Biomedical Research **30** (3) 149-156, 2009

# Expression of short-chain fatty acid receptor GPR41 in the human colon

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*(Received 26 August 2008; and accepted 23 February 2009)*

**ABSTRACT**

Short-chain fatty acids (SCFAs), including acetate, propionate and butyrate, are the most commonly found anions found in the monogastric mammalian large intestine, and are known to have a variety of physiological and pathophysiological effects on the gastrointestinal tract. We investigated the protein and mRNA expression levels of GPR41, a possible G protein coupled receptor for SCFA, using Western blot analysis and reverse transcriptase-polymerase chain reaction. We found that GPR41 protein and mRNA are expressed in human colonic mucosa. Immunohistochemistry for GPR41 showed that mucosal GPR41 protein is localized in cytoplasm of enterocytes and enteroendocrine cells. Moreover, GPR41-immunoreactive endocrine cells contained peptide YY but not serotonin or GPR43. The cellular population of GPR41 ($0.01 \pm 0.01$ cells/crypt) was much smaller than that of GPR43 ($0.33 \pm 0.01$ cells/crypt) in the human colon. However, the potency order of SCFA-induced phasic contraction of colonic smooth muscle that we previously reported is consistent with GPR41 (propionate $\geqq$ butyrate > acetate) but not GPR43 (propionate = butyrate = acetate). Therefore, the present study suggests that GPR41 expressed in human colonic mucosa may function as a sensor for luminal SCFAs.

Short-chain fatty acids (SCFA) are a major anion present in the large intestinal lumen of monogastric mammals including humans. SCFAs are produced during anaerobic bacterial fermentation of unabsorbed carbohydrates and dietary fibers. The concentration of SCFAs in human feces is reported to be approximately 100 mM, and are primarily comprised of acetate, propionate, and butyrate. Molar ratios of SCFAs in human fecal content are 50–60, 15–20, and 10–20 for acetate, propionate, and butyrate respectively (3, 22). Through absorption and metabolism of SCFAs, the host is able to salvage energy from food not digested in the upper intestine. Furthermore, luminal SCFAs have various physiological and pathophysiological effects in the gastrointestinal (GI) tract (4, 10, 17, 19).

In our previous studies, we demonstrated that SCFAs evoke two different effects on rat colonic smooth muscle *in vitro* (13–15). In the early phase, propionate and butyrate induced concentration-dependent phasic contractions in circular and longitudinal muscle, but acetate had no such effect (13–15). In the late phase, propionate induced a concentration-dependent increase in frequency of spontaneous contractions in circular (13) and longitudinal muscle (15), but butyrate did not affect the frequency of spontaneous longitudinal muscle contractions (15). On the other hand, acetate induced a

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concentration-dependent decrease in the frequency of spontaneous longitudinal muscle contractions independent of early phase contractions (15). Taken together, these studies suggest that individual SCFAs induce different physiological responses through varying receptive mechanisms. In addition, we showed that the propionate-induced contractions of both early and late phases on colonic smooth muscle are abolished by mucosa-free preparation (13, 14). We therefore hypothesized that SCFA-induced physiological effects are mediated by more than one specific receptor for each SCFA in the colon.

In 2003, two research groups independently identified GPR41 and GPR43 orphan G-protein coupled receptors (GPCRs) (1, 11) that vary in specificity for individual SCFAs, intracellular signaling, and tissue localization. GPR41 appears to be coupled exclusively to the $G_{i/o}$, whereas GPR43 displays dual coupling to $G_q$ and $G_{i/o}$ (11). GPR43 exhibits similarly potent agonist activities for acetate, propionate and butyrate, whereas GPR41 shows notable differences; that is, the rank order of potency for GPR41 is propionate $\geqq$ butyrate > acetate. Gene expression levels of both GPR41 and GPR43 in the stomach and small intestine are comparatively lower than other tissues including spleen, bone marrow and other tissues (1, 11). On the other hand, quantitative real-time PCR revealed that GPR43 gene expression in the large intestine was higher than in other regions of the GI tract (5). Furthermore, we recently reported the presence of GPR43 in the rat terminal ileum and colon (8) and human colon as well as its gene and protein expression patterns (9). Using immunohistochemistry techniques, we demonstrated that GPR43 is expressed in peptide YY (PYY)-containing enteroendocrine cells. PYY is released following the luminal addition of SCFAs, and induces inhibition of upper GI motility, the so called "ileal/colonic brake" (2), leading us to hypothesize that GPR43 might function as a specific receptor for SCFAs. On the other hand, different studies suggest that GPR41 might contribute to at least local regulation of colonic motility induced by SCFAs, according to the potency order (13–15). However, little is known about the expression and distribution of GPR41 in large intestine. It is important to learn the expression and precise localization patterns of GPR41 in order to further our understanding of the physiological role of SCFAs in colonic motility.

