The Reduced Folate Carrier (RFC) Is Cytotoxic to Cells under Conditions of Severe Folate Deprivation

RFC AS A DOUBLE EDGED SWORD IN FOLATE HOMEOSTASIS

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The reduced folate carrier (RFC), a bidirectional anion transporter, is the major uptake route of reduced folates essential for a spectrum of biochemical reactions and thus cellular proliferation. However, here we show that ectopic overexpression of the RFC, but not of folate receptor α, a high affinity unidirectional folate uptake route serving here as a negative control, resulted in an ~15-fold decline in cellular viability in medium lacking folates but not in folate-containing medium. Moreover to explore possible mechanisms of adaptation to folate deficiency in various cell lines that express the endogenous RFC, we first determined the gene expression status of the following genes: (a) RFC, (b) ATP-driven folate exporters (i.e. MRP1, MRP5, and breast cancer resistance protein), and (c) poly(glu)-γ-glutamyl synthetase and γ-glutamyl-hydroxylase (GGH), enzymes catalyzing folate polyglutamylation and hydrolysis, respectively. Upon 3–7 days of folate deprivation, semiquantitative reverse transcription-PCR analysis revealed a specific ~2.5-fold decrease in RFC mRNA levels in both breast cancer and T-cell leukemia cell lines that was accompanied by a consistent fall in methotrexate influx, serving here as an RFC transport activity assay. Likewise a 2.4-fold decrease in GGH mRNA levels and ~19% decreased GGH activity was documented for folate-deprived breast cancer cells. These results along with those of a novel mathematical biomodeling devised here suggest that upon severe short term (i.e. up to 7 days) folate deprivation RFC transport activity becomes detrimental as RFC, but not ATP-driven folate exporters, efficiently extrudes folate monoglutamates out of cells. Hence down-regulation of RFC and GGH may serve as a novel adaptive response to severe folate deficiency.

Reduced folate cofactors play an essential role as one-carbon donors and acceptors in several crucial intracellular metabolic reactions (1–6). However, mammalian cells are devoid of the enzymatic capacity for folate biosynthesis and thus are absolutely dependent on folate uptake from exogenous dietary sources (7). Therefore, folate deficiency may impair the de novo biosynthesis of purines and thymidylate and thereby disrupt DNA and RNA metabolism, homocysteine remethylation, methionine biosynthesis, and subsequent formation of S-adenosylmethionine, the universal methyl donor, which in turn may lead to the impairment of methylation reactions (1–6). Based on their key role in cellular metabolism, folate cofactors are efficiently retained in cells via polyglutamylation, an ATP-dependent reaction in which up to 9 eq of glutamate units are added to the γ-carboxyl residue of folate cofactors (8, 9) (see Fig. 1A). Whereas this reaction is catalyzed by the enzyme folylpoly-γ-glutamate synthetase (9), the enzyme γ-glutamyl hydroxylase (GGH)² catalyzes the hydrolysis of these terminal γ-glutamyl residues from polyglutamylated folates (10). Importantly the long chain (n > 3) folate polyglutamate derivatives can no longer be extruded via ATP-dependent efflux transporters such as the multidrug resistance proteins (MRPs/ABCCs) (11, 12) and breast cancer resistance protein (BCRP/ABCG2) (13) as well as through the reduced folate carrier (RFC), a bidirectional folate transporter (14).

The molecular mechanisms underlying adaptation to folate deficiency are generally associated with alterations in folate uptake, ATP-driven folate efflux, intracellular folate retention, and folate-dependent metabolism. These mechanisms include (a) altered activity of various folate-dependent enzymes including dihydrofolate reductase (15) and thymidylate synthase (16), (b) augmented polyglutamylation via increased folylpoly-γ-glutamate synthetase activity (17, 18), (c) overexpression of folate influx systems including the RFC (SLC19A1) (16, 19) and the folate receptor (FR) (15, 17, 20), and (d) down-regulation of ATP-dependent folate exporters of the MRP/ABCC family (21, 22) as well as BCRP/ABCG2 (18, 23).

