Aquaporin-8 Is Involved in Water Transport in Isolated Superficial Colonocytes from Rat Proximal Colon\textsuperscript{1,2}

Umberto Laforenza,\textsuperscript{3} Emanuela Cova, Giulia Gastaldi, Simona Tritto, Monica Grazioi, Nicholas F. LaRusso,\textsuperscript{*} Patrick L. Splinter,\textsuperscript{*} Patrizia D’Adamo,\textsuperscript{†} Marisa Tosco,\textsuperscript{**} and Ulderico Ventura

Dipartimento di Medicina Sperimentale, Sezione di Fisiologia Umana, Università di Pavia, Italy; \textsuperscript{*}Center for Basic Research in Digestive Diseases, Division of Gastroenterology and Hepatology, Mayo Medical School, Clinic and Foundation, Rochester, MN; \textsuperscript{1}DIBIT, San Raffaele Scientific Institute, Milan, Italy; and \textsuperscript{**}Dipartimento di Scienze Biomolecolari e Biotecnologie, Università di Milano, Italy

ABSTRACT Water is an essential nutrient because it must be introduced from exogenous sources to satisfy metabolic demand. Under physiologic conditions, the colon can absorb and secrete considerable amounts of water even against osmotic gradients, thus helping to maintain the body fluid balance. Here we describe studies on both aquaporin (AQP) expression and function using cells isolated from the superficial and lower crypt regions of the rat proximal colon. The expression of AQP-3, -4, and -8 in isolated colonocytes was determined by semiquantitative RT-PCR and by immunoblotting. The localization of AQP-8 in the colon was evaluated by immunohistochemistry. A stopped-flow light scattering method was used to examine osmotic water movement in isolated colonocytes. Moreover, the contribution of AQP-8 to overall water movement through isolated colonocytes was studied using RNA interference technology. Colonocytes from the proximal colon express AQP-3, -4, and -8 with increasing concentrations from the lower crypt cells toward those on the surface. Osmotic water permeability was higher in surface than in crypt colonocytes ($P < 0.05$); it was significantly inhibited by the water channel blocker dimethyl sulfoxide, and reversed by β-mercaptoethanol ($P < 0.05$). Immunohistochemistry revealed a strong AQP-8 labeling in the apical membrane of the superficial colonocytes. Inhibition of aquaporin-8 expression by small interfering RNA significantly decreased osmotic water permeability (~38%; $P < 0.05$). Current results indicate that aquaporin-8 may play a major role in water movement through the colon by acting on the apical side of the superficial cells. J. Nutr. 135: 2329–2336, 2005.

KEY WORDS: \textbullet{} aquaporins \textbullet{} water channel \textbullet{} small interfering RNA \textbullet{} colon

Water is an essential macronutrient because it must be introduced with foods to satisfy metabolic demand. The colonic epithelium plays a major role in salt and water transport and contributes to body fluid homeostasis. About 1.5–2 L of water (1) are absorbed daily by the colon against an osmotic gradient, producing dehydrated feces. Moreover, the colon may secrete water and electrolytes under certain physiologic and pathophysiologic conditions (2). In the past, the accepted paradigm was that fluid absorption takes place in the surface epithelium and fluid secretion in the crypt. In contrast, it is now established that colonic crypts can absorb as well as secrete so that most fecal consolidation is probably not due to crypt absorption (3,4).

Theoretically, water can cross the epithelia either by para-cellular (i.e., across cell junctions) or transcellular (i.e., through the apical and basolateral membranes) routes. Transcellular transport may involve 3 different mechanisms: 1) diffusion across the lipid bilayer; 2) diffusion across the water channels i.e., aquaporins (AQPs)\textsuperscript{4}; and 3) cotransport with ions and nutrients (5).

Because the colon is a tight epithelium characterized by high electrical resistance, it is conceivable that water flux occurs mainly by transporters and/or AQPs. As in other tissues of the gastrointestinal tract, AQPs were identified in the colon epithelium (6). AQP-2, -3, -4, and -8 were found along the crypt-villus axis (abstract). Pfluegers Arch. 2003; 445:R39).

\textsuperscript{1}Preliminary data appeared in Abstract form [Laforenza U, Gastaldi G, Cova E, Tritto S, Ventura U. Involvement of aquaporins 3 and 8 in water transport in rat colonocytes isolated along the crypt-villus axis (abstract). Pfluegers Arch. 2003; 445:R39].