Therefore, in the present study, we investigated the expressions patterns of GPR41 protein and mRNA in human colon through utilization of Western blot analysis and RT-PCR respectively, and then examined the localization of GPR41 protein in human colonic mucosa using immunohistochemical techniques.

## MATERIALS AND METHODS

*Human tissue preparation*. This study was approved by the Institutional Review Board of Shizuoka General Hospital and the University of Shizuoka. Segments of human ascending colon were obtained (following informed consent) from patients undergoing colectomy for carcinoma. A nonpathological region was cut from the surgical specimen, and then placed in ice-cold Krebs-Ringer solution (composition: (in mmol/L), 117 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 1.2 $NaH_2PO_4$, 25 $NaHCO_3$, 2.5 $CaCl_2$, and 11 glucose saturated with 95% $O_2$−5% $CO_2$) for transport to the laboratory.

*Peptide synthesis and production of anti-GPR41 antiserum*. Peptide synthesis and antiserum production methods were described previously (8). In brief, human GPR41 (1–19)-Cys (MDTGPDQSYFSGNHW FVFS) was synthesized (purity > 95%) using the Fmoc-strategy for solid-phase methodology in an automated peptide synthesizer (Pioneer; Applied Biosystems, Foster, Calif., USA). The synthesized peptide was used for antigen, and was injected intradermally into multiple sites of three Japanese white female rabbits (2.0–2.5 kg). Immunization was then performed at 2-week intervals, using half the dose of immunogen used for the primary immunization. The rabbits were bled from the marginal ear vein, ten days after each immunization. After six immunizations, one of the three rabbits revealed a high titer antiserum (RY1494) against human GPR41 (1–19), and was used for the present study.

*Western blot analysis*. Human intestinal specimens were divided into mucosa, submucosa, and muscle. These tissues were immediately frozen in liquid nitrogen, and stored at −80°C until protein isolation. Western blot analysis was performed as previously reported (9).

We also investigated N-linked glycosylation of GPR41 by using PNGase F (New England BioLabs Japan Inc., Tokyo, Japan), according to manual instructions. Negative control was added only to buffers without enzyme.

*RT-PCR analysis*. Human colonic mucosa was immediately immersed in RNAlater RNA Stabilization Reagent (Qiagen Inc., Tokyo, Japan). Total RNA

was isolated by RNeasy Micro Kit (Qiagen Inc.) according to the manufacturer's directions. To prevent possible contamination with DNA, samples were treated with deoxyribonucrease (RT-grade; Wako Pure Chemical Industries Ltd., Osaka, Japan) as recommended by the manufacturer. Isolated total RNA was transformed into cDNA by Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Tokyo, Japan), and stocked at −20°C. Polymerase chain reaction (PCR) was performed using Premix Taq Ex Taq™ (Takara Bio, Shiga, Japan) according to manufacturer instructions. A set of primers (Forward; 5'-ACCTGCTGGCCCTGGTG-3', Reverse; 5'-GGTCAGGTTGAGCAGGAGCA-3') for PCR of human GPR41 was based on the human GPR41 mRNA sequence (Refseq ID: NM_005304). PCR cycles consisted of denaturing at 94°C for 30 s, annealing at 55.5°C for 30 s, and extension at 72°C for 1 min; reactions were repeated for 35 cycles. Finally, amplification products were stored at 4°C until use. Amplification products and OneSTEP Maker 5 (Nippon Gene, Toyama, Japan) were separated by electrophoresis on 2% agarose gel in 0.5 × TRIS-borate-EDTA buffer and stained with SYBR Green 1 (Molecular Probes, Eugene, Oregon, USA). Fluorescence-sensitive images of the gels were captured by Pharos FX (Bio-Rad Laboratories, Tokyo, Japan).