RFC, the main focus of this study, serves as the major uptake route of folates in mammalian cells (14). Whereas RFC exhibits a relatively wide pattern of tissue expression, the expression of the additional folate uptake systems including the FR family (19, 24–35) and the proton-coupled folate transporter (PCFT/SLC46A1) (36–40), both of which are unidirectional transport systems, is rather restricted to a limited number of tissues. Consistently in vivo studies have revealed that whereas RFC-null embryos died in utero prior to embryonic day 9.5 the rescue of the nullizygous embryonic lethal phenotype was achieved by

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supplementation of pregnant monoallelic RFC dams with 1-mg daily subcutaneous doses of folic acid (41). Furthermore the rescued RFC nullizygous embryos died within 12 days after birth due to a failure of hematopoietic organs (41).

RFC functions as a bidirectional anion exchanger (36, 42) with a high affinity ($K_m = 1 \mu M$) for reduced folates and hydrophilic antifolates such as methotrexate (MTX; $K_m = 5–10 \mu M$) but very low affinity ($K_m = 200–400 \mu M$) for folic acid (42–44). RFC can neither bind nor hydrolyze ATP to drive its folate transport activity across the plasma membrane. Rather the RFC-dependent uphill influx of folates is coupled to the downhill efflux of organic phosphates including thiamine monophosphate and pyrophosphate (45) that are readily available in the cytoplasm.

In the current study we hypothesized that under conditions of severe folate deprivation the folate efflux component of RFC transport activity may result in intracellular folate depletion and consequently decreased cellular viability. Based upon experimental results as well as on novel mathematical simulation data, we show here for the first time that upon short term (i.e. up to 7 days) exposure of cells to folate-free growth conditions, RFC-dependent folate efflux activity becomes detrimental as RFC extrudes folate monoglutamates out of cells, a process facilitated by the GGH-dependent conversion of folate polyglutamates to monoglutamates. Moreover this cytotoxic folate efflux activity may be abrogated by specific adaptive down-regulation of both RFC and GGH via decreased gene expression and subsequently decreased catalytic activities; indeed this novel survival response to folate-deprived conditions has been established here for T-cell leukemia CEM/7A cells with overexpression of the endogenous RFC (19) as well as for the breast cancer MCF7/MR cells that expresses only moderate levels of this double edged sword transporter.

**EXPERIMENTAL PROCEDURES**

**RFC-dependent Cellular Viability in the Presence or Absence of Folates**

The Chinese hamster ovary cell line deficient in RFC activity termed C5 (46) as well as its human RFC- and human FR-overexpressing transfectants (i.e. C5/RFC and C5/FR, respectively (46)) were trypsinized and washed three times with folic acid-free growth medium. Then cells ($6 \times 10^5$) were seeded in each of two T25 flasks in 5 ml of folic acid-free growth medium. The sublines in the first set of flasks were termed C5/NF, C5/RFC-NF, and C5/FR-NF (i.e. no folate), whereas the sublines in the second set of flasks were supplemented with folate derivatives according to their initial growth conditions (46), resulting in the sublines C5/HF (i.e. high folate; supplemented with 2.3 $\mu M$ folic acid), C5/RFC-3nMLCV (i.e. supplemented with 3 nm leucovorin, which was used as a folate source because of its high affinity to the RFC when compared with folic acid), C5/FR-3nMFA (i.e. supplemented with 3 nm folinic acid, which was used as a folate source because of its high affinity to the FRα when compared with Leucovorin (LCV)). All six flasks were simultaneously incubated for 6 days in a humidified CO$_2$ incubator without medium replenishment in each of three independent experiments. Following these 6 days of incubation, cells were detached by trypsinization, and the number of viable cells was determined by hemocytometer counting after trypan blue staining.

**Folate Deprivation**

The following are the folate deprivation protocols that we have developed for the following cell lines to explore possible mechanisms of adaptation to folate deficiency.

- **MCF7/MR Cells (with Moderate Levels of the RFC)**—MCF7/MR cells were grown as described previously (18). Following trypsinization, cells were washed three times with folic acid-free growth medium containing 10% dialyzed fetal calf serum and antibiotics. Then cells ($2.3 \times 10^5$) were seeded in each of two T75 flasks in 15 ml of folic acid-free growth medium; the subline in the first flask was therefore termed MCF7/MR-NF, whereas the second flask was supplemented with 2.3 $\mu M$ folic acid and was thus termed MCF7/MR-HF. Following 3 days of incubation, cells were then ready for the various analyses including semi-quantitative RT-PCR and determination of initial rates uptake of [$^3$H]MTX and GGH activity.

