Role of reduced folate carrier in intestinal folate uptake

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Balamurugan, Krishnaswamy, and Hamid M. Said. Role of reduced folate carrier in intestinal folate uptake. Am J Physiol Cell Physiol 291: C189–C193, 2006. First published February 22, 2006; doi:10.1152/ajpcell.00594.2005.—Studies from our laboratory and others have characterized different aspects of the intestinal folate uptake process and have shown that the reduced folate carrier (RFC) is expressed in the gut and plays a role in the uptake process. Little, however, is known about the actual contribution of the RFC system toward total folate uptake by the enterocytes. Addressing this issue in RFC knockout mice is not possible due to the embryonic lethality of the model. In this study, we describe the use of the new approach of lentivirus-mediated short hairpin RNA (shRNA) to selectively silence the endogenous RFC of the rat-derived intestinal epithelial cells (IEC-6), an established in vitro model for folate uptake, and examined the effect of such silencing on folate uptake. First we confirmed that the initial rate of [3H]folic acid uptake by IEC-6 cells was pH dependent with a markedly higher uptake at acidic compared with alkaline pH. We also showed that the addition of unlabeled folic acid to the incubation buffer leads to a severe inhibition (~95%) in [3H]folic acid (16 nM) uptake at buffer pH 5.5 but not at buffer pH 7.4. We then examined the effect of treating (for 72 h) IEC-6 cells with RFC-specific shRNA on the levels of RFC protein and mRNA and observed substantial reduction in the levels of both parameters (~80 and 78%, respectively). Such a treatment was also found to lead to a severe inhibition (~90%) in initial rate of folate uptake at buffer pH 5.5 (but not at pH 7.4); uptake of the unrelated vitamin, biotin, on the other hand, was not affected by such a treatment. These results demonstrate that the RFC system is the major (if not the only) folate uptake system that is functional in intestinal epithelial cells.

Intestinal epithelial cells

IT IS WELL KNOWN that an adequate supply of folate is necessary for normal human health and well-being due to the critical roles played by this vitamin in the synthesis of purine and pyrimidine (precursors of nucleic acids), in the metabolism of several amino acids (including homocysteine), and in the initiation of protein synthesis in mitochondria (1). Folate deficiency is a significant nutritional problem and occurs under a variety of conditions, including impairment in intestinal folate uptake due to intestinal disease (5), drug interaction (7), and a potential genetic defect in the uptake process (6). Humans and other mammals cannot synthesize folate, and thus must obtain the vitamin from exogenous sources via intestinal absorption. Therefore, the intestine plays a central role in controlling and regulating folate body homeostasis. The intestine is exposed to two sources of folate: 1) a dietary source, which 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 in that region of the gut. Studies (see Refs. 12, 16, and 18 for reviews) from our laboratory and others have shown that in both the small and the large intestine, folate uptake occurs via an efficient, acidic pH-dependent and specialized carrier-mediated mechanism. Cloning studies from our laboratory have identified the reduced folate carrier (RFC) as an intestinal folate transporter (9, 15). Subsequent studies by us and others have shown that the level of expression of the RFC system parallels changes in intestinal folate uptake observed under a variety of conditions, including dietary folate deficiency (13, 19), developmental maturation during early stages of life (2), and cell differentiation along the crypt/villus axis (3, 15). Despite these findings, there is no definitive study demonstrating the actual contribution of the RFC system toward total folate uptake in intestinal epithelial cells (IECs). Addressing this issue using a RFC knockout approach in mice is not possible; this is due to the embryonic lethality of the model (21). Addressing the issue of contribution of the RFC system toward total intestinal folate uptake is of physiological importance due to the critical role played by folate in normal cellular metabolism and because of the many conditions that are associated with impairment in the intestinal folate absorption process (5, 6, 8). It is also important in light of a recent study (20) that suggested the lack of a role for RFC in intestinal folate uptake at the physiological acidic pH of the intestinal surface (17). Rather, the study postulated that intestinal folate uptake takes place via an RFC-independent system, although nothing was provided about the molecular identity of the putative system (20). The latter study was performed using IECs that were chemically mutagenized with ethylmethanesulfonate, and then subjected to selective pressure with anti-folate.

