Cloning and Functional Expression of a New Water Channel Abundantly Expressed in the Testis Permeable to Water, Glycerol, and Urea*

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A new member of the aquaporin (AQP) family has been identified from rat testis. This gene, referred as aquaporin 7 (AQP7), encodes a 269-amino acid protein that contained the conserved NPA motifs of MIP family proteins. AQP7 has the amino acid sequence homology with other aquaporins (~30%), and it is highest with AQP3 (48%), suggesting that both AQP3 and AQP7 belong to a subfamily in the MIP family. Injection of AQP7-cRNA into Xenopus oocytes expressed a 26-kDa protein detected by immunoblotting. The expression of AQP7 in oocytes stimulated the osmotic water permeability by 10-fold which was not inhibited by 0.3 mM mercury chloride. The Arrhenius activation energy for the stimulated water permeability was low (2.1 kcal/mol). AQP7 also facilitated glycerol and urea transport by 5- and 9-fold, respectively. The activation energy for glycerol was also low (5.3 kcal/mol after the correction of the endogenous glycerol permeability of oocytes). Northern blot analysis revealed a 1.5-kilobase pair transcript expressed abundantly in testis. In situ hybridization of testis revealed the expression of AQP7 at late spermatids in seminiferous tubules. The immunohistochemistry of testis localized the AQP7 expression at late spermatids and at maturing sperms. AQP7 may play an important role in sperm function.

Recent studies have identified several water channels (aquaporins) that belong to the MIP family (reviewed in Ref. 1). The MIP family proteins are widely expressed in almost all organisms. In Escherichia coli, there are two MIP family proteins; one is a glycerol facilitator (GlpF) (2), and the other is a water channel (AQP Z) (3). A yeast (Saccharomyces cerevisiae) has four MIP family proteins in its genome. Plants have many MIP family proteins. For example, there are more than 12 MIP family proteins in Arabidopsis thaliana (4). In mammals, more than eight members have been reported; most of them transport water and those have been named “aquaporins.” Seven members of aquaporins have been reported in rat and human (1). AQP0 (originally named MIP26) is present exclusively at lens epithelium. AQP1 is present in many tissues including red blood cells, kidney, eye, lung, choroid plexus, bile duct, and vascular endothelium. AQP2 is solely present at the apical membrane of kidney collecting duct cells. AQP3 and AQP4 are localized at the basolateral membranes in some tissues such as kidney, colon, and trachea. AQP3 is present in urinary bladder, skin, and sclera of eye, and AQP4 is present in stomach, skeletal muscle, spinal chord, brain, and retina. AQP5 is present at the apical membranes of exocrine tissues. AQP6 (originally named WCH3 or hKID) is present only in kidney (5).

Aquaporins are usually found in the selected tissues where water movements are abundant and/or physiologically important (6). Unexpectedly, a high water permeability of human and ram sperm has been reported (7). It is also reported that the high water permeability of human sperm is mercury-resistant and not mediated by AQP1 (8). The molecular basis for this high water permeability is unknown. The sperm water channel may be veterinary important for the cryopreservation of sperms. Here we report the cloning and the functional expression of a new aquaporin (AQP7) from rat testis.

EXPERIMENTAL PROCEDURES

Reverse-transcribed PCR—One microgram of rat testis total RNA was reverse-transcribed and used for PCR with 5 µM set of degenerative primers as previously reported (9); sense strand, 5′-CGGAAATTCTTGGNNAAYCCNGCNGTNAC-3′, and antisense strand, 5′-CCGGATCCTAATCNCNNGNNNGGTGRTT-3′ (the abbreviation recommended by the IUPAC-IUB). The primers were derived from the consensus amino acid sequences of the MIP family (4) (Leu-Asn-Pro-Ala-Val-Thr and Asn-Pro-Ala-Arg-Asp-Phe, respectively). The PCR was conducted in the following profile: 94°C for 1 min, 46°C for 1 min, and 72°C for 3 min for 30 cycles. The PCR products were cut with EcoRI and BamHI on both ends, ligated into EcoRI- and BamHI-cut pSPORT (Life Technologies, Inc.), and sequenced.

Library Construction and Screening—An oligo(dT)-primed rat testis cDNA library in λZAP (Stratagene) was screened under a stringent condition (6 × SSPE, 5 × Denhardt’s solution, 0.2% SDS, 100 µg/ml salmon sperm DNA, 50% formamide at 42°C) with a PCR clone labeled with [32P]dCTP (random priming; Amersham Corp.). A positive clone (AQP7) was isolated and in vitro excised as described in the Stratagene protocol and sequenced by the dyeoxy chain termination method (Sequenase version 2; U. S. Biochemical Corp.).

