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# A water channel closely related to rat brain aquaporin 4 is expressed in acid- and pepsinogen-secretory cells of human stomach**

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**Abstract We isolated a cDNA clone encoding a water channel protein, aquaporin (AQP), from human stomach. The encoded protein consisted of 323 amino acid residues, containing six putative transmembrane domains. The protein was designated human aquaporin 4 (hAQP4) because of its 94% sequence similarity to rat brain AQP4. Expression of hAQP4 cRNA in *Xenopus* oocytes resulted in a significant increase in osmotic water permeability, indicating that this protein functions as a water channel. Northern blot analysis demonstrated a strong signal of hAQP4 mRNA in brain, lung, and skeletal muscle as well as in stomach. Immunohistochemical experiments with human stomach tissues showed that hAQP4 as a protein is expressed mainly in cells located in the glandular portion of the fundic mucosa. These include chief cells which secrete pepsinogen and parietal cells which secrete hydrochloric acid. These results strongly indicate that hAQP4 is a principal factor involved in the osmotic regulation of pepsinogen and acid secretion in the stomach.**

*Key words:* Aquaporin; Water channel; Human stomach; Gastric parietal cell; Gastric chief cell

## 1. Introduction

Water channels are known to be protein molecules that mediate water transport into and out of cells [1–3]. These are divided into several subtypes in terms of primary structures, pharmacological sensitivities, and abilities for transport of non-ionic small solutes such as urea and glycerol [4]. To date, several water channel molecules have been identified. These include aquaporin 1 (AQP1) [5–7], AQP2 [8], AQP3 [9,10], AQP4 [11], and AQP5 [12], all of which show a high degree of similarity to one another.

Expression patterns of aquaporins in organs or tissues are quite different from one another; AQP1 is expressed in a wide variety of organs and tissues including kidney, eye, spleen, lung, and choroid plexus [13–17], AQP2 exclusively in the renal collecting duct [18,19], AQP3 in the kidney, colon, and small intestine [9,10], AQP4 in rat brain [11], and AQP5 in salivary glands [12]. These observations suggested that different species of aquaporin exhibit different organ-specific functions.

In mammalian stomach, a large quantity of gastric juice is secreted, so it is probable that water channels are deeply involved in its active exocrine function. However, there has been little information in this respect. Although AQP1 is known to be expressed in various tissues and organs [13–17], it has not been detected in stomach [20,21].

The purpose of this study is to disclose the structure and function of water channels expressed in the stomach. Here we report the cDNA cloning and molecular characterization of a human stomach water channel that may take part in the secretion of digestive fluids.

## 2. Materials and methods

### 2.1. RT-PCR

Human stomach mRNA (1 μg; Clontech) was reverse-transcribed using oligo-dT primer and a first-strand cDNA synthesis kit (Pharmacia). The three oligonucleotide primers: *W-1* (5′-CA(CT)IT(CA)-AA(CT)CCIGCIGTIAC-3′), *W-2* (5′-AGIGGIG(GC)ICACI-(TC)CAA(CT)CC-3′), and *W-A* (5′-CCIA(CT)CCA(AG)(AT)-AIA(CT)CCA(AG)TG-3′), were prepared according to the amino acid sequences H(IL)NPAVT, SG(AG)H(SIA)NP, and HW(IV)-(FY)W(IV)G commonly conserved in aquaporin family members [22]. PCR amplification was performed using degenerate primers (1 mM each) and reverse-transcribed cDNA as a template (1 min at 96°C, 1 min at 50°C, 3 min at 72°C, 30 cycles). A band of about 400 bp was isolated by agarose gel electrophoresis and subcloned into pUC 18 vector. The insert DNA fragment was sequenced using a 373A DNA sequencer (Perkin Elmer). 5′-RACE was carried out to determine the nucleotide sequence of the 5′-end as follows [23]. Human stomach mRNA (1 μg) was reverse transcribed into cDNA using an oligonucleotide primer, *W-B* (5′-TTTCCCAT-GATAAC-3′), derived from the PCR fragments. An oligo(dA)-tail was added by terminal deoxynucleotidyltransferase. The (dA)-tailed cDNA was amplified using an oligo(dT) primer, *W-T* (5′-CGGCTGCAGTTTTTTTTTTTTTTTTTT-3′), and a specific primer in the PCR fragment, *W-C* (5′-GCAGGTCCAAAGGATCGG-3′). For 3′-RACE, oligo-dT primed human stomach cDNA was amplified using a specific primer in the PCR fragment, *W-3* (5′-CTGTCTTCTACATCGCAG-3′), and W-T (above).

