcDNA4/His Max Vector (Invitrogen), and the cDNA including the Xpress tag was transcribed to cRNA as described above.

Stage V and VI oocytes were removed from female *Xenopus laevis* and defolliculated oocytes were prepared as described elsewhere [24]. The oocytes were injected with 26 nl of water or 17 ng cRNA in 26 nl water by a microinjector (Drummond Nanoject Au-

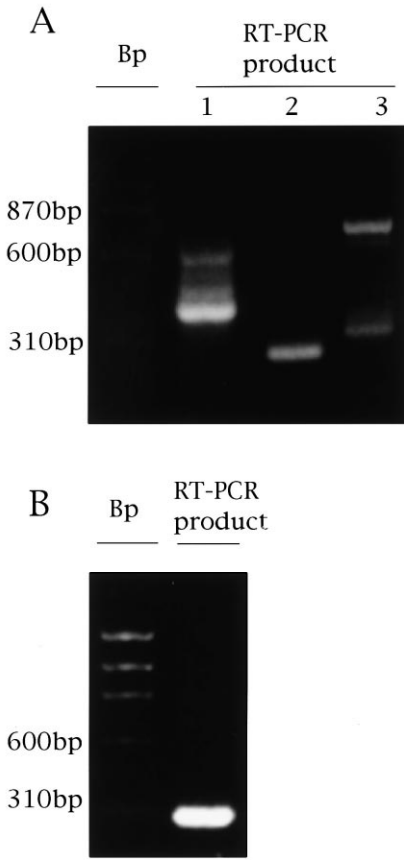
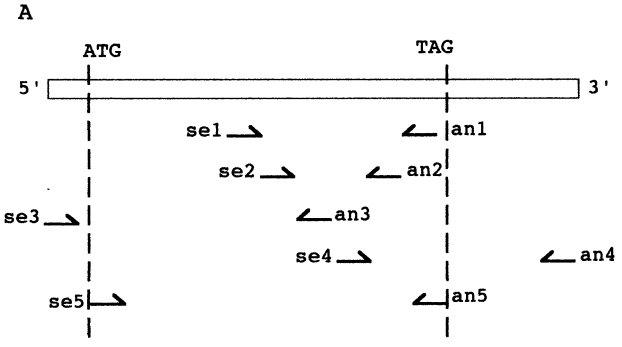


Fig. 2. The electrophoresis of the RT-PCR products for AQP1 from kidney (A) and red blood cell progenitors (B) with the primers se1 and an1 (1), se2 and an2 (2), and se3 and an3 (3) in dogs (see Fig. 1 for the primers). The ca. 300-bp band in B is the product of nested PCR. The first PCR was done with primers se1 and an1, and the nested PCR with primers se2 and an2.



B

numbers	Oligonucleotide
se1	5'-CCTGGCTGATGGTGTGAACT-3'
an1	5'-ACCCTGGAGTTGATGTCGTC-3'
se2	5'-TCGAGATCATTGGCACCCTG-3'
an2	5'-GGCCAGGATGAAGTCGTAGA-3'
se3	5'-GCAGCGGTCTCAGGCCAA-3'
an3	5'-TAGTAGCCAGCACGCATAGCACCA-3'
se4	5'-CACACAACTTCAGCAACCACTGGA-3'
an4	5'-TTGGTCAGCTTGTCAGAGTGTCTA-3'
se5	5'-ACGATGGCCAGCGAGTTCAAGAAG-3'
an5	5'-CTACTTGGGCTTCATCTCCACCCG-3'

