Ontogenic regulation of folate transport across rat jejunal brush-border membrane

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Submitted 25 April 2003; accepted in final form 26 June 2003

Balamurugan, Krishnaswamy and Hamid M. Said. Ontogenic regulation of folate transport across rat jejunal brush-border membrane. Am J Physiol Gastrointest Liver Physiol 285: G1068–G1073, 2003. First published July 3, 2003; 10.1152/ajpgi.00188.2003.—Folate is an essential micronutrient that in mammals must be obtained from exogenous sources via intestinal absorption. Previous studies from our laboratory and others have demonstrated that folate absorption from the small intestine is mediated via the reduced-folate carrier (RFC). The goal of this study was to determine whether the initial step of folate uptake by intestinal epithelial cells, i.e., transport across the brush-border membrane (BBM) of the polarized enterocytes, is ontogenically regulated, and if so, to determine the molecular mechanism involved. Purified BBM vesicles (BBMV) isolated from suckling, weanling, and adult rats were used in this study. The initial rate of carrier-mediated uptake of a physiological concentration of folic acid (0.1 μM) by jejunal BBMV was found to be significantly (P 0.01) higher in suckling compared with weanling rats, which was, in turn, significantly (P < 0.01) higher than that in adult rats. This decline in carrier-mediated folate uptake with maturation was found to be mediated via a decrease in the maximum velocity of the folate uptake process (6.55 ± 0.87, 2.16 ± 0.10, 0.90 ± 0.16 pmol·mg protein·1·10 s−1 for suckling, weanling, and adult rats, respectively), with no changes in its apparent Km.

Branch analysis of BBM protein and real-time PCR showed RFC protein and mRNA levels, respectively, to be significantly (P < 0.01 for both) higher in suckling compared with weanling rats, which were in turn significantly (P < 0.01 for both) higher than that in adult rats. These changes were found by nuclear run-on assay to be associated with a parallel decline in the RFC transcriptional rate in jejunal epithelia with maturation. In situ hybridization showed a similar pattern of RFC message distribution along crypt/villus axis in suckling and adult rat jejunum. These results demonstrate for the first time that folate transport across the intestinal BBM is under ontogenic regulation during early stages of life and that this regulation involves a transcriptional regulatory mechanism(s).

It is well known that an adequate supply of folate is necessary for normal human health and well-being. Folate is required for the synthesis of purine and pyrimidine precursors of nucleic acids, for the metabolism of several amino acids (including homocysteine), and for the initiation of protein synthesis in mitochondria (2). Humans and other mammals cannot synthesize folate and thus must obtain the vitamin from exogenous sources via absorption in the intestinal tract. Therefore, the intestine plays a central role in controlling and regulating folate body homeostasis in all ages. The intestine is exposed to folate from two sources: 1) a dietary source, where the vitamin is absorbed in the small intestine, and 2) a bacterial source in which the vitamin is synthesized by the normal microflora of the large intestine and is absorbed there. In both the small and large intestine, an efficient pH-dependent, specialized, carrier-mediated mechanism is involved in folate uptake (reviewed in Ref. 15). The molecular identity of the system involved has been delineated after its cloning from human and mouse small intestine and shown the involvement of the so-called reduced-folate carrier (RFC) (4, 8, 13).

Compared with our current understanding of the physiology and molecular identity of the intestinal folate absorption process, relatively little is known about the regulation of the absorption event. Previous studies from our laboratory (9) have shown that the intestinal transepithelial transport of folate in rat intestine is regulated by substrate levels in the diet. This adaptive regulation was found to involve the transport event of the vitamin across the enterocyte brush-border membrane (BBM) and appears to involve transcriptional regulatory mechanism(s) (9). In other investigations (10), we have shown, by using rat intact intestinal tissue preparations (everted sacs), that the overall transepithelial transport process of folate is ontogenically regulated. It is unclear, however, whether the ontogenic regulation involves the initial step of folate uptake from the lumen into the enterocytes, i.e., the transport events across the BBM domain of the polarized absorptive epithelial cells. Also not clear is the molecular mechanism(s) involved in this ontogenic regulation. Our aim in this study was, therefore, to address these issues by using the rat as an animal model. Addressing these issues is of physiological and nutritional importance, because folate is essential for normal growth and development, processes that are most active during early stages of life. The results showed that the carrier-mediated folate transport across the intestinal BBM was under ontogenic...
regulation during early stages of life and that this regulation involves a transcriptional mechanism(s).