### 2.2. Construction of a cDNA Library and cDNA cloning

Double-strand cDNA was synthesized from human stomach mRNA (2 μg) using a random primer and inserted into λgt10 phage vector. One million plaques from a constructed human stomach cDNA library were screened with a probe of a PCR fragment labeled with [α-$^{32}$P]dCTP. Hybridization was performed at 60°C, and washing at 60°C with 1×SSC containing 0.1% SDS. Positive clones were subcloned into pBluescript KS vector and sequenced. The sequence was determined on both strands.

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**The nucleotide sequence data reported in this paper has been submitted to the GenBank/EMBL/DDBJ Data Bank with accession number D63412.

*Abbreviations:* AQP, aquaporin; hAQP4, human aquaporin 4; MIWC, mercurial-insensitive water channel; nt, nucleotide(s); *Pf* coefficient of osmotic water permeability; RACE, rapid amplification of cDNA ends.


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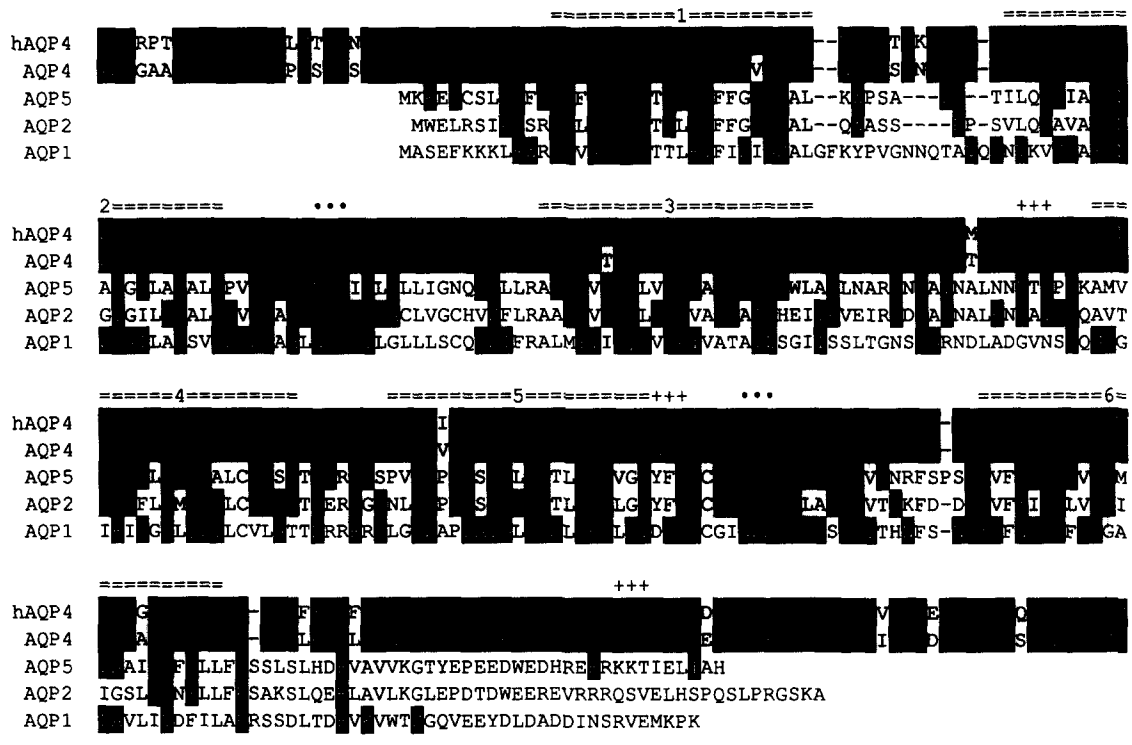