\textsuperscript{2} Funded by COFIN 99 (GR, 9905021792, 002) and by the University of Pavia 2002.

\textsuperscript{3}To whom correspondence should be addressed. E-mail: lumberto@unipv.it.

\textsuperscript{4}Abbreviations used: AQP, aquaporin; β-ME, β-mercaptoethanol; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; Pf, osmotic water permeability; siRNA, small interfering RNA.
shown to be present in the colon, the function and physiologic importance of these AQPs in water transport has not been addressed. Interestingly, studies on AQP-4 null mice demonstrated that fluid absorption was not impaired by AQP-4 deletion, although AQP-4 null mice had a slightly higher water content in feces and reduced water osmotic permeability compared with normal mice (13). A more recent investigation surprisingly found no significant impairment in colonic fluid absorption or fecal dehydration in AQP-8 null mice colon (14).

The purpose of this work was to study the expression and functional role of AQPs in water transport through colonic cells and to determine whether any differences existed between superficial (mature) and lower crypt cells (immature and proliferative).

For this study, we examined the expression of AQP-3, -4, and -8 mRNA and protein in colonocytes isolated along the crypt-lumen regions in the rat proximal colon using RT-PCR and immunoblotting. We performed functional experiments by assaying osmotically induced water permeability in freshly isolated cells in both the absence and presence of the well-known water channel inhibitor dimethyl sulfoxide (DMSO) to distinguish the contribution of AQP-3 and -8 to water permeability from that of AQP-4 (DMSO-insensitive). To determine the relative contribution of the AQP-8 toward the overall osmotic water permeability of isolated colonocytes, we used a well-established approach to selectively and specifically silence the AQP-8 gene by means of small interfering RNA (siRNA) (15,16).

MATERIALS AND METHODS

Cell isolation and characterization. Adult Wistar albino rats (350–400 g body weight) were housed at the animal facility of the Department of Experimental Medicine, Section of Human Physiology in Pavia. They were cared for and killed according to the current European legal Animal Practice requirements. Rats were used after 16 h of food deprivation with free access to water. The isolation of superficial and lower crypt colonocytes from the proximal colon was performed as described previously (17); 5 cell fractions were obtained and identified as superficial (fraction 1 and 2), middle (fraction 3), and lower crypt colonocytes (fraction 4 and 5) by assaying thymidine kinase activity. Thymidine kinase activity, a typical marker of proliferating cells, was determined by measuring the [3H]-thymidine (specific activity, 925 GBq/mmol; Amersham Biosciences Europe) phosphorylation rate (18). Enzyme activity was expressed as pmol/(mg protein · 30 min). Cell viability was assessed by the trypan blue (0.2%) dye exclusion test for each cell fraction and different experimental condition (19).

RT-PCR assay. Total cellular RNA was extracted from each cell fraction using the SV total RNA Isolation System (Promega). Single cDNA was synthesized from RNA (1 mg) using random hexamers and IMPROM II reverse transcriptase (Promega). Specific primers for rat $\alpha$-actin (7), AQP-3 (20), AQP-4 (sense, 5'-TTTCAAGCGCTCCTGAGCTC-3'; antisense, 5'-TTCCATGAACCCTGCTGCTGTCAGAGTTT-3'), and AQP-8 (sense, 5'-GGGATCTCTGTTCCATGAAC-3'; antisense, 5'-CTGCTGCTGTAGAGTGGCTC-3') were synthesized based on their published sequences. cDNA amplification was performed by Taq DNA polymerase (Promega) and for all PCR assays consisted of an initial denaturation of 5 min at 96°C followed by 30 s at 96°C, 30 s at different annealing temperatures (AQP-3, 56°C; AQP-4, 55°C; AQP-8, 61°C; $\beta$-actin, 62°C), 30 s at 72°C, and for an extension of 7 min at 72°C. Reverse transcription was always performed either in the presence or in the absence of reverse transcriptase enzyme. First, the sequences of AQP bands were checked using the Big dye terminator cycle sequencing kit (Applied Biosystem). PCR products were separated on a 3% NuSieve® (2:1) gel agarose, stained with ethidium bromide, and acquired with the Image Master VDS (Amersham Biosciences Europe). Densitometric analysis of the bands was performed by the Total Lab v 1.11 computer program (Amersham Biosciences Europe). The molecular weight of the PCR products was compared with the DNA molecular weight marker VIII (Roche Molecular Biochemicals).