**MATERIALS AND METHODS**

[^H]folic acid (specific activity 25–30 Ci/mmol; radiochemical purity 97%) and all other radioactive materials were obtained from New England Nuclear (Boston, MA) and Amersham Pharmacia Biotech (Piscataway, NJ). All other chemicals, reagents, and kits used in this study were of analytical/molecular biology grade and were obtained from commercial sources. Cellulose nitrate filters (0.45-μm pore size) for use in uptake studies was purchased from Millipore, (Bedford, MA). Adult (males, 60 days old), weanling (25–27 days old), and suckling (13 days old) Sprague-Dawley rats were used in the study (Harlan Sprague Dawley, Indianapolis, IN) and were allowed free access to food and water. The National Council’s guidelines for the care and use of laboratory animals were followed and the Animal Subcommittee of our Institution approved the studies.

**Preparation of intestinal BBM vesicles and uptake studies.** Animals were euthanized by CO₂ inhalation and cervical dislocation, and the entire small intestine was removed and thoroughly flushed with ice-cold 0.9% NaCl. The jejunum (representing the proximal half of the small intestine) was placed on a glass plate maintained at 4°C and cut open; mucosa was harvested by scraping with a microscope glass slide. All subsequent procedures were performed at 4°C. BBM vesicles (BBMV) were isolated from the jejunum of rats of different age groups by a modification of Kessler’s divalent cation (Mg²⁺) precipitation technique (6) as described by us previously (7, 9, 11, 12). The final vesicular pellets were suspended in a buffer of 280 mM mannitol and 20 mM HEPES-Tris (pH 7.4) to achieve a final protein concentration of 5–10 mg/ml. Uptake studies were performed on the day of isolation by a rapid filtration technique (5) as described previously (7, 9, 11, 12). All incubations were performed at 37°C in the following buffer final concentrations (in mM): 100 NaCl, 80 mannitol, 10 HEPES, and 10 MES, pH 5.5. Protein concentrations were determined by using a protein assay kit (Bio-Rad, Hercules, CA). The suitability of BBMV for studying ontogeny of a transport process has been previously established (1, 7, 12). This has been confirmed in the present study by demonstrating a similar enrichment in the activity of the intestinal BBM marker enzyme, alkaline phosphatase, in final protein concentration by 10.2±0.33.6 on April 3, 2017 http://ajpgi.physiology.org/ Downloaded from

