**Cloning and characterization of porcine aquaporin 1 water channel expressed extensively in gastrointestinal system**

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**INTRODUCTION**

Aquaporins (AQPs) are water channel proteins located on membranes of various cell types where they create high water permeability. So far at least 12 mammalian members of AQP family have been molecularly localized in diverse fluid transporting tissues have been molecularly identified. Recent studies on human subjects with AQP gene mutations using transgenic AQP knockout mice indicate that AQP have important functions in multi-tissue physiology and pathophysiology[1, 2]. In the gastrointestinal (GI) system, several aquaporins (including AQPs 1, 3-5, 7-10) are localized on various epithelial and endothelial cell membranes of human and rodent GI organs and provide a trans-cellular pathway for fast water movement during fluid secretion and absorption[3-6]. Studies on AQP knockout mice demonstrated that AQP5 plays a role in saliva secretion[7], AQP1 a role in dietary fat processing, and AQP4 role in colonic fluid absorption and fecal dehydration[8]. On the other hand, localization of AQPs permeability that is inhibitable by HgCl₂ was detected in porcine erythrocytes and CHO cells stably transfected with pAQP1 cDNA. Immunoblot analysis of porcine erythrocytes and pAQP-transfected CHO cells revealed an unglycosylated 28 ku band and larger glycosylated proteins.

**CONCLUSION:** pAQP1 is the first porcine aquaporin that can be molecularly identified so far. The broad distribution of pAQP1 in epithelium and endothelium of porcine digestive organs may suggest an important role of channel-mediated water transport in fluid secretion/absorption as well as in digestive function and pathophysiology of the gastrointestinal system.

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**Key words:** Aquaporin; Molecular cloning; Porcine gastrointestinal organs; Water transport; Digestive function

**RESULTS:** An 813 bp cDNA encoding a 271 amino acid porcine aquaporin (designated pAQP1) was cloned from liver mRNA (pAQP1 has a 93% identity with human AQP1 and contains two NPA motifs conserved in AQP family, one consensus sequence for N-linked glycosylation, and one mercury-sensitive site at cysteine 191). RT-PCR analysis revealed extensive expression of pAQP1 mRNA in porcine digestive glands and gut. Northern blot showed a single 3.0 kb transcript in selected digestive organs. pAQP1 protein was localized at central lacteals of the small intestine, microvessels of salivary glands, as well as epithelium of intrahepatic bile ducts by immunoperoxidase. High osmotic water

**METHODS:** A PCR-based cloning strategy and RACE were used to clone full-length AQP coding sequence from reversely transcribed pig liver cDNA. Stopped-flow light scattering and a YFP-based fluorescence method were used to measure the osmotic water permeability of erythrocytes and the stably transfected CHO cells. RT-PCR, Northern blot, and immunohistochemistry were used to determine the gastrointestinal expression and localization of cloned AQPs. Protein expression in transfected cells and red blood cells was analyzed by Western blot.

**AIM:** To clone and characterize the porcine aquaporins (AQPs) in the gastrointestinal system.

**RESULTS:**

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in some mouse GI organs does not indicate physiological importance. For example, AQP4 deletion in gastric parietal cells does not affect gastric acid secretion\(^\text {[10]}\), AQP1 deletion in micro-vessels of salivary glands does not affect saliva secretion, AQP1 deletion in intra-hepatic bile ducts does not affect the flow rate and components of bile\(^\text {[8]}\). GI phenotype in AQP1-null human subjects has not been reported so far, which may indicate species differences of AQPs in GI organs of mammalian species other than in those of human and rodents have been poorly studied. The pig GI system more resembles the human GI system both structurally and physiologically in model animals\(^\text {[11]}\). However, no porcine AQP member has been identified so far. In the present study, we cloned the first porcine aquaporin, pAQP1, from pig liver, by a PCR-based homologous cloning strategy. Its functional properties and distribution in pig GI system were analyzed.