Membrane preparation and immunoblotting. Freshly isolated colonocytes were homogenized with a Teflon glass Potter-Elvehjem homogenizer (Kontes) in a solution containing 100 mmol/L NaCl, 1 mmol/L EDTA, 10 mmol/L Tris-HCl, pH 6.8, and 0.1 g/L phenylmethylsulfonil fluoride. After centrifugation at 100,000 g for 60 min at 4°C, the pellets were suspended in the homogenization buffer and treated as previously described (21). Solubilized proteins (70 µg) were subjected to 12.5% SDS-PAGE and transferred to Hybond-P polyvinylidene fluoride membranes (Amersham Biosciences Europe) by electroelution. After 3 h of blocking with TBS containing 5% nonfat dry milk and 0.1% Tween, the membranes were incubated overnight with affinity-purified antibodies to AQP-3, -4, -8 (Alpha Diagnostic International) diluted 1:2000 in the blocking solution. The membranes were washed and incubated with peroxidase-conjugated goat anti-rabbit IgG (1:3000 in the blocking solution). ChemiBlot™ Molecular Weight Markers were used to accurately estimate molecular weight and as a positive control for immunoblot (Chemicon International).

Immunohistochemistry. Rats were anesthetized with halothane and perfused intracardially with acetate-buffered 4% formalin. The colon was removed, postfixed for 1 h, and processed into paraffin. Serial paraffin sections (5 µm) were brought to water and treated with 3% hydrogen peroxide for 10 min at room temperature to block the endogenous peroxidases. After being washed for 5 min with PBS, sections were blocked with 3% bovine serum albumin (BSA) in PBS for 30 min at room temperature. Sections were incubated for 2 h at room temperature with affinity purified anti-AQP8 antibody diluted 1:2000 in PBS containing 1% BSA. After three 5-min washes with PBS containing 1% BSA, the sections were first incubated for 15 min at room temperature with biotinylated anti-rabbit IgG; after 3 washes with PBS containing 1% BSA, they were incubated for 15 min at room temperature with HRP-conjugated streptavidin (Universal DAKO LSAB® + kit, peroxidase, K0679, DakoCytomation). The reaction was visualized by incubation with the DakoCytomation 3,3’-diaminobenzidine chromogen solution. The sections were counterstained with hematoxylin and mounted in DPX (distyrene-plasticizer-xylene; Merck Eurolab). Control experiments were performed simultaneously using antibodies preadsorbed with an immunizing peptide or omitting primary antibody. The immunostained slides were examined by light microscopy using an Olympus BX41, and digital images were captured with an Olympus Camedia C-5030 zoom digital camera (Olympus Italia).

Water permeability measurements. Osmotic water permeability (PF) was measured in a suspension of cells freshly isolated from the crypt-lumen regions of the proximal colon by the stopped-flow light scattering method (22,23). The experiments were performed at 25°C on a stopped flow apparatus (RX2000, Applied Photophysics) with a pneumatic drive accessory (DA.1, Applied Photophysics) straightforward coupled with a Varian Cary 50 spectrometer (Varian Australia). Scattered light was measured at 600 nm in time intervals of 10 ms at a wavelength of 450 nm. The time course of cell swelling caused by exposure to the hypotonic gradient (150 mosm/L) was measured for 60 s at the acquisition rate of 1 point/0.1 s. The initial rate constant (k) of cell volume changes was obtained by setting the time course light scattering with a single exponential equation (GraphPad Prism 4.00, 2003). The water permeability coefficient, Pf, was calculated as previously described by Wiener et al. (24) from the following equation:

$$ Pf = k \cdot V_v / \Delta C \cdot V_w \cdot A $$

where $\Delta C$ is the osmotic gradient, $V_w$ the molar water volume, $V_v$ the cell volume, and A is the cell surface area. $V_v$ and A were obtained by light microscopy using 4.75-µm beads as an internal standard. Water transport was evaluated in: 1) normal untreated colonocytes; 2) cells treated for 5 min with 500 mmol/L DMSO; and 3) cells treated for 5
min with DMSO followed by 10 min of treatment with 5 mmol/L β-mercaptoethanol (β-ME).

