

Aquaporin-9 is expressed in a mucus-secreting goblet cell subset in the small intestine

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Abstract We analyzed the expression of aquaporins (AQPs) in the small intestine to elucidate their functions, and found that AQP9, which had not previously been detected there, is present in duodenum, jejunum, and ileum. AQP9 is expressed in colon as well, but not in stomach. Also, its expression in these intestinal sections is limited to the basolateral membranes of a goblet cell subset. Our finding that AQP9 is present specifically in goblet cells as mucus-secreting cells suggests its involvement in the synthesis and/or secretion of a certain kind of mucus which may protect the intestinal surface and smooth the flow of intestinal contents.

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Key words: Aquaporin; Water channel; Goblet cell; Small intestine; Large intestine

1. Introduction

Aquaporins (AQPs) refer to water channels involved in water transport across the plasma membrane [1,2]. In humans, they comprise 11 subtypes that constitute the AQP family. Not only water but also small solutes can be transported by some subtypes of this family [2]. We have suggested the involvement of AQP(s) in digestion after food intake by finding the expression of AQP4 in acid- and pepsinogen-secreting cells of human stomach [3]. The small intestine as well may have some functional AQP(s) involved in food digestion, since this organ has an activity for absorbing the major part of imbibed and food-borne water and for excreting undigested residues by secretion [4,5]. Indeed, information is available that AQP1, AQP3, AQP4, AQP5, AQP7, AQP8 and AQP10 are expressed in the small intestine [4,6,7], but their functions remain unknown.

In the present study, we investigated the expression of other AQPs in the small intestine, particularly in the ileum, and found that AQP9 as a new intestinal AQP subtype is ex-

pressed specifically in a subset of the mucus-secreting goblet cells. Here we also report on its possible functions deduced from histochemical studies.

2. Materials and methods

2.1. RT-PCR

Human small intestine poly A RNA was purchased from Clontech. Mouse small intestine total RNA was extracted from adult male mouse (C57BL/6J, 10 weeks old) using ISOGEN (Nippon Gene) and then treated with DNase I. The RNA samples obtained were reverse-transcribed using a random primer and a first-strand cDNA synthesis kit (Amersham Biosciences). The following primer sets were prepared for amplification of cDNA fragments of AQPs: human AQP1 (hAQP1), 5'-GCATCACCTCCTCCTG-3' and 5'-CATC-CAGGTCATACTCCT-3' (forward and reverse primer, respectively) for a 429 bp fragment; hAQP2, 5'-CCATTACACCGGCTGCTC-3' and 5'-GGAGAAGCCACCACTAGC-3' for a 523 bp fragment; hAQP3, 5'-ATCGTGTGTGTGCTGGCC-3' and 5'-AGGGTGGAT-CGTGAAGGG-3' for a 462 bp fragment; hAQP4, 5'-CTGGTCAT-GGTCTCCTGG-3' and 5'-CCTTCTTCTCCTCTCCCC-3' for a 468 bp fragment; hAQP5, 5'-GTGGGCAACCAGATCTCG-3' and 5'-CGTCAGGCTCATACGTGC-3' for a 509 bp fragment; hAQP7, 5'-GCCCTGGAGGAAGTTCC-3' and 5'-ACCCACCACCAAGT-CTCC-3' for a 447 bp fragment; hAQP8, 5'-GTGACAGTCCAGG-AGCAG-3' and 5'-AAGCAGGCCAAGCAGTG-3' for a 490 bp fragment; hAQP9, 5'-CTGAACAGTGGCTGTGCC-3' and 5'-GCG-TTATTCTGGGGGTG-3' for a 478 bp fragment; and mouse AQP9 (mAQP9), 5'-TTGTCTGGGGCTGCAACTG-3' and 5'-CCCA-CTACATGATGACGC-3' for a 552 bp fragment. PCR amplifications (30 s at 94°C, 30 s at 60°C, 1 min at 72°C, 40 cycles) were performed in the presence of 0.5 µM of each primer, 800 µM dNTPs, 1 × ExTaq buffer and 25 U/ml ExTaq DNA polymerase (Takara Bio). The PCR products obtained were cloned into the pBluescript II SK (–) vector (Stratagene) and their nucleotide sequences were confirmed using a DNA sequencer (Applied Biosystems).