Our goal in this study was, therefore, to determine the actual contribution of the RFC system toward total folate uptake in IECs. We used the nontransformed rat-derived intestinal epithelial IEC-6 cell line, which we previously demonstrated as a suitable in vitro model system to study cellular and molecular aspects of intestinal folate uptake (14). Among the clear advantages of this intestinal epithelial cellular system is that it does not express the folate receptor (i.e., it is like the native mammalian intestine) (14), and thus no complications in interpretation of uptake data are expected from this source. We also used the new approach of lentivirus-mediated short hairpin RNA (shRNA) to selectively silence the rat RFC gene (via degradation of its mRNA), an approach that has been well established in recent years (11). After verifying the effectiveness of this approach in selectively silencing the RFC gene, our studies showed that the RFC system is the main (if not the only) folate uptake system that operates in IECs.

MATERIALS AND METHODS

[3H]Folic acid (specific activity, 26.2 Ci/mmol; radiochemical purity 98.0%) was obtained from Moravek Biochemicals (Brea, CA).
The culture medium, trypsin, and other cell culture ingredients were obtained from Sigma (St. Louis, MO). Most of the fine quality analytic grade chemicals used in this investigation were obtained from either Sigma or Fisher Scientific.

**Cell culture and uptake studies.** The rat-derived IEC-6 cells were obtained from the American Type Culture Collection (Rockville, MD). IEC-6 cells were maintained in culture in DMEM containing 5% (vol/vol) fetal bovine serum, 3.7 g sodium bicarbonate, 2 mg/l insulin, 50 IU/ml penicillin, and 50 μg/ml streptomycin (complete medium) at 37°C and 5% (vol/vol) CO2. Cells between passages 7 and 14 were used for all experiments. Cells (4×10^6) were plated in 2 ml of complete medium in 12-well culture plates. At this concentration, cells were ~1-day preconfluent at 24 h after being plated. After plating of cells, medium was changed at 16 h postplating and every 24 h thereafter throughout experiments unless otherwise noted. Uptake studies were performed on confluent cell monolayers 3–4 days after confluence.

\[\text{[H]}\text{Folic acid uptake was examined in cells incubated in Krebs-Ringer buffer containing (in mM) 133 NaCl, 4.93 KCl, 1.23 MgSO}_4, 0.85 CaCl}_2, 5 glucose, 5 glutamine, 10 HEPES, and 10 MES, pH 5.5 (unless otherwise stated).}\]
\[\text{[H]}\text{Folic acid was added to the incubation medium at the outset of the uptake experiment, and the reaction was terminated after 3 min (initial rate; Ref. 14) by the addition of 1 ml of ice-cold buffer, followed by immediate aspiration. Cells were then rinsed twice with ice-cold buffer and lysed with 1 ml of 1 N NaOH.}\]
\[\text{Lysates were neutralized with HCl, and then radioactivity was measured in a scintillation counter. The protein content of cell digest was measured in parallel wells using a protein assay kit (Bio-Rad, Richmond, VA). On an average, 0.5% of the total [H]folic acid added to the incubation medium at the onset of incubation was taken up by control cells after the standard 3-min incubation.}\]

**Pretreatment of IEC-6 cells with gene-specific shRNA.** Pretreatment of IEC-6 cells with rat RFC gene-specific shRNA was performed as described by the manufacturer (Invitrogen, Carlsbad, CA). Three custom-made shRNAs (Invitrogen) were tested for silencing the endogenous RFC carrier in IEC-6 cells. Briefly, the targeted regions for silencing of rat RFC were selected from the GenBank RFC cDNA stream from the start codon ATG. Comparable results were obtained and 1,796 (GCCTACTGCTTGCCAAGAAGA) nucleotides downstream (GCCTACTGCTTGCCAAGAAGA), 839 (GCTACTACCTGATCACCTACT), and 1,796 (GCCTACTGCTTGCCAAGAAGA) nucleotides downstream from the start codon ATG. Comparable results were obtained with the three shRNAs, and thus data presented in this report was obtained using the shRNA that targeted the region starting at 839 nt downstream of the start codon ATG. Viral stock was prepared using RFC-specific shRNA and the Block-iT inducible H1 lentiviral RNAi kit (Invitrogen) as per the manufacturer’s protocols. IEC-6 cells (40–50% confluent) were transiently transfected with 10 μl rat RFC-shRNA virus per well of a 12-well plate in the presence of polybrene (Fisher Scientific, Tustin, CA). Assays for silencing were performed on confluent monolayers 2–3 days after transfection.