*Immunohistochemistry*. Immunohistochemistry techniques were based those used in our previous report (9). In brief, the human colonic tissues were immersed in Zamboni's fixative at 4°C overnight. The fixed tissues were washed in phosphate-buffered saline (PBS), and further cryoprotected by immersing in PBS containing 30% sucrose and 0.1% sodium azide at 4°C. The cryoprotected tissues were rapidly frozen with optimal cutting temperature (OCT) compound (Tissue-Tek; Sakura Finetechnical, Tokyo, Japan) in liquid nitrogen, and stored at −80°C until use. The frozen block was cut into 10 μm-thin sections in a cryostat (CM1100; Leica Microsystems, Wetzlar, Germany). Tissue sections were preblocked with 0.5% BSA in PBS, and incubated at 4°C, overnight, with primary antibodies diluted with PBS: rabbit anti-GPR41 (RY1494), 1 : 10,000; goat anti-5-HT (ImmunoSter Inc., Hudson, Wisconsin, USA), 1 : 4,000; chicken anti-PYY (GenWay Biotech Inc., San Diego, Calif., USA), 1 : 500; or goat anti-GPR43 (Santa Cruz Biotechnology Inc., Delaware, Calif., USA), 1 : 250. After washing in PBS (3 × 10 min), sections were incubated at room temperature for 1 h with secondary antibodies diluted with PBS: donkey anti-rabbit IgG-Alexa Flour 594 (Molecular Probes Inc., Leiden, Netherlands), 1 : 200; donkey anti-goat IgG-Alexa Flour 488 (Molecular Probes), 1 : 200; or donkey anti-chicken IgY-FITC (Chemicon International, Billerica, Massachusetts, USA), 1 : 200. In the secondary antibody diluents, 4',6-diamidino-2-phenylindole (DAPI) solution (1 : 200; Dojindo Laboratories, Kumamoto, Japan) were also added. Sections were then washed in PBS (3 × 10 min) and cover-slipped with a mounting medium (DakoCytomation, Glostrup, Denmark). Immunoreactivity was then visualized by using a fluorescence microscope (IX70; Olympus, Tokyo, Japan), and images were captured by means of a cooled charge-coupled digital camera system (AxioVision 135; Zeiss, Munich-Hallbergmoos, Germany). In order to remove out-of focus fluorescence, captured images were deconvoluted using image-processing software (Photoshop; Adobe, San Jose, Calif., USA). Methods for checking specificity of secondary antibody were previously reported (9).

To check the specificity of antiserum for GPR41, an absorption test was performed as follows. After neat antigen solution was mixed with neat anti-GPR41 serum 10 : 1, the mixture was diluted with PBS to a working concentration of GPR41 serum (1 : 10,000). Sections were incubated with this diluted mixture of antibody and antigen as per standard immunohistochemical procedure.

*Populations of the GPR41-/GPR43-immunoreactive enteroendocrine cells*. To investigate the populations of GPR41- and/or GPR43-immunoreactive enteroendocrine cells, the cell numbers of GPR41- only, GPR43- only, and GPR41 and GPR43 combined-immunoreactive enteroendocrine cells per vertical crypt section (from top to bottom) were estimated by counting immunoreactive cells in each section by the following method using double-immunostaining for GPR41 and GPR43. The number of immunoreactive cells and number of crypts were counted in three to four different sections from ascending colon removed from one patient, and the number of immunoreactive cells per crypt was then calculated. Next, the data from three patients were averaged, and expressed as means ± S. E. M.

## RESULTS

### *GPR41 protein expression*

Tissue distribution of GPR41 protein was analyzed by Western blotting using the specific antiserum against the N-terminal peptide of human GPR41.

Fig. 1A shows the expression of GPR41 protein in human ascending colon. Protein expression levels were higher in colonic mucosa than submucosa or muscle (Fig. 1A, left panel). The signal was weakened by blocking peptide mixed with primary antibody (Fig. 1A, right panel).