2.2. Tissue sampling

For *in situ* hybridization, adult male mice (C57BL/6J, 7–10 weeks old) were sacrificed by cervical dislocation. Tissue samples were rapidly excised on ice, washed in ice-cold phosphate-buffered saline (PBS), and immersed in O.C.T. compound (Sakura). After freezing in liquid nitrogen, the samples were cut into 7 µm thick sections with a cryostat (Leica). Sections were mounted on glass slides coated with 3-aminopropyltriethoxysilane (Matsunami Glass Ind.). For immunohistochemistry and periodic acid Schiff's stain (PAS)/alcian blue staining, mice were deeply anesthetized with sodium pentobarbital and then perfused with 4% paraformaldehyde in PBS. Tissue samples were excised, post-fixed in 4% paraformaldehyde in PBS (30–60 min on ice), and cryoprotected with 30% (w/v) sucrose in PBS on ice. Frozen sections were prepared as described above.

2.3. *In situ* hybridization

A 570 bp cDNA fragment for mAQP9 was amplified from the first strand cDNA of mouse liver using the following primer set: forward

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Abbreviations: AQP, aquaporin; DIG, digoxigenin; hAQP, human aquaporin; mAQP, mouse aquaporin; PAS, periodic acid Schiff's stain; PBS, phosphate-buffered saline

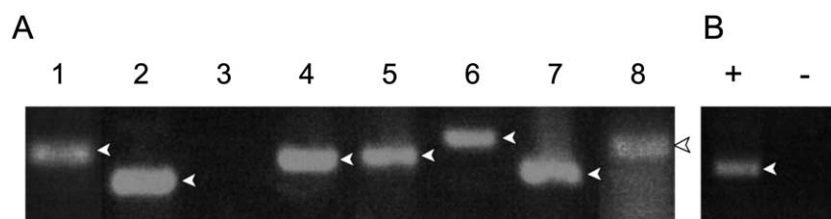


Fig. 1. AQP expression in the small intestine. A: RT-PCR of hAQPs in the small intestine. PCR amplification was performed as described in Section 2. Primer sets used for reaction and estimated lengths of amplified fragments are as follows: lane 1, hAQP9 (478 bp); lane 2, hAQP1 (429 bp); lane 3, hAQP2 (523 bp); lane 4, hAQP3 (462 bp); lane 5, hAQP4 (468 bp); lane 6, hAQP5 (509 bp); lane 7, hAQP7 (447 bp); and lane 8, hAQP8 (490 bp). Fragments corresponding to the cDNA for hAQP9 (lane 1) and several AQP subtypes were amplified from human small intestine cDNA (white arrowheads). B: RT-PCR of mAQP9 in the small intestine. PCR amplification was performed using template-derived mouse small intestine total RNA after (lane +) or before (lane -) the reverse transcription reaction. Fragments corresponding to mAQP9 were amplified only when the RNA sample was reverse transcribed (white arrowhead).

primer with *Bam*HI site-added 5'-TTGGATCCCTTTGTCGGGGC-TGCAACTG-3' and reverse primer with *Hind*III site-added 5'-CCAAGCTTCCCCTACATGATGACGCTG-3'. A 485 bp cDNA fragment for mouse MUC2 was amplified from the first strand cDNA of mouse small intestine using the following primer set: forward primer with *Bam*HI site-added 5'-GTGGATCCGGGAAGATGCACT-CATGG-3' and reverse primer with *Hind*III site-added 5'-CCAA-GCTTTCTCCAGGTACACCATG-3'. PCR amplifications were performed as described above, and the fragments obtained were cloned into pBluescript II SK (-) vector. Digoxigenin (DIG)-labeled riboprobe preparation and hybridization were basically according to Matsumoto et al. [8,9]. In brief, DIG-labeled antisense and sense riboprobes were synthesized using DIG RNA Labeling MIX (Roche Applied Science) from *Bam*HI-linearized template by T7 RNA polymerase (Stratagene) and from *Hind*III-linearized template by T3 RNA polymerase (Stratagene), respectively. These probes were then treated with alkaline 'sizing' buffer (42 mM NaHCO₃, 63 mM Na₂CO₃ and 5 mM dithiothreitol (DTT)) for 40 min at 60°C to yield cRNA fragments with an average length of about 150 bases. Fresh frozen tissue sections were fixed with 4% paraformaldehyde in PBS for 15 min, prehybridized for 2 h at 58°C with 50% (v/v) formamide, 5× SSC

and 40 µg/ml denatured salmon testis DNA (Sigma). Hybridization with a riboprobe was carried out for about 40 h at 58°C in 50% (v/v) formamide, 5× SSC, 5× Denhardt's solution, 500 µg/ml denatured salmon testis DNA (Sigma), 250 µg/ml *Escherichia coli* tRNA (Sigma), and 1 mM DTT. After hybridization, the sections were washed twice for 5 min each at 58°C with 5× SSC, and twice for 30 min each at 58°C with 0.2× SSC. Samples were incubated for 1 h with blocking reagent (Roche Applied Science) and then for 1 h with anti-DIG antibody conjugated with alkaline phosphatase (Roche Applied Science) (1:500) in the same blocking reagent. For color reaction, 5-bromo-4-chloro-3-indolyl phosphate (Sigma) and nitro-blue-tetrazolium (Sigma) were used.