**Western blot analysis.** Western blot analysis was performed as described previously (2) with the use of anti-rat RFC polyclonal antibodies. Briefly, IEC-6 cells were lysed with 20 mM Tris-HCl, pH 7.4, containing 100 mM KCl, 0.9% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 0.5 μg/ml leupeptin. Membranous extracts (100 μg protein) were subjected to SDS-8% PAGE and electroblotted on Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). After being blocked with 5% powdered nonfat milk in PBS-Tween 20, blots were incubated with rabbit anti-rat RFC polyclonal antibodies, whose specificities have been previously established (13). Immunodetection was performed with goat anti-rabbit IgG conjugated to horseradish peroxidase using an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL). Specific bands were quantitated with the use of the Eagle Eye II system (Stratagene, La Jolla, CA).

**Quantitative and semiquantitative PCR analysis.** Oligo(dT) primers and 5 μg of total RNA isolated from IEC-6 cells were used with a SuperScript RT-PCR kit (Life Technologies, Rockville, MD) to synthesize first-strand cDNA. To amplify a portion of the open reading frame of rat RFC, we used two gene-specific primers corresponding to the sequence in the open reading frame of rat RFC to produce a 162-bp product. To determine the level of endogenous rat RFC in shRNA-pretreated and control cells, we performed real-time as well as semiquantitative PCR within the linear range of amplification. The conditions for both PCR were 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min. The semiquantitative PCR products were analyzed on 2% agarose gels, the images were captured using an Eagle Eye II system, and the amplified PCR products were normalized to amplified β-actin controls. To confirm the specificity of the RFC-shRNA effect on rat RFC, we also examined the effect of such treatment on the mRNA level of the unrelated biotin transporter, Na+-dependent multivitamin transporter (SMVT), and that of the housekeeping gene β-actin. The final quantitation for real-time PCR was achieved by comparing the threshold cycles with β-actin as described earlier (2). For the real-time PCR analysis, the threshold cycle value obtained with control RFC mRNA level over the expression of β-actin was considered to be 100%.

**Data presentation and statistical analysis.** Transport data presented in this report are means ± SE of multiple separate uptake determinations and are expressed in femtomoles per milligram protein per unit time. Data were analyzed using the Student’s t-test or ANOVA, with statistical significance set at 0.05. All semiquantitative RT-PCR and Western blot analyses were performed on at least three separate occasions with comparable results. Representative data are presented in this report.

**RESULTS**

**General characteristics of folate uptake by intestinal epithelial IEC-6 cells.** We confirmed our previous findings on the effect of extracellular buffer pH and Na+, as well as that of unlabeled folic acid added to the incubation medium, on the initial rate of [H]folic acid (16 mM) uptake by IEC-6 cells due to their relevance to the subject of the present report. Our results showed the uptake of [H]folic acid to be significantly (P < 0.01) higher at acidic buffer pH of 5.5 compared with buffer pH 7.4 (364 ± 25 and 55 ± 8 fmol/mg protein·min⁻¹·3 min⁻¹, respectively). Uptake of [H]folic acid was also found to be independent of the presence of Na+ in the incubation medium at both pH 5.5 and 7.4 (Fig. 1A). In addition, the presence of unlabeled folic acid (1 mM) in the incubation medium significantly (P < 0.01) decreased the initial rate of folic acid uptake at buffer pH 5.5, but not at buffer pH 7.4 (Fig. 1B). These results are very similar to those observed previously in our laboratory with IEC-6 cells and with other intestinal preparations (12, 14, 16) and show that folate uptake by the IECs at acidic buffer pH is predominantly carrier mediated in nature, whereas it is nonmediated at buffer pH 7.4.

Although IEC-6 cells do not express the folate receptor (14), we also examined in this study the level of binding/diffusion of the [H]folic acid to the IEC-6 cells. This was performed by examining the folic acid uptake in cells incubated at 4°C (representing binding and diffusion) and 37°C (total uptake). The results showed the uptake at 4°C to be <13% of the total uptake at 37°C (67 ± 15 and 496 ± 27 fmol/mg protein·min⁻¹·3 min⁻¹, respectively). This clearly demonstrates the minor role played by binding/diffusion in folate uptake by these cells.