*Glycosylation for GPR41 protein*

The theoretical molecular weight of GPR41 is speculated to be 38 kDa, but the predominant immunoreactive band of Fig. 1A was located near 53 kDa. Therefore, we considered that the human GPR41 expressed in human colonic mucosa was glycosylated because glycosylation of protein results in a higher molecular weight than theoretical weight in SDS-PAGE due to the addition of oligosaccharides. To investigate whether the human GPR41 protein expressed in intestinal mucosa was N-linked glycosylated or not, we treated mucosal protein with PNGase F, and detected GPR41 protein by Western blotting (Fig. 1B). In tissues treated with PNGase F, the antibody detected two distinct immunoreactive bands, a major 50 kDa and a minor 53 kDa ($n$ = 3).

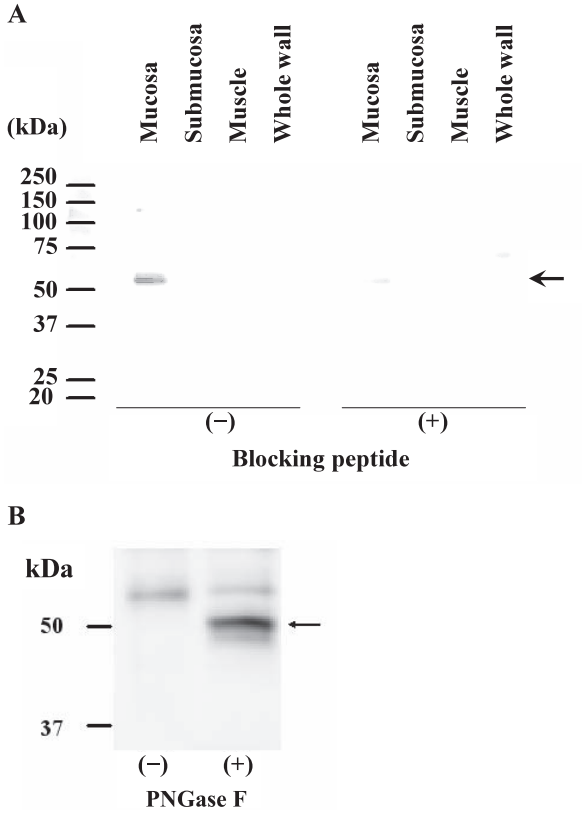


**Fig. 1** Analysis of GPR41 protein expression in the human ascending colon. Panel **A**: Western blot analysis of GPR41 protein expression. Proteins were extracted from mucosa, submucosa, muscle, and whole wall obtained from human ascending colon. Equal amounts of protein samples were separated by SDS-PAGE, transferred to PVDF (polyvinylidene diflouride) membrane, and stained with anti-GPR41 antibody. An absorption control experiment using blocking peptide was also performed. Panel **B**: Analysis of glycosylation of GPR41. N-linked glycosylation of the mucosal protein sample isolated from human ascending colon was digested by PNGase F treatment. (–) lanes showed negative control (only buffers without enzyme were added to these controls and they were otherwise incubated under identical conditions to enzyme-added (+) samples).

*GPR41 mRNA expression*

To confirm GPR41 expression in mucosa, we performed RT-PCR analysis. The signal band of the expected base pair size (150 bp) was detected using a RT (+) cDNA sample from total RNA extracted from human ascending colonic mucosa (Fig. 2). No signal band was detected from the RT (−) sample, indicating that the signal is not an amplification product of genomic DNA.

*Immunohistochemistry*

To identify the cellular distribution of GPR41 protein in human colon, immunohistochemical staining was performed using anti-GPR41 antiserum. GPR41-immunoreactivity was observed as dotted staining in the apical cytoplasm of enterocytes (Fig. 3A arrow and 3B), enteroendcrine cells (Fig. 3A arrowhead and 3C), and several lamina propria cells (Fig. 3A). The apical cytoplasm of enterocytes showed two staining patterns; fine granular staining dispersed in the apical cytoplasm and coarse-granular staining close to nuclei may correspond respectively to the endoplasmic reticulum and Golgi apparatus. In negative control staining, utilizing secondary antibody alone and in absorption control, such staining was not observed (data not shown), however, many lamina propria cells were also stained in absorption control. Thus, staining in the epithelium was specif-

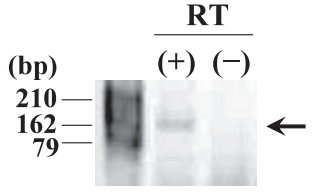


**Fig. 2** RT-PCR analysis of GPR41 mRNA expression in the human ascending colon. Total RNA sample extracted from human ascending colonic mucosa was transformed into cDNA, and amplified by PCR. PCR products were separated by electrophoresis and stained with SYBR Green 1. Reverse transcription (–) was performed as a negative control to confirm that there was no genomic DNA contamination.

ic for GPR41, but most staining of lamina proprial cells seemed to be non-specific.