2.4. Western blot analysis

For Western blotting, ileum, liver and kidney were excised from adult male mice (C57BL/6J, 7–10 weeks old) and homogenized in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, containing 1 mM phenyl-methylsulfonyl fluoride (Nacalai tesque), 1 µg/ml leupeptin (Sigma) and 1 µg/ml pepstatin A (Sigma). Homogenates were centrifuged at 800×g for 10 min. The supernatants were applied to SDS-PAGE and transferred to Immobilon P membranes (Millipore). The membranes

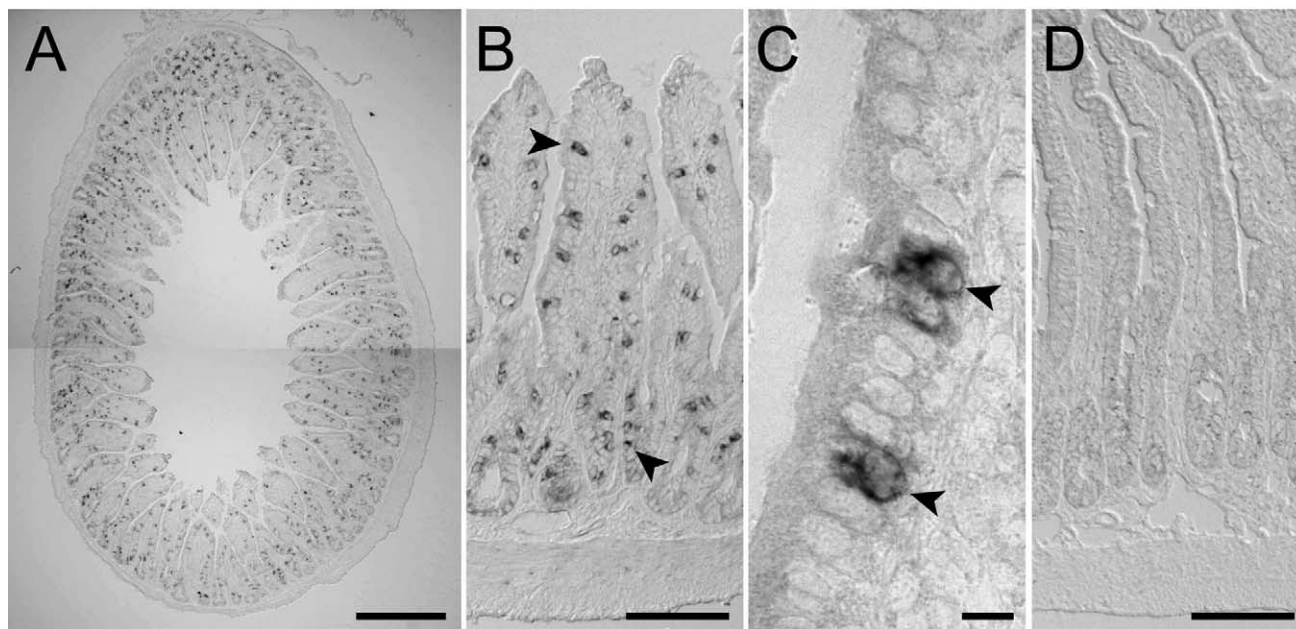


Fig. 2. Localization of mAQP9 mRNA in mouse ileum. A–C: In situ hybridization for mAQP9 mRNA expression in the ileum was carried out using a DIG-labeled antisense riboprobe. A: An ileum section as a whole is shown at low-magnification. Expression of the mAQP9 mRNA appeared in a scattered fashion throughout the section. Note that two images from the same sample are combined into one. B: High-magnification image of A. Signals indicating the expression of mAQP9 are detected in epithelial cells of both villi and crypts (arrowheads). C: High-magnification image of part of a villus. Signals are localized in several limited epithelial cells (arrowheads). D: No specific signal was detected with a sense riboprobe. Scale bars indicate 300 µm (A), 100 µm (B,D), and 10 µm (C).