### MATERIALS AND METHODS

#### cDNA cloning of pAQP1

Full-length coding sequence of pig AQP1 mRNA was cloned using a PCR-based homologous cloning strategy\(^\text {[12]}\). Total RNA was extracted from pig liver using TRIZOL reagent (Invitrogen). mRNA was isolated from the total liver RNA using an Oligotex mRNA kit (Qiagen). cDNA was reversely transcribed from the mRNA using a first strand cDNA amplification kit (Invitrogen) and used as template for 30 cycles of PCR amplification at 94 °C for 30 s, at 55 °C for 30 s, at 72 °C for 1 min with degenerate oligonucleotide primers designed according to amino acid sequences around the two NPA motifs of aquaporin family: sense: 5'-CA(C-T)IT(CA)AA(CT)CCGGTGGTAC-3'; antisense: 5'-(G-C) G I (A G) I (A -G)(AT)GC(TG)IGC(GG)TT-3'. PCR products were subcloned into a PCR-based homologous cloning vector and sequenced. The longest open reading frame was designated as pAQP1.

#### RT-PCR and Northern blot

Total RNA was extracted from pig salivary glands, liver, pancreas, esophagus, stomach, small intestine and colon respectively, using TRIZOL reagent. For RT-PCR analysis, cDNAs were reversely transcribed from 5 µg each of total RNA using first strand cDNA amplification kit and 30 cycles of PCR amplification were performed at 94 °C for 30 s, at 62 °C for 30 s, at 72 °C for 2 min with primers flanking the coding sequence of pAQP1 (sense: 5'-CG-GATCCATGGCCACGAGTTCAAGAG-3'; antisense: 5'-GCTCTAGATTCTGGGTTCATCTCCACC-3'). For Northern blot, total RNA (20 µg) from liver, salivary gland, small intestine and colon was resolved on 1.2% formaldehyde gel and transferred to a Hybond-N+ nylon membrane (Amersham). The membrane was hybridized at 68 °C for 1 h in a rapid hybridization buffer (Amersham) and then hybridized for 1 h with a 32P-labeled probe corresponding to the full-length pAQP1 coding sequence. The membrane was washed three times in 0.2 x SSC, 0.1% SDS at 68 °C, each for 15 min, and autoradiographed for 16 h with double intensifying screens. The membrane was boiled in water and re-probed with a 600 bp pig β-actin cDNA sequence.

#### Transfection and water permeability measurement of pAQP1 in CHO cells

A cell-based fluorescence microassay employing a Cl\(_{-}\)-sensitive EYFP mutant\(^\text {[13]}\) was developed to measure the channel-mediated water permeability of plasma membrane. The 720 bp EYFP-H148Q-V163S DNA fragment encoding the Cl\(_{-}\)-sensitive EYFP mutant was PCR-amplified using Vent DNA polymerase (New England Biolabs) and primers with engineered restriction sites HindIII (5' primer) and XbaI (3' primer). The fragment was digested with HindIII/XbaI and ligated to the mammalian expression vector pcDNA3.1 Hygro (hygromycin-resistant, Invitrogen) predigested with HindIII/XbaI to form expression plasmid pcDNA3.1 Hygro-EYFP-H148Q-V163S. For pAQP1 expression, the 813 bp pAQP1 full length coding sequence was PCR-amplified using Vent DNA polymerase and primers with engineered restriction sites BamHII (5' primer) and Xhol (3' primer). The amplified pAQP1 fragment was digested with BamHII/Xhol and ligated to the mammalian expression vector pcDNA3.1 (+) (G418-resistant, Invitrogen) predigested with BamHII/Xhol to form expression plasmid pcDNA3.1 pAQP1.

CHO-K1 cells were first transfected with expression plasmid pcDNA3.1Hygro-EYFP-H148Q-V163S using Lipofectin reagent (Invitrogen). After selection by hygromycin (Roche) at 500 µg/mL, highly expressed CHO clones were isolated by selecting survival cell colonies under fluorescence microscope. The YFP-expressing cell clone, CHO-K1/EYFP-H148Q-V163S, was then transfected with pcDNA3.1 pAQP1 and selected with G418 (Invitrogen) at 500 µg/mL. pAQP1 expression in surviving CHO cell clones was analyzed by immunoblot. CHO cell clones with high cytoplasmic expression of Cl\(_{-}\)-sensitive EYFP mutant and plasma membrane expression of pAQP1 were selected for water permeability measurement.
For water permeability measurement, the CHO-K1/ EYFP-H148Q-V163S/pAQP1 cells were plated in 96-well clear bottom of black-walled microplates (Costar) at the density of 20000 cells per well in F-12 Ham’s medium supplemented with 10% FBS and 0.5 mg/mL G418. After incubation at 37 °C in an atmosphere containing 5% CO₂ and 95% air with 90% humidity for 18 h, the cells in 96-well plates were washed twice in PBS buffer (200 μL/wash), leaving 100μL of cell suspension for measurement. Water permeability was determined spectrophotometrically. Protein concentrations in CHO cell and ghost lysates were determined by the method of Lowry et al. [14].