Expression of AQP7 in Xenopus Oocytes—EcoRI fragment (1.3 kb) of AQP7 cDNA (containing open reading frame and the untranslated sequences) was blunt-end-ligated into the BglII site of a pSP64T-derived BlueScript vector containing 5′- and 3′-untranslated sequences of β-globin gene of Xenopus (pXBG-ev1; a generous gift from Dr. Peter Agre). Capped cRNA was synthesized using T3 RNA polymerase after a digestion with BamHI to linearize the plasmids. The defoliciated Xenopus oocytes were injected with 50 nl of water or of AQP7 cRNA and incubated at 18°C for 48 h in modified Barth’s buffer.

* This work was supported by a grant-in-aid from the Ministry of Education, Science and Culture (Japan) and a grant from The Salt Education, Science and Culture (Japan) and a grant from The Salt

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‡ The abbreviations used are: GlpF, glycerol facilitator; AQP, aquaporin; PCR, polymerase chain reaction; kb, kilobase pair(s).
Immunodetection of AQP7 Expressed in Xenopus Oocytes—Two polyclonal anti-AQP7 antibodies were raised in rabbits against a synthesized N-terminal peptide (MAGSVLENIQSVLQK) and a C-terminal peptide (GLIHAGIPPQGS), respectively. The enzyme-linked immunosorbent assay titers of each antiserum were 13,200 for N-terminal antibody and 15,200 for C-terminal antibody. The oocytes membranes were isolated as described previously (10). The membrane fraction was resuspended in a loading buffer containing 3% SDS, 65 mM Tris-HCl, 10% glycerol, and 5% 2-mercaptoethanol. After being heated at 70 °C for 10 min, the solubilized proteins were separated by SDS-polyacrylamide gel electrophoresis. Membrane proteins from 3 oocytes were applied in each lane. The proteins were transferred to Immobilon-P filter (Millipore) using a semi-dry system. The filters were incubated for 1 h with the above polyclonal antibodies (100-fold dilution). The filters were further incubated for 1 h with125I-protein A solution, followed by autoradiography.

Osmotic Water Transport Assay—Water permeability was measured as described previously (9). In brief, oocytes were transferred from 200 to 70 mOsm of modified Barth's buffer at 25 °C, and oocyte swelling was monitored by video microscopy. The coefficient of osmotic water permeability ($P_f$, $\text{nm}^2/	ext{sec}$) was calculated from the initial slope of oocyte swelling as previously reported (9). The Arrhenius activation energy was calculated by measuring $P_f$ at 5 and 25 °C.

Glycerol and Urea Uptake Assay—The oocytes were incubated in Barth's solution either with [14C]glycerol (specific activity, 5.88 GBq/mm; Amersham Corp.) or [14C]urea (specific activity, 2.02 GBq/mm; Amersham Corp.) at room temperature for 2–10 min. The oocytes were then rapidly washed four times with ice-cold Barth's solution. The individual oocytes were lysed in 200 $\mu$l of 10% SDS solution overnight for liquid scintillation counting. The Arrhenius activation energy for glycerol was calculated by measuring $P_f$ at 10, 20, and 30 °C.

Northern Blots—A rat multiple tissue Northern blot (CLONTECH) was hybridized under high stringency condition with a 1.3-kb AQP7 cDNA labeled with [384a-32P]dCTP. Each lane of poly(A)$^+$ RNA from rat tissues. The filter was washed under high stringency conditions.

In Situ Hybridization of AQP7 in Testis—Cryosections (10 $\mu$m) were cut from rat testis from 12-week-old Harlan Sprague Dawley strain rats, fixed with 4% formaldehyde in phosphate-buffered saline, and treated with 0.25% acetic anhydride, 0.1 M triethanolamine HCl (pH 8.0) for 10 min. Digoxigenin-labeled antisense and sense riboprobes were made with T7 or T3 RNA polymerase from 0.5-kb cDNA C-terminal fragment of AQP7 by DIG RNA labeling kit (Boehringer Mannheim) following the manufacturer's instruction. DIG nucleic acid detection kit (Boehringer Mannheim) was used for the detection of AQP7 RNA in the tissues. In brief, sections were hybridized with digoxigenin-labeled RNA probes (0.5 ng/ml) for 24 h at 37 °C. After the blocking buffer treatment, sections were incubated in anti-DIG-AP conjugate for 5 h at room temperature. Following the incubation in detection buffer and subsequent color-substrate solution, sections were immersed in quenching buffer, and photographed.