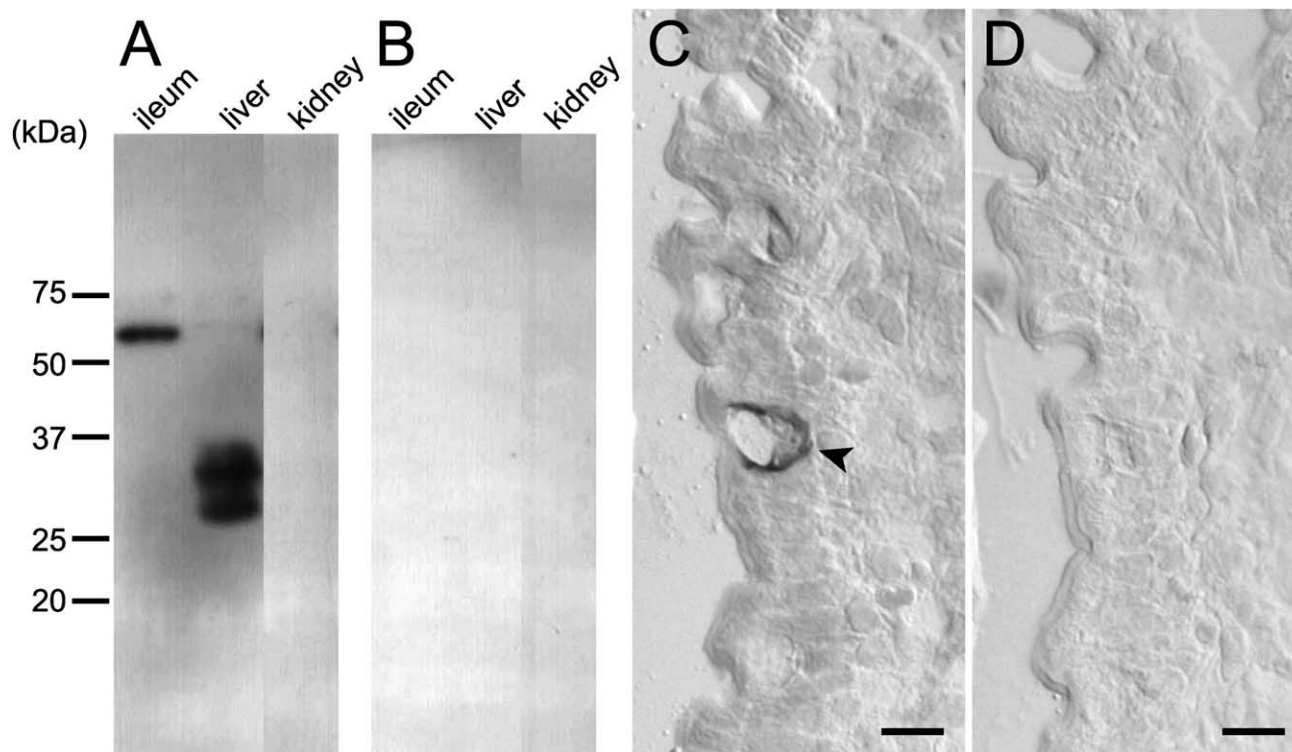


Fig. 3. Detection of mAQP9 protein in mouse ileum. A,B: Western blot analysis of a roughly purified membrane fraction of the ileum, liver and kidney was carried out using affinity-purified anti-AQP9 antibody (A) or anti-AQP9 antibody preabsorbed with an antigenic peptide (B). A: In the liver lane, there are bands assigned to the monomeric 32 and 35 kDa forms of AQP9, and a faint band consistent with a dimeric form of 65 kDa. In the ileum lane only the form with lower mobility was seen. No signal was detected in the kidney lane. B: No band was detected when preabsorbed anti-AQP9 antibody was used. C,D: Immunostaining was carried out with a mouse ileum section using anti-AQP9 antibody (C) or anti-AQP9 antibody preabsorbed with an antigenic peptide (D). High magnification image of a villus is shown. C: Basolateral membranes of several apical expanded epithelial cells are stained. D: No positive staining result was obtained when preabsorbed anti-AQP9 antibody was used. Scale bars indicate 10 μ m (C,D).

were incubated for 1 h at room temperature with blocking solution (5% skim milk in Tris-buffered saline containing 0.05% Tween 20) prior to incubation overnight at 4°C with affinity-purified rabbit anti-AQP9 antibody (Alpha Diagnostics) (1:200) in blocking solution. The membranes were then incubated for 1 h at room temperature with secondary horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Biosciences) in blocking solution. Detection by chemiluminescence was performed using SuperSignal West Dura Extended Duration Substrate (Pierce) and Hyperfilm MP (Amersham Life Science). For preabsorption of anti-AQP9 antibody, the antibody was incubated with an excess amount of antigenic peptide for 24 h at 4°C. Control experiments were carried out using the preabsorbed anti-AQP9 under the same conditions as described above.