Dotted stainings of GPR41 in the enterocyte cytoplasm possibly at the endoplasmic reticulum and Golgi apparatus were stronger at surface epithelium than at the bottom of the crypt (Fig. 3D). The GPR41-immunoreactive endocrine cells were open-type with thin cell bodies that extended to the lumen surface (Fig. 3C). However, number of GPR41-immunoreactive endocrine cells were fewer than GPR43 cells.

We next investigated whether GPR41-immunoreactive endocrine cells colocalized with 5-HT, and found that GPR41-immunoreactive endocrine cells were not 5-HT-immunoreactive (Fig. 4A). Since GPR43 immunoreactivity is reported to colocalize with PYY (8, 9), we also checked whether GPR41 was colocalized with PYY. Indeed we found that all GPR41-immunoreactive endocrine cells were also immunoreactive for PYY (Fig. 4B), but not *vice versa* (Fig. 4C).

Double-immunostaining for GPR41 and GPR43 revealed that immunoreactive GPR41 and GPR43 were not colocalized in endocrine cells (Fig. 5A),

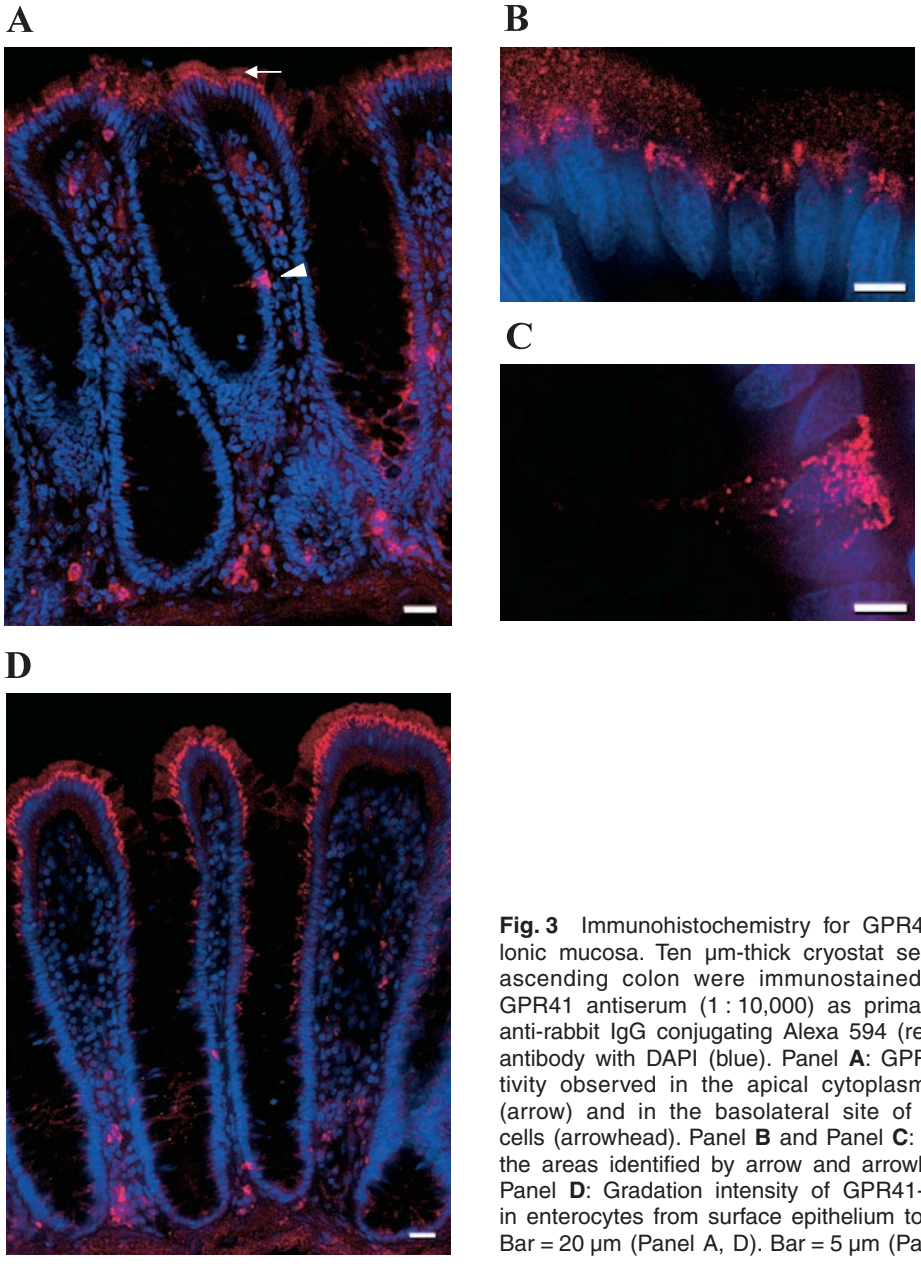


**Fig. 3** Immunohistochemistry for GPR41 in human colonic mucosa. Ten μm-thick cryostat sections of human ascending colon were immunostained by rabbit anti-GPR41 antiserum (1 : 10,000) as primary antibody, and anti-rabbit IgG conjugating Alexa 594 (red) as secondary antibody with DAPI (blue). Panel **A**: GPR41-immunoreactivity observed in the apical cytoplasm of enterocytes (arrow) and in the basolateral site of enteroendocrine cells (arrowhead). Panel **B** and Panel **C**: Magnifications of the areas identified by arrow and arrowhead in panel A. Panel **D**: Gradation intensity of GPR41-immunoreactivity in enterocytes from surface epithelium to bottom of crypt. Bar = 20 μm (Panel A, D). Bar = 5 μm (Panel B, C)

