Expression of the reduced folate carrier SLC19A1 in IEC-6 cells results in two distinct transport activities

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Rajgopal, Arun, Esteban E. Sierra, Rongbao Zhao, and I. David Goldman. Expression of the reduced folate carrier SLC19A1 in IEC-6 cells results in two distinct transport activities. Am J Physiol Cell Physiol 281: C1579–C1586, 2001.—Intestinal absorption of folates has been characterized as a facilitative process with a low pH optimum. Studies with intestinal epithelial cells have suggested that this activity is mediated by the reduced folate carrier (RFC1). In this paper, we report on folate transport characteristics in an immortalized rat IEC-6 cell line that was found to exhibit the predominant influx activity for methotrexate (MTX) at pH 5.5 with a low level of activity at pH 7.4. Transfection of this cell line with an RFC1 construct resulted in clones exhibiting increased MTX uptake at both the pHs and high folic acid uptake only at the low pH. For the two clones with the highest level of transport activity, relative MTX influx at the two pHs was reversed. Moreover, the low pH MTX influx activity ([MTX]o = 0.5 μM) was markedly inhibited by 20 μM folic acid while influx at neutral pH was not. Furthermore, in the presence and absence of glucose at low pH, MTX and folic acid influx activity was inhibited by azide, while MTX influx at pH 7.4 was stimulated by azide in the absence of glucose but was unchanged in the presence of glucose and azide. This was contrasted with the results of transfection of the same RFC1 construct into an L1210 murine leukemia cell line bearing a nonfunctional endogenous carrier. In this case, the activity was expressed only at pH 7.4. These data indicate that RFC1 can exhibit two distinct types of folate transport activities in intestinal cells that must depend on tissue-specific modulators.

Folates play a key role as cofactors for one-carbon metabolism in cells, and consequently, they are required for the de novo synthesis of purines, pyrimidines, some amino acids, and for the initiation of mitochondrial protein synthesis (1). Mammals cannot synthesize folate and must depend on dietary sources through intestinal absorption (1). Impaired intestinal absorption of folic acid is common in a variety of diseases of the small intestine, and it is also caused by congenital defects (13, 36). Folate deficiency is the most prevalent vitamin deficiency in the Western hemisphere (1). Folate deficiency is associated with megaloblastic anemia; more subtle deficiency has been associated with increased risk of cardiovascular disease, cancer, and defects in neural tube closure in developing embryos (2, 7, 38).

Dietary folates are a complex mixture of pteroylpolyglutamates of various chain lengths (21). Absorption of folates in the proximal small intestine is a multistep process. First is the enzymatic hydrolysis of polyglutamates by folate hydrolase; this is followed by transport of monoglutamates across the luminal membrane into the enterocyte, followed by transport across the basolateral membrane and entry into the portal capillary system (21). Intestinal absorption of folates and antifolates is optimal at low pH (30); for instance, folate absorption is high in patients with pancreatic insufficiency due to the low intestinal pH. Conversely, patients who lack the ability to acidify their gastric fluids have high small intestinal pH and low folate absorption (26, 27). There is a high hydrogen ion concentration adjacent to the luminal surface of the small intestine, the “intestinal surface acid microclimate,” where the absorption of nutrients is most active, in contrast to the bulk intestinal fluid pH (29).

The reduced folate carrier (RFC1) mediates the bidirectional fluxes of reduced folates and antifolates in many different mammalian cell types (14, 34). Folates are also transported in some tissues by receptors, anchored in the plasma membrane through a glycosylphosphatidylinositol moiety, which translocates folates unidirectionally into cells via an endocytotic pathway (14). RFC1 has been cloned from human, murine, and other species (34). There are four different splice variant forms of murine RFC1 (4, 35). RFC1 from intestine incorporates exon 1a in place of exon 1, and its 3′-untranslated region (UTR) is longer by 250 bases compared with RFC1 cDNA from murine leukemia cells, but the open reading frames are identical (28).

RFC1 has been implicated as the major transport route for folates and antifolates in intestinal cells (6, 23, 28). However, the properties of transport are different from RFC1-mediated processes in other tissues...