Fig. 1. Alignment of the amino acid sequences of hAQP4 and other known aquaporins: AQP4 [11]], AQP5 [12], AQP2 [8], and AQP1 [7]. Gaps are inserted to maximize matching. White letters in black boxes denote amino acid residues identical to hAQP4. Predicted transmembrane domains of the hAQP4 protein are overlined (double dashed lines). The consensus sites for *N*-glycosylation are denoted by plusses (+). The conserved NPA motifs are denoted by closed circles.

*2.3. Expression and function assays*

The fragment containing the entire coding sequence of the mRNA, which corresponded to the nucleotide sequence from −3 to +1102 nt, was amplified from subcloned cDNA by PCR using *W-4*, 5′-GGCATGAGTGACAGACCC-3′ (underlined ATG indicating the initiation methionine), as the sense primer and *W-D*, 5′-CCTCTAGACTGAGTAATATGACATG-3′ (underlined sequence indicating a *Xba*I site), as the antisense primer. The PCR product containing the entire open reading frame of hAQP4 was digested with *Xba*I and subcloned into the *Stu*I-*Xba*I site of pCS2$^+$ (kindly supplied by Dr. Z. Honda), which contained the 5′ region of *Xenopus* β-globin mRNA. The resultant plasmid, designated pCShAQP4, was digested with *Not*I, and capped mRNA was synthesized in vitro using SP6 RNA polymerase. Fifty nl of cRNA solution (1 μg/μl) or an equal volume of water as a negative control was microinjected into defolliculated *Xenopus* oocytes (stage V-VI). After incubation for 72 h in 200 mosM modified Barth's solution (MBS) at 18°C, the oocyte water permeability (*Pf*) was determined by measuring the rate of initial swelling of the oocyte in response to a 3-fold dilution of MBS with water at 20°C as described by Preston et al. [24]. Oocyte swelling was monitored every 10 s. The measurements of the uptake of urea and glycerol into the oocytes were performed as described by Ishibashi et al. [9].

*2.4. Northern blot analyses*

A 5 μg portion of human stomach poly(A)$^+$ RNA was electrophoresed in an agarose/formaldehyde gel and transferred to a nylon filter. For Northern blot analysis of other tissues, a human multiple Northern blot (Clontech) was used. Filters were hybridized with a $^{32}$P-labeled cDNA fragment of hAQP4 at 42°C for 16 h with 100 μg/ml denatured salmon sperm DNA in 10×Denhardt's solution, 2% SDS, 5×SSPE, 50% deionized formamide, and washed in 0.1×SSC containing 0.1% SDS at 50°C. The filters were also hybridized under the same conditions with an AQP1 cDNA fragment (+1 to +810 nt) [7] amplified from stomach cDNA as a template using a pair of primers, 5′-GCGGATCCATGGCCAGCGAGTTCAAG-3′ and 5′-GCGAATTCCTATTTGGGCTTCATCTC-3′ (*Bam*HI and *Eco*RI sites underlined).

