Molecular Cloning of a New Aquaporin from Rat Pancreas and Liver*

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A new water channel (aquaporin-8, gene symbol AQP8) was isolated from rat pancreas and liver by homology cloning. Ribonuclease protection assay showed intense expression of the gene in pancreas and liver, less intense in colon and salivary gland, and negligible in other organs. The full-length cDNA was obtained by ligation of ~1.4-kilobase (kb) cDNA isolated from the rat liver cDNA library to ~0.5 kb of the 5’-end fragment obtained by the rapid amplification of cDNA ends method. A major transcript of ~1.45 kb was demonstrated in liver and colon by Northern blot analysis. Expression of the cRNA in Xenopus oocytes markedly enhanced osmotic water permeability in a mercury-sensitive manner, indicating a water channel function of this molecule. The open reading frame encoded a 263-amino acid protein with a predicted molecular size of 28 kDa. Hydropathy analysis represented six membrane-spanning domains and five connecting loops containing two sites of NPA motif as preserved in other aquaporins. Unlike other mammalian aquaporins, AQP8 has an unusual structure with a long N terminus and a short C terminus, which are found in plant aquaporin, γ-tonoplast intrinsic protein. By in situ hybridization, AQP8 mRNA expression was assumed in hepatocytes, acinar cells of pancreas and salivary gland, and absorptive colonic epithelial cells. The physiological role(s) of AQP8 remain to be elucidated.

The aquaporins are water-selective membrane channels found in many species of animals and plants as the family of major intrinsic protein (MIP). Aquaporin-1 (AQP1) is the first protein recognized as a channel-forming integral membrane protein of 28 kDa. Hydropathy analysis represented six membrane-spanning domains and five connecting loops containing two sites of NPA motif as preserved in other aquaporins. Unlike other mammalian aquaporins, AQP8 has an unusual structure with a long N terminus and a short C terminus, which are found in plant aquaporin, γ-tonoplast intrinsic protein. By in situ hybridization, AQP8 mRNA expression was assumed in hepatocytes, acinar cells of pancreas and salivary gland, and absorptive colonic epithelial cells. The physiological role(s) of AQP8 remain to be elucidated.

The nucleotide sequence reported in this paper has been submitted to GenBank™/EBL Data Bank with accession number AF007775 and the gene has been designated AQP8 by the HUGO/GDB Nomenclature Committee.

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1 The abbreviations used are: MIP, major intrinsic protein; AQPn, aquaporin n; TIP, tonoplast intrinsic protein; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase(s); P/I, coefficient of osmotic water permeability.

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The previous studies showed the presence of unique aquaporins in various exocrine glands such as salivary gland and lacrimal gland (16). Since pancreas is a secretory organ, secreting various digestive enzymes such as lipase, amylase, and protease in a volume of about 2,000 ml/day in humans (20), it may be reasonable to speculate the presence of an aquaporin family in pancreas. In the present study, we attempted to isolate aquaporins expressed in pancreas by polymerase chain reaction (PCR) using degenerate primers for the aquaporin family (15, 16). Through the analysis, AQP1 and AQP4 were cloned and coincidentally a new member of the aquaporin family, AQP8, was identified as a water channel prominently expressed in pancreas and liver.