In AQP-8 gene silencing experiments, osmotic water permeability was evaluated 24 h after the cells' transfection by exposing colonocytes transfected with AQP8-siRNA or with scrambled siRNA to the hypertonic gradient (150 mosm/L). The time course of cell swelling was measured for 60 s by light microscopy using an Olympus BX41 while digital images were captured with an Olympus Camedia C-5050 zoom digital camera (Olympus Italia). Serial images were taken at 400X magnification and cell diameters measured in a randomized manner without knowledge of cell treatment using 4.75-μm beads as an internal standard. The cell surface area was evaluated by the Scion Image Beta 4.02 computer program (Scion Corporation) and the Pf values calculated as described above.

Osmotic water permeability in the gene-silencing experiments was not measured by the stopped-flow light scattering method because trypsinization of cells from culture matrix could damage the colonocytes.

Silencing of AQP-8 expression using siRNAs. Cell fractions 3, 4, and 5 were pooled and seeded onto polystyrene tissue culture dishes (35 mm diameter) pretreated with collagen IV (6 mg/mL) (Sigma C 0543). The growth medium was a DMEM/Nutrient mixture F-12 HAM supplemented with 10% fetal calf serum, 0.5 kU/L penicillin, and 50 mg/mL streptomycin.

An siRNA duplex corresponding to nucleotides 165–185 of rat AQP-8 cDNA was chemically synthesized by a commercial vendor (Ambion). The siRNA duplex consisted of a 21-nucleotide sense strand (5'-AUGGUAACUGUCAGGCAUGGdTdT-3'), a scrambled siRNA (sense strand, 5'-UACUAACCGUCCGAGAUCdTdT-3'; antisense strand, 5'-AUGUACUCCGACGGAUAdTdT-3'), and a 21-nucleotide antisense strand (5'-AUGGUAACUGUCAGGCAUGGdTdT-3'). A scrambled siRNA (sense strand, 5'-UACUAACCGUCCGAGAUCdTdT-3'; antisense strand, 5'-AUGUACUCCGACGGAUAdTdT-3'), which showed no significant homology to any known protein (analyzed using BLAST), was used as a control.

After 24 h from seeding, cells were transciently transfected by incubation with a growth medium containing siRNA oligonucleotides or scrambled siRNA (20 nmol/mL) and siPORTTM lipid siRNA transfection agent (Ambion, Europe), according to the manufacturer's instructions. After 90 min, the incubation medium was diluted 1:1 (v:v) with fresh growth medium.

To determine the effectiveness of siRNA in silencing AQP-8 gene expression, 24 h after transfection, cells were harvested and analyzed for AQP-8 protein levels by immunoblotting.

Protein content. The protein content was determined using the method of Lowry et al. (25) with BSA as a standard.

Statistics. All data are expressed as means ± SEM. The significance of the differences of the means was evaluated using 1-way ANOVA followed by Newman-Keuls’s Q test, 2-way ANOVA followed by the Bonferroni post test, or Student’s t test. All statistical tests were carried out with statistical software programs (Primer of Biotastistics 1.0 (26); GraphPad Prism 4.00, 2003).

RESULTS

Characterization of the colonocytes. The 5 epithelial cell populations isolated by sequential time incubation of proximal colonic loops showed that thymidine kinase activity in fractions 1, 2, and 3 was 5% of that in fractions 4 and 5 (P < 0.05; Fig. 1). Because undifferentiated cells have high proliferative activity, which decreases with the state of differentiation, fractions 1 and 2 were identified as superficial, mature, and differentiated cell populations (upper crypt cells), whereas fractions 4 and 5 were considered to be proliferating and undifferentiated cell populations (lower crypt cells) (17). Because the cells from fraction 3 could consist of both immature and mature cells, functional studies were performed only on differentiated upper (fractions 1 and 2) and proliferating lower cells (fractions 4 and 5). The transcript and protein expression experiments were performed in all 5 fractions of the proximal colon.

