Distribution and Dietary Induction of Cellular Retinol-Binding Protein Type II along the Villus-Crypt Axis of the Rat Jejunum

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Summary Cellular retinol-binding protein type II (CRBP-II) is exclusively expressed in the small intestinal absorptive cells. We previously reported that dietary fat induces CRBP-II expression within 12 h of fat intake. To examine at which locus of the villus-crypt axis this response to dietary fat occurs, 6-wk-old rats were fed a low-fat diet (7% energy) for 7 d, and then given free access to a high-fat diet (70% energy) for the subsequent 12, 24 or 48 h. Cryostat sectioning of jejunal segments followed by RNA blot hybridization of the transcripts revealed that CRBP-II mRNA was expressed maximally in the lower villus, and the immunoreactive protein of CRBP-II was expressed maximally in the mid-villus. Feeding the high-fat diet caused a pronounced increase in CRBP-II mRNA level from the lower- to middle-villus within 12 h. These results suggest that the CRBP-II gene is maximally expressed in the lower villus, and that dietary fat causes an enhancement of CRBP-II gene expression in the villus cells.

Key Words CRBP-II, high-fat diet, rats, villus, crypt

Cellular retinol-binding protein type II (CRBP-II) is exclusively expressed in the small intestinal villus cells and plays a pivotal role in intestinal absorption and metabolism of retinol and β -carotene. CRBP-II not only directs retinal reductase to convert retinal to retinol in enterocytes, but also directs lecithin:retinol acyltransferase (L-RAT) to convert retinol to retinyl esters, which leads to incorporation of vitamin A into chylomicron (1). Like many other genes involved in intestinal nutrient absorption (2–5), the CRBP-II gene is thought to be expressed at certain stages of the differentiation or maturation of intestinal absorptive cells, which are derived from intestinal stem cells in the crypt and continuously differentiate into the cells destined for nutrient absorption. We have previously demonstrated that CRBP-II protein and the enzyme activities involved in vitamin A absorption, e.g. β -carotene cleavage enzyme, retinal reductase and L-RAT, are coordinately expressed along the villus-crypt axis in the small intestine (6, 7). However, the precise distribution of CRBP-II mRNA along the villus-crypt axis was unclear. We previously showed that the distribution of the gene transcripts for lactase-phlorizin hydrolase (LPH) is more apical and broader than that of sucrase-isomaltase (SI) along the crypt-villus axis, and that dietary sucrose specifically enhances SI mRNA level in the lower villus and LPH mRNA level in the middle and upper villi (5). These results suggest

that nutrients enhance intestinal gene expression in specific phase(s) of differentiation in absorptive cells along the villus-crypt column. Our previous studies showed that dietary fat is a major regulator of CRBP-II gene expression (8, 9), and that the fat-induced increase in CRBP-II mRNA occurs within 12 h of fat exposure, via one of the nuclear receptors, PPAR α (10, 11). It is most likely that expression of the CRBP-II gene is regulated by dietary fat at a certain stage of maturation in the absorptive cells. However, it is unclear at which region along the villus-crypt axis of the jejunum the CRBP-II expression is induced by dietary fat.

In this study, we employed the cryostat slicing technique to quantitatively determine the distribution of CRBP-II mRNA level along the villus-crypt axis of the jejunum of rats which were fed either a low-fat diet (7% fat as energy) or high-fat diet (70% fat as energy).

MATERIALS AND METHODS

Animals and diet. Six-week-old male Sprague-Dawley (SD) rats (Japan SLC, Hamamatsu, Japan) had free access to a standard chow diet (MF; Oriental Yeast, Co., Ltd., Tokyo, Japan) and water until the experiment began. To examine the basal localization of CRBP-II mRNA along the villus-crypt columns the rats were killed at 10:00 a.m. To investigate the diet-induced changes in the expressions of the CRBP-II gene and protein, rats were fed a low-fat diet (7% energy as corn oil) for 7 d, and they were subsequently given free access to a high-fat diet (70% energy as corn oil) for 12, 24 or

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Table 1. The composition of diets (g/kg).