MATERIALS AND METHODS

cDNA Cloning—Rat pancreas total RNA (1 μg) was reverse-transcribed at 37 °C for 1 h by using an oligo(dT) primer and reverse transcriptase in a volume of 20 μl (Superscript II, Life Technologies, Inc.). Nested, degenerate primers reported previously (15, 16) were synthesized: sense primers, WCU-1 (5’-STBGNCAYRTGYANGAANGCNCA-3’) and WCU-2 (5’-GGGATCCCGHCAYNTNAAYCCHGYNGTNTAC-3’); antisense primers, WCD-1 (5’-GGCTGCSRNGCWGCN- GCNGGC-3’) and WCD-2 (5’-CCGATTCGCGGDIR1NNHTNNTSNHD- SMNCC-3’). The reverse-transcribed RNA was amplified with Taq DNA polymerase (5 units/μl, AmpliTag; Perkin-Elmer Corp.) by PCR for 30 cycles (1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C) using 0.2 mM dNTP and 1.5 mM MgCl2. The PCR products were re-amplified using 100 pmol of WCU-1 and WCD-1 in a total volume of 50 μl of buffer containing 0.2 mM dNTP and 1.5 mM MgCl2. The PCR products were re-amplified using 100 pmol of WCU-2 and WCD-2. The amplified products of ~360 bp were subcloned into pGEM 3Z vector (Promega Corp., Madison, WI) at the EcoRI/BamHI site and sequenced by a DNA sequence (Applied Biosystems, Perkin-Elmer Corp.) using ~21M13 and M13R dye primers (Perkin-Elmer Corp.). A clone was isolated as a novel aquaporin cDNA with 41% nucleotide sequence identity to a tonoplast aquaporin, γ-TIP (19).

Ribonuclease Protection Assays—The plasmids inserted with the ~360-bp PCR product and a partial sequence of the full-length cDNA described below (315 bp, 701–1015) were linearized with HindIII and EcoRI, respectively, and used as a template for in vitro transcription of [32P]labeled antisense cRNA probe. To detect a housekeeping gene, a rat glyceraldehyde-3-phosphate dehydrogenase cDNA of 123 bp was used as reported previously (21). Total RNA was extracted from rat systemic organs by the acid/guanidinium/phenol/chloroform method, and 10 μg...
of total RNA were hybridized with the \textsuperscript{3}P-labeled probes for 16 h at 48.5 °C. The unhybridized probes were digested with ribonuclease A (4 μg/ml) and ribonuclease T\textsubscript{1} (120 units/ml) for 60 min at 30 °C, and the ribonuclease were digested with proteinase K (0.5 μg/ml) at 37 °C for 30 min. After phenol/chloroform extraction, the hybridized probes were precipitated with ethanol and heat-denatured for electrophoresis on 6% polyacrylamide gels. The gels were exposed to x-ray films for 3 days.

**Northern Blot—**Total RNA (15 μg) isolated from rat liver, colon, kidney, and cerebrum were electrophoresed on a 1.2% formaldehyde gel, transferred to a nylon membrane, and hybridized with a \textsuperscript{3}P-labeled cRNA probe (10\textsuperscript{6} cpm/ml) transcribed from the cloned cDNA (315 bp, 701–1015) at 55 °C in 50% formamide, 5 × Denhardt’s solution, 0.2% SDS, and 5% dextran for 18 h. The membrane was washed twice in 2 × SSC containing 0.1% SDS at 25 °C, twice in 1 × SSC containing 0.1% SDS at 65 °C, and twice in 0.1 × SSC containing 0.1% SDS at 65 °C and was exposed to an x-ray film for 36 h.

**Library Screening—**Since mRNA expression was intense in liver and pancreas by a preliminary ribonuclease protection assay, ~2 × 10\textsuperscript{6} colonies of rat liver cDNA library (Life Technologies, Inc.) transferred to nylon membranes were hybridized with the \textsuperscript{3}P-labeled antisense cRNA probe (10\textsuperscript{6} cpm/ml) for 16 h at 50 °C, and the membranes were washed at 65 °C in 0.1 × SSC containing 0.1% SDS for autoradiography. One positive clone of ~1.4 kb was isolated and sequenced. The 5'-end of the entire cDNA was isolated by rapid amplification of cDNA ends by heminested PCR. Two types of oligonucleotide primer for the 5'-end sequence of the 1.4-kb cDNA were synthesized: gene-specific primers GSP-1 (5'-CCTCCAAACGAGTGACTGCG-3') and GSP-2 (5'-GGTGCACCCGGCTGATGTTCC-3'). Plasmids isolated from the rat liver cDNA library (100 pg) were amplified by PCR using 12.5 pmol of the SP6 promoter primer and the GSP-2 primer for 40 cycles (1 min at 94 °C, 1 min at 47 °C, 1 min at 72 °C). The heminested PCR products were subcloned into pGEM-T vector (Promega Corp.) and sequenced. The longest cDNA obtained by 5'-RACE was ligated to the ~1.4-kb clone to construct the full length of ~1.45-kb cDNA. The hydrophylicity of the putative protein was analyzed by DNAsis software (Hitachi Software Engineering Co., Yokohama, Japan), identities to other aquaporins were searched using the FASTA program (GenomeNet), and phylogenesis was analyzed by PILEUP software (Genetic Computer Group, Madison, WI).