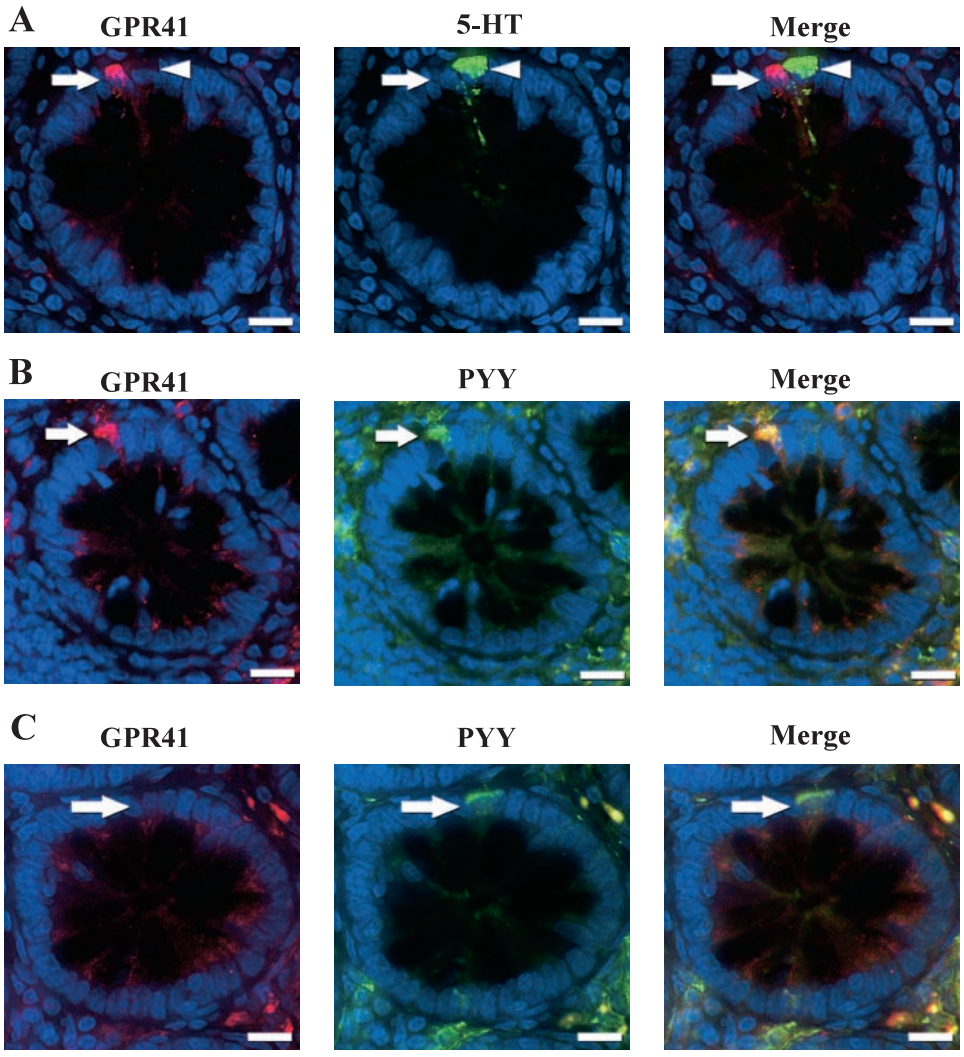


**Fig. 4** Double-immunostaining of GPR41 and 5-HT/PYY in enteroendocrine cells. Panel **A**: Lack of colocalization of GPR41 with 5-HT. A GPR41-immunoreactive endocrine cell (arrow) was not colocalized with 5-HT (arrowhead). Panel **B**: GPR41-immunoreactive (red) and PYY-immunoreactive (green) endocrine cell (arrow). Colocalization of GPR41 and PYY was shown as yellow staining. Panel **C**: PYY-immunoreactive, but not GPR41-immunoreactive endocrine cell (arrow). All GPR41-containing endocrine cells also contained PYY, but a part of PYY-containing endocrine cells did not express GPR41. Bar = 20 μm.

and GPR41- or GPR43-immunoreactive endocrine cells were estimated to be 0.01 ± 0.01 or 0.33 ± 0.01 cells per crypt, respectively (Fig. 5B).

## DISCUSSION

GPR41 protein was expressed in human colonic mucosa, but not in submucosa or muscle (Fig. 1A). RT-PCR analysis indicates that GPR41 mRNA is expressed in human colonic mucosa (Fig. 2). Furthermore, GPR41 protein was localized in the cytoplasm of enterocytes in areas representative of endoplasmic reticulum and Golgi apparatus (Fig. 3B) and in PYY-containing enteroendocrine cells (Fig. 4B).