Ingredient	Low-fat-diet	High-fat-diet
Vitamin-free casein	157	159
Corn starch	526	36
Corn oil	24	247
Mineral mix ^a	28	28
Vitamin mix ^a	8	8
DL-Methionine	2.4	2.4
Choline bitartrate	1.6	1.6
2% agar	254	518
Total	1,000	1,000

^a AIN-76 mineral mix and AIN-76 vitamin mix (1977) purchased from Oriental Yeast, Co., Ltd., Tokyo, Japan.

48 h. The composition of the diets is shown in Table 1. The rats were killed by decapitation at 10:00 a.m. to avoid any variation in intestinal gene expression due to circadian fluctuation. The experimental procedures used in the present study met the guidelines of the animal usage committee of the University of Shizuoka.

Tissue preparation. The entire small intestine was removed, and the duodenum extending from the pylorus to the ligament of Treitz was discarded. The jejunoleum was divided into three segments of equal length. The proximal one-third (jejunum) of the jejunoleum was flushed with diethylpyrocarbonate (DEPC)-treated, ice-cold 0.154 mol/L of NaCl solution. A 1-cm segment (100 mg) was excised from the middle region of the jejunal segment, and total RNA was extracted. An adjacent segment (2 cm) was separated into two parts, one of which was opened longitudinally, flattened on a glass slide, serosal-side down, frozen in liquid nitrogen, and immediately subjected to cryostat sectioning. A frozen tissue block of 7×7 mm² was covered with a supporting medium (OCT Compound, Miles Laboratories, Elkhart, IN, USA) and transferred, serosal-side down, to a pre-flattened supporting surface of 1 g/L agar within a cryostat at -18°C. The tissue was sectioned transversely at 10 µm thickness through the submucosa into the muscular layer as described previously (12). Sections taken from various depths in the villus-crypt column were attached to microscope slides and examined for the presence of villus and crypt architecture after staining with 3 g/L methylene blue. The other segment was fixed in 4% paraformaldehyde/phosphate buffer (18.4 mmol/L NaH₂PO₄, 61.1 mmol/L Na₂HPO₄, pH 7.4) and dehydrated in ethanol. This tissue was used for immunohistochemical and in situ hybridization experiments. The mucosa was scraped from the remaining part of the jejunal segment with a glass microscope slide. The intestinal mucosa was homogenized in 4 vol/wt of ice-cold 10 mmol/L sodium phosphate/140 mmol/L NaCl (pH 7.2), containing 0.1 mmol/L phenylmethyl sulfonyl fluoride. After centrifugation of homogenates at 105,000 ×g for 1 h (4°C), the clear supernatant fraction (cytosol) was frozen at -20°C and used for assay of CRBP_{II}.

RNA extraction and RNA blot hybridization. To deter-

mine CRBP_{II} mRNA levels along the villus-crypt columns, 20 consecutive sections were combined and homogenized in 200 µL of 25 mmol/L sodium citrate buffer (pH 7.0) containing 4 mol/L guanidine thiocyanate, 17 mmol/L sodium N-lauroylsarcosine and 13 mmol/L mercaptoethanol, using a polytron homogenizer at 15,000 rpm for 15 s. The homogenate was immediately subjected to RNA extraction, as described by Chomczynski and Sacchi (13). Dot-blot analysis was performed using the ³²P-labeled cDNA probe for rat CRBP_{II} as described previously (5). Control hybridization was carried out for β-actin.

Immunological assays. To assay the immunoreactivity of CRBP_{II} at different heights of the villus-crypt columns, tissue blocks were sectioned as described above, and 10 consecutive sections were combined and homogenized in 1 mL of 10 mmol/L sodium phosphate/140 mmol/L NaCl (pH 7.2) by sonication at 4°C for 30 s using a Branson Sonifier 250 (Branson Sonic Power, Danbury, CT, USA) at dial position 3. The homogenate was centrifuged at 4°C at 105,000 ×g for 60 min, and the resulting supernatant was stored at -20°C. The amounts of CRBP_{II} were measured by enzyme-linked immunosorbent assay with a mono-specific antiserum, as described previously (14).