Fig. 1. Location of nucleotide primers for PCR (A), and the sequences of sense (se) and antisense (an) oligonucleotide primers for PCR (B).

```
-31                                                          GCAGCGGTCTCAGGCCAAGCCCCCTGCCAGC    -1

  1   ATG GCC AGC GAG TTC AAG AAG AAG CTC TTC TGG AGG GCG GTG GTG GCC GAG TTC CTG GCC    60
  1    M   A   S   E   F   K   K   K   L   F   W   R   A   V   V   A   E   F   L   A    20

 61   ATG ATC CTC TTC GTC TTC ATC AGC ATC GGT TCT GCC CTG GGC TTC AAC TAC CCG GTG AGG   120
 21    M   I   L   F   V   F   I   S   I   G   S   A   L   G   F   N   Y   P   V   R    40

121   AAC AAC CAG ACA GCA GGT GCG GCC CAG GAT AAC GTG AAG GTG TCA CTG GCC TTC GGG CTG   180
 41    N   N   Q   T   A   G   A   A   Q   D   N   V   K   V   S   L   A   F   G   L    60

181   AGC ATT GCC ACC CTG GCC CAG AGC GTG GGC CAC ATT AGT GGC GCC CAC CTC AAC CCA GCT   240
 61    S   I   A   T   L   A   Q   S   V   G   H   I   S   G   A   H   L   N   P   A    80

241   GTC ACG CTG GGG CTG CTG CTC AGT TGC CAG ATC AGC ATC CTC AGG GCT GTC ATG TAC ATC   300
 81    V   T   L   G   L   L   L   S   C   Q   I   S   I   L   R   A   V   M   Y   I   100

301   ATC GCC CAG TGC GTG GGG GCC ATC GTG GCC ACC GCC ATC CTC TCG GGC ATC ACC TCC TCC   360
101    I   A   Q   C   V   G   A   I   V   A   T   A   I   L   S   G   I   T   S   S   120

361   CTT CCG GAC AAC TCC CTC GGC CGA AAC GAG CTG GCC CCT GGT GTG AAC TCC GGT CAG GGC   420
121    L   P   D   N   S   L   G   R   N   E   L   A   P   G   V   N   S   G   Q   G   140

421   TTG GGC ATC GAG ATC ATT GGC ACC CTG CAG CTG GTG CTG TGC GTG CTG GCC ACC ACG GAC   480
141    L   G   I   E   I   I   G   T   L   Q   L   V   L   C   V   L   A   T   T   D   160

481   AGG AGG CGG CGT GAC CTC GGG GGC TCG GGC CCC CTG GCC ATC GGC CTC TCT GTG GCC TTG   540
161    R   R   R   R   D   L   G   G   S   G   P   L   A   I   G   L   S   V   A   L   180

541   GGA CAC CTG CTG GCG ATC GAC TAC ACA GGC TGC GGT ATC AAC CCC GCC CGG TCC TTC GGC   600
181    G   H   L   L   A   I   D   Y   T   G   C   G   I   N   P   A   R   S   F   G   200

601   TCC TCG GTG ATC ACA CAT AAC TTC AAG GAC CAC TGG ATT TTC TGG GTC GGG CCG TTC ATC   660
201    S   S   V   I   T   H   N   F   K   D   H   W   I   F   W   V   G   P   F   I   220

661   GGG GGA GCC CTG GCG GTG CTC ATC TAC GAC TTC ATC CTG GCC CCC CGC AGC AGC GAC CTC   720
221    G   G   A   L   A   V   L   I   Y   D   F   I   L   A   P   R   S   S   D   L   240

721   ACG GAC CGC GTG AAG GTG TGG ACC AGC GGC CAG GTG GAG GAG TAT GAG CTG GAC GGC GAC   780
241    T   D   R   V   K   V   W   T   S   G   Q   V   E   E   Y   E   L   D   G   D   260

781   GAC ATC AAC TCC CGG GTG GAG ATG AAG CCC AAG TAG AGAGGCCTTGGCCCGGGCACCCACGCGGGGG   847
261    D   I   N   S   R   V   E   M   K   P   K   *                                  271

848   TGGGCAGGGGCGGGCGGAGGGCGGGAGGGGTGCAATCACGTCCTAGACACTCTGACAAGCTGACCAA              914
```

Fig. 3. Sequences of cDNA and deduced amino acids of AQP1 from dog kidney. The oligonucleotide sequences of the primers for full length PCR are underlined (se5 and an5).

tomatic Injector). On day 3 after the injection, the oocytes were transferred from 200 mOsm to 70 mOsm Barth's buffer diluted with water [5]. A microphotograph with a scale of the oocytes was obtained during the first 5 min of incubation. Oocyte volume was calculated from their diameter [5]. After the volume measurement, the Xpress tag was detected with an antibody (Invitrogen). Osmotic water permeabilty (*Pf*) was computed by the following formula according to Zhang et al. [25]:

$$Pf = [V_o \times d(V_t/V_o)/dt]/[S \times V_w \times (O_i - O_o)].$$

Pf was expressed as cm/s, whereas V_o is the initial oocyte volume; V_t the volume at time t; S the initial oocyte surface area; V_w the molar ratio of water (18 cm^3/mol); O_o the medium osmotic pressure; and O_i the intracellular osmotic pressure. Statistical analysis for the volume and water permeability were performed using Student's t-test.