Western blot analysis revealed a single, sharp band in the mucosa sample of human colon, suggestive specificity for GPR41, as per results of the absorption test (Fig. 1A). However, the signal band indicative of GPR41 protein was detected at near 53 kDa, a higher molecular weight than the theoretical molecular weight of human GPR41 (38 kDa). It has been reported that the human GPR41 protein includes one N-linked glycosylation site in the second extracellular loop (N166), one PKC site (S216), two combined PKA/PKC (T328, T329) phosphorylation

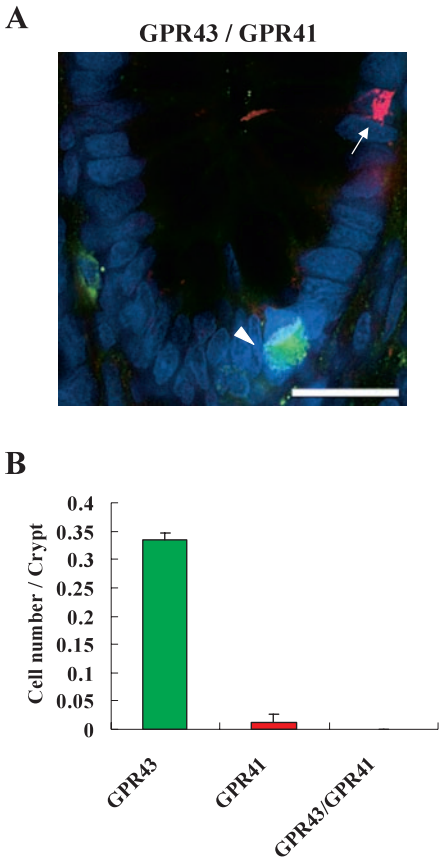


**Fig. 5** Double-immunostaining of GPR41 and GPR43 in enteroendocrine cells. Panel **A**: GPR41-immunoreactive (red, arrow) and GPR43-immunoreactive (green, arrowhead) endocrine cells. GPR41 and GPR43 were not colocalized. Bar = 20 μm. Panel **B**: Cell counts of GPR41- or GPR43-immunoreactive enteroendocrine cells.