Immunohistochemistry of AQP7 in Testis—The testis from Wistar rats (9-month-old) was fixed with Bouin's fixative. The testis was embedded in paraffin. The sections of 8 $\mu$m were stained with a polyclonal antibody against the C terminus of AQP7 in 1:3000 dilution after blocking with 5% normal goat serum and 3% bovine serum albumin. Subsequently, the sections were treated with anti-rabbit IgG conjugated with peroxidase (Sigma). Following the diaminobenzidine reaction and the counter staining with hematoxylin, the sections were mounted with Permafluor (Lipshaw, Pittsburgh, PA).

**Fig. 1. Sequence analysis of testis AQP7.** A, nucleotide sequence and deduced amino acid sequence of the clone isolated from a rat testis cDNA library. Probable transmembrane domains are underlined. A polyadenylation consensus is double-underlined. B, hydrophy analysis of deduced amino acid sequence using a 13-residue window (19). The average local hydrophobicity at each residue was plotted on the vertical axis and the residue number on the horizontal axis. C, alignment of the amino acid sequences of E. coli GlpF (2), rat AQP3 (13), and rat AQP7. Gaps are inserted to maximize matching. White letters in black boxes denote the amino acid residues conserved at least two of them. The predicted transmembrane domains of AQP7 are underlined. The conserved NPA motifs are overlined.
RESULTS

Cloning of the cDNA and Analysis of the Amino Acid Sequence of AQP7—We used the two highly conserved NPA boxes of MIP family proteins for designing a set of degenerative oligonucleotide PCR primers. Searches for new aquaporins from rat testis cDNA using these primers led to the identification of a new clone. We screened a rat testis cDNA library with this PCR clone as a probe. We isolated 16 partially overlapping clones. A cDNA clone of 1.3 kb, AQP7 (Fig. 1A), was chosen for further study. The translation initiation site was assigned to the first ATG triplet that is downstream of nonsense codons found in-frame and similar to a good Kozak initiation of the translation site (ACCATGG). cDNA consists of a 5′-untranslated region of 260 base pairs and 3′-untranslated region of 199 base pairs followed by a poly(A) tail. An open reading frame codes a protein of 269 amino acids with a relative molecular mass calculated as 28,876 kDa. Hydropathy analysis predicts six transmembrane regions with N terminus and C terminus localized in the cytosol similar to other MIP family members (Fig. 1B). The C terminus, however, is exceptionally short with little hydrophilic residues. The human AQP7 also had an identical stop site.1 The 3′ noncoding se-

2 K. Ishibashi, unpublished observations.

Fig. 2. Western blot analysis of oocyte membrane proteins probed with polyclonal antibodies against AQP7. Oocytes were injected with water (cRNA(−)) or 5 ng of cRNA of AQP7 (cRNA(+)). Membranes prepared from three oocytes were loaded in each lane. The blots were probed with anti-AQP7 antiserum against the C-terminal peptide (A) or against the N-terminal peptide (B). The specific bands for AQP7 cRNA injection were indicated by arrows. The positions of the molecular markers (kDa) are indicated.

Fig. 3. Functional expression of AQP7 in Xenopus oocytes. A, osmotic water permeability ($P_o$) of oocytes injected with 50 nl of water or 5 ng of AQP7 cRNA. Bars show mean ± S.E. of five determinations of oocytes. Hg indicates that the assay was performed after 5 min incubation in 0.3 mM mercury chloride. B, time course of [14C]glycerol uptake into oocytes injected with water (○) or 20 ng of AQP7 cRNA (●). C, time course of [14C]urea uptake into oocytes injected with water (○) or 20 ng of AQP7 cRNA (●). D, glycerol permeability ($P_{gly}$) of oocytes expressing AQP7 at different temperatures. Each point represents means of 7-8 measurements. $P_{gly}$ was measured in water-injected oocytes (○) and in AQP7-cRNA-injected oocytes (●) at 4, 20, and 20 °C. To estimate AQP7-dependent $P_{gly}$, $P_{gly}$ of control oocytes was subtracted from $P_{gly}$ of AQP7 oocytes at each temperature. AQP7-dependent $P_{gly}$ was calculated to be 5.3 kcal/mol from the fitted line.
sequence contains a consensus polyadenylation signal (double underlined) with poly(A) tail. No potential N-linked glycosylation site (NX(S/T)) nor the consensus protein kinase C phosphorylation site is present in the predicted amino acid sequence of AQP7. The cytoplasmic second loop contains a potential phosphorylation site by protein kinase C (residue Thr-174). This phosphorylation site is present in the predicted amino acid sequence (Fig. 1). The toxicity of mercury chloride to sperm precluded further study with higher concentration of mercury chloride. In this study of the heterologous expression system, we examined the effect of much higher concentrations of mercury chloride. 0.3 mM HgCl₂ did not affect the increase of Pᵣ (Fig. 3A) (179 ± 15 μm/s; n = 12). To examine the temperature dependence of Pᵣ, Pᵣ values at 5 and 25 °C were measured (n = 12 each). The determined activation energy from the Arrhenius equation of Pᵣ was 2.1 kcal/mol, a value in the range expected for a water channel.