**Effect of treating IEC-6 cells with lentivirus-mediated RFC-specific shRNA on the level of expression of the RFC and on folate uptake.** We used the lentivirus-mediated RFC-specific shRNA approach to silence the RFC system and then examined...
the effect of such silencing on specific parameters of the folate uptake process in the IEC-6 cells. First, we confirmed that treatment with RFC-specific shRNA indeed leads to silencing of the RFC gene. This was performed by determining the effect of such a treatment on the level of RFC mRNA using real-time and semiquantitative PCR with rat RFC-specific primers. The results showed a significant ($P < 0.01; \sim 78\%$) reduction in RFC mRNA level in shRNA-treated cells compared with control cells (Fig. 2A). This reduction in RFC mRNA level was found to be specific for RFC because no changes in the levels of mRNA of the unrelated biotin transporter, SMVT, and that of the housekeeping gene, $\beta$-actin, were observed (as determined by semiquantitative PCR; Fig. 2A, inset). We also examined the effect of treating the IEC-6 cells with RFC-specific shRNA on the level of RFC protein by means of Western blot analysis using specific RFC polyclonal antibodies (13). The results showed a significant ($P < 0.01; \sim 80\%$) reduction in RFC protein level in shRNA-treated cells compared with untreated controls (Fig. 2B). Again, the effect was found to be specific for the RFC protein because the level of unrelated SMVT protein and that of $\beta$-actin were not affected by such a treatment. These results clearly demonstrate the efficiency and specificity of the shRNA silencing effect on the RFC gene in the IEC-6 cells.

After the establishment of the effectiveness and specificity of the shRNA approach in silencing the RFC gene, we examined the functional consequences of such silencing on folate uptake by IEC-6 cells at both buffer pHs 5.5 and 7.4. The results showed significant ($P < 0.01; 90\%$) inhibition in the initial rate of folic acid (16 nM) uptake in the shRNA-treated cells compared with untreated controls at buffer pH 5.5 (Fig. 2A). Again, the effect was found to be specific for the RFC protein because the level of unrelated SMVT protein and that of $\beta$-actin were not affected by such a treatment. These results clearly demonstrate the efficiency and specificity of the shRNA silencing effect on the RFC gene in the IEC-6 cells.

Fig. 1. A: role of Na$^+$ in folic acid uptake by intestinal epithelial cells (IEC-6). Confluent monolayers of IEC-6 cells were incubated at 37°C for 3 min (initial rate; Ref. 14) at pH 5.5 (left) and at pH 7.4 (right) in the presence and absence of Na$^+$ in the incubation buffer (in the absence of Na$^+$, an equimolar concentration of K$^+$ was used). [3H]Folic acid (16 nM) was added at the onset of incubation. Data are means ± SE of 3 independent experiments. B: effect of unlabeled folic acid on uptake of [3H]folic acid by IEC-6 cells. Confluent monolayers of IEC-6 cells were incubated for 3 min in Krebs-Ringer buffer, pH 5.5 or 7.4, at 37°C in the presence or absence of unlabeled folic acid (1 mM) and the presence of [3H]folic acid (16 nM). Data are means ± SE of 3 independent experiments.

Fig. 2. Effect of treatment of IEC-6 cells with reduced folate carrier (RFC)-specific short hairpin RNA (shRNA) on mRNA and protein levels of the endogenous RFC. A: real-time PCR analysis of mRNA of RFC and $\beta$-actin were performed as described in MATERIALS AND METHODS. Inset shows the semiquantitative PCR analysis for the levels of RFC, Na$^+$-dependent multivitamin transporter (SMVT), and $\beta$-actin in shRNA-treated (lane 2) and untreated control (lane 1) IEC-6 cells. Notice the lack of effect of shRNA treatment on SMVT and $\beta$-actin mRNA expression. B: Western blot analysis of RFC protein in membranous fractions of IEC-6 cells. Levels of expression of RFC protein in control and RFC-specific shRNA-pretreated IEC-6 cells are shown. Also shown is the lack of effect of shRNA treatment on protein levels of SMVT and $\beta$-actin. Data are representative of 3 separate sets of experiments.
No such inhibition, however, was observed when folate uptake was measured at buffer pH 7.4 (Fig. 3B). We also examined the effect of the RFC shRNA treatment on the initial rate of uptake of the unrelated biotin and found the uptake to be similar in the RFC shRNA-treated and untreated cells (47.94 \pm 5.9 and 50.69 \pm 12.3 fmol/mg protein^{-1} min^{-1}, respectively).