**Expression in Vitro—**In vitro protein synthesis from the cloned cDNA was performed in a cell-free translation system according to the manufacturer's protocol (Promega Corp.) with \textsuperscript{35}S-methionine and canine pancreas microsomes at 30 °C for 90 min. The reaction products were electrophoresed on a 12.5% SDS-polyacrylamide gel and examined by autoradiography (15, 16).

**Oocyte Expression Studies—**A 1.45-kb \textit{Sal}I/BamHI fragment containing the 5'-untranslated sequence and the entire open reading frame of the cloned cDNA was ligated into pSPORT2 vector (Life Technologies, Inc.). Capped eRNA was synthesized in vitro after linearization with \textit{Not} I as described previously (22). \textit{Xenopus laevis} (stage IV–VI) were defolliculated by collagenase and microinjected with 50 nl of eRNA probe (10\textsuperscript{6} cpm/ml) for 16 h at 24 °C. The oocytes were transferred to 70 mosM modified Barth’s buffer at 24 °C, and their swelling was monitored by videomicroscopy. The coefficient of osmotic water permeability (P\textsubscript{f}) was assessed by a real-time quantitative imaging method as described recently (4). The uptake of \textsuperscript{14}C]urea or \textsuperscript{14}C]glycerol was measured by incubating oocytes in 200 mosM modified Barth’s buffer for 10–20 min at 24 °C followed by washing and SDS solubilization. No increase in ion conductance was found when control oocytes were compared to RNA-injected oocytes by the two-electrode voltage clamp method (16). The percentage of amino acid identity between AQP8 and each of the other aquaporins searched by the FASTA program (GenomeNet service) is presented.

**Fig. 1.** Sequence and predicted structure of AQP8. A, nucleotide and deduced amino acid sequences of AQP8. The putative six transmembrane domains are underlined, and an adjacent putative N-glycosylation site is identified (asterisk). Two NPA motifs conserved in the majority of aquaporin members are boxed. A polyadenylation consensus sequence is double-underlined. B, Kyte-Doolittle hydrophy line profile of the deduced amino acid sequence analyzed by DNAsis (Hitachi Software Engineering Co.). C, Kyte-Doolittle hydrophy profile of the deduced amino acid sequence analyzed by DNAsis (Hitachi Software Engineering Co.).
Fig. 2. In vitro translation and functional expression of AQP8. 
A, synthesized protein by a cell-free expression system in the presence of cRNA transcribed from AQP8 cDNA and microsomes. B, osmotic water permeability (Pf) of oocytes injected with 10 ng of AQP8 cRNA or water (50 nl) without cRNA. Shown are the mean values and standard deviations of 3–5 oocytes receiving no further treatment (black bar), or oocytes incubated for 5 min in 1 mM HgCl₂ (stippled bars), or oocytes incubated for 5 min in 1 mM HgCl₂ followed by 30 min in 5 mM β-mercaptoethanol (open bar).