Measurement of erythrocyte water permeability

Pig blood was drawn from the ear by venipuncture, and the washed erythrocytes were diluted into phosphate buffered saline (PBS) at a 0.1 vol %. Osmotic water permeability was measured by a stopped-flow light scattering technique in which the diluted erythrocyte suspension was mixed rapidly with PBS containing 100 mmol/L sucrose.[12] Erythrocyte volume was measured continuously by 90 s light scattering for 90 s at a 520-nm wavelength.

**Immunohistochemistry**

Paraffin-embedded blocks were prepared from fresh normal pig salivary gland, liver, and small intestine fixed in 4% formalin. For immunohistochemistry, 3-μm sections of pig tissues were prepared by standard procedures and exposed to the primary affinity-purified rabbit-antirat AQ1 antibody (AB3065, Chemicon International) diluted at 1:1000 for 1 h at room temperature. In some experiments, the primary AQ1 antibody was preincubated with the immunizing peptide before administered to the tissue sections. After washed, the sections were exposed to a horseradish peroxidase-conjugated goat antirabbit IgG (A6154, Sigma) secondary antibody at 1:2000 dilution followed by development with 3,3'-diaminobenzidine (DAB) liquid substrate dropper system (D7679, Sigma). The sections were counterstained with haematoxylin as the last step.

**RESULTS**

**Cloning and analysis of pAQP1 cDNA**

The full-length cDNA coding sequence and the predicted amino acid sequence of pAQP1 (Genbank accession no. AY585335) are shown in Figure 1A. The 813 bp open reading frame encoded a 271-amino acid protein (Costar) at the density of 20000 cells per well in F-12 Ham’s medium supplemented with 10% FBS and 0.5 mg/mL G418. After incubation at 37 °C in an atmosphere containing 5% CO₂ and 95% air with 90% humidity for 18 h, the cells in 96-well plates were washed twice in PBS buffer (200 μL/wash), leaving 100μL of cell suspension for measurement. Water permeability was determined spectrophotometrically. Protein concentrations in CHO cell and ghost lysates were determined by the method of Lowry et al. [14].

**Western blotting**

Stably transfected CHO cells were lysed in 10 mM HEPES buffer containing 0.5% SDS, 100 mmol/L DTT and 57.4 μmol/L PMSF (pH 7.5). Red blood cells from pigs, human beings and mice were first burst in hypotonic solution, and then the ghosts were centrifuged and lysed in SDS buffer. Protein concentrations in CHO cell and ghost lysates were determined spectrophotometrically. For immunoblotting, 20 μg proteins from the CHO cell lysate or 5 μg proteins from the ghost lysate of red blood cells from pigs, human beings and mice were dissolved in protein sample buffer, heated at 65 °C for 10 min, and then resolved on 12% SDS-PAGE minigels. Proteins were blotted into PVDF membranes, blocked for 1 h, washed with TBS-T (pH 7.4), and incubated for 1 h at room temperature with anti-AQP-1 antibody diluted at 1:1000. After washed, membranes were incubated with HRP-conjugated goat anti-rabbit IgG (1:3000, Sigma), and immunoreactive sites in membranes were revealed by enhanced chemiluminescence (Amersham).
pAQP1 had a 29 - 52% similarity in amino acid sequences to other mammalian aquaporin members[15, 16]. There were two consensus sequences for phosphorylation by protein kinase-C like in human and mouse AQP1. However, pAQP1 contained only one consensus site at residue 42 for N-linked glycosylation compared to two sites in human and mouse AQP1 sequences at residues 47 and 48 instead of the human and mouse sequences.