2.5. Immunohistochemistry and PAS/alcian blue staining

For immunohistochemistry, paraformaldehyde-fixed tissue sections were incubated in methanol containing 0.3% H₂O₂ for 30 min at 4°C. Sections were incubated for 1 h at room temperature with 3% normal goat serum (NGS) and 1% bovine serum albumin (BSA) in PBS containing 0.1% Triton X-100 prior to incubation overnight at 4°C with affinity-purified rabbit anti-AQP9 antibody (1:600) in dilution solution (1% NGS, 1% BSA in PBS containing 0.1% Triton X-100). Sections were incubated for 1 h at room temperature with secondary biotinylated goat anti-rabbit IgG (Vector Laboratories) in dilution solution and then incubated for 30 min with avidin–biotin–peroxidase complex (Vector Laboratories). A metal enhanced 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate kit (Pierce) was used as a chromogenic substrate. For alcian blue counter staining, DAB-stained sections were incubated for 5 min with alcian blue staining solution (Nacalai tesque). For PAS/alcian blue staining, paraformaldehyde-fixed tissue sections were incubated in methanol for 30 min at 4°C, reacted with a Schiff's PAS kit (PolyScience), and then incubated for 5 min with alcian blue staining solution.

3. Results

3.1. RT-PCR analysis of the expression of AQPs in the small intestine

Using an RT-PCR technique, we first examined the expression of AQP subtypes in human small intestine. The already reported intestinal AQP subtypes, hAQP1, hAQP3, hAQP4, hAQP5, hAQP7 and hAQP8, were found as amplified bands at the expected positions (Fig. 1A). In addition, hAQP9, which had not been identified by Northern blot analysis probably because its expression level is very low, was found to be present in the small intestine (Fig. 1A). Since, however, it was technically and ethically difficult to obtain human small-intestinal sections for histological analysis, we carried out further studies with mouse tissue, which permits in-depth experimentation. It was then necessary to confirm the expression of AQP9 in the mouse small-intestinal sample as well, prior to carrying out the following histochemical staining studies. We conducted an RT-PCR experiment using mouse small-intestinal mRNA and an mAQP9-specific primer set, and readily detected an amplified band at the position expected for mAQP9 mRNA (Fig. 1B). For confirmation, the fragments from the bands were submitted to nucleotide sequence analyses (data not shown).