- **CEM/7A Cells (with Overexpression of the RFC)**—CEM/7A cells were cultured in growth medium containing 0.2 nm LCV as has been described previously (19). Cells were then washed three times with folic acid-free growth medium containing 10% dialyzed fetal calf serum and antibiotics and transferred ($10^7$ cells) to each of two T75 flasks in 50 ml of folic acid-free growth medium containing 10% dialyzed fetal calf serum and antibiotics; the subline in the first flask was termed CEM/7A-NF, whereas the second T75 flask was supplemented with 0.2 nm LCV and was therefore termed CEM/7A-HF. Following 7 days of incubation, cells were then ready for the various analyses including semi-quantitative RT-PCR and determination of initial rates uptake of [$^3$H]MTX and GGH activity.

**RNA Extraction and Semiquantitative RT-PCR**

Total RNA extraction followed by cDNA synthesis was undertaken as described previously (47). To evaluate the levels of β-actin, glyceraldehyde-3-phosphate dehydrogenase, BCRP, MRP5, MRP1, GGH, folypoly-γ-glutamate synthetase, PCFT, FRα, and RFC gene expression, semiquantitative RT-PCR analysis was used. PCR was carried out in a total volume of 30 μl in the presence of the following cDNA quantities (using 6-fold serial template dilutions): 100, 16.7, and 2.8 ng. Each PCR contained a 0.4 $\mu M$ concentration of the sense and antisense primers (see Table 1) and 1× REDTaq™ ReadyMix™ PCR reaction mixture (Sigma). Following an initial denaturation at 95 °C for 10 min, 24–35 cycles each of 1 min of denaturation at 95 °C, 1 min of annealing at 50–61 °C (Table 1), and 1 min of elongation at 72 °C as well as a final extension period of 10 min at 72 °C were carried out. PCR products were analyzed by electrophoresis on 1–2% agarose gels. Representative results of three independent experiments are shown.

**Determination of Initial Rates of [$^3$H]MTX Uptake**

Following the folate deprivation protocols, [$^3$H]MTX influx was determined as described previously (46). The advantage of using MTX rather than folic acid is its 40–80-fold higher affinity to RFC when compared with folic acid (42–44).
**Table 1**