**Western blot analysis.** Western blot analysis was performed by using specific polyclonal anti-rat RFC antibodies as described previously (9). Briefly, BBM protein was isolated from the jejunum of suckling, weanling, and adult rats as described earlier in the presence of 1 mM PMSF, 1 mg/ml aprotinin, and 0.5 mg/ml leupeptin. Identical BBM protein amounts (100 μg) for suckling, weanling, and adult rats were treated with Laemmli’s buffer and resolved on 10% SDS-PAGE. After electrophoresis, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane at 35 mA for overnight at 4°C in wet transfer buffer. The membranes were blocked for 2 h at room temperature with PBS-Tween 20 (PBS-T) buffer that contained 5% (wt/vol) nonfat dry milk powders. The membranes were then incubated overnight at 4°C with specific polyclonal anti-rat RFC antibodies (1:10,000 in PBS-T buffer; Alpha Diagnostic, San Antonio, TX), and washed four times with PBS-T buffer. The specificity of the rat RFC antibody was demonstrated in enriched BBMVs isolated from the jejunum, where the immunoblot analysis identified an ~65-kDa band, which was blocked after preadsorption with the synthetic peptide (see RESULTS). The secondary antibody (anti-rabbit IgG-peroxidase conjugate diluted in 1:5,000 in PBS-T buffer) was applied for 2 h at room temperature followed by four washes with PBS-T buffer. As an internal control, the blots were stripped and then treated with β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were detected by using enhanced chemiluminescence substrate (ECL; Amersham Pharmacia Biotech) according to the manufacturer’s instructions and exposed to KODAX-ray film for periods of 30 s to 5 min. **Real-time PCR analysis.** Total RNA was isolated from the jejunal mucosa of suckling, weanling, and adult rats by using TRIzol reagent after the protocol described by the manufacturer (Life Technologies, Rockville, MD). Five micrograms of total RNA were reverse transcribed by using Superscript II (Life Technologies) enzyme and the manufacturer’s procedures. Each total RNA sample was DNase-treated before reverse transcription. After the reverse transcription, all of the samples were diluted with sterile water, and three different dilutions were used for each SYBRgreen real-time PCR. The real-time PCR amplification program was carried out using Light Cycler technology, to accurately analyze the differences in rat RFC gene expression in the suckling, weanling, and adult rats. Gene-specific primers corresponding to the PCR targets were designed by using the specifications given by the vendors (Bio-Rad). Amplions were for rat RFC (162 bp long) and β-actin (116 bp long). The amplions chosen were specifically located inside the open reading frame of the rat RFC gene to allow use of plasmid DNA to generate standard curves. Each SYBRgreen reaction (50 μl total volume) contained 5 μl of diluted cDNA (or plasmid DNA standard) as a template. The amplification program consisted of 1 cycle of 95°C with a 60-s hold (“hot start”) followed by 40 cycles of 95°C for 1 min, specified annealing temperature with 30-s hold, 72°C with 1-min hold for extension, and data acquisition. Amplification was followed by melting curve analysis program run for one cycle at 95°C with 0-s hold, 65°C with 10-s hold, and 95°C with 0-s hold at the step-acquisition mode. A negative control without cDNA template was run with every assay to assess the overall specificity. The final quantitation was achieved by a relative standard curve.

**Nuclear run-on assay.** To determine whether ontogeny is associated with changes in the transcription rate of the RFC gene, we performed nuclear run-on assays by using nuclei isolated from the jejunum of suckling, weanling, and adult rats as described by us recently (7). Briefly, rat intestine was excised, and intestinal contents were washed with mucosal wash solution (0.15 M NaCl, 5 mM DTT). The jejunum was cut longitudinally, and the mucosa was scraped, then suspended in 10 volumes of homogenization buffer (in mM: 250 sucrose, 10 MgCl₂, 2 DTT, 10 HEPES, pH 8.0, 2.5% Nonidet P-40, and 0.1 PMSF) and allowed to stand on ice for 1 h. After homogenization using a Potter-Elvehjem homogenizer, the suspensions were centrifuged at 1,500 g for 15 min and the pellets were resuspended in 35 ml of sucrose cushion buffer (2.5 M sucrose, 1 mM MgCl₂, 1 mM HEPES, pH 8.0, and 0.1 mM PMSF) and centrifuged at 50,000 g for 2 h at 4°C. The nuclei pellet was resuspended in 500 μl of nuclear storage buffer (in mM: 50 Tris, pH 8.0, 0.1 EDTA, 5 MgCl₂, 0.1 PMSF, and 40% glycerol) and counted by using a methylene blue stain. Nuclear transcription reactions were carried out as follows: equal amounts of nuclei were incubated for 30 min at 37°C in a reaction buffer of (in mM) 150 KCl, 5 MgCl₂, 3.5 DTT, 50 EDTA, 1 of ATP, CTP, and GTP, 20% glycerol, 100 μCi of [³²P]UTP (ICN, Irvine, CA), and 20 mM Tris, pH 8.0, in a final volume of 300 μl. Labeled RNA transcripts were

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isolated after passing through a Sephadex G-50 spin column. The labeled RNA transcripts (∼5 × 10^6 counts·min⁻¹·ml⁻¹) were used for hybridizing to rat RFC cDNAs immobilized on nylon filters. The hybridizations and the subsequent washes were done at 42°C by a standard method. Membranes were exposed to X-ray film, and the relative band intensities were determined by densitometry. Data were normalized relative to rat β-actin cDNA, which was used as a negative control.