3.2. Expression of mAQP9 in the small intestine

We then carried out detailed histochemical analyses for

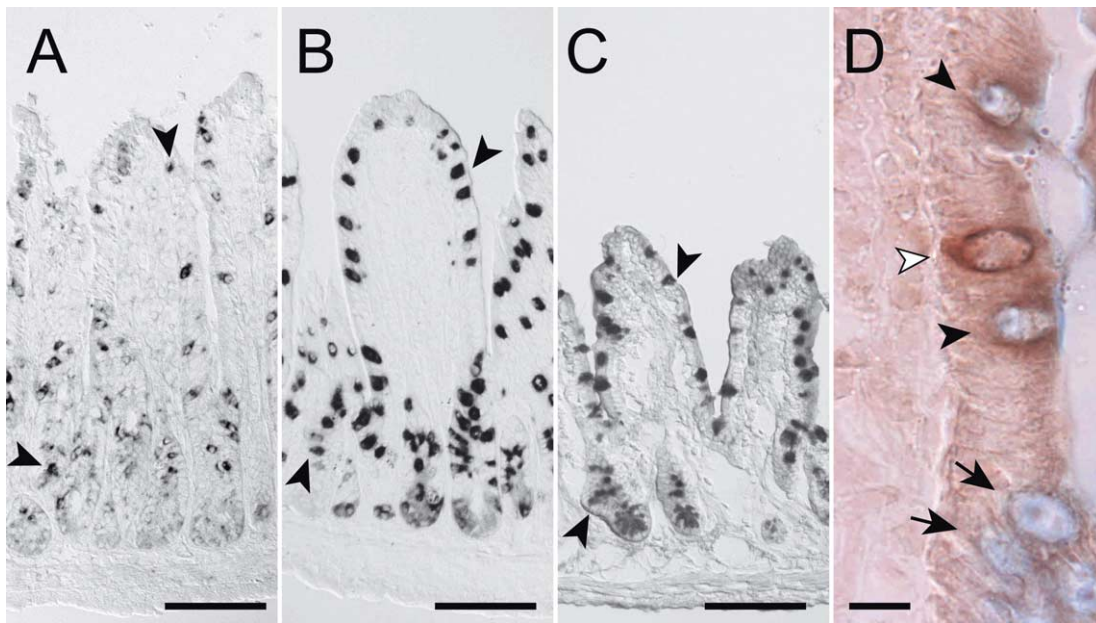


Fig. 4. Specification of mAQP9 expressing-cells in mouse ileum by staining of goblet cells. A,B: In situ hybridizations of mouse ileum sections were carried out using antisense riboprobes for mAQP9 (A) or mMUC2 (B). Adjacent ileum sections were used for A and B. Staining of both mAQP9 and mMUC2 is seen in several epithelial cells of both villi and crypts (arrowheads). C: PAS/alcian blue staining was carried out with a mouse ileum section. PAS/alcian blue-stained goblet cells are also present in both villi and crypts (arrowheads). D: Ileum section after immunostaining with anti-mAQP9 antibody and counterstaining with alcian blue. mAQP9-immunoreactivity (IR) (brown) is sometimes overlapped with alcian blue staining (blue) in some apically expanded epithelial cells (arrowheads). Apical expanded epithelial cells are also detected showing only mAQP9-IR (white arrowhead) or only alcian blue staining (arrows). Scale bars indicate 100 μ m (A–C) and 10 μ m (D).

AQP9 using mouse samples. First, in situ hybridization was performed to examine the mAQP9 expression in the ileum. Using a DIG-labeled antisense riboprobe, signals were observed throughout the ileum (Fig. 2A), especially in both villi and crypt epithelia (Fig. 2B). However, the signals were scattered in the epithelia (Fig. 2A–C). No signal was seen in a control experiment with a sense probe in any part of the ileum (Fig. 2D).

Subsequently, immunohistochemical analysis of mAQP9 protein was conducted using anti-AQP9 antibody. First, we carried out a Western analysis using a roughly purified membrane fraction from mouse ileum. A similar sample from liver was used as a positive control because it is known to express AQP9 [10,11], and another sample from kidney was used as a negative control. In the liver lane, there were major bands corresponding to ca. 32 kDa and to ca. 35 kDa, and a faint band corresponding to ca. 65 kDa (Fig. 3A). A band corresponding to ca. 32 kDa is due to the mAQP9 monomer, and a band corresponding to ca. 35 kDa to the sugar adduct mAQP9 monomer. A band corresponding to ca. 65 kDa is probably due to the mAQP9 dimer because it was reported that mAQP9 dimer undissociated by the SDS was detected in the same position [12]. Furthermore, none of the bands observed in the liver lane could be detected when a similar analysis was conducted using anti-AQP9 antibody that had been preabsorbed with the antigenic peptide (Fig. 3B). These results are consistent with the published data [12,13], and indicate that this antibody can be useful to detect mAQP9 protein. In the ileum lane, there was only a major band corresponding to ca. 65 kDa and bands corresponding to ca. 32 kDa and to ca. 35 kDa were not detected (Fig. 3A). The presence of this major band indicates the expression of mAQP9 protein in ileum.

Next, this antibody was used for immunohistochemical analysis of mouse ileum sections. Staining of the ileum clearly showed the presence of mAQP9 in some epithelial cells (Fig. 3C). Preabsorption with the antigenic peptide abolished the signal (Fig. 3D). The frequency of the immunoreactive cells, i.e. mAQP9-IR(+) cells, was similar to that of cells recognized as positive by in situ hybridization (Fig. 2A–C). Each of the mAQP9-IR(+) cells was found to exhibit an expanded shape at the apical portion, and to be stained by the mAQP9 antibody in the basolateral membrane only (Fig. 3C). Both the frequency and the shape of the cells led us to speculate that these mAQP9-positive cells are goblet cells.

We focused our investigation on goblet cells in the mouse ileum and employed the characterized marker MUC2 as a mucoprotein secreted from goblet cells. In situ hybridization for MUC2 and PAS/alcian blue staining for glycoproteins showed signals scattered in the epithelia of both villi and crypts (Fig. 4B,C). A scattered distribution profile of MUC2-positive cells in epithelia was found to resemble that of mAQP9-positive cells as described above (Fig. 4A). This result suggests that the mAQP9-positive cells are goblet cells, although the population of mAQP9-positive cells was smaller than that of MUC2-positive cells (Fig. 4A–C).

Furthermore, we reconfirmed our findings by double-staining with anti-AQP9 antibody and acidic mucus staining alcian blue on the same ileum section. Most of the mAQP9-IR(+) cells were positively stained by alcian blue (Fig. 4D, arrowheads). Most of these cells were thus identified as goblet cells, although a few of the mAQP9-IR(+) cells were alcian blue-negative (Fig. 4D, white arrowhead).