Immunohistochemical analysis. Immunohistochemical analysis was performed using an ABC high-HRP immunostaining kit (Toyobo, Tokyo, Japan), according to the manufacturer's instructions. Tissue sections were deparaffinized in xylene and ethanol, and incubated for 30 min in a 1 : 200 dilution of rabbit anti-rat CRBP_{II} in PBS containing normal goat serum (14). After rinsing in PBS three times for 5 min each, sections were incubated for 30 min in a 1 : 100 dilution of biotin-conjugated goat anti-rabbit IgG in PBS, mounted in glycerol and photographed. To verify the specificity of immunostaining, companion sections were processed as above using rabbit pre-immune serum.

In situ hybridization. As the cRNA probe of rat CRBP_{II} for in situ hybridization, a fragment corresponding to +1 to +303 from the transcription start site (15) was prepared using rat CRBP_{II} cDNA cloned in pGEM-Teasy as a template. Antisense and sense riboprobes were labeled with digoxigenin using a DIG RNA labeling kit (Roche Molecular Biochemicals, Tokyo, Japan), according to the manufacturer's instructions. Tissue sections were deparaffinized in xylene and ethanol and treated with 0.2 mol/L HCl for 8 min, 0.1 mol/L triethanolamine-HCl (pH 8.0) for 1 min and 0.26% (v/v) acetic anhydride in 0.1 mol/L triethanolamine (pH 8.0) for 15 min. After rinsing for 10 min with 2×SSC (0.03 mol/L sodium citrate, 0.3 mol/L NaCl, pH 7.0), the sections were incubated with a hybridization solution which contained 6 ng/µL DIG-labeled probe in hybridization buffer (50% (v/v) formamide, 2×SSC, 10% (w/v) dextran sulfate, 1 µg/µL yeast t-RNA, 1 µg/µL sonicated salmon sperm DNA and 1 µg/µL BSA) at 50°C for 16 h. After hybridization, the slides were sequentially washed with 5×SSC (0.075 mol/L sodium citrate, 0.75 mol/L NaCl, pH 7.0) solution at 60°C for 10 min,

Fig. 1. Distribution of CRBP II mRNA and the immunoreactive CRBP II protein along the villus-crypt axis of the rat jejunum. Rats had free access to a laboratory chow diet throughout the experimental period. (A) Dot blots for CRBP II mRNA in serial sections of villus-crypt columns and the graphic representation of the distribution of CRBP II mRNA along the villus-crypt axis of the rat jejunum. Total RNA was extracted from serial sections of the jejunal segments, and 600 ng of total RNA were subjected to dot-blot hybridization. Autoradiographic images were quantified using an image analyzer, and the results for each sample, normalized for β -actin mRNA abundance, were expressed as arbitrary units. The abscissa depicts the total height of the intestinal wall, with 100% representing the top part of the villus and 0% the bottom part of the serosal side. Values represent means $\pm$ SE, $n=4$. Relative positions of the villus and crypt are depicted with rectangles. (B) In situ hybridization of CRBP II mRNA using DIG-labeled CRBP II antisense or sense RNA. Bars=100 μ m. (C) Distribution of CRBP II levels along the villus-crypt axis measured using ELISA. Immunoreactive CRBP II protein was normalized for total protein abundance. Abscissa depicts the total height of the intestinal wall, with 100% representing the top part of the villus and 0% the bottom part of the serosal side. Values are means $\pm$ SE, $n=4$. (D) Immunohistochemical analysis of jejunal CRBP II protein. Bars=100 μ m.