Oligonucleotides used for semiquantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers: sense primer (S), anti-sense primer (AS)</th>
<th>Annealing temperature</th>
<th>Number of cycles</th>
<th>Product length</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFC</td>
<td>5’-AGCTCTCTGGAGCAGATC–3’ (S), 5’-ACTCTTGGGCACTGTCT–3’ (AS)</td>
<td>58</td>
<td>24–30</td>
<td>619</td>
<td></td>
</tr>
<tr>
<td>FRA</td>
<td>5’-CCAGACGCTTGGACGACGATC–3’ (S), 5’-CCAGACGCTTGGACGACGATC–3’ (AS)</td>
<td>61</td>
<td>34</td>
<td>510</td>
<td></td>
</tr>
<tr>
<td>PCFT</td>
<td>5’-TGCTTCTGGACTGCTGCTGACAC–3’ (S), 5’-TGCTTCTGGACTGCTGCTGACAC–3’ (AS)</td>
<td>55</td>
<td>35</td>
<td>100</td>
<td>56</td>
</tr>
<tr>
<td>FPGS</td>
<td>5’-CCAGACGCTTGGACGACGATC–3’ (S), 5’-CCAGACGCTTGGACGACGATC–3’ (AS)</td>
<td>60</td>
<td>28</td>
<td>449</td>
<td>63</td>
</tr>
<tr>
<td>GGH</td>
<td>5’-GAGCTTCTTGGACTGCTGCTGACAC–3’ (S), 5’-GAGCTTCTTGGACTGCTGCTGACAC–3’ (AS)</td>
<td>50</td>
<td>30</td>
<td>79</td>
<td>64</td>
</tr>
<tr>
<td>MRP1</td>
<td>5’-CCAGACGCTTGGACGACGATC–3’ (S), 5’-CCAGACGCTTGGACGACGATC–3’ (AS)</td>
<td>60</td>
<td>28</td>
<td>280</td>
<td>63</td>
</tr>
<tr>
<td>MRP5</td>
<td>5’-GAGCTTCTTGGACTGCTGCTGACAC–3’ (S), 5’-GAGCTTCTTGGACTGCTGCTGACAC–3’ (AS)</td>
<td>58</td>
<td>30</td>
<td>112</td>
<td>65</td>
</tr>
<tr>
<td>BCRP</td>
<td>5’-GCTCTTCTTTGACTGCTGCTGACAC–3’ (S), 5’-GCTCTTCTTTGACTGCTGCTGACAC–3’ (AS)</td>
<td>60</td>
<td>27</td>
<td>172</td>
<td>66</td>
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<tr>
<td>GAPDH</td>
<td>5’-AACGTTGCGTGGATGAGCTGCAAGTGGTGAAC–3’ (S), 5’-AACGTTGCGTGGATGAGCTGCAAGTGGTGAAC–3’ (AS)</td>
<td>60</td>
<td>35</td>
<td>514</td>
<td>63</td>
</tr>
<tr>
<td>β-actin</td>
<td>5’-CCAGACGCTTGGACGACGATC–3’ (S), 5’-CCAGACGCTTGGACGACGATC–3’ (AS)</td>
<td>56</td>
<td>28</td>
<td>576</td>
<td>63</td>
</tr>
</tbody>
</table>

GGH Activity Assay

Catalytic GGH activity assay was measured according to the original protocol described by O’Connor et al. (48) with some slight modifications (49). These protocols are based on the ability of GGH to convert MTX-Glu2 to MTX (48, 49). GGH activity is expressed as nmol of MTX formed/h/mg of protein.

Mathematical Biomodeling of Intracellular Folate Depletion

Here we devised a mathematical biomodel aiming at evaluating the intracellular folate pool-depleting effect of the RFC as well as of several representatives of the ATP-binding cassette (ABC) transporter superfamily (i.e. MRP3 and MRP4) under folate-free growth conditions (Fig. 1B).

The above mentioned folate efflux systems transport intracellular monoglutamylated folates to the extracellular compartment, which is literally infinite relative to the intracellular volume. Thus, the influx of the transported folates is negligible and can be omitted in the model. We applied the Michaelis-Menten equations (67) to simulate these efflux transport activities.

Hence the efflux velocity ($V_e$) can be derived from the basic enzymatic reaction kinetics where a substrate $S$ is converted to a product $P$ as follows according to the equation

$$V_e = \frac{V_{max} \cdot [S]}{K_m + [S]} \quad \text{(Eq. 1)}$$

where $S$ is the concentration of the substrate, $V_{max}$ is the limiting velocity value at substrate saturation (i.e. when $[S] \gg K_m$), and $K_m$ is the substrate concentration when $V = V_{max}/2$. In our model, $t = \text{The duration of time (in minutes) in which the cells have been exposed to the folate-free growth conditions.}$ $V_e = \text{The efflux velocity (μmol/liter/min which is μM/min) of the examined transporter at time t.}$ $[S] = \text{The intracellular concentration (μM) of the monoglutamylated folate pool at time t.}$ $V_{max} = \text{The maximum folate efflux velocity of the examined transporter (μM/min).}$ $K_m = \text{The monoglutamylated reduced folate concentration (μM) of the examined transporter}$

in which $V = V_{max}/2$. Inserting the last series of definitions into Equation 1 results in the following.

$$V_e = \frac{V_{max} \cdot [\text{MonoFP}]_t}{K_m + [\text{MonoFP}]_t} \quad \text{(Eq. 2)}$$

[TFP]$_t$ = the intracellular concentration of the total folate pool (μM) at time $t$. However, the cytosolic fraction of folates in mammalian cells is only 38% of the total folate pool size (50, 51). Moreover the cytosolic monoglutamate folate fraction, which serves as the available folate efflux fraction for RFC as well as for several ABC transporters, is only 2% of the total cytosolic folate pool in mammalian cells (50, 51). Therefore, the cytosolic monoglutamylated folate fraction is only 0.76% of the total intracellular folate pool size under folate-replete conditions.