To exclude the possibility that the higher folate concentration (~9 \mu M) present in the growth medium has biased our results on the apparent predominant role of the RFC in folate uptake system by IEC-6 cells, we also repeated the shRNA pretreatment experiment using IEC-6 cells maintained in growth medium containing a physiological level of folate (~0.5 \mu M; the folate level in the intestinal lumen after a meal is estimated to be in the range of 0.1–0.5 \mu M). The results again showed a significant (P < 0.01) inhibition in folate uptake by cells pretreated with RFC-specific shRNA compared with untreated (control) cells (89 \pm 17 and 625 \pm 0.009 fmol/mg protein^{-1} min^{-1}, respectively).

**DISCUSSION**

Our goal in this study was to determine the relative contribution of the RFC system toward total folate uptake by intestinal epithelial cells. We did so using confluent monolayers of the rat-derived IEC-6 cells as a model because previous studies from our laboratory (14) have established their suitability as an excellent in vitro model system with which to study different aspects of the intestinal folate uptake process. An interesting feature of these cells is their lack of the folate receptor, a mechanism that can also transport folate in certain cell types; thus they are similar to native mammalian enterocytes in this regard. We also used the lentivirus-mediated RFC-specific shRNA approach to silence the RFC gene of IEC-6 cells, and then examined the effect of such silencing on folic acid uptake. The gene-specific shRNA approach (a DNA vector-mediated expression that is predominantly driven by the U6 or H1 RNA polymerase III promoter) was selected for our investigations because it is superior to the transient duplex siRNA approach and produces a prolonged silencing effect on the targeted gene (10). The use of plasmid vectors instead of retroviral vectors improved the utility of the shRNA gene silencing approach in biological studies (reviewed in Ref. 4).

To begin our study, we first reconfirmed the main characteristics of the folate uptake process in IEC-6 and showed the uptake to be very low at buffer pH 7.4 but increased markedly (~8-fold) at buffer pH 5.5; uptake was also Na\(^+\) independent at both pHs. Also, the uptake at buffer pH 5.5 was predominantly carrier mediated while it is nonmediated at pH 7.4. These results are very similar to those observed previously with IEC-6 cells and other intestinal preparations (12, 14, 16). We then examined the effect of treating the IEC-6 cells with RFC-specific shRNA on the level of expression of the RFC mRNA and protein. Our results showed that both of these RFC parameters were substantially and specifically reduced in shRNA-treated compared with nontreated cells. After the verification of the effectiveness and specificity of the shRNA approach in silencing the RFC gene, we then examined the effect of such silencing on folate uptake at both buffer pHs 5.5 and 7.4. The results showed severe and specific inhibition in folate uptake in the shRNA-treated cells compared with controls at buffer pH 5.5; no effect, however, was observed at buffer pH 7.4. The shRNA-mediated effect on folate uptake was found to be specific because uptake of the unrelated biotin was similar in the shRNA-treated and control cells. These findings clearly show that the RFC system is the main (if not the only) folate uptake mechanism that operates in the nontransformed (normal) IEC-6 cells, and that for this system to function properly, it requires an acidic extracellular environment. The latter conclusion is in line with our previous observations that the function of RFC expressed in IEC-6 cells is highly dependent on acidic extracellular pH (7). These findings provide direct evidence for the involvement of RFC in intestinal folate uptake process and support the previous indirect findings that concluded a significant role for this system in intestinal folate uptake. Examples of the previous findings that...
pointed to a role for the RFC system in intestinal folate uptake include the following: 1) the cloning of the RFC cDNA from mammalian intestinal mucosa (9, 15), 2) the parallel changes in the level of expression RFC and folate uptake in dietary folate deficiency (13, 19), during intestinal maturation (early developmental) (2), and during cell migration along the crypt-villus axis (3, 15), and 3) the inhibition of intestinal folate uptake by RFC specific polyclonal antibodies (3). The conclusions drawn from our present study and that from the previous investigations, however, are in contrast to the conclusion drawn from a recent study by Wang et al. (20).

In the latter study the authors used chemically mutagenized IEC-6 cells (described as being RFC null) to conclude that the RFC system does not have a role in folate uptake at the previously described physiological acidic pH of the intestinal surface (17). Instead, they suggested the existence of an RFC-independent system, but nothing was provided regarding the molecular identity of the claimed putative system. The cause(s) of this discrepancy between our present study and that of Wang et al. (20) is not clear to us, but may be due to the use of chemically mutagenized vs. normal IEC-6 cells in the two studies.

In summary, results of the present investigation provide direct evidence for the involvement of RFC as the main folate uptake system in intestinal epithelial cells. In addition, the results show that this system requires an acidic extracellular environment for its optimal function in these epithelia.

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REFERENCES