3. Results

3.1. RT-PCR of AQP1 from dog kidney and erythroblasts

Fig. 2 shows the electrophoresis of RT-PCR products from the cDNA of AQP1 in the kidney (A) and

the erythroblasts (B). Firstly, the product of ca.400-bp cDNA fragment was amplified in the kidney, but not in the erythroblasts, by PCR with the primers of se1 and an1. By nested PCR with the primers se2 and an2 after the first PCR, a 300-bp PCR product was detected in the erythroblasts (B). The cDNA sequences of these PCR products were determined, and they were nearly identical (80–90%) to the corresponding sequences reported for AQP1 in the other species. This suggests that these PCR products were the cDNA fragments of AQP1. The oligonucleotides of 5′- and 3′-ends were amplified from the template by PCR with primers of se3 and an3, and se4 and an4, respectively. The 21-bp oligonucleotide of the 5′-end sequence in dog AQP1 was 100% identical to that in humans and bovines, and 22 of 24 nucleotides of the 3′-end in dog AQP1 were identical to those in humans, bovines and rodents. After these determinations of the cDNA sequences, we performed PCR for the full-length of the AQP1 cDNA with the primers including the 5′- and 3′-end regions, se5 and an5. The full-length cDNA was ca. 800-bp on the electrophoresis (Fig. 2A).

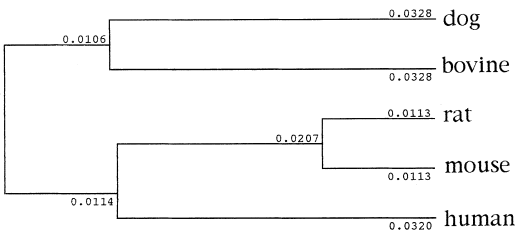
3.2. cDNA and deduced amino acid sequences of dog AQP1

Fig. 3 shows the cDNA and deduced amino acid sequences of the AQP1 from the dog kidney. The full-length cDNA sequence of the AQP1 from dog kidney was 816 bp, the same number as in bovine, but longer by 6 bp than in humans, rats and mice [20,21]. The fragment cDNA cloned from erythroblasts corresponded to that in the kidney from 448 to 682 in the cDNA sequence, and from 150 to 227 in the amino acid sequence. Though the cloning of the full-length cDNA failed in the erythroblasts, the fragment cDNA of the AQP1 was cloned and the cDNA was 100% identical to the corresponding sequence in the kidney. Nucleotide sequence data reported here are registered in the DDBJ data base under the accession number AB011373 and AB011374.

3.3. Comparison of cDNA and deduced amino acid sequences among dog and other species

Fig. 4 shows the genetic tree (A), and a comparison of the cDNA and the deduced amino acid sequences among the dog and the other species (B). The cDNA sequences among these species were very similar at 85–90%, and the order of the homology with dogs was bovines, humans and rodents. The homology of the deduced amino acid sequences among these species was also high, 91–94%, and the order of the homology with dogs was the same as for the cDNA sequences.

A



B

Amino Acids

	dog	bovine	human	mouse	rat
dog		93.7	92.6	91.5	90.8
bovine	89.8		91.1	90.4	90.0
human	88.6	88.5		94.1	93.7
mouse	85.5	86.9	88.1		97.8
rat	85.4	85.6	88.3	95.2	

Nucleotides

Fig. 4. The genetic tree (A) and a comparison of the homologies in the cDNA and the deduced amino acid sequences of AQP1 among dogs, bovines, humans, mice and rats (B). The analysis was performed using a Genetix Mac computer program.

3.4. Membrane topology and characteristic of amino acid sequence in dog AQP1

Fig. 5 shows the hydropathy analysis (A) and predicted membrane topology model (B) in the dog AQP1. The topology model was drawn according to the hydropathy analysis and previous reports [7,22]. The TMs and the loops (LP) between the two TMs were numbered as shown in Fig. 5A. The peptide sequence of dog AQP1 had six TM domains.