Based upon the essential lack of folates in the extracellular medium under folate-free growth conditions along with the continuous efflux of cytosolic folate monoglutamates via the examined transporter as well as on the Le Chatelier principle, one could predict a continuous conversion of intracellular folate polyglutamates to monoglutamate congeners via lysosomal GGH activity (see Fig. 1B) in an attempt to retain the original fraction (i.e. 0.76%) of the monoglutamylated folate pool (i.e. [MonoFP])$_t$ relative to the total intracellular folate pool (i.e. [TFP])$_t$. Whereas the kinetic understanding of mitochondrial influx as well as efflux of folates is limited and based upon the above mentioned experimental data as well as on the Le Chatelier principle, we used the calculated cytosolic monoglutamylated folate fraction of 0.76% in this theoretical modeling as follows.

$$V_e = \frac{0.0076 \cdot V_{max} \cdot [\text{TFP}]_t}{K_m + 0.0076 \cdot [\text{TFP}]_t} \quad \text{(Eq. 3)}$$

The [TFP]$_t$ is equal to the initial total folate pool (i.e. [TFP]$_t = a$) from which the amount of monoglutamylated folates that has been transported via the examined transporter was subtracted until time $t$ (minutes) as described in the following equation.
RFC and Detrimental Folate Efflux

\[ \frac{d[\text{TFP}]}{dt} = \frac{0.0076 \cdot V_{\text{max}} \cdot [\text{TFP}]_{i}}{K_m + 0.0076 \cdot [\text{TFP}]_{i}} \]  
(Eq. 4)

This is a separable differential equation that can be written as follows.

\[ dt = \frac{K_m + 0.0076 \cdot [\text{TFP}]_{i}}{0.0076 \cdot V_{\text{max}} \cdot [\text{TFP}]_{i}} \cdot d[\text{TFP}]. \]  
(Eq. 5)

Integration of both sides of the equations results in the following equation.

\[ t = -\frac{0.0076 \cdot [\text{TFP}]_{i} + K_m \cdot \ln[\text{TFP}]_{i}}{0.0076 \cdot V_{\text{max}}} + \text{constant} \]  
(Eq. 6)

The total intracellular folate pool size of cultured mammalian tumor cells (i.e. \([\text{TFP}]_{i} = 0\)) was experimentally found to be \(\sim 11.3 \mu M\) (52); using these empirical data results in the following equation.

\[ 0 = -\frac{0.0076 \cdot 11.3 + K_m \cdot \ln^{11.3}}{0.0076 \cdot V_{\text{max}}} + \text{constant} \]  
(Eq. 7)

Hence the following relation describes each biosimulation.

\[ \text{constant} = \frac{0.0859 + K_m \cdot 2.4248}{0.0076 \cdot V_{\text{max}}} \]  
(Eq. 8)

The mathematical expression that describes the constant (i.e. Equation 9) can be used in Equation 7 as follows.

\[ t = -\frac{0.0076 \cdot [\text{TFP}]_{i} + K_m \cdot \ln[\text{TFP}]_{i}}{0.0076 \cdot V_{\text{max}}} + \frac{0.0859 + K_m \cdot 2.4248}{0.0076 \cdot V_{\text{max}}} \]  
(Eq. 10)

Rearrangement of Equation 10 results in the following equation.

\[ t = \frac{0.0859 + K_m \cdot 2.4248 - 0.0076 \cdot [\text{TFP}]_{i} - K_m \cdot \ln[\text{TFP}]_{i}}{0.0076 \cdot V_{\text{max}}} \]  
(Eq. 11)

This final implicit function (i.e. Equation 11) demonstrates the relation between the total intracellular folate pool (i.e. \([\text{TFP}]_{i}\)) and the duration of folate deficiency (in minutes) for an examined transporter with affinity for reduced folate (i.e. \(K_m\) in \(\mu M\)) and the maximum folate efflux velocity (i.e. \(V_{\text{max}}\) in \(\mu M/min\)).