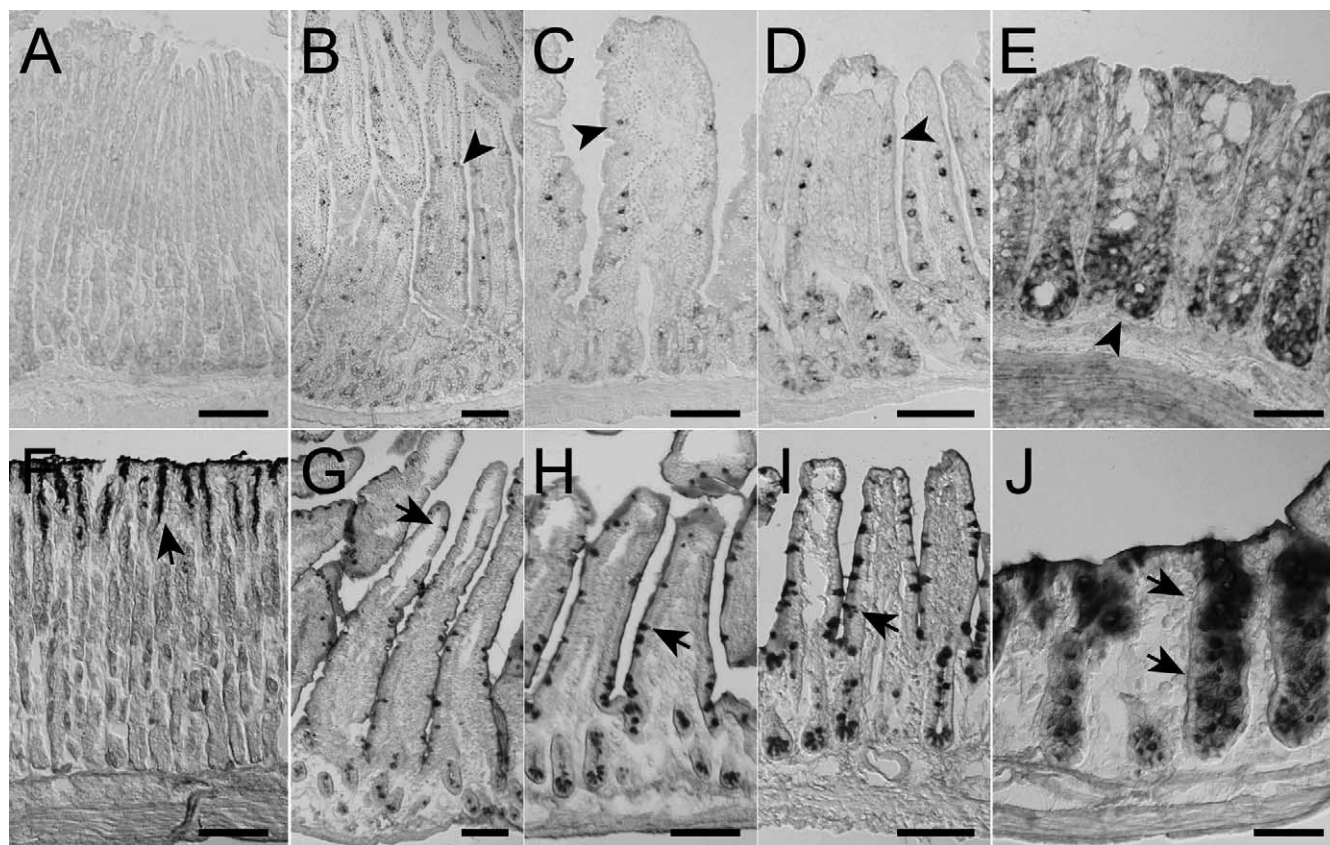


Fig. 5. mAQP9 mRNA localization in mucus-secreting cells of mouse gastrointestinal tract. In situ hybridization using mAQP9 antisense riboprobe (A–E) and PAS/alcian blue staining (F–J) were carried out with stomach (A,F), duodenum (B,G), jejunum (C,H), ileum (D,I), and colon (E,J) sections. In the stomach, no mAQP9 signal is found even in a mucous secretion cell (A,F, arrow). In duodenum, jejunum, ileum, and colon, mAQP9 signals are localized in goblet cells (arrowheads in B–E, arrows in G–J). Note that the population of mAQP9 expressing-cells in these tissues is smaller than that of goblet cells (B–E, G–J). mAQP9 expressing-cells in duodenum, jejunum and ileum are scattered in goblet cells of both villi and crypts (B–D), whereas colon mAQP9 expressing-cells are localized only in goblet cells at the bottom portion of crypts (E). Scale bars indicate 100 μ m (A–D,F–I) and 50 μ m (E,J).