There were two tripeptide motifs (Asn-Pro-Ala or NPA) at LP 3 and 5, and a mercury inhibition site at LP 5, which were strongly conserved among AQP1 and the other members of the AQP family. The mercury inhibition site, Cys-191 in dog and bovine, corresponded to Cys-189 in humans and the rodents. There were four consensus sites for phosphorylation; one protein kinase C (PKC) site at the intracellular loop (LP 4), one other PKC, one caseine kinase II (CKII) and one cAMP-dependent kinase (AK) site in the C-terminal region. There was also a glycosylation site at LP 1.

The TMs and LP 2, 3 and 4 were highly conserved among the species compared, but LP 1 and 5 showed remarkable variation. At LP 1 in dog AQP1, five, four and seven of 13 amino acids were different than those in bovines, humans and rodents, respectively. Furthermore, both Ala-45 and Gly-46 at LP1 in dogs and bovines AQP1 were missing in humans and rodents. Thus, the peptide sequences in dog and bovine were longer by two amino acids than in humans and rodents. At LP 5, one, three and five of 26 amino acids in dog AQP1 were different than in bovines, humans and rodents, respectively. These mutations caused defects in a *N*-linked glycosylation site at LP 5 in dog and bovine AQP1, though there was a glycosylation site with an Asn-Phe-Ser-Asp (NFSN) motif at the LP in human and rodent AQP1.

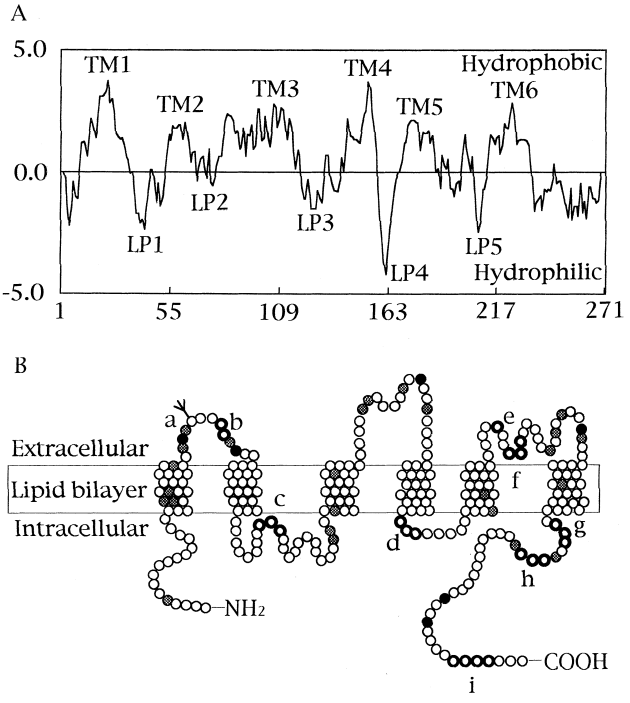


Fig. 5. Hydropathy analysis according to the method of Kyte and Doolittle [23] (A) and the predicted transmembrane topology (B) of the deduced amino acid sequence in dog AQP1. a, consensus sites for *N*-linked glycosylation; d, g, h, i with bold circles, consensus sites of PKC (d, h), CKII (i) and AK (g), respectively. c and f, Asp-Pro-Ala (NPA) motif. e, Cys-191. b, Two additional amino acid residues; closed circles, amino acid residues substituted only in dog; shaded circles, amino acid residues substituted in dog and some of the other animals.