*2.5. Immunohistochemistry of hAQP4 protein*

The hAQP4 COOH-terminal decapeptide, DQSGEVLSSV, was synthesized and a cysteine residue was added to the $NH_2$-terminal in order to conjugate the peptide to keyhole limpet hemocyanine. Rabbit antiserum was immunized with the resultant undecapeptide conjugated to hemocyanine. The antibody was affinity-purified by passage of the antiserum through a peptide column prepared by conjugating the same antigen peptide to epoxy-activated Sepharose 6B (Pharmacia). Surgically obtained human stomach tips were fixed in Bouin's solution fixative (Sigma), dehydrated in a graded series of ethanol, and embedded in paraffin. Sections (4 μm in thickness) were pretreated with hydrogen peroxide (3 μl/ml in methanol) for 30 min, blocked with normal goat serum (1 : 20 in PBS) for 30 min, and immunoreacted with diluted affinity purified IgG (in PBS) overnight. The immunoreacted sections were treated with a biotinylated secondary antibody (goat anti-rabbit IgG, 1 : 100 in PBS) for 30 min, and incubated with an avidin-biotin-peroxidase complex (Vector Laboratories) for 30 min at room temperature. The signals were developed by treatment with 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in 10 mM sodium phosphate (pH 7.4). The sections were examined under a light microscope.

## 3. Results

*3.1. cDNA cloning of human stomach aquaporin (hAQP4)*

We first tried to obtain cDNA fragments encoding human stomach aquaporins by RT-PCR. The use of primer pairs, W-1 and W-A or W-2 and W-A, yielded distinct fragments about 400 bp in length. Since the length was thought to be reasonable in light of the known sequences of aquaporins, we determined the nucleotide sequences of these RT-PCR fragments. As a result, only one nucleotide sequence was obtained that encoded a protein highly similar in partial amino acid se-

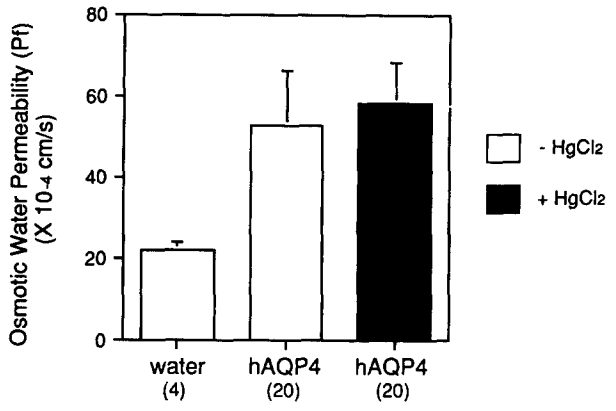


Fig. 2. Function of hAQP4 in *Xenopus* oocytes. *Xenopus* oocytes were injected with 50 nl of water or 50 ng of the cRNA for hAQP4. Osmotic water permeability (*Pf*) was measured 72 h after injection. Swelling was monitored at 20°C. When indicated, the assay was performed after 5 min incubation in 1 mM $HgCl_2$ (black bars). Each bar shows the mean and the standard deviation. The numbers of oocytes subjected to *Pf* measurement are shown in parentheses.

quence to rat AQP4 expressed in brain [11] and rat MIWC expressed in lung [25].

To obtain a full-length cDNA corresponding to the RT-PCR clone, a random-primed human stomach cDNA library of $10^6$ plaques was screened. Simultaneously, 5′- and 3′-RACE were performed. The nucleotide sequence of the obtained cDNA clone, termed λcW4, contained a total coding region of 969 bp (323 amino acid residues). From 5′-RACE analysis, the nucleotide position at −20 was concluded to be located near the 5′-end of the mRNA.

### 3.2. Sequence homology and overall structure

The protein encoded by the cDNA clone most resembled rat brain AQP4 [11], with 94% similarity in amino acid sequence. Thus, the encoded protein was designated human aquaporin 4 (hAQP4). As shown in Fig. 1, hAQP4 shows significant similarities to other known aquaporins: 54% to AQP5 [12], 49% to AQP2 [8], 47% to AQP1 [7], and 30% to AQP3 [9]. hAQP4 contains six putative transmembrane domains, with both the $NH_2$ and COOH termini located in the cytosol (Fig. 1). The amino acid sequence includes two NPA (Asn-Pro-Ala) motifs that are known to be present in all aquaporins [22]. Of three potential *N*-glycosylation consensus sites located in the middle region, Asn-153 is conserved in AQP2 [8] and AQP5 [12], while the others, Asn-206 and Asn-283, are specific to hAQP4 and AQP4 [11].