3.3. Expression of mAQP9 mRNA in gastrointestinal goblet cells

Not only ileum but also other intestinal sections, i.e. duodenum, jejunum, and colon, have goblet cells. Other digestive tissues also contain mucus-secreting cells, e.g. surface mucous cells in the stomach. It therefore seemed possible that mAQP9 would be expressed in these mucus-secreting cells and also in goblet cells of different origins. We therefore investigated the expression of mAQP9 in various digestive organs. In situ hybridization with an mAQP9 antisense probe was applied to mouse stomach, duodenum, jejunum, and colon sections, and then PAS/alcian blue staining of these sections was carried out to identify goblet and mucus-secreting cells. No positive results were obtained with stomach (Fig. 5A,F), while mAQP9 was clearly detected in duodenum and jejunum as well as in ileum epithelia (Fig. 5B–D). In situ hybridization with the mAQP9 sense probe gave no signal in slices of mouse stomach, duodenum, jejunum, or colon (data not shown). Positive cells expressing mAQP9 were scattered from the tips of villi to the bases of crypts (Fig. 5B–D). With respect to the population of the mAQP9-positive cells, the observed relationship is as follows: duodenum < jejunum < ileum (Fig. 5B–D), probably reflecting that the goblet cell population increases in the same order (Fig. 5G–I). Our data also show that the population of mAQP9-positive cells is not equivalent to that of goblet cells, indicating the existence of mAQP9 in a portion of

goblet cells. In the colon, however, goblet cells resided throughout the crypt epithelia (Fig. 5J), though mAQP9 signals appeared on epithelial cells in the bottom portion of the crypt (Fig. 5E,J). mAQP9 was found to be expressed in a subset of goblet cells, although its expression pattern in the colon differs from that in the small intestine. These results suggest that mAQP9 is expressed only in a subset of mucus-secreting goblet cells, not in all the mucus-secreting cells.

4. Discussion

Our present study is the first that reports on the presence of AQP9 in mouse colon as well as human and mouse small intestine, and also on its specific localization in a subset of intestinal goblet cells. It has been reported that AQP9 is expressed in hepatocytes, leukocytes, immature spermatocytes, Leydig cells and astrocytes, among other cells types [11,14,15], which have well-developed smooth endoplasmic reticula (ERs). In contrast to these observations, our present report concerns the presence of AQP9 in goblet cells which does not have developed smooth ERs, but have well-developed rough ERs. This report also describes that AQP9 in the goblet cell subset is localized in the basolateral membrane (Fig. 3C), whereas AQP9 is localized in the apical membrane of the above-mentioned non-intestinal cell types [11,15].

AQP9 is expressed in only a subset of goblet cells in small

intestine and colon (Fig. 5). Possible explanations for this limited domain of expression may be as follows: First, in goblet cells not expressing AQP9, some other AQP subtype(s) may possibly be expressed and exert a similar function. Second, the AQP9-expressing goblet cells may be functionally different from the other goblet cells. At present, we think that the second possibility is more likely because different goblet cells show different drug responses [16,17], and the mucus contained in goblet cells has a variety of mucin subtypes [18,19], pH [19] and response toward immunostaining [20].

AQP9 is a recently reported AQP subtype, also being an aquaglyceroporin which can transport glycerol, urea, etc., as well as water [10,11,21]. Though the physiological function of AQP9 is a subject of speculation for many research groups [12,13,15,22], its real roles in vivo are still unclear. However, our finding clarifies that the expression of AQP9 takes place specifically in goblet cells whose functions are well defined. Probably, therefore, AQP9 may function for the synthesis and/or secretion of mucus. It is reported that, unlike the other AQPs, this aquaporin AQP9 may have a characteristic ability to permeate glycerol, mannitol, sorbitol, and other low-molecular-weight solutes [11,21]. Because of the ability, Nihei et al. [15] have suggested that AQP9 in hepatocytes and testis Leydig cells participates in the rapid cellular uptake and release of various small-size lipid and/or cholesterol metabolites through their apical membrane. It is known that goblet cells in the intestine can take up extracellular glucose, galactose, galactosamine, etc., through their basolateral membranes and use them for the synthesis of mucus [23]. This means that AQP9 may participate in the transport these solutes, together with water, to and from goblet cells.

The mucus secreted from the goblet cells is known to take part in protecting the intestinal surface, smoothing the flow of intestinal contents [24,25]. Such important functions of goblet cells have long been known and, in addition, a recent finding that tumor frequency is higher in MUC2-knockout mice [26] accentuates the importance of these cells. Also, it has been reported that goblet cells, which are characterized by their synthesis and secretion of mucus, can be regulated by hormones, drugs, etc. [16,27–29]. On the other hand, the expression, localization and function of AQPs are also known to be hormone-dependent [30,31], as in the case of AQP9 [32,33]. There may thus be a close correlation between mucus and AQP9. The present study is thus the first that clearly suggests the involvement of the newly found intestinal AQP, AQP9, in the synthesis and secretion of a certain kind of mucus which may protect the intestinal surface and smooth the flow of intestinal contents.

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