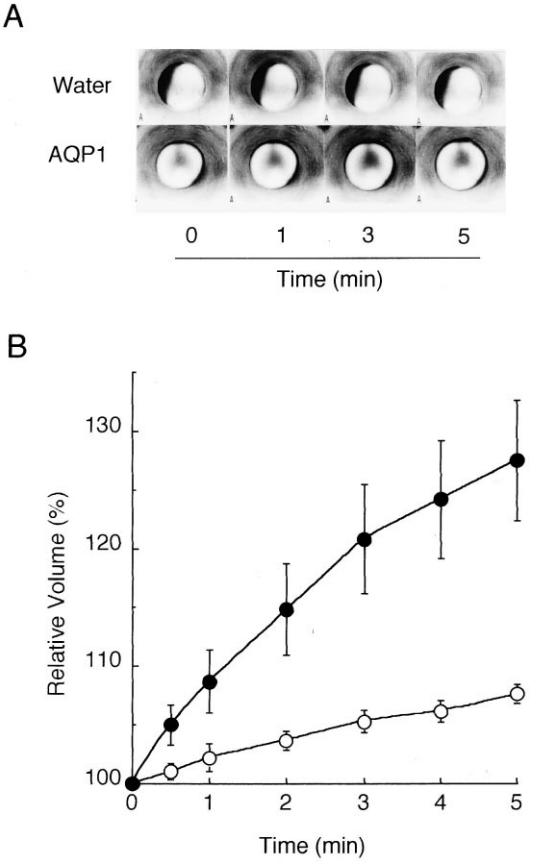


Fig. 6. Microphotograph (A) and relative volume (B) of *Xenopus* oocytes injected with AQP1 cRNA (AQP1 or closed circles) and water (Water or open circles) in the hyposmotic medium for the first 5 min of incubation. The figure presents a typical case from four experiments, and the data were means and S.D. of five oocytes.

3.5. Expression of AQP1 in Xenopus oocytes

Fig. 6 shows the volume change as shown by microphotograph (A) and relative volume (B) in the *Xenopus* oocytes which were injected with the

AQP1 cRNA in the hyposmotic medium. The oocytes injected with AQP1-cRNA were swollen to $128 \pm 5.1\%$ when incubated in a hyposmotic medium for 5 min, but the oocytes injected with water were swollen to only $108 \pm 0.8\%$. The osmotic water permeability was $8.7 \times 10^{-3} \pm 2.1 \times 10^{-3}$ cm/s when the cRNA was injected, against $2.6 \times 10^{-3} \pm 0.9 \times 10^{-3}$ cm/s when only water was injected. There were clear statistical differences between the oocytes injected with the cRNA and water in both relative volume change and water permeability. Thus, the gene product of cDNA from dog kidney AQP1 functioned to increase water permeability of the cellular membrane in *Xenopus* oocytes.

4. Discussion

The AQP1 cDNA in dog kidney was very homologous to that in bovine, human, mouse and rat; the cDNA in bovines was the closest to that in dogs among the species reported. Dog AQP1 was constructed with 271 amino acids, the same as in bovines, and two amino acids were added at LP 1, compared to that in humans and rodents. The dog AQP1 had two NPA motifs which were completely conserved in AQP and the aquaglyceroporin family; moreover, the dog AQP1 possessed a mercury inhibition site, Cys-191, which was also highly conserved among the family [7,26,27]. An examination of the expression of AQP1 mutants constructed by site-directed mutagenesis showed both NPA motifs and Cys-189 were essential for water permeability in human AQP1 [28]. Dog AQP1 also possessed the essential motifs or the amino acid residue(s) for water permeability.

The amino acid sequence homology between dog and bovine was very high at 94%. However, some LP regions in these animal AQP1 showed remarkable substitutions with different amino acids. LP1 showed considerable variation between these species: 38% of amino acids in the peptide sequence of the LP 1 in dog AQP1 were different from those in bovines which had the closest homology with dogs [21]. Furthermore, two additional hydrophobic amino acids were added in the LP1 of dog AQP1, and one hydrophobic and one hydrophilic amino acid existed at the LP1 in bovine AQP1 that were not present in humans and rodents. Therefore, the LP1 in dog AQP1 might be rather hydrophobic compared with other species. At Arg-40 in the LP1 of dog AQP1, there was a substitution with a different amino acid than in the other species: the amino acid was basic in dogs and bovines, but it was neutral or acidic in other animals [13,20–22]. Thus, since LP1 was the most variable region, these mutations might not be important for water transport itself. However, these amino acid substitutions could cause different functions of the LP1 among species.

The glycosylation site at the LP1 showed ABH antigenicity in human AQP1, so the site also might act as a ligand site in dog AQP1. Human AQP1 has Colton antigen [29], and the mutation of Ala-45 in human AQP1 changed the Colton antigen type [29]. The penta peptide sequence of the NH_2-terminal side of the Ala-45 in human AQP1 was NNQTA, and was the same as dog AQP1. Thus, the peptide sequence in the vicinity of the Ala-45 involved with Colton antigen in human AQP1 was also conserved in dog AQP1, and these sequences might have some competence as a ligand site.