50% (v/v) formamide/2 $\times$ SSC at 60°C for 30 min and TNE buffer (0.5 mol/L NaCl, 10 mmol/L Tris-HCl, pH 7.6) at 37°C for 10 min. The sections were then treated with RNase A (2 μ g/mL) in TNE buffer at 37°C for 30 min, followed by washing with TNE buffer at 37°C for 30 min, with 2 $\times$ SSC at 48°C (30 min, twice) and 0.2 $\times$ SSC at 48°C (30 min, twice). After washing with 0.1 mol/L Tris-HCl, pH 7.5, at room temperature for 10 min, the slides were incubated with a blocking buffer containing 1% (w/v) blocking reagent (Roche) in 0.1 mol/L Tris-HCl, pH 7.5, at room temperature for 60 min. The slides were incubated for 18 h at 4°C with a blocking buffer containing anti-digoxigenin-AP, Fab fragments (Roche). These slides were washed four times with 0.1 mol/L Tris-HCl, pH 7.5, for 10 min and soaked in buffer containing 0.1 mol/L Tris, 0.1 mol/L NaCl and 0.2 mol/L MgCl₂, pH 9.5, at room temperature for 5 min. Finally, immunoreactivity of sections was visualized by incubating for 18 h at 4°C with 450 μ g Nitroblue tetrazolium chloride (NBT)/175 μ g 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt (BCIP) solution in 1 mL of buffer containing 0.1 mol/L Tris, 0.1 mol/L NaCl and 0.2 mol/L MgCl₂, pH 9.5.

Statistical analysis. All results were subjected to one-way ANOVA. Differences in mean values at the same height of the villus-crypt columns among groups were tested using Tukey's multiple range test and were considered significant at $p<0.05$.

RESULTS

Distribution of CRBP II expression along the villus-crypt axis of the rat jejunum

To quantitatively compare expression of CRBP II along the jejunal villus-crypt axis, segments were fractionated along the axis using cryostat sectioning. First, we extracted total RNA from fractions taken from various heights in the villus-crypt columns and quantified the CRBP II mRNA by RNA dot-blot hybridization (Fig. 1A). Dot-blot analysis showed that the CRBP II mRNA level was markedly elevated at the base of the villi, reaching a maximum at the lower villus. It reached a maximum level at 45% of the height of the villus-crypt columns, and gradually declined toward the top of the villi (Fig. 1A). In situ hybridization analysis showed that the CRBP II transcript was strongly expressed from the top of the crypt to the base of the villi (Fig. 1B). We mea-

sured the distribution of the amount of immunoreactive CRBP II protein along the villus-crypt axis and found that it increased gradually from the base of the villi to the middle-villus region. It reached a maximum level at 75% of the height of the villus-crypt columns and the CRBP II protein level decreased towards the top of the villi (Fig. 1C). Immunohistochemistry showed that staining for the CRBP II protein was most dense from the base of the villi to the middle villus region (Fig. 1D).

Fig. 2. Effects of dietary fat on CRBP II expression in the rat jejunum. Rats were fed a high-fat diet for 12, 24 and 48 h after feeding a low-fat diet for 7 d. (A) CRBP II mRNA levels were analyzed by Northern blot hybridization using 20 μ g jejunal total RNA. CRBP II mRNA levels were normalized by signals of β -actin mRNA. (B) CRBP II protein levels were measured using ELISA. Values are means $\pm$ SE, $n=4$. Asterisks indicate significant differences compared with the levels in rats at 0 h (* $p<0.05$, ** $p<0.01$).

Effects of dietary fat on CRBP II expression in the rat jejunum

We examined the time-dependent effects of dietary fat on jejunal CRBP II expression. CRBP II mRNA and protein levels were not changed until 3 h after feeding a high-fat diet (data not shown). However, the levels of CRBP II mRNA and protein increased at 12, 24 or 48 h after feeding a high-fat diet compared with values at 0 h. CRBP II mRNA levels at 12, 24 and 48 h had increased 2.5-, 3- and 4-fold, respectively, compared with the level at 0 h (Fig. 2A). Similarly, CRBP II protein levels at 12, 24 and 48 h were 1.5-, 1.6- and 2.1-fold greater, respectively, than the level at 0 h (Fig. 2B).