In Situ Hybridization—In situ hybridization was done by a procedure as described previously (23). In brief, 32P-labeled antisense and sense RNA probes were synthesized with T7 and SP6 RNA polymerases using a linearized cDNA template encoding 81–889 nucleotides. Cryostat sections (10 μm thickness) of rat liver, pancreas, salivary gland, and colon were fixed in 4% paraformaldehyde in phosphate-buffered saline and treated with 5 μg/ml proteinase K (Promega Corp.) in 500 mM NaCl, 10 mM Tris-HCl, pH 8.0, for 10 min at room temperature and hybridized with each probe (1 × 10⁶ cpm/section) overnight at 55 °C. After washing in 2 × SSC, 1 mM EDTA, 10 μM β-mercaptoethanol and at room temperature, the sections were treated with 20 μg/ml proteinase A in 500 mM NaCl, 10 mM Tris-HCl, pH 8.0, for 10 min at room temperature and hybridized with each probe (1 × 10⁶ cpm/sec) overnight at 55 °C. After washing in 2 × SSC, 1 mM EDTA, 10 mM β-mercaptoethanol at 55 °C for 4 h and washing in 0.5 × SSC at room temperature. After dehydration, the sections were exposed to photographic emulsion for 5–7 days in the dark at 4 °C, developed, and counterstained with hematoxylin to observe under a bright or dark field microscope.

RESULTS AND DISCUSSION

Isolation and Distribution of the Cloned cDNA—AQP1 and AQP4 cDNAs were mainly amplified from rat pancreas cDNA by PCR using the degenerate primers. However, some were similar but completely different in nucleotide sequence to aquaporin family genes reported previously. Analysis of the PCR product revealed high nucleotide sequence homology to aquaporin family members: AQP1, 34%; AQP2, 40%; AQP3, 36%; AQP4, 34%; AQP5, 37%; γ-TIP, 41%. Preliminary ribonuclease protection assay using the PCR-cloned cDNA as a template showed intense expression of the mRNA in pancreas and liver and weak expression in colon and salivary gland. The expression profile of this gene in systemic organs was apparently distinct from those of other aquaporins.

Cloning of the Full-length cDNA—The ~1.4-kb cDNA was obtained after screening of the rat liver cDNA library at high stringency with the cRNA probe in vitro transcribed with the PCR-cloned cDNA. By the 5'-rapid amplification of cDNA ends method ~150 bp of the 5'-end of the cDNA was isolated and ligated to the ~1.4-kb cDNA. The cDNA sequence was composed of a 101-bp 5'-untranslated sequence preceding an initiation site (Kozak consensus) (24), a 789-bp open reading frame, and a 589-bp 3'-untranslated sequence containing a polyadenylation consensus (Fig. 1A).

Deduced Amino Acid Sequence and Structure—Analysis of the GenBank data base revealed that the cloned cDNA was a new member of the aquaporin family. The open reading frame encodes a polypeptide of 263 amino acids (Mr = ~28,000) with homology to aquaporins: γ-TIP, 37%; AQP2, 37%; MIP, 37%; α-TIP, 35%; AQP1, 34%; AQP5, 33%; AQP4, 30%; AQP3, 26%, evaluated by identity using the FASTA program. Interestingly, the deduced amino acid sequence was highly homologous with the plant aquaporin family (γ-TIP and α-TIP). Kyte-Doolittle hydropathy analysis confirmed six putative membrane-spanning domains (I–VI) and five connecting loops containing two NPA motif sites of NPA motif (Asn-Pro-Ala) preserved in other aquaporin family members (Fig. 1B). The predicted polypeptide has an unusual structure with a longer N terminus (37 residues) and a shorter C terminus (10 residues) compared with those of other mammalian aquaporins. The long N terminus and short C terminus have been observed in plant aquaporin family (γ-TIP and α-TIP). Near the NPA motifs, the deduced amino acid sequence contains a sequence highly conserved in other aquaporins. The gene has been designated aquaporin-8 (symbol AQP8) by the HUGO/GDB Nomenclature Committee (University College of London, London, UK) and registered in the GenBank data base (accession number AF007775). A phylogenetic analysis showed that AQP8 developed in a different branch from other aquaporins (Fig. 1C).