After isolation, the cells’ viability was assayed by the trypan blue exclusion test and was >90% in each cell fraction. Moreover, treatment with DMSO or DMSO followed by β-ME did not modify cell viability. Mercurial compounds, well recognized inhibitors of AQP-3 and -8, could not be used in this study because they strongly affected cell viability even at low concentrations, as previously demonstrated (27,28).

mRNA and protein expression of AQPs. The presence of mRNA of AQP-3, -4, and -8 was explored by semiquantitative RT-PCR using specific primers for the different AQPs. The results of gel agarose electrophoresis of representative PCR reaction products are shown in Figure 2A. Single bands of the expected size of cDNA fragments were amplified. The sequences of the PCR products revealed complete matching with the published sequences for the related AQPs.

All of the AQPs investigated were expressed in rat proximal colonocytes (Fig. 2). Densitometric analysis of the bands showed that AQP-3 and AQP-8 mRNA was significantly higher in the surface cell fractions than in lower crypt cells (fraction 1, 99.8% ± 5 vs. fraction 5, 60.5 ± 4% AQP3:β-actin, P < 0.004; fraction 1, 113.6 ± 4% vs. fraction 5, 66.3 ± 6% AQP8:β-actin, P < 0.001, Student’s t test). In contrast, the expression pattern of the AQP-4 transcript had a distribution along the crypt-lumen regions opposite to that of AQP-3 and -8, with the lower crypt cell fractions displaying significantly higher levels of AQP-4 mRNA than the more superficial ones (fraction 1, 23.3 ± 4% vs. fraction 5, 102.3 ± 7% AQP4:β-actin, P < 0.001, Student’s t test) (Fig. 2A).

Total membrane preparations from proximal colonocytes isolated along the crypt-lumen regions were analyzed by immunoblotting with affinity-purified antibodies against rat AQP-3, -4, and -8. Isolated colonocytes expressed the AQPs investigated with increasing intensity from the lower crypt toward the upper crypt regions (Fig. 2B). When antibodies were preadsorbed with large amounts of immunizing peptides, the protein bands disappeared completely, indicating the specificity of the reaction (not shown).

Two bands were observed in immunoblots probed with anti-AQP3 antibodies at 75 and 36 kDa, consistent with a dimer and a glycosylated monomeric form, respectively (29,30). Immunoblotting experiments for AQP-4 showed a 45-kDa glycosylated and a 30-kDa form, as previously reported (8,31,32). The AQP-8 protein pattern revealed a glycosylated monomeric form (33 kDa) and a dimeric form (60–68 kDa) (7,28,33).
Immunohistochemical localization of AQP-8 protein in the rat colon. The localization of AQP-8 protein in the colon was further investigated by immunohistochemistry because its cellular and subcellular distribution is presently unclear. In the proximal colon, the intensity of AQP-8 immunostaining decreased from the tip toward the base of the crypt (Fig. 3a). No labeling was detectable in the intercalated goblet cells (Fig. 3a and inset). At high magnification, the epithelial cells of the proximal colon showed strong AQP-8 immunostaining of the apical membranes (Fig. 3a inset; arrows). Intracellular staining was also observed, whereas basolateral membranes were negative. Immunolabeled controls were negative (Fig. 3b).

Water permeability measurements. Examination of morphometric parameters showed that isolated cell fractions were consistently spherical, with a shape factor of ~1. The upper and the lower crypt colonocytes exposed to the hypotonic buffer behaved as a functional osmometer, showing a sudden swelling recorded as a rapid decrease in light scattering (Fig. 4A). In control experiments, little or no signal change was observed in the absence of an osmotic gradient (data not shown). Figure 4A shows representative curves from light scattering measurements of the superficial and lower crypt cells from the proximal colon. The cells belonging to fractions 4 and 5 changed their volume slowly compared with fractions 1 and 2, as indicated by the slower variation of the light scattering signal. Water permeability was significantly higher in the upper compared with the lower crypt colonocytes (P < 0.05, 2-way ANOVA followed by the Bonferroni post test; Fig. 4B). In upper cell fractions, Pf values were ~30% higher than in lower crypt fractions. The AQPs' involvement in cell swelling was confirmed by the observation that cells pretreated with the AQP water channel inhibitor DMSO (28,34,35) significantly reduced their Pf values when exposed to a hypotonic environment (P < 0.05, 2-way ANOVA followed by the Bonferroni post test). This inhibitory effect was greater in the superficial cells (30–40%) than in the lower crypt cells (12–20%) of the proximal colon. The DMSO-induced inhibition of the osmotic water permeability was reversed by β-ME treatment. Finally, the values of the DMSO-independent osmotic permeability were similar in both lower crypt and surface cells from the proximal colon (~55 μm/s).