Dietary fat-induced increases in CRBP II mRNA and protein levels along the villus-crypt columns

To determine at which locus of the villus-crypt axis the CRBP II mRNA levels were elevated following the dietary change from a low-fat diet to a high-fat diet, RNA blot hybridizations were performed using the total RNA extracted from the homogenates of the crypt at sections collected at various heights of the villus-crypt columns. In the control rats fed a low-fat diet (at time 0), CRBP II mRNA peaked at the mid-villus, decreasing both in the lower- and the upper-villus (Fig. 3A). Feeding the high-fat diet caused a significant increase of CRBP II mRNA levels only in the crypt and the lower-villus (i.e. at 15, 25, 35 and 45% of the height of the villus-crypt columns) at 12 h after the dietary change. In the rats fed the high-fat diet for 24 h and 48 h, significantly greater CRBP II mRNA levels were also observed in the mid-villus than in the rats fed the low-fat diet. In the rats fed the high-fat diet, maximal CRBP II mRNA levels were attained at 45% of the overall height of the villus-crypt column, and the CRBP II levels at this loca-

Fig. 3. Effects of dietary fat on CRBP II mRNA levels and the immunoreactive CRBP II protein in second sections of villus-crypt columns of the rat jejunum. Rats were fed a high-fat diet for 12, 24 and 48 h after feeding a low-fat diet for 7 d. (A) Dot blots for CRBP II mRNA in serial sections of villus-crypt columns and the graphic representation of the distribution of CRBP II mRNA along the villus-crypt axis of the rat jejunum. Total RNA was extracted from the serial sections of jejunal segments and analyzed for CRBP II mRNA abundance by dot blot hybridization. The results for each sample, normalized for β -actin mRNA abundance, were expressed as arbitrary units, representing the mean value of the maximal levels along the villus-crypt columns of control animals as 100%. Values are means $\pm$ SE, $n=4$. (B) Distribution of CRBP II levels along the villus-crypt axis measured using ELISA. Immunoreactive CRBP II protein was normalized for total protein abundance. Values are means $\pm$ SE, $n=4$. Asterisks indicate significant differences compared with the levels in rats at 0 h (* $p<0.05$).

tion at 12, 24 and 48 h after being offered a high-fat diet were 1.4-, 2.1- and 2.2-fold greater, respectively, than the control rats fed the low-fat diet (Fig. 3A). CRBP-II protein levels in the control rats fed a low-fat diet (at time 0) gradually increased from the base of the villus to the mid-villus region, reaching maximal levels at 65% of the height of the villus-crypt column, and decreasing to a lower level in the upper villus. In rats fed the high-fat diet for 12 h, CRBP-II protein levels were significantly greater only in the lower-villus region than in rats fed the low-fat diet (Fig. 3B). In the rats fed the high-fat diet for 12 h and 24 h, maximal CRBP-II protein levels were attained at 55% of the height of the villus-crypt column, and decreased towards the top of the villus. Further increases in CRBP-II protein levels were observed in the mid-villus in rats fed the high-fat diet for 48 h, and in this case maximal protein levels were found at 65% of the height of the villus-crypt column, and CRBP-II protein levels decreased towards the top of the villus. The CRBP-II protein levels at 65% of the height of the villus-crypt columns at 12, 24 and 48 h after being offered a high-fat diet were 1.8-, 1.7- and 2.7-fold greater, respectively, than in the control rats at time 0 (Fig. 3B).

DISCUSSION

In this study, we employed the technique of cryostat sectioning to examine the distribution of CRBP-II mRNA in the small intestine of rats which were fed a laboratory chow diet. The dot-blot hybridization of the RNA extracted from the tissue slices, which was obtained at various heights of villus-crypt axis, enabled us to quantitatively determine the distribution of CRBP-II mRNA. Results demonstrated that the CRBP-II mRNA level was minimal in crypt regions, abruptly elevated at the villus-crypt junction and then gradually declined towards the top of the villi. In addition, using an enzyme-linked immunosorbent assay we showed that the amount of immunoreactive CRBP-II protein along the villus-crypt axis was gradually elevated from the base to middle villus region, and decreased toward the top of the villi. This result indicates that CRBP-II protein expression is following that of mRNA expression. Many gene products that are characteristic of the differentiated enterocyte are known to be abruptly expressed at the villus-crypt junction; these include the sodium-glucose cotransporter 1 (SGLT1) (16), the fructose transporter GLUT5 (4), aminopeptidase N (2) and liver fatty acid-binding protein (3). These enterocyte-specific genes seem to be separated into two groups in terms of the mRNA distribution along the villus-crypt axis. One group of genes including genes encoding for SI, liver fatty acid-binding protein and aminopeptidase N were shown to exhibit maximal mRNA accumulation in the lower- to mid-villus regions, followed by a marked decrease in the mRNA levels in the top portions of the villus (2, 3, 5). Another group of genes including LPH, SGLT1 and glucose transporter 2 (GLUT2) are expressed persistently as the enterocyte migrates up to the villus tip (5, 16). The present study suggests that CRBP-II