### 3.3. Water permeability evoked by hAQP4 protein expressed in Xenopus oocytes

To evaluate the function of hAQP4, the mRNA coding region for the hAQP4 protein was injected into *Xenopus* oocytes and expressed. Following cRNA injection (50 ng), the oocytes were incubated for 72 h and then subjected to a hypotonic medium. Swelling was monitored and the coefficient of osmotic water permeability (*Pf*) was calculated. As shown in Fig. 2, expression of hAQP4 induced an increase in *Pf* from $22 \pm 1.8 \times 10^{-4}$ cm/s (control) to $52 \pm 13 \times 10^{-4}$ cm/s; this increase was statistically significant ($P < 0.05$). The *Pf* values were not inhibited by $HgCl_2$ in the hAQP4-expressing oocytes (Fig. 2). Moreover, hAQP4-expressing oocytes exihibited no increase in the uptake of [$^{14}$C]urea or [$^{14}$C]glycerol over that of control oocytes (data not shown). These data demonstrate that hAQP4 functions as a water channel in *Xenopus* oocytes.

### 3.4. Tissue-specific expression of hAQP4 mRNA

The cDNA insert was used as a probe to evaluate the expression of hAQP4 mRNA in human stomach. As shown in Fig. 3A, a major band was detected at approximately 5.5 kb and two minor bands were detected at 3.7 and 1.4 kb. Also, hAQP4 mRNA was expressed clearly in peripheral tissues of the brain, lung, and skeletal muscle, and, to a much lesser extent, in those of ovary; no expression was detected in other organs including kidney, small intestine, and colon (Fig. 3A). On the other hand, AQP1 mRNA was expressed in nearly all tissues as well as in stomach (Fig. 3B). Thus, different aquaporin mRNAs show different tissue-specific distributions, whereas, at least in humans, hAQP4 is expressed distinctly, if not exclusively, in the stomach.

### 3.5. Immunohistochemical analysis of hAQP4 protein in stomach

To obtain precise information about the localization of the hAQP4 protein in human stomach, immunohistochemical experiments were carried out using a polyclonal antibody raised against the COOH-terminal decapeptide of hAQP4. Strong signals were found distributed along the glandular base region of the fundic gland, but not in the epithelial regions facing the

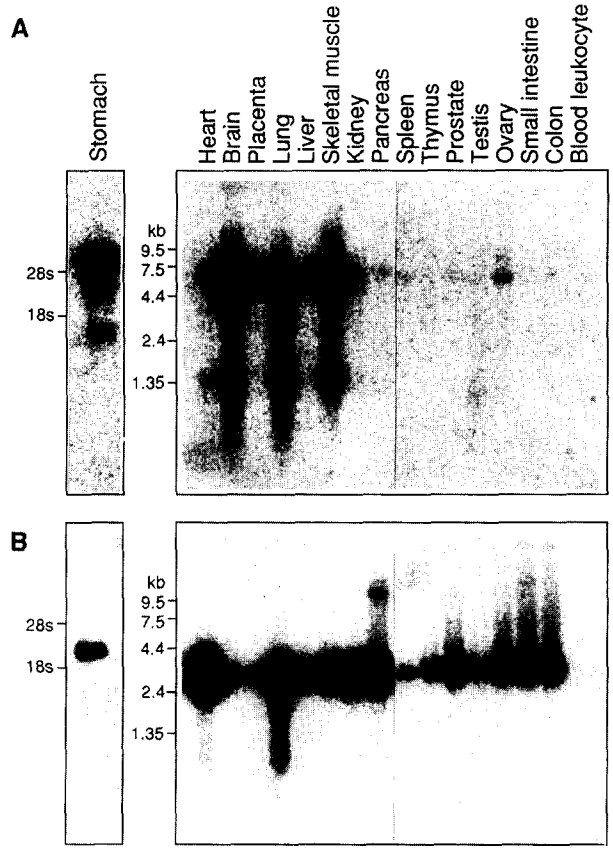


Fig. 3. Northern blot analysis of hAQP4. Human poly(A)$^+$ RNA (5 μg for stomach; 2 μg for other tissues) was hybridized with $^{32}$P-labeled probes corresponding to the open reading frame of hAQP4 cDNA (A) or AQP1 cDNA (B). The positions of the markers used are indicated.