Expression and Water Transport Function—Cell-free translation of the cloned cDNA in the presence of microsomes yielded a major band at ~27 kDa (Fig. 2A), corresponding to the pre-
FIG. 4. In situ localization of AQP8 mRNA in rat pancreas, liver, salivary gland, and colon. Bright field (A–D) and dark field views of sections of pancreas (A–A’), liver (B–B’), salivary gland (C–C’), and colon (D–D’) examined by the antisense (A’–D’) or sense (A”–D”) cRNA probe for AQP8. i, pancreatic islet; p, portal area; cv, central vein; d, excretory duct. Magnification, × 250.
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dicted mass (28 kDa) with slightly more rapid electrophoretic mobility as described previously (16, 25).

Transmembrane water flow through the protein translated from the cloned cDNA was evaluated in Xenopus oocytes. After 36 h of incubation in 200 mmol buffer at 20 °C, the oocytes were transferred in 70 mmol buffer, and swelling of the oocytes was monitored by videomicroscopy. Injection of oocytes with the cRNA synthesized from the cloned cDNA increased the Pf by approximately 14-fold, qualifying the characteristics of the translated protein as a new member of the aquaporin family (Fig. 2B).

The increase in Pf mediated by expression of AQP8 was blocked by treatment of oocytes with 1 mm HgCl₂ and restored by incubation in β-mercaptoethanol similar to most of the already known aquaporins (Fig. 2B). Likewise, oocytes expressing AQP8 exhibited no increase in the membrane transport of [¹⁴C]urea or [¹⁴C]glycerol and no increase in the membrane conductance measured by an electrophysiological method (data not shown).

**mRNA Expression in Systemic Organs**—Northern blot analysis using total RNA extracted from rat liver and colon showed a single band of ~1.5 kb (Fig. 3A), equivalent to the size of other aquaporin family members reported previously (14, 16). No bands were detected in kidney and cerebrum RNA samples. The mRNA expression detected by Northern blot analysis was comparable with the finding obtained by the ribonuclease protection assay, confirming the predominant mRNA expression in pancreas and liver and weak expression in colon and salivary gland (Fig. 3B). The unique distribution of the expression suggested a crucial role(s) of this water channel in those organs.

**In Situ Hybridization**—Intense AQP8 mRNA expression was demonstrated in glandular lobules but not in islets of pancreas by in situ hybridization (Fig. 4A). No significant signals were observed in pancreas with the sense probe. No aquaporin family members except AQP1 have been shown in pancreas although the presence of water channels may be presumed as pancreas secretes a large volume of water. The predominant expression of AQP8 in pancreas suggested an important role of this aquaporin in the secretion of pancreatic juice.

In situ hybridization of the liver showed AQP8 mRNA expression in parenchymal cells and not in the portal area, predicting the expression in hepatocytes. Previous studies showed that osmotic flow of water across hepatocyte membranes occurred mainly via a water channel-independent pathway whereas cholangiocytes transported water probably through AQP1 (26, 27). The observation may be consistent with negligible mRNA expression of aquaporin family members in hepatocyte (26). Contrasting with these studies, a new aquaporin family, AQP8, was isolated from liver cDNA, and its mRNA expression was shown to be intense in liver by ribonuclease protection assay and Northern blot analysis. Furthermore, in situ hybridization predicted the expression of AQP8 mRNA in hepatocytes. The role(s) of AQP8 gene products in liver may be apparent when the subcellular localization is elucidated by immunohistochemistry.

The mRNA expression of AQP8 was also localized in acinar cells in the salivary gland and absorptive columnar epithelial cell layer in colon, suggesting a cooperative role of this aquaporin with other aquaporins in the secretion of saliva and in water absorption.

The AQP8 mRNA expression of narrow tissue distribution may indicate important role(s) of AQP8 in these tissues. Further studies are required to elucidate the cellular and subcellular localization and function of this aquaporin.

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