Silencing of AQP-8 expression using siRNA. Our aim in these experiments was to examine the effect on osmotic water permeability of selectively knocking down the AQP-8 of colonocyte primary cultures by siRNA technology. In this manner, the role of AQP-8 in colonocyte transmembrane water permeability could therefore be assessed.

First, we verified that the siRNA used was able to silence...

**FIGURE 2** AQP-3, -4, and -8 mRNA (A) and protein (B) expression in surface and lower crypt colonocytes of the rat proximal colon. (A) Representative semiquantitative RT-PCR of AQP-3, -4, and -8. Reverse transcription was performed in the presence (+) or absence (−) of reverse transcriptase enzyme. AQP mRNA expression was normalized to β-actin. The 485-, 382-, 305-, and 509-bp bands correspond to the AQP-3, -4, -8, and β-actin–specific PCR products, respectively. (B) Representative immunoblotting of AQP-3, -4, and -8. AQP-3 immunoblot: 36- and 45-kDa bands. AQP-8 immunoblot: 33- and 60-kDa bands. F1, F2, F3, F4, and F5, cell fractions.

**FIGURE 3** Immunohistochemical localization of AQP-8 protein in the rat proximal colon. The labeling is present in the surface epithelial cells of the crypt and almost absent in the epithelium at the base of the crypt (a). Intense immunoreactivity is observed in the apical part of the epithelial cells, but intracellular staining is also present (inset). Arrows in the inset show the staining of apical membranes at high magnification. Goblet cells are not stained. Controls in which the primary antibody was omitted show an absence of labeling (b). Original magnifications: (a, b) X400, (inset) X1000.
AQP-8-siRNA by 78.5% (analysis showed that AQP-8 protein levels were inhibited by the endogenous AQP-8 protein level compared with controls. Treatment with siRNA strongly reduced the AQP-8 protein by immunoblotting cultured colonocytes transfected with AQP8-siRNA (Silenced) or with scrambled siRNA (Control). Treatment with siRNA strongly reduced the AQP-8 gene. This was done by determining the levels of AQP-8 mRNA can be explained as an early transcription of the AQP-8 gene, as previously suggested for another AQP (11).

These results demonstrate the direct involvement of AQP-8 in the overall water transport through isolated colonocytes and suggest a leading role for AQP-8 in DMSO-sensitive water permeability.

**DISCUSSION**

Water is a basic component of the human organism, accounting for ~60% of adult body weight with variations according to age and sex. The largest share of water in the human organism is introduced into the body through the intake of food and drink: for this reason water is considered an essential nutrient. Water has various essential biological functions; in the gastrointestinal tract, in particular, water is needed to digest and absorb nutrients.

The main function of the colonic epithelium is to contribute to body fluid homeostasis by salt and water transport and to produce dehydrated feces. Recent studies demonstrated that water absorption in the colon occurs in both surface and crypt epithelia (4). The main difference between the small and the large intestine is that in humans, the small intestine absorbs ~9 L of water daily by a near-isosmotic mechanism, whereas colonic crypt epithelia absorb an additional 1.5 L of water even against high hydraulic resistance to produce a hypertonic adsorbate (3,6). Nevertheless, these differences in absorptive capacity (mechanisms) between the small and the large intestine cannot be ascribed to differences in AQP expression (1,6).

The mechanisms underlying water transport in the colon and the role of surface epithelial cells, in particular, are still not fully clear (1). To our knowledge, this is the first work assessing both the expression of AQP's and functional studies on water permeability in the surface and lower crypt cells from the proximal colon.

The cell fractions, obtained from the proximal colon by sequential time incubation, represent surface (fractions 1 and 2) and lower crypt (fractions 4 and 5) cell populations, respectively, as results from the thymidine kinase activity assay; this validates the cell fraction isolation procedure (Fig. 1).