In situ hybridization. This procedure was performed as described previously (8) with minor modifications. Briefly, a 1-cm tissue sample was collected from the midjejunal area of suckling and adult rats fixed for 2 h in 4% paraformaldehyde at 4°C, washed in ice-cold PBS containing 30% sucrose overnight at 4°C, and embedded in optimum cutting temperature. Frozen sections (10 μm thick) were blocked with PBS containing 1% powerblock solution for 2 h. The 158-bp fragment of the rat RFC subcloned into the pGEM-T easy vector (Promega, Madison, WI) was linearized for the probe preparations. Sense and antisense cRNA probes were prepared by using an in vitro transcription kit (Roche, Mannheim, Germany), according to the manufacturer’s recommendations. In situ hybridization was performed by using the single-stranded degoxigenin-labeled RNA probe on a cryostat section of rat small intestine according to the method described by an in situ hybridization fluorescence detection kit (InnoGenex, San Ramon, CA). Finally, sections were washed three times in cold PBS, mounted by using slowfade antifade mounting medium (Molecular Probes, Eugene, OR), and analyzed by inverted fluorescence microscopy (Nikon, Melville, NY). Adjacent sections were also hybridized with a sense probe that had the same nucleotide sequence as the mRNA and these were used as negative controls.

Statistical analysis. All transport measurements presented in this paper are the results of multiple separate uptake determinations from different BBMVs isolated from separate occasions from rats of different age groups. Uptake by the carrier-mediated process was determined by subtracting uptake by diffusion [calculated from the slope of the uptake line at high pharmacological concentrations (1 mM) and the point of origin] from total uptake at each concentration examined. Data are expressed as means ± SE in femtomoles or picomoles per milligram protein per unit time. Data were analyzed by using the Student’s t-test. Kinetic parameters of the saturable component of the intestinal folate uptake process (i.e., V_max and apparent K_m) were determined by using a computerized model of the Michaelis-Menten equation as described by Wilkinson (18). Western blotting, real-time PCR, nuclear run-on assay, and in situ hybridizations were performed on three separate occasions by using three independently isolated samples from different rats; representative data and/or blots/sections are presented in this report.

RESULTS

Carrier-mediated uptake of folate by jejunal BBMV of suckling, weanling, and adult rats. The initial rate of carrier-mediated uptake of a physiological concentration of folic acid (0.1 μM) was examined in BBMV’s prepared form the jejunum of suckling, weanling, and adult rats. Incubation was performed at an incubation buffer pH of 5.5, a preferential pH for the functioning of the intestinal carrier-mediated folate uptake system (11, 14, 16, 17). Our results showed folic acid uptake by jejunal BBMV to be significantly (P < 0.01) higher in suckling rats compared with weanling, which was, in turn, significantly (P < 0.01) higher than that of adult rats (109 ± 2.0, 37 ± 1.0, and 20 ± 1.0 fmol·mg protein⁻¹·s⁻¹, respectively).

To determine whether the ontogenic changes in intestinal carrier-mediated folate uptake process are mediated via changes in the V_max and/or the apparent K_m of the process, we examined the initial rate of folic acid uptake as a function of concentration by BBMV isolated from the jejunum of suckling, weanling, and adult rats. Kinetic parameters of the saturable component were then determined as described in MATERIALS AND METHODS. Results showed folic acid uptake to be saturable as a function of concentration in all age groups (Fig. 1). V_max of the saturable process was found to be highest in suckling rats but decreased with maturation (V_max of 6.55 ± 0.87, 2.16 ± 0.11, 0.96 ± 0.16 pmol·mg protein⁻¹·s⁻¹ for suckling, weanling, and adult rats, respectively). In contrast, no significant changes in the apparent K_m were observed with maturation (apparent K_m of 0.93 ± 0.07, 1.17 ± 0.20, and 1.37 ± 0.70 μM for suckling weanling and adult rats, respectively).