LP 5 was another region with many variations. The human AQP1 mutant artificially inserted with two additional amino acids on the COOH-terminal side of Val-201 was found to reduce water permeability [29], so the amino acid sequence before or behind Val-201 must be important for the water permeability function. Therefore, the sequence of Cys-189 to Val-201 in human AQP1 corresponding to Cys-191 to Val-203 in dog and bovine AQP1 might also be essential for water permeability. The Ser-202 in dog and bovine AQP1 corresponded to Ala-200 in humans and rodents, and the character of the amino acid was changed to hydrophilic in dog and bovine compared to that in human and rodents. The amino acid residues neighboring Val-201 or -203, which are important for water permeability, varied among the species, so these differences could well reflect their functions.

Lys-209 and Asp-210 found at LP5 in dog AQP1 are basic and acidic amino acids, respectively, though the amino acids corresponding to the sequence in human AQP1, Ser and Asn, are neutral. These mutations at the positions of 209 and 210 in dog and bovine caused the defect of an *N*-linked glycosylation site which existed in human and rodent AQP1.

Hence, these mutations may result in some conformational change and/or functional difference among the species. However, the substitution at amino acid residue 205, which had a position closer to the critical residue, Val-201, than the positions of the above mutations, failed to affect the function [30]. Thus, the mutations of Lys-209 and Asp-210 in dog AQP1 may not be essential for water permeability.

Two His residues were found at LP 5 in dog, bovine and human AQP1, though only one His residue existed in the rodent AQP1. The pK_a of imidazole residue of His was pH 6, and sensitive to pH change in the physiological condition. Thus, the His residues may be sensitive to extracellular pH change and may affect the LP5 conformation.

Four consensus kinase sites were conserved in all species compared: two PKC, one CKII and one AK site. The consensus AK site in AQP1 was only tripeptide, Arg-Ser-Ser, while high affinity for the AK was usually found with a tetrapeptide, Arg-Arg-X-Ser/Thr (X can substitute with any amino acid, and Ser/Thr is either of the amino acids) [22,31]. In human AQP1, cAMP-dependent phosphorylation was observed, and cAMP and arginine vasopressin activated water permeability in *Xenopus* oocytes injected with AQP1 cRNA [32,33]. Thus, it was strongly suggested that AQP1 was stimulated by AK [33]. Therefore, vasopressin is considered to serve as a stimulator for AQP1, just as for AQP2 [5,34].

Water permeability through the gene product of the dog AQP1 was shown in *Xenopus* oocytes the same as other AQP1 [5,13,32]. Thus, it was confirmed that dog AQP1 also functioned as a water channel. The water permeability was slightly lower in this experiment than reported elsewhere [5,28]. This might be due to the batches of *Xenopus* oocytes or the amount or quality of cRNA injected. In one experiment, the oocytes injected with the cRNA in fact suddenly increased to 140% within 2–3 min and burst (data not shown).

Though the full-length cDNA of AQP1 was not detected in the dog erythroblasts, a fragment cDNA was cloned and the sequence was 100% identical to that in kidney. It was therefore suggested that mRNA of AQP1 is probably expressed in the progenitor cells of dog red blood cells, and that AQP1 might exist in the dog red blood cell membrane as reported in other species [8,22]. Erythroblasts or reticulocytes synthesize hemoglobin almost entirely, so sometimes it is difficult to detect mRNA other than hemoglobin [35]. Actually, a fragment cDNA of K-Cl cotransporter, a red blood cell membrane protein, was not detected in reticulocytes, but was found from the early stage of erythroblasts differentiated from erythroleukemia cells [35]. These findings might explain why only a fragment cDNA was cloned in erythroblasts in this experiment, and not full-length cDNA.

These results contribute not only to adding dog AQP1 to the reported gene family of AQP1; the variation found in dog AQP1 will also be useful in order to examine the role of the water channel. The authors intend to use this gene to examine water permeation during RVD in dog red blood cells in the future.

Acknowledgements

This work was partly supported by a Grant-in-Aid for Scientific Research from The Ministry of Education, Science, Sports and Culture of Japan to H.F. (09660331).