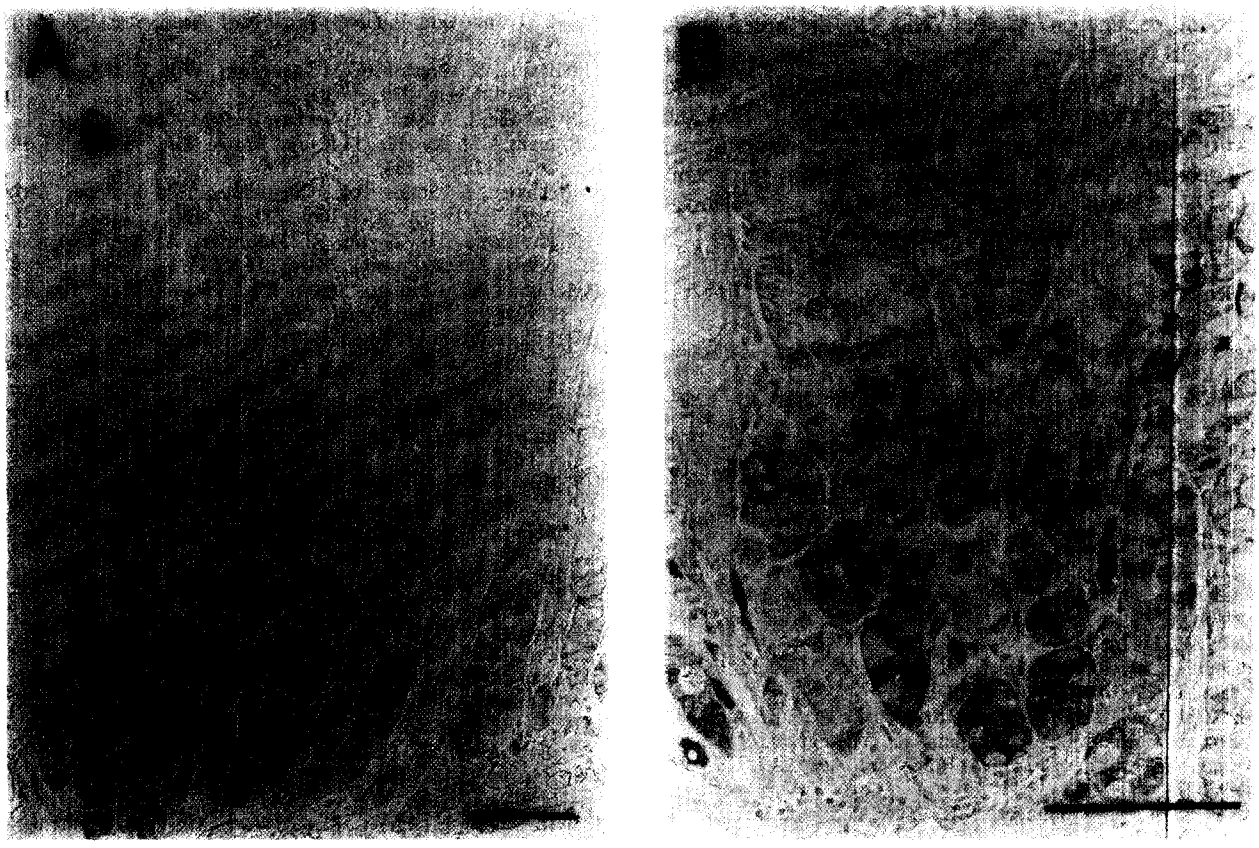

Fig. 4. Immunostaining of human stomach tissue samples using hAQP4 antibody. Human stomach sections were stained using an affinity-purified anti-hAQP4 antibody. (A) Low-magnification micrographs showing the fundic gland mucosa of human stomach. (B) High magnification micrographs showing the base region of the fundic gland. (Bars=15 μm) (A) Section stained using anti-hAQP4 antibody and observed at low magnification. Strong signals are distributed along the glandular base region of the fundic gland, but not in the epithelial or glandular neck regions. (B) Glandular base regions observed at high magnification, with hAQP4 protein detected mainly in chief cells and with scattered signals clearly distributed in the basolateral membrane of parietal cells. c, gastric chief cell; gl, gastric lumen; and p, parietal cell.