Expression and immunolocalization of pAQP1 in digestive organs

Multitissue RT-PCR analysis indicated broad expression of pAQP1 mRNA in pig digestive organs including salivary glands, pancreas, liver and different segments of the gut (Figure 3A). Figure 3B shows Northern blot analysis of total RNA from pig salivary glands, liver, small intestine, and colon respectively using the full length pAQP1 cDNA as a probe. A single transcript of about 3.0 kb was seen in liver > small intestine > colon > salivary glands. Figure 4 shows an immunoperoxidase staining of pAQP1 in selected digestive organs. Specific AQP1 labeling was seen both in the endothelia of central lacteals in villi of the small intestine (Figures 4A and 4B) and in the endothelia of periductal microvessels in salivary glands (Figure 4C). In the liver, AQP1 staining was mainly seen both in the endothelia of central lacteals in villi of the liver and in the endothelia of periductal microvessels. A consecutive section of E showing immunostaining with AQP1 antibody preabsorbed with the immunizing peptide (F).

Functional characterization of pAQP1

Since AQP1 is a erythrocyte water channel, osmotic water permeability of pig red blood cells was analyzed using stopped-flow light scattering and compared with human and mouse red blood cells. Figure 5A shows the time course of osmotic cell shrinking in response to a 100 mmol/L inwardly directed osmotic gradient.
Two contiguous time scales were used to plot the full
time course of decreasing cell volume (increasing light
intensity). The computed osmotic water permeability of
pig, human and mouse erythrocytes is summarized in
Figure 5B. The pig erythrocyte membrane showed a high
osmotic water permeability that was inhibited > 90% by
0.3 mmol/L HgCl₂, similar to that of human and mouse
erythrocytes. Water channel function of pAQP1 was also
analyzed in heterologous expression system. Figure 5C
shows the time course of YFP fluorescence of CHO cells
stably transfected with pAQP1 cDNA in response to extra-
cellular hypotonicity (50% PBS). The t1/2 of fluorescence
dynamics for pAQP1-transfected cells was 1.2 s compared
to 4.6 s for the same cells incubated with 0.1mmol/L
HgCl₂ and > 20 s for mock-transfected cells, indicating
that a high membrane water permeability was mediated by
pAQP1 water channel inhibited by mercurial compounds.
Figure 5D shows Western blot analysis of red blood cells
and pAQP1-transfected CHO cells using an affinity-
purified AQP1 antibody. An unglycosylated 28 ku band
glycosylated proteins at a higher molecular weight were
determined both in red blood cells of human beings, pigs,
wildtype mice and in pAQP1-transfected CHO cells, but
missed in AQP1-knockout mouse erythrocytes. There was
a significant decrease of glycosylated protein in pAQP1
compared to human AQP1 and mouse AQPI in red blood
cells. Western blot analysis of pig gastrointestinal tissues
was not done because of the difficulty to remove red
blood cell contamination.

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Figure 5 Functional properties of pAQP1 in erythrocytes and stably transfected CHO cells. A. Osmotic water permeability of pig, human and mouse erythrocytes measured by stopped-flow light scattering from the time course of erythrocyte volume in response to a 100 mmol/L inwardly directed sucrose gradient in the absence (left panel) and presence (right panel) of 0.3 mmol/L HgCl₂. B. Summary of osmotic water permeability coefficient (Pf) for erythrocytes from pig, human and mouse measured in A (mean ± SE, n=4); C. Osmotic water permeability of CHO cells stably transfected with pAQP1 cDNA measured by YFP-based fluorescence assay. Water permeability of CHO cells was expressed as half time (t1/2) needed from water injection to the point when the cytoplasmic fluorescence reached the maximum. t1/2 of pAQP1 transfected CHO cells in the absence and presence of 0.1 mmol/L HgCl₂ was 1.2 s and 4.6 s separately. t1/2 > 20 s in mock-transfected CHO cells; D. Immunoblot of erythrocytes and transfected
CHO cells. Lanes 1-4: erythrocytes from AQPI-/- mice, AQPI +/+ mice, pigs and human beings. Lane 5: CHO cells stably transfected with pAQP1 cDNA.
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