The \(K_m\) and \(V_{\text{max}}\) of the various folate efflux systems have been derived in previous publications from cells that overexpress the examined transporter. These experiments yielded reliable results for the \(K_m\); however, the calculated \(V_{\text{max}}\) does not represent a physiologic value. Hence we used the human leukemia CCRF-CEM cells that express normal levels of the RFC along with several members of the ABC superfamily including substantial levels of MRP1 and MRP4 (21) to derive the total typical capacity of folate efflux in mammalian cells. Hence given that the experimental \(V_{\text{max}}\) is 4 pmol/(10^7 cells × min) for a folate-based compound (19) and that the reported cell volume for CCRF-CEM cells is 4 \(\times 10^{-10}\) ml/cell (36), we found that the typical maximal capacity (i.e. \(V_{\text{max}}\)) of folate efflux in these mammalian cells is exactly 1 \(\mu M/min\). During previous studies, a reduction of 60% in the folic acid efflux rate constant was documented in the presence of the RFC transport inhibitor N-hydroxysuccinimide ester of MTX (21). Therefore, the estimated capacity of RFC is 0.6 \(\mu M/min\), whereas the remaining folate efflux systems have a cellular folate efflux capacity of 0.4 \(\mu M/min\). These estimated folate efflux capacities were used to evaluate the folate efflux contribution of RFC relative to the remaining ATP-driven folate efflux systems in the current bio-simulation. However, to thoroughly investigate the folate depleting nature of these efflux transporters, four hypothetical efflux capacities were used for several transporters as follows: 1) 1 \(\mu M/min\), 2) \(10^{-1}\) \(\mu M/min\), 3) \(10^{-2}\) \(\mu M/min\), and 4) \(10^{-3}\) \(\mu M/min\). The theoretical experiments were conducted to characterize the folate-depleting effect of RFC with a high affinity (\(K_m = 1 \mu M\)) for reduced folates, which serve as the main intracellular folate derivatives, when compared with two representatives of the ABC transporters including MRP3 with \(K_m = 1.74\) mM for reduced folates (11, 53) and MRP4 with \(K_m = 0.64\) mM for reduced folates (53, 54). These representatives of the ABC transporters were chosen because of available kinetics data (i.e. \(K_m\)) for the reduced folate derivatives that serve as the dominant intracellular folate fraction. The various graphs were plotted in a single coordinate system using the Graph 4.3 software.

**Statistical Analysis**

We used a paired Student’s \(t\) test to examine the significance of the difference between two populations for a certain variable. A difference between the averages of two populations was considered significant if the \(p\) value obtained was \(<0.05\).