gastric lumen or in the glandular neck regions (Fig. 4A). No signal was observed when control serum was used or when the antibody was preabsorbed with antigen peptide (data not shown). The major staining regions contained gastric chief cells (Fig. 4B), which secrete pepsinogen. Strong but scattered signals were also found in a specific region of the cell surface of parietal cells (Fig. 4B), which secrete hydrochloric acid. In some cases, the signals in parietal cells seemed to face the basolateral membrane.

## 4. Discussion

In the present study, we isolated a cDNA clone encoding a water channel, hAQP4, expressed in human stomach. As shown in Fig. 1, hAQP4 shows 94% similarity to AQP4 expressed in rat brain [11], with much less similarity (about 30–50%) to other aquaporins. Thus, hAQP4 expressed in human stomach is believed to be a structural counterpart of rat AQP4.

Expression of the hAQP4 mRNA was detected in stomach, brain, lung, and skeletal muscle (Fig. 3A). In all tissues where hAQP4 was expressed, three bands of hAQP4 mRNA were detected (Fig. 3A). Since genomic Southern analysis indicated that hAQP4 gene is a single copy in the human genome (data not shown), the multiple bands probably result from alternative splicing. As mentioned above, hAQP4 is not as widely distributed as AQP1 but rather expressed in tissue-specific manner; its expression in the gastrointestinal tract is limited to the stomach.

We demonstrated that the mRNA for both hAQP4 and AQP1 are expressed in human stomach (Fig. 3A,B). Ishibashi et al. [9] showed that AQP3 mRNA is also expressed in stomach. However, when RT-PCR was carried out using several oligonucleotide primers corresponding to amino acid sequences conserved in all known aquaporins, we obtained RT-PCR clones coding only for hAQP4. On the other hand, no immunohistochemical data are available confirming the presence of AQP1 [20,21]. In our experiments using hAQP4 antibody, the localization of the hAQP4 protein was clearly demonstrated (Fig. 4). Taken together, it appears likely that hAQP4 protein occurs more abundantly than other aquaporins in stomach.

Next, we considered the relationship between hAQP4 protein distribution in stomach and water channelling function. In stomach, gastric juice is secreted at a rate of about 2 liters per day, so it is probable that some water transport system is involved in this secretion. There are many fundic glands in the mucosa of the stomach body where major stomach functions are exerted. Fundic glands are composed of several types of cells including chief, parietal, mucous neck, and endocrine cells. The major immunoreactive signals of hAQP4 are observed in chief cells (Fig. 4B) which secrete pepsinogen to be converted autocatalytically into pepsin at low pH. It is thus postulated that the function of hAQP4 is also related to the

secretion of pepsinogen in chief cells. On the other hand, parietal cells secrete hydrochloric acid by the action of $H^+,K^+$-ATPase. In basolateral membranes, hAQP4 seemed to be distributed sporadically (Fig. 4B). There is thus the possibility that hAQP4 is involved in water transport driven by acid secretion that causes an osmotic gradient. In conclusion, hAQP4 is believed to function as a major water channel involved in acid and pepsinogen secretion in stomach.