Effect of ontogeny on level of expression of rat RFC protein. In this study, we examined and compared by means of Western blot analysis, the level of expression of the RFC protein in purified BBMV preparations isolated from the jejunum of suckling, weanling, and adult rats. In all age groups, a band was observed with an apparent molecular weight of ~65 kDa (Fig. 2A, left). The specificity of the antibodies was determined by treating the antibodies with the antigenic peptide, which led to the disappearance of the specific band (Fig. 2B, right). Densitometric analysis revealed the level of RFC protein to be significantly (P < 0.01) higher in suckling (60 ± 3.3) compared with weanling (36 ± 1.6) rats, which was, in turn, higher than that in adult (26 ± 2.3) rats.

Fig. 1. Carrier-mediated folate uptake by jejunal brush-border membrane (BBM) vesicles (BBMV) of suckling, weanling, and adult rats as a function of concentration. Uptake studies were performed using BBMV as described in MATERIALS AND METHODS in the presence of the specific concentrations of unlabeled folate shown. Incubation was performed for 10 s (initial rate; Refs. 9, 11) at 37°C. Each data point represents the means ± SE of 4–6 separate uptake determinations from different BBMV preparations isolated from 6–8 rats.

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Effect of ontogeny on steady-state mRNA level of RFC in jejunal mucosa. In this study, we examined and compared the steady-state mRNA level of RFC in jejunal mucosa of suckling, weanling, and adult rats. This was performed by real-time PCR analysis by using total RNA and specific primers designed from the cDNA of RFC as described in MATERIALS AND METHODS. Transcript levels of the RFC gene were measured individually before and after pooling the total RNA from each rat group, and both experiments gave the same results (Table 1). PCR generated a single product of the expected size as estimated by agarose gel electrophoresis (data not shown); this was verified by using appropriate controls. Results (Table 1) showed that the steady-state level of RFC mRNA was significantly \( P < 0.01 \) higher in suckling rats compared with weanling rats, which was in turn significantly \( P < 0.01 \) higher than that of adult rats.

Effect of ontogeny on transcription rate of RFC in jejunal epithelia. In this study, we examined the effect of ontogeny on the transcription rate of RFC. This was performed by means of a nuclear run-on assay. In these assays, all RNAs undergoing transcription at the time of nuclear isolation are labeled (Fig. 3A) and probed against cDNAs of rat RFC and \( \beta \)-actin. Densitometric results (Fig. 3B) showed the transcriptional rate declined from suckling \( (81 \pm 3.5) \) to weanling \( (60 \pm 1.5) \) to adult \( (38 \pm 2.1) \) rats after adjusting for background and equilibrating to \( \beta \)-actin signals. In fact, the nascent transcription rate of rat RFC was approximately twofold higher \( (P < 0.01) \) in suckling compared with adult rats.

Distribution of the RFC message level along the crypt/villus axis of rat jejunum with maturation. It has been previously suggested that nutrient uptake could occur along the entire crypt/villus axis during early stages of life but become confined to the villus region with maturation (3). This implies possible changes in message distribution of nutritional transporters along the vertical axis of the gut with maturation. To test whether such redistribution occurs in the case of the RFC message with maturation, we performed in situ hybridization on sections of suckling and adult rat’s jejunum. Although the suckling rats showed a slightly higher expression of RFC in the crypt compared with adult rats, the overall results (Fig. 4) showed a similar pattern of expression of the RFC message along the jejunal villi in the two age groups.