As AQP3 transport glycerol (9, 11, 12) and urea (9, 13), we examined the glycerol and urea uptake in AQP7-expressing oocytes. Oocytes were incubated in the presence of 165 μM [¹⁴C]glycerol, and intracellular radioactivity was measured after 2 min. AQP7 cRNA (20 ng) injection stimulated glycerol uptake by 5-fold (Fig. 3B) (1020 ± 183 cpm; n = 5 versus 202 ± 42; n = 6) and for 10 min by 16-fold (1717 ± 107 cpm; n = 4 versus 107 ± 5; n = 9) (the calculated Pᵣ was 18.9 × 10⁻⁹ cm/s); which was comparable with the previous reports of glycerol uptake by AQP3 (9, 13). Urea uptake was also stimulated with AQP7 expression (Fig. 3C). The incubation in 22.6 μM [¹⁴C]urea for 5 min resulted in the increase of urea uptake through AQP7 by 9-fold (824 ± 238 cpm; n = 5 versus 93 ± 4; n = 6) and for 10 min by 16-fold (1717 ± 107 cpm; n = 4 versus 107 ± 5; n = 9) (the calculated Pᵣ was 12.0 × 10⁻⁶ cm/s). The degree of stimulation of urea uptake by AQP7 is much higher than that of AQP3 (9, 11, 13). The activation energy for glycerol permeability was calculated by measuring Pᵣ at different temperatures. However, the result was complicated by the endogenous glycerol permeability of oocytes. AQP7-dependent Pᵣ was obtained by subtracting Pᵣ of control oocytes from Pᵣ of oocytes expressing AQP7 at each temperature (Fig. 3D). The activation energy for AQP7-dependent Pᵣ was calculated to be 5.3 kcal/mol. As both activation energies for water and glycerol are low, water and glycerol permeate AQP7 by channel mechanism and may share the same pathway.

AQP7 Expression and Tissue Distribution—Northern blot analysis revealed that AQP7 mRNA (1.5 kb) was expressed most abundantly in testis (Fig. 4). The size of the bands of heart and kidney (2.4 and 1.35 kb) seemed to be different from that of testis. Much weaker bands were detected in skeletal muscle.
that this is a functionally important region of the protein (10). The mercury-sensitive Cys is located just in front of the second NPA box in AQP1, AQP2, AQP5, and AQP6 indicating that there are three Cys (Cys-169, -185, -195) in deduced amino acid sequence of AQP7, they may not be localized near the AQP7 aqueous pore.

The previous studies on water permeability of sperms of human, ram, fowl, and bull showed their high water permeability with low activation energy, which is insensitive to mercury chloride (7, 8, 16). The character of the sperm water channel agrees well with that of AQP7, and the immunohistochemical study revealed that AQP7 is present at sperms. The physiological role of such a high water permeability in sperm is not clear at present. In E. coli, its glycerol transporter (GlpF) is localized in the operon with glycerol kinase (2), and the functional coupling between GlpF and glycerol kinase has been shown (17). Interestingly, testis has a unique glycerol kinase encoded by a different gene from the systemic one (18). It is possible that testis-specific glycerol kinase and AQP7 are functionally coupled and that the major function of AQP7 is glycerol transport rather than water transport. As glycerol has been used as an almost universally effective cryoprotectant for sperm, the glycerol permeability of sperm may be important as a determinant of optimal cooling rate. Whether the activity of AQP7 is critical for sperm cryopreservation remains to be clarified.

Acknowledgments—We thank Kouji Takahashi, Hiroyuki Ooshima, Masanobu Kawasaki, and Kiyohide Fushimi for helpful discussions.

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DISCUSSION
We have cloned a new water channel (AQP7) from rat testis. AQP7 has the sequence and functional similarity to AQP3. Thus, AQP3 and AQP7 comprise a new subfamily in aquaporins. On the other hand, AQP0, -1, -2, -4, -5, and -6 have amino acid homology with each other on the order of 40–50% and comprise the other subfamily. The latter group seems to be more selective in the permeation of water, and the former group seems to be less selective and also permeable to glycerol. However, the nature and the precise localization of water and/or glycerol pore(s) within each group are not yet determined. The discovery of another member of the AQP3 group will facilitate the comparison of sequence-related functional differences between two groups.

The water permeability of aquaporins is inhibited by mercury chloride except for AQP4 (14) and plant aquaporin RD28 (15). The mercury-sensitive Cys is located just in front of the second NPA box in AQP1, AQP2, AQP5, and AQP6 indicating that this is a functionally important region of the protein (10).