The results clearly show that all of the AQP proteins investigated were expressed in the proximal colon with a distribution along the crypt-lumen regions decreasing from superficial to lower crypt colonocytes (Fig. 2). These findings are essentially in agreement with those previously reported (7,8,11). Indeed, the expression of AQP-3 and AQP-4 was demonstrated previously in the basolateral membrane of the colonic surface epithelium of rats (8,11). However, Koyama et al. (36) did not detect AQP-4 protein or AQP-4 mRNA in either the proximal or distal colon. The present results clearly demonstrate the presence of both AQP-3 and AQP-4, whose protein expression decreases progressively from the superficial to the lower crypt regions in the proximal colon (Fig. 2). The pattern of distribution along the crypt-lumen regions was similar for AQP-3 protein and transcript, whereas AQP-4 mRNA increased from the superficial to the lower crypt colonocytes. This reverse pattern of distribution for AQP-4 mRNA can be explained as an early transcription of the AQP-4 gene, as previously suggested for another AQP (11).

![Normalized light scattering changes (A) and Pf (B) of rat proximal colonocytes exposed to a 150 mosm/L osmotic gradient.](image)

**FIGURE 4** Normalized light scattering changes (A) and Pf (B) of rat proximal colonocytes exposed to a 150 mosm/L osmotic gradient. Control, untreated cells; DMSO, dimethyl sulfoxide-treated cells; β-ME, dimethylsulfoxide-treated cells reversed with β-mercaptoethanol treatment. F1, F2, F3, F4, F5, cell fractions. Each bar represents the mean ± SEM of the Pf values obtained from at least 4 different preparations. Means with different letters differ, P < 0.05 (2-way ANOVA followed by the Bonferroni post test).

The mechanisms underlying water transport in the colon and the role of surface epithelial cells, in particular, are still not fully clear (1). To our knowledge, this is the first work assessing both the expression of AQP's and functional studies on water permeability in the surface and lower crypt cells from the proximal colon.

The cell fractions, obtained from the proximal colon by sequential time incubation, represent surface (fractions 1 and 2) and lower crypt (fractions 4 and 5) cell populations, respectively, as results from the thymidine kinase activity assay; this validates the cell fraction isolation procedure (Fig. 1).

The results clearly show that all of the AQP proteins investigated were expressed in the proximal colon with a distribution along the crypt-lumen regions decreasing from superficial to lower crypt colonocytes (Fig. 2). These findings are essentially in agreement with those previously reported (7,8,11). Indeed, the expression of AQP-3 and AQP-4 was demonstrated previously in the basolateral membrane of the colonic surface epithelium of rats (8,11). However, Koyama et al. (36) did not detect AQP-4 protein or AQP-4 mRNA in either the proximal or distal colon. The present results clearly demonstrate the presence of both AQP-3 and AQP-4, whose protein expression decreases progressively from the superficial to the lower crypt regions in the proximal colon (Fig. 2). The pattern of distribution along the crypt-lumen regions was similar for AQP-3 protein and transcript, whereas AQP-4 mRNA increased from the superficial to the lower crypt colonocytes. This reverse pattern of distribution for AQP-4 mRNA can be explained as an early transcription of the AQP-4 gene, as previously suggested for another AQP (11).
AQP-8 distribution in the colon has been studied but not clearly defined (7,12,36). Indeed, mRNA expression in the distal colon previously demonstrated by ribonuclease protection assay and in situ hybridization (36) was not confirmed by Calamita et al. (7). Our results (not shown) demonstrate that AQP-8 is distributed along the entire large intestine without significant differences between the proximal and distal portions. Moreover, immunohistochemical experiments (12) showed that in the colon, AQP-8 was expressed in both the superficial and lower crypt cells, whereas Calamita et al. (7) found consistent labeling only in superficial cells. Furthermore, according to Koyama et al. (36) and Calamita et al. (7) but not Elkjaer et al. (12), the AQP-8 protein localized is mainly in the superficial cells (Figs. 2 and 3). The differences in AQP-8 distribution can be explained by the different experimental techniques utilized or the different conditions of the animals before killing (i.e., food deprived or fed). The results of our immunohistochemical studies on the subcellular localization of AQP-8 reveal particularly prominent expression in the apical membrane (Fig. 3a, inset), which agrees in part with the previously reported localization of the AQP-8 near to and at the apical membrane (7,12). These results strongly suggest that water movement in the proximal colon can utilize AQP-8. Because AQP-8 intracellular staining was also present (Fig. 3) (7,12), we speculate that a cAMP-induced redistribution of AQP-8 from intracellular vesicles to the plasma membrane similar to that already demonstrated in cultured hepatocytes may also occur (28). The membrane localization of AQP-8 was further confirmed by siRNA experiments. Indeed, primary cultured colonocytes silenced for the AQP-8 gene showed a significant reduction in water transport properties (Fig. 5). This suggests for the first time that AQP-8 under normal conditions may represent an important route for water transport through the apical membrane domain of rat colonic superficial cells.