References

[1] P.M. Deen, M.A. Verdijk, N.V. Knoers, B. Wieringa, L.A. Monnens, C.H. van Os, B.A. van Oost, Requirement of human renal water channel aquaporin-2 for vasopressin-dependent concentration of urine, Science 264 (1994) 92–95.

[2] H. Hasegawa, T. Ma, W. Skach, M.A. Matthay, A.S. Verkman, Molecular cloning of a mercurial-insensitive water channel expressed in selected water-transporting tissues, J. Biol. Chem. 269 (1994) 5497–5500.

[3] K. Ishibashi, S. Sasaki, K. Fushimi, S. Uchida, M. Kuwahara, H. Saito, T. Furukawa, K. Nakajima, Y. Yamaguchi, T. Gojobori et al., Molecular cloning and expression of a member of the aquaporin family with permeability to glycerol and urea in addition to water expressed at the basolateral membrane of kidney collecting duct cells, Proc. Natl. Acad. Sci. USA 91 (1994) 6269–6273.

[4] K. Ishibashi, M. Kuwahara, Y. Gu, Y. Kageyama, A. Tohsaka, F. Suzuki, F. Marumo, S. Sasaki, Cloning and functional expression of a new water channel abundantly expressed in the testis permeable to water, glycerol, and urea, J. Biol. Chem. 272 (1997) 20782–20786.