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protein. Transport of MTX in murine L1210 leukemia cells was evaluated as described previously (40).

Primers and plasmids. Two primers, RFC1 5′-CAATCT-GCTGTGAGTGACCT 3′ (sense) and RFC2 5′-AACCATT-TAAACAGACCTCG 3′ (antisense), comprising the 5′- and 3′-UTR of intestinal RFC1, were designed from the published sequence (28) and used for PCR amplification and subsequent cloning. PCR-Blunt vector (Invitrogen) was used for the initial cloning of intestinal RFC1; it was then subcloned into the pcDNA 3.1(−) expression vector for transfection studies (Invitrogen).

Cloning of intestinal RFC1. RFC1 from intestine was cloned by RT-PCR. Total RNA was isolated from mouse intestine, and first-strand cDNA was synthesized by reverse transcriptase (SuperScript II RT; GIBCO BRL) and oligo(dT). The RFC1 cDNA was amplified using the RFC1 and RFC2 primers noted earlier. The amplified product was first cloned in PCR-Blunt vector and then subcloned in pcDNA 3.1(−) vector at the XhoI and BamHI sites.

Transfections. Transfections were carried out by electroporation using the Cell-Porter (GIBCO BRL) according to the instruction manual, with some modifications. IEC-6 cells in log phase were trypsinized and suspended in DMEM without serum and antibiotics. The cells were washed twice with serum-free DMEM. Approximately 1 × 10⁷ cells were resuspended in this serum-free medium with 50 μg of plasmid DNA in a final volume of 900 μl. Electroporation was performed at room temperature (330 μF and 320 V). After electroporation, the cells were distributed on a six-well plate in DMEM containing 10% FBS and antibiotics and incubated at 37°C. After 2 days, the medium was replaced with DMEM containing serum, antibiotics, and G418, and the plates were screened for resistant clones after 2 wk. The same RFC1 construct was transfected into the MTX'A L1210 murine leukemia cell line, which lacks functional carrier activity (3). After this, cells were selected in RPMI 1640 medium containing G418 (750 μg/ml), and the surviving cells were isolated by dilution cloning.

Western blot. Cells growing in 75-cm² flasks were trypsinized, washed twice with ice-cold HBS, and suspended in 100 μl of the same solution containing 10 μl of protease inhibitor (P-8340, Sigma). The cell suspension was sonicated for 20 s in ice, mixed with 100 μl of 2x SDS-PAGE sample buffer without dithiothreitol, and loaded in the gel without heating. Western blot analysis was carried out according to the ECL Plus protocol from Amersham, as previously described using a polyclonal antibody to the COOH-terminus of RFC1 (39).

Statistical analysis. All experiments were performed at least three times with each data point in duplicate. Results are presented as means ± SE. Conditions were compared by analysis of variance (ANOVA). The observed results from ANOVA were confirmed by conducting a nonparametric ANOVA followed by comparisons between control and other conditions. Where appropriate, contrasts were corrected with multiple comparisons by Dunnett’s t-test. All analyses were carried out using SAS software (SAS Institute, Cary, NC).

RESULTS

pH profiles of folate influx in IEC-6 cells. Folic acid and MTX influx were evaluated in IEC-6 cells using an extracellular concentration of 0.5 μM. MTX and folic acid influx were highest at pH 5.0 (0.365 ± 0.022 and 0.645 ± 0.035 nmol·g protein−1·min−1, respectively), as indicated in Fig. 1. For MTX influx, there was a second, smaller peak at pH 7.0–7.5 (Fig. 1A). For folic
acid, influx at the highest pH was barely detectable, and influx increased as the pH decreased (Fig. 1B). At pH 7.4, influx of MTX and folic acid were 0.149 ± 0.024 and 0.028 ± 0.011 nmol/g protein/min, respectively, a 5.3-fold difference. At pH 5.0, influx of folic acid exceeded that for MTX by a factor of 1.7. Hence, uptake of folic acid and its analog MTX are highest at low pH, which mimics the in vivo conditions of the small intestine.