## References


[1] Agre, P., Sasaki, S. and Chrispeels, M.J. (1993) Am. J. Physiol. 265, F461.
[2] Knepper, M.A. (1994) Proc. Natl. Acad. Sci. USA 91, 6255–6258.
[3] Chrispeels, M.J. and Agre, P. (1994) Trends Biol. Sci. 19, 421–425.
[4] Agre, P., Brown, D. and Nielsen, S. (1995) Curr. Opin. Cell Biol. 7, 472–483.
[5] Denker, B.M., Smith, B.L., Kuhajda, F.P. and Agre, P. (1988) J. Biol. Chem. 263, 15634–15642.
[6] Smith, B.L. and Agre, P. (1991) J. Biol. Chem. 266, 6407–6415.
[7] Preston, G.M. and Agre, P. (1991) Proc. Natl. Acad. Sci. USA 88, 11110–11114.
[8] Fushimi, K., Uchida, S., Hara, Y., Hirata, Y., Marumo, F. and Sasaki, S. (1993) Nature 361, 549–552.
[9] Ishibashi, K., Sasaki, S., Fushimi, K., Uchida, S., Kuwahara, M., Saito, H., Furukawa, T., Nakajima, K., Yamaguchi, Y., Gojobori, T. and Marumo, F. (1994) Proc. Natl. Acad. Sci. USA 91, 6269–6273.
[10] Echevarria, M., Windhager, E.E., Tate, S.S. and Frindt, G. (1994) Proc. Natl. Acad. Sci. USA 91, 10997–11001.
[11] Jung, J.S., Bhat, R.V., Preston, G.M., Guggino, W.B., Baraban, J.M. and Agre, P. (1994) Proc. Natl. Acad. Sci. USA 91, 13052–13056.
[12] Raina, S., Preston, G.M., Guggino, W.B. and Agre, P. (1995) J. Biol. Chem. 270, 1908–1912.
[13] Sabolic, I., Valenti, G., Verbavatz, J.-M., Van Hoek, A.N., Verkman, A.S., Ausiello, D.A. and Brown, D. (1992) Am. J. Physiol. 263, C1225–C1233.
[14] Nielsen, S., Smith, B.L., Christensen, E.I., Knepper, M.A. and Agre, P. (1993) J. Cell Biol. 120, 371–383.
[15] Hasegawa, H., Zhang, R., Dohrman, A. and Verkman, A.S. (1993) Am. J. Physiol. 264, C237–C245.
[16] Bondy, C., Chin, E., Smith, B.L., Preston, G.M. and Agre, P. (1993) Proc. Natl. Acad. Sci. USA 90, 4500–4504.
[17] Folkesson, H.G., Matthay, M.A., Hasegawa, H., Kheradmand, F. and Verkman, A.S. (1994) Proc. Natl. Acad. Sci. USA 91, 4970–4974.
[18] Nielsen, S., DiGiovanni, S.R., Christensen, E.I., Knepper, M.A. and Harris, H.W. (1993) Proc. Natl. Acad. Sci. USA 90, 11663–11667.
[19] Fushimi, K., Sasaki, S., Yamamoto, T., Hayashi, M., Furukawa, T., Uchida, S., Kuwahara, M., Ishibashi, K., Kawasaki, M., Kihara, I. and Marumo, F. (1994) Am. J. Physiol. 267, F573–F582.
[20] Hasegawa, H., Lian, S.-C., Finkbeiner, W.E. and Verkman, A.S. (1994) Am. J. Physiol. 266, C893–C903.
[21] Nielsen, S., Smith, B.L., Christensen, E.I. and Agre, P. (1993) Proc. Natl. Acad. Sci. USA 90, 7275–7279.
[22] Van Os, C.H., Deen, P.M.T. and Dempster, J.A. (1994) Biochim. Biophys. Acta 1197, 291–309.
[23] Frohman, M.A., Dush, M.K. and Martin, G.R. (1988) Proc. Natl. Acad. Sci. USA 85, 8998–9002.
[24] Preston, G.M., Carroll, T.P., Guggino, W.B. and Agre, P. (1992) Science 256, 385–387.
[25] Hasegawa, H., Ma, T., Skach, W., Matthay, M.A. and Verkman, A.S. (1994) J. Biol. Chem. 269, 5497–5500.