DISCUSSION

The aim of the present study was to determine whether the initial step of intestinal folate uptake by the polarized absorptive epithelial cells, i.e., transport...
across the BBM domain, is ontogenically regulated, and if so, to determine the molecular mechanism(s) involved in such a regulation. The rat was used as the experimental animal model in these investigations because of the similarity of its intestinal transport physiology (including that of folate uptake) to that of humans. The results showed that the initial step of carrier-mediated folate uptake across the intestinal BBM to be indeed ontogenically regulated and that this regulation involves transcriptional mechanism(s).

With the use of an established BBMV technique, we first measured and compared the initial rate of carrier-mediated uptake of a physiological concentration of folate in suckling, weanling, and adult rats. Results showed the uptake to be significantly higher in suckling rats but decreased with maturation. This down-regulation in folate uptake with maturation was found to be mediated via a significant decrease in the $V_{\text{max}}$ of the folate uptake process with no change in the apparent $K_{\text{m}}$ of the uptake system. These findings suggest that developmental maturation is associated with a decrease in the number (and/or activity) of the intestinal folate uptake carriers with no changes in their affinity. Because the RFC is the carrier system involved in folate absorption in the gut (4, 8, 13), we examined the effect of ontogeny on the level of RFC protein at the jejunal BBM by Western blot analysis. The results showed significantly ($P < 0.01$) higher levels of RFC protein in suckling compared with weanling rats, which was higher than that in adult rats. These findings provide support for the above stated suggestion that the decrease in carrier-mediated folate uptake by jejunal BBMV with maturation is due to a decrease in the number of transport carriers involved. The higher level of RFC protein, in jejunal BBM of suckling compared with weanling and adult rats, was associated with a higher level of RFC mRNA in the former compared with the latter age groups. This suggests that the higher RFC protein level during early stages of life is, at least in part, due to a higher rate of RFC protein synthesis.

To determine whether transcriptional mechanism(s) are involved in the upregulation of the level of RFC mRNA during early stages of life, we performed nuclear run-on assay using nuclei isolated from the jejunum of suckling, weanling, and adult rats. The results showed a higher transcriptional rate for RFC in suckling compared with weanling rats, which was in turn higher than that of adult rats. These studies clearly show that ontogenic regulation of the intestinal folate uptake process across the luminal BBM of the absorptive enterocyte is mediated, at least in part, via transcriptional regulatory mechanism(s) of RFC. Further studies are required to determine the exact nature of this mechanism(s), and whether alternation(s) in RFC mRNA stability is also involved in this ontogenic regulation of folate uptake across the intestinal BBM.

It has been previously suggested that nutrient uptake in the suckling period could occur along the entire length of the crypt/villus axis but become confined to the villus region in adulthood (3). This suggestion implies possible changes in the distribution of mRNA of the involved transporters along the crypt-villus axis with maturation. To determine whether RFC mRNA undergoes such a redistribution during ontogeny, we performed in situ hybridization on jejunal sections of suckling and adult rats. Although the suckling rats showed a slight early expression of RFC in the crypt compared with adult rats, the overall results (Fig. 4) showed a similar pattern of expression of the RFC message along the jejunal villi in the two age groups. Assuming that the RFC message level is a reflection of functional folate uptake across the BBM, these findings suggest that the ontogenic regulation of folate transport across jejunal BBM is due to an upregulation of RFC message in the same villus cells of suckling and adult rat intestine, i.e., it does not involve regional redistribution of the message along the crypt/villus axis with maturation.

The higher folate uptake during the early stages of life is most probably designed to ensure a sufficient supply of this essential micronutrient to the animal for use in the synthesis of precursors of DNA, RNA, and protein. In summary, our results demonstrate for the first time that folate transport across intestinal BBM is ontogenically regulated, and that this regulation is...
associated with parallel changes in RFC protein and mRNA levels. In addition, the results provide evidence for the involvement of transcriptional mechanism(s) in this ontogenic regulation.

DISCLOSURES

This study was supported by grants from the Department of Veterans Affairs and National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-56061 and DK-58057.

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