**pH-Dependent inhibitory effects of folic acid on MTX influx in IEC-6 cells.** The presence of two distinct peaks of MTX influx activity at neutral and low pH, with only one peak of activity for folic acid at low pH, suggested that folic acid might be useful as a discriminator for transport at the different pH levels. As shown in Fig. 2, 20 μM of folic acid virtually abolished MTX influx at pH 5.5 but had no effect at pH 7.4.

**MTX influx in IEC-6 clones transfected with RFC1.** To further elucidate the mechanism(s) underlying the low pH transport route, the murine RFC1 cDNA construct bearing the intestinal 3' and 5' UTR (28) was transfected in IEC-6 cells. Twenty resistant clones (in G418) were identified; six showed variable degrees of MTX influx activity that exceeded the rate in the vector control cell lines. Two clones, AR5 and AR13, showed the highest transport activities relative to the vector control at pH 5.5 (P < 0.05) and pH 7.4 (P < 0.05) (Fig. 3). However, the pattern of activity between the clones was different at the two pHs. AR5 showed higher MTX influx relative to AR13 at pH 7.4 (Fig. 3B), whereas AR13 showed higher influx relative to AR5 at pH 5.5 (Fig. 3A). These differences in AR5 and AR13 activities at the two pHs were highly significant (P < 0.0001 and P = 0.0021, respectively). AR14 exhibited higher influx at pH 7.4 relative to the vector control (P < 0.05) but no significant increase in activity at pH 5.5 compared with vector-only transfected cell lines. This difference in pH effect was significant at P < 0.028. Western blot (Fig. 4) of the total cell lysates confirmed the expression of murine RFC1 protein in all six clones. The AR5 and AR13 transfected lines with the highest transport activities also showed the highest levels of RFC1 expression. These two clones were used for further experiments. No protein was detected in the vector control, consistent with the fact that the antibody was raised against the COOH-terminal residue of mouse RFC1, which is considerably different from, and does not cross-react with, the endogenous rat carrier (39).

Folic acid influx in IEC-6 clones transfected with RFC1. Because folic acid influx is a better discriminator of low vs. neutral pH transport activities, folic acid influx was assessed in the AR5, AR13, and AR14 cell lines at both pHs (Fig. 5). Folic acid influx activity was increased in the AR13 and AR5 lines compared with
the vector control cell line at pH 5.5, ~2.7- and ~1.8-fold, respectively. These relative rates are similar to the pattern observed for MTX (Fig. 3). AR14 showed only a small increase at pH 5.5 with only a negligible increase in influx activity for folic acid at pH 7.4, consistent with the apparent high affinity of folic acid for RFC1 only at the low pH.

MTX influx in AR5 and AR13 RFC1 transfectants. In Fig. 6, where a full time course of MTX influx over 2 min is shown, both the AR5 and AR13 cell lines showed increased influx at both neutral and acid pH compared with the vector-only transfected cells. AR5 exhibited three- and sixfold increases in influx at pH 5.5 (Fig. 6A) and at pH 7.4 (Fig. 6B), respectively, whereas AR13 showed a fourfold increase at both pHs. Again, AR13 activity was greater than that of AR5 at pH 5.5, whereas the pattern was reversed at pH 7.4. Hence, the ratio of MTX influx in AR13 to AR5 was 1.4 at pH 5.5 and 0.64 at pH 7.4.

Pattern of inhibition of MTX influx by folic acid in RFC1-transfected IEC-6 cells. As shown in Fig. 7A, 20 μM nonlabeled folic acid inhibited [3H]MTX influx by
70% and 80% at pH 5.5 in AR5 and AR13 cells, respectively. However, at pH 7.4, folic acid had no inhibitory effect in AR5 cells and only slight inhibition (14%) in AR13 cells (P > 0.347).