The functional experiments performed in the presence of osmotically induced water fluxes showed that Pf values of the superficial cells were \(~30–40\%\) higher than those of lower crypt cells (Fig. 4). Our functional observations agree well with the immunoblotting and RT-PCR results, which show higher AQP expression in superficial vs. lower crypt cells. The water permeability coefficient gradually decreased from fraction 1 to fraction 5; it was \(~100\ \mu\text{m/s}\) in the surface cells and \(60–70\ \mu\text{m/s}\) in the lower crypt cells. These values indicate that in the surface cell populations, a large amount of water moves through AQP channels. Treatment with the water channel inhibitor DMSO, which blocks the AQP-3 and -8 (10,30,37,38), reduced the Pf by \(30–40\%\) in superficial cells but only by \(12–21\%\) in lower crypt cells. Thus, permeability inhibition due to DMSO is not the same in the upper and lower crypt colonocytes, even though AQP distribution along the crypt axis is the same. Indeed, we expected the same inhibition rate in the presence of a maintained ratio between the DMSO-sensitive and insensitive AQPs in superficial and lower crypt colonocytes. This finding could be explained by the presence of another not yet identified DMSO-insensitive water channel expressed more prominently in lower crypt colonocytes or, alternatively, by the higher diffusional water permeability of lower crypt cells. Interestingly, an increased ratio of cholesterol:phospholipid content in mature vs. immature proliferative cells that decrease membrane fluidity and permeability was already demonstrated in the rat small intestine (39).

To distinguish the contribution of different AQPs to overall osmotic water transport, we performed functional experiments in a primary culture colonocyte knockdown for AQP-8. The results clearly demonstrate that AQP-8 gene suppression is associated with a reduction in water transport similar to that obtained by DMSO treatment (Fig. 5). Therefore, this suggests that functioning AQP-8 can mediate most of the DMSO-sensitive water permeability of the colonocytes.

However, the different sensitivity of AQPs to DMSO and the relative permeability of the AQPs to water confirm that the DMSO-sensitive water permeability in isolated colonocytes is probably related almost entirely to the AQP-8 content. Indeed, the DMSO-sensitive AQP-3s have relative permeability to water \((0.33)\) that is lower than that of the AQP-8 (1.35) (6). Overall, we speculate that transcellular water movement in rat colonocytes occurs mainly via AQP-8 in the apical pole.
and via AQP-3 and, especially, AQP-4 in the basolateral pole (8,11).

Recently, AQP-7 was also shown to exist in colonic tissue (40,41). Intense AQP-7 immunoreactivity was observed in the apical membrane of the superficial colonic cells, but intracellular staining was also present (41). Thus, its involvement in the DMSO-insensitive fluid transport in the colon could be advanced as a hypothesis.

In conclusion, our results strengthen the hypothesis of a pivotal role for AQP-7 and -4 in water transport in colonic superficial cells. The smaller numbers of water channels in the lower crypt cells confirm the ability of this epithelium to produce dehydrating capacity (3). An integrative hypothetical model of the channels and transporters responsible for colonic fluid and ion transport is depicted in Figure 6. Results summarized for ion transport in transgenic mice lacking aquaporin-4 water channels are shown. A hypothetical regulated translocation of intracellular AQP-7 and -8 to the apical membrane is also suggested.

LITERATURE CITED

29. Ecelbarger CA, Terris J, Frindt G, Echevarria M, Bastidas JA. Colon water transport is depicted in Figure 6. Results summarized for ion transport in transgenic mice lacking aquaporin-4 water channels are shown. A hypothetical regulated translocation of intracellular AQP-7 and -8 to the apical membrane is also suggested.