[5] G.M. Preston, T.P. Carroll, W.B. Guggino, P. Agre, Ap-

pearance of water channels in Xenopus oocytes expressing red cell CHIP28 protein, Science 256 (1992) 385–387.
[6] P. Agre, S. Sasaki, M.J. Chrispeels, Aquaporins: a family of water channel proteins [letter], Am. J. Physiol. 265 (1993) F461.
[7] P. Agre, M. Bonhivers, M.J. Borgnia, The aquaporins, blueprints for cellular plumbing systems, J. Biol. Chem. 273 (1998) 14659–14662.
[8] S. Nielsen, B.L. Smith, E.I. Christensen, M.A. Knepper, P. Agre, CHIP28 water channels are localized in constitutively water-permeable segments of the nephron, J. Cell. Biol. 120 (1993) 371–383.
[9] Y. Maeda, B.L. Smith, P. Agre, M.A. Knepper, Quantification of Aquaporin-CHIP water channel protein in microdissected renal tubules by fluorescence-based ELISA, J. Clin. Invest. 95 (1995) 422–428.
[10] S. Nielsen, B.L. Smith, E.I. Christensen, P. Agre, Distribution of the aquaporin CHIP in secretory and resorptive epithelia and capillary endothelia, Proc. Natl. Acad. Sci. USA 90 (1993) 7275–7279.
[11] T.L. Pallone, B.K. Kishore, S. Nielsen, P. Agre, M.A. Knepper, Evidence that aquaporin-1 mediates NaCl-induced water flux across descending vasa recta, Am. J. Physiol. 272 (1997) F587–596.
[12] I. Sabolic, G. Valenti, J.M. Verbavatz, A.N. Van Hoek, A.S. Verkman, D.A. Ausiello, D. Brown, Localization of the CHIP28 water channel in rat kidney, Am. J. Physiol. 263 (1992) C1225–1233.
[13] R. Zhang, W. Skach, H. Hasegawa, A.N. van Hoek, A.S. Verkman, Cloning, functional analysis, cell localization of a kidney proximal tubule water transporter homologous to CHIP28, J. Cell. Biol. 120 (1993) 359–369.
[14] T. Ma, B. Yang, A. Gillespie, E.J. Carlson, C.J. Epstein, A.S. Verkman, Severely impaired urinary concentrating ability in transgenic mice lacking aquaporin-1 water channels, J. Biol. Chem. 273 (1998) 4296–4299.
[15] H. Fujise, I. Yamada, M. Masuda, Y. Miyazawa, E. Ogawa, R. Takahashi, Several cation transporters and volume regulation in high-K dog red blood cells, Am. J. Physiol. 260 (1991) C589–C597.
[16] H. Fujise, K. Abe, M. Kamimura, H. Ochiai, K-Cl cotransport and volume regulation in the light and the dense fraction of high-K dog red blood cells, Am. J. Physiol. 273 (1997) R991–R998.
[17] H. Fujise, M. Mori, E. Ogawa, Y. Maede, Variant of canine erythrocytes with high potassium content and lack of glutathione accumulation, Am. J. Vet. Res. 54 (1993) 602–606.
[18] H. Wada, T. Suda, Y. Miura, E. Kajii, S. Ikemoto, Y. Yawata, Expression of major blood group antigens on human erythroid cells in a two phase liquid culture system, Blood 75 (1990) 505–511.
[19] K. Tebbi, S. Rubin, D.H. Cowan, E.A. McCulloch, A comparison of granulopoiesis in culture from blood and marrow cells of nonleukemic individuals and patients with acute leukemia, Blood 48 (1976) 235–243.
[20] A. Lanahan, J.B. Williams, L.K. Sanders, D. Nathans, Growth factor-induced delayed early response genes, Mol. Cell. Biol. 12 (1992) 3919–3929.
[21] R.V. Patil, X. Yang, I. Saito, M. Coca-Prados, M.B. Wax, Cloning of a novel cDNA homologous to CHIP28 water channel from ocular ciliary epithelium, Biochem. Biophys. Res. Commun. 204 (1994) 861–866.
[22] G.M. Preston, P. Agre, Isolation of the cDNA for erythrocyte integral membrane protein of 28 kilodaltons: member of an ancient channel family, Proc. Natl. Acad. Sci. USA 88 (1991) 11110–11114.
[23] J. Kyte, R.F. Doolittle, A simple method for displaying the hydropathic character of a protein, J. Mol. Biol. 157 (1982) 105–132.
[24] L. Lu, C. Montrose-Rafizadeh, T.C. Hwang, W.B. Guggino, A delayed rectifier potassium current in Xenopus oocytes, Biophys. J. 57 (1990) 1117–1123.
[25] R.B. Zhang, K.A. Logee, A.S. Verkman, Expression of mRNA coding for kidney and red cell water channels in Xenopus oocytes, J. Biol. Chem. 265 (1990) 15375–15378.
[26] L.S. King, P. Agre, Pathophysiology of the aquaporin water channels, Annu. Rev. Physiol. 58 (1996) 619–648.
[27] J.H. Park, M. Saier Jr., Phylogenetic characterization of the MIP family of transmembrane channel proteins, J. Membr. Biol. 153 (1996) 171–180.
[28] J.S. Jung, G.M. Preston, B.L. Smith, W.B. Guggino, P. Agre, Molecular structure of the water channel through aquaporin CHIP. The hourglass model, J. Biol. Chem. 269 (1994) 14648–14654.
[29] B.L. Smith, G.M. Preston, F.A. Spring, D.J. Anstee, P. Agre, Human red cell aquaporin CHIP. I. Molecular characterization of ABH and Colton blood group antigens, J. Clin. Invest. 94 (1994) 1043–1049.
[30] G.M. Preston, J.S. Jung, W.B. Guggino, P. Agre, Membrane topology of aquaporin CHIP. Analysis of functional epitope-scanning mutants by vectorial proteolysis, J. Biol. Chem. 269 (1994) 1668–1673.
[31] P.J. Kennelly, E.G. Krebs, Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases, J. Biol. Chem. 266 (1991) 15555–15558.
[32] R.V. Patil, Z. Han, M.B. Wax, Regulation of water channel activity of aquaporin 1 by arginine vasopressin and atrial natriuretic peptide, Biochem. Biophys. Res. Commun. 238 (1997) 392–396.
[33] A.J. Yool, W.D. Stamer, J.W. Regan, Forskolin stimulation of water and cation permeability in aquaporin 1 water channels [see comments], Science 273 (1996) 1216–1218.
[34] T. Yamamoto, S. Sasaki, K. Fushimi, K. Ishibashi, E. Yaoita, K. Kawasaki, F. Marumo, I. Kihara, Vasopressin increases AQP-CD water channel in apical membrane of collecting duct cells in Brattleboro rats, Am. J. Physiol. 268 (1995) C1546–C1551.
[35] C.M. Pellegrino, A.C. Rybicki, S. Musto, R.L. Nagel, R.S. Schwartz, Molecular identification and expression of erythroid K:Cl cotransporter in human and mouse erythroleukemic cells, Blood Cells Mol. Dis. 24 (1998) 31–40.