Energy dependence of MTX and folic acid influx in RFC1-transfected IEC-6 cells. Influx of MTX and folic acid in AR5 cells was assessed for energy dependence at both the acidic and neutral pH. As shown in Figs. 8A and 9A, in the absence of glucose, MTX and folic acid influx was decreased by 33% and 35% at pH 5.5, respectively, in the presence of 10 mM azide. In contrast, MTX influx was increased >70% by azide at pH 7.4 in the absence of glucose (Fig. 8A); however, there was no change in folic acid influx at this pH (Fig. 9A). In the presence of glucose (Figs. 8B and 9B), the inhibitory effects of azide were essentially unchanged at pH 5.5 for MTX (Fig. 8) and folic acid (Fig. 9). At pH 7.4, the stimulatory effect of azide on MTX influx was abolished (Fig. 8) in the presence of glucose, but there was no change in folic acid influx at this pH both in the presence and absence of glucose (Fig. 9). This is consistent with the very low level of folic acid transport activity at neutral pH.

MTX influx in murine L1210 leukemia cells transfected with RFC1. To assess the tissue specificity of MTX influx at low pH, the same RFC1 construct with the intestinal 3' and 5'-UTR was transfected into MTX'rA murine L1210 leukemia cells, which lack carrier function due to a mutation (Ala-130-Pro) in the third transmembrane domain (3). Of four clones, only one showed a statistically significant increase in activity with a fivefold greater influx than that of vector control cells at pH 7.4 (Fig. 10). At pH 5.5, these cells did not show a statistically significant increase in MTX influx.

Fig. 7. Effects of folic acid on MTX influx in AR5 and AR13 transfectants. MTX influx was assessed in the presence or absence of 20 μM folic acid at pH 5.5 (A) and at pH 7.4 (B) after 2 min. The extracellular [3H]MTX concentration was 0.5 μM. PC is the vector control-transfected cells. Results are averages of 3 separate experiments ± SE.

Fig. 8. Effect of energy inhibition on MTX influx in the AR5 transfectant. MTX influx was assessed in the presence or absence of 10 mM azide at pH 5.5 and at pH 7.4 in the presence (B) or absence (A) of glucose. MTX (0.5 μM) influx was measured after 2 min. Results are averages of 3 separate experiments ± SE.

Fig. 9. Effect of energy inhibition on folic acid influx in the AR5 transfectant. Folic acid influx was assessed in the presence or absence of 10 mM azide at pH 5.5 and at pH 7.4 in the presence (B) or absence (A) of glucose. Folic acid (0.5 μM) influx was measured over 2 min. Results are averages of 3 separate experiments ± SE.
influx activity ($P > 0.366$). Figure 11 compares the expression levels of the transfected clone with that of the vector-only transfectant. Both the Northern (Fig. 11A) and the Western (Fig. 11B) blots show that the transfected clone has higher RFC1 expression levels than vector-only transfected cells. The transcript length of endogenous RFC1 is similar to that of the transfected construct.

**DISCUSSION**

A variety of studies over the past few decades have characterized aspects of the intestinal transport of folates. After cloning of the murine and human RFC1 (34), reports suggested that intestinal folate transport is mediated, at least in part, by this carrier (6, 23, 28). Hence, RFC1 is highly expressed in intestinal cells, and transport was shown to be inhibited by an antibody specific to this carrier (6). Furthermore, transfection of RFC1 into IEC-6 cells increased folate transport at pH 5.5 (20). However, confounding these observations was the demonstration that the characteristics of transport in intestinal cells are quite different from that of RFC1-mediated processes in other tissues. In particular, intestinal folate transport has a very low pH optimum (30), whereas transport mediated by RFC1 in other tissues has a neutral pH optimum (32). Furthermore, while folic acid has an affinity for the folate transporter in intestinal cells that is comparable to that of MTX, it has an affinity for RFC1 in cells of other tissue origins that is two orders of magnitude lower than that of MTX and reduced folates (30). Consistent with this is the lack of influx activity for folic acid at neutral pH, a folate with a very low affinity for RFC1. However, when these cells were transfected with an intestinal RFC1 construct, influx of MTX was augmented at both pH 5.5 and pH 7.4 in at least some of the transfected clones, but only the low pH component was abolished by folic acid. Moreover, folic acid transport activity was stimulated only at the low pH. This is unlike other studies in which a similar construct transfected into IEC-6 cells produced only a low pH component for folic acid and 5-methyltetrahydrofolate, although expression in *Xenopus* oocytes resulted in transport activity of the same substrates only at the high pH (20). Of particular interest in our studies was the variability in the relative extent to which neutral and low pH activities were expressed in the transfec-
tants. For two clones, relative transport activities for MTX were reversed at pH 7.4 vs. pH 5.5, and in a third, transport activity for MTX was induced only at pH 7.4. Western blot analyses directed specifically against the murine RFC1 confirmed expression of protein that is identical to the carrier in leukemia cells. When the same RFC1 construct used for transfection in IEC-6 cells was transfected in the murine L1210 leukemia MTX rA line, activity was stimulated only at pH 7.4 (15, 33) (see below).