belongs to the former group of genes which show a sharp decline in mRNA levels in the upper half of the villus-crypt columns. This result may indicate that the activation of CRBP-II gene transcription in the villus cells is transient, being restricted to the lower- to mid-villus. The reason why the CRBP-II protein is highly expressed at the mid-villus is not clear. Our previous study revealed that the enzyme activities of proteins related to retinol and β -carotene absorption/digestion in the small intestine such as L-RAT, β -carotene cleavage enzyme and retinal reductase were also detected at the highest expression levels at the mid-villus (7). It is speculated that retinol/ β -carotene digestion/absorption related proteins including CRBP-II may address their digestion/absorption by synchronously expressing in the villus. The present study extends our previous finding which showed that dietary fat elicited or enhanced the levels of CRBP-II mRNA in the rat jejunum (8, 9). The high-fat diet induced an increase in CRBP-II mRNA as early as 12 h after consumption (Fig. 2), while it is already confirmed that feeding a low-fat diet does not induce CRBP-II gene expression in adult (8) or weaning rats (17). The cryostat slicing technique enables us to determine the precise locus of villus cells where the rapid mRNA accumulation occurred. This study demonstrated that the initial rise in CRBP-II mRNA levels was most prominent in the cells which were accumulating the gene transcripts, i.e. at the lower villus. This result suggests that dietary fat enhances the efficiency of transcription of the CRBP-II gene. This notion is consistent with our previous studies which used a nuclear run-on assay to show that the transcription of the CRBP-II gene is enhanced in rats fed a high-fat diet as compared with those fed a low-fat diet (10). The molecular mechanism underlying the dietary fat-mediated regulation of CRBP-II gene expression at the specific regions along the villus-crypt axis is still unclear. We have previously demonstrated that dietary fat, especially unsaturated fatty acids (oleic, linoleic and alpha-linolenic acids), induced CRBP-II gene expression in the rat jejunum (9). The rat CRBP-II promoter region contains nuclear response elements, i.e. RXRE and RE3. These elements are known to be bound to peroxisome proliferator-activated receptors (PPARs) and retinoid X receptor (RXRs) heterodimers (18). Our recent study demonstrated that long-chain polyunsaturated fatty acids caused conformational changes of PPAR α , which led to parallel increases in both DNA binding to the PPAR α -response element and DNA binding-independent transactivity (19). Additionally, our previous studies revealed that a diet containing high triacylglycerol with polyunsaturated fatty acids induces gene expression of PPAR α as well as that of CRBP-II (20). Furthermore, we have shown not only that the PPAR α -RXR heterodimer in nuclei of the jejunum of rats fed a diet containing high triacylglycerol with polyunsaturated fatty acids was strongly bound to CRBP-II-RE3 in vitro (10), but also that promoter activity of the CRBP-II-promoter and enhancer activity of CRBP-II-RE3 co-transfected with PPAR α and RXR α were enhanced by linoleic acid and

arachidonic acid in intestinal cell line Caco-2 cells (21). Thus, it appears likely that the polyunsaturated fatty acids in the high-fat diet stimulate transcription of the CRBP II gene through the activator of PPAR α in the lower villus cells. The expression of PPAR α and RXR α in the villus cells was reported by Braissant et al. (22). They showed by in situ hybridization that PPAR α transcription was expressed in the small intestine villus cells. We also confirmed by immunohistochemical analysis that both PPAR α and RXR α are expressed in the villus cells (unpublished result). Further studies are needed to investigate whether PPAR α contributes to induction of CRBP II expression by dietary fat along the villus-crypt axis of the rat jejunum.

In conclusion, the results of the present study suggest that the CRBP II gene is maximally expressed in the lower villus, and that dietary fat elicits enhancement of CRBP II gene expression in the villus cells which are accumulating the transcripts.

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