sites, and one palmitoylation site (C295) in the C-terminus (18). Thus, it is probable that the actual molecular weight is higher molecular than the theoretical weight calculated from its amino acids sequence. In the present results, PNGase F-treated GPR41 protein shifted to a smaller molecular weight at about 50 kDa (Fig. 1B), indicating that human GPR41 protein expressed in the human colonic mucosa does indeed have an N-linked glycosylation site. N-linked glycosylation of cell surface receptors is generally recognized to increase molecular stability, promote cell surface expression, and often modulate ligand binding activity (6, 21), but the specific function of N-linked glycosylation of GPR41 is unclear as of yet; it may be related to higher ligand specificity as compared to GPR43. The fact that PNGase F-treated GPR41 protein still showed a higher molecular weight compared to its theoretical weight suggests that GPR41 protein might have other, unknown, modifications in addition to N-linked glycosylation.

Immunohistochemistry analysis revealed that GPR41 was localized in the apical cytoplasm of enterocytes (Fig. 3B), but not in the apical membrane. Dotted staining of GPR41 in enterocyte cytoplasm was stronger at surface epithelium than at the bottom of the crypt (Fig. 3D); further, the GPR41-immunoreactivity was located in endoplasmic reticulum and Golgi apparatus between the apical membrane and the nuclei. We therefore speculate that GPR41 may be transported to the apical membrane and/or internalized from the apical membrane. GPR41 has been reported to couple with $G_{i/o}$ protein to increase intracellular $Ca^{2+}$ and to decrease cAMP concentrations, and both intracellular $Ca^{2+}$ and cAMP can function as second messengers to induce synergistic fluid secretion (7). It is therefore possible that luminal SCFAs modulate colonic ion transport via GPR41-mediating second messengers.

GPR41 protein was expressed in enteroendocrine cells containing PYY but not 5-HT (Fig. 4A, 4B). We previously reported that all PYY-containing endocrine cells expressed GPR43 in rat (8) and human (9) colon, but found in the present study that GPR41-immunoreactivity localized in PYY-containing endocrine cells only to a small degree. Interestingly, GPR41 and GPR43 are not colocalized in endocrine cells, seemingly raising a discrepancy that PYY-immunoreactive but GPR43-immunonegative endocrine cells are present in human ascending colon. It is possible that we did not find GPR41-immunoreactive enteroendocrine cells in the previous study, because that population was quite small (about 4% of the population of GPR43-immunoreactive endocrine cells in the current study).

Do enteroendocrine cells containing GPR41, an acknowledged minority, play any physiological role in the ascending colon? In previous studies (23, 24), the potency order of individual SCFA-induced physiological responses, including peristaltic and secretory responses, has been reported to be propionate ≧ butyrate >> acetate, as seen in GPR41, but different than GPR43 (propionate = butyrate = acetate). It is possible that these physiological functions are mediated via GPR41 rather than GPR43 even at the low GPR41 expression levels found in this study.

Although Mitsui *et al.* reported that 5-$HT_4$ receptor antagonist blocked the propionate-induced contraction of colonic smooth muscle (14), the present results indicate that GPR41 is colocalized with PYY, not with 5-HT (Fig. 4A, 4B). Moreover, exogenous addition of PYY in the same physiological experi-

ments did not induce the previously reported responses with SCFAs (20, 24). This discrepancy is not resolved in the present study, but it is possible that GPR41-expressing enteroendocrine cells contain other transmitter(s) that activate enterochromaffin cells containing 5-HT, mucosal mast cells and/or enteric neurons. Further investigation is needed to identify such transmitters and/or to investigate other as yet unknown mechanisms in the human colon.

In conclusion, GPR41 is expressed in the human ascending colon, and is N-linked glycosylated. Moreover, GPR41 is localized in epithelial cells and PYY-containing enteroendocrine cells, but not 5-HT- or GPR43-immunoreactive enteroendocrine cells. The present study indicates that GPR41 expressed in human colonic mucosa may function as a sensor for luminal SCFAs.

## Acknowledgements

This work was supported in part by funding from the Japan Society for the Promotion of Science (no. 18590207), by the Smoking Research Foundation, by the Salt Research Foundation, and by the Food Science Institute Foundation awarded to A. Kuwahara.

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