To account for the transport activity at low pH requires that either RFC1 is posttranslationally modified to a form with very different transport properties or this carrier associates with other protein(s) to result in a complex with very altered transport characteristics. RFC1 has consensus phosphorylation sites for protein kinase C, cAMP-dependent kinase, and casein kinase, but there is no information as to whether alterations in phosphorylation of these sites modulate its function. Certainly phosphorylation modulates activities of RFC1.

Fig. 11. Expression of murine intestinal RFC1 in the MTX rA-transfected cell line. A: Northern blot; 30 μg of total RNA was loaded in each lane and probed with the full-length RFC1. B: Western blot; 30 μg of protein from total cell lysate was loaded in each lane. The COOH-terminal antibody was used to detect RFC1.
other transporters, such as the rat organic cation transporter (rOCT1) (22) and the sodium/glucose cotransporter (SGLT1) (16). There are also reports of protein-protein interactions that modulate transport function. For instance, the interaction between syntaxin-1A and the glycine transporter (10) inhibits substrate translocation. Similar findings were observed for the γ-aminobutyric acid transporter (8).

There is evidence of a transport route for folates with a low pH optimum in retinal pigment epithelial cells (5). In Fisher rat 3T3 cells, the major transport route for folate compounds is an acid pH-dependent carrier-mediated system independent of RFC1 expression (19). Low levels of folate transport activity with a low pH optimum has also been well characterized in L1210 murine leukemia cell lines and manifests properties of transport that are observed at pH 5.5 in intestinal cells. However, this transport activity cannot be related in its entirety to RFC1, since influx at low pH is present when RFC1 expression is abolished (Ref. 41 and unpublished observations).

The energy dependence of MTX influx at pH 5.5 and pH 7.4 in the transfected IEC-6 cell lines is also consistent with transport mediated by different mechanisms. As observed for MTX transport in leukemia cells (11, 12), influx at pH 7.4 in the transfected IEC-6 cells is enhanced when energy metabolism is blocked by azide, a phenomenon that is reversed when glucose is present. This stimulatory effect of azide may be due to inhibition of a multidrug resistant protein (MRP)-related efflux component that exports folates from cells (17, 18), and there is evidence that MRPs are expressed in intestinal epithelial cells (24, 37). Folic acid influx at neutral pH was unchanged by azide in the transfec-
tants, which correlates with the low affinity of folic acid for RFC1 at this pH. The effects of energy inhibition with and without energy substrate were entirely different at the low pH. The unidirectional flux of MTX and folic acid into the cells was inhibited by azide, and the addition of glucose did not alter this effect. Hence, transport at the low pH cannot, apparently, be sustained by anaerobic glycolysis. The mechanism by which this influx process is inhibited by azide is unclear but could be related to abolition of the transmembrane proton gradient linked to folate entry.

In summary, this study provides further information regarding folate transport in intestinal cells using a spontaneously immortalized IEC-6 cell line in which both neutral and low pH transport activities are associated with RFC1 expression and can be differentiated by folic acid inhibition and energy requirement. These data indicate further that this low pH activity is tissue specific and can vary among clones of the same cell origin. These transfec-
tants represent a useful model system to delineate the molecular mechanism underlying the two intestinal folic acid transport activities. Further studies are in progress to clarify the basis for these observations.

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