**RESULTS AND DISCUSSION**

In mammalian cells, the transport of tetrahydrofolate cofactors (i.e. a reduced folate derivative) proceeds primarily via the RFC, a high affinity transporter of naturally occurring reduced folates (e.g. \(K_m = 1 \mu M\) for 5-methyltetrahydrofolate). RFC is a non-concentrative, facilitative transporter with the characteristics of a bidirectional anion exchanger that equally displays influx and efflux of reduced folates. We hence postulated here that the high affinity folate efflux activity component of the RFC may be detrimental to cells subjected to folate-free conditions. The rationale behind this hypothesis was that under these conditions of folate deprivation RFC would extrude reduced folate monoglutamates out of cells. Given the lack of folates in the extracellular...
medium under conditions of severe folate deprivation along with the high affinity RFC-dependent efflux of folate monoglutamates and based upon the Le Chatelier principle, one could predict a continuous conversion of intracellular folate polyglutamates to monoglutamate congeners via lysosomal GGH activity (Fig. 1B). This should result in the continuous efflux of folate monoglutamates via RFC thereby leading to decreased intracellular folate pool and consequent loss of cellular viability (Fig. 1B and Fig. 2). Furthermore the folate influx component of the RFC becomes useless when folates are absent from the growth medium, hence rendering RFC a high affinity unidirectional folate exporter (Fig. 1B). To explore this hypothesis that RFC exerts a cytotoxic effect under conditions of folate deficiency, we first compared the viability of three sublines in medium containing or lacking folates; these cell lines included RFC-null Chinese hamster ovary C5 cells, their stable C5/RFC transfectants overexpressing the RFC (46), and C5/FR transfectants overexpressing FRα, the latter of which lacks folate efflux activity and thus serves as a negative control to the efflux component of the RFC (Fig. 2). The principal advantage of using this particular panel of cell lines is that they are devoid of endogenous RFC transport activity (46). Moreover to investigate the possible cytotoxic effect of the RFC we preferred to use C5/RFC cells with ectopic RFC overexpression driven by a dominant cytomegalovirus promoter (46) rather than an endogenously overexpressed RFC that may be down-regulated via a protective mechanism and thereby may compromise this cytotoxic effect. The results revealed a similar viability of C5, C5/RFC, and C5/FR cells in folate-replete growth medium (i.e. C5-HF, C5/RFC-3nMFA, and C5/FR-3nMFA, respectively). All six flasks were simultaneously incubated for 6 days in a humidified CO₂ incubator at 37 °C. Following these 6 days of incubation, cells were detached by trypsinization, and the number of viable cells was determined by hemocytometer counting after trypan blue staining. The star denotes a statistically significant difference. Error bars indicate ± S.D. of each series of measurements for each cell line.
transporters may be down- or up-regulated upon short-term folate deprivation. Semiquantitative RT-PCR analysis revealed a specific 2.4-fold ($p = 0.01$) and 2.6-fold ($p = 0.005$) decrease in RFC and GGH mRNA levels, respectively, upon 7 days of folate deprivation in breast cancer MCF7/MR cells (i.e. MCF7/MR-NF versus MCF7/MR-HF); 7 days were the minimal duration time of folate deficiency that enabled us to detect a statistically significant difference in the expression of one or more genes in this cell line (Fig. 3). Similarly RFC-overexpressing CCRF-CEM/7A leukemia cells (19) displayed a specific 2.5-fold decrease ($p = 0.009$) in RFC mRNA levels after 3 days of incubation in folate-free medium (i.e. CEM/7A-NF versus CEM/7A-HF; Fig. 3); in these cells, 3 days were the minimal duration time of folate deficiency that yielded a statistically significant difference in RFC gene expression. The stable gene expression status of the additional folate uptake systems including FRα (19, 24–35) and PCFT (36–40) (Fig. 3) strongly suggests that the folate efflux component of RFC was detrimental to cells upon folate deficiency rather than its folate influx component. This 2.5-fold decrease in RFC mRNA levels in both breast cancer cells and T-cell leukemia lines under folate deficiency was then examined at the transport activity level (Fig. 4); the initial rates of $[^3H]$MTX uptake were determined in the two folate-deprived cell lines. Consistent with the decreased RFC transcript levels in folate-deprived cells, both MCF7/MR (expressing low levels of RFC) and CCRF-CEM/7A cells (overexpressing the RFC) showed a 49% ($p = 0.03$) and 44% ($p = 0.004$) decrease in the influx of $[^3H]$MTX under conditions of folate deprivation, respectively (Fig. 4, A and B). Thus, the cytotoxic effect of the RFC upon folate-deficient conditions was probably minimized because of the decreased RFC transport activity. Likewise the 2.4-fold decrease in GGH mRNA levels was accompanied with ~19% decreased GGH activity ($p = 0.025$; Fig. 5A) for folate-deprived MCF7/MR-NF cells relative to their folate-replete counterparts. This specific down-regulation of both RFC and GGH gene expression and activity may serve as a novel cellular adaptive-protective response under folate-deficient conditions aimed at counteracting the detrimental conversion of folate polyglutamates to monoglutamates and their subsequent high affinity extrusion via the RFC. Further studies are warranted to pinpoint the putative RFC and GGH promoter elements that may respond to folate deprivation and thereby result in repression of gene expression in medium lacking...
olfates. The fact that neither of the ATP-driven folate exporters including MRPs, BCRP, and GLS (11, 12, 53, 55) underwent down-regulation under these folate-depleted conditions (Fig. 3) suggests an augmented detrimental role for RFC-dependent high-affinity folate efflux activity relative to the above ATP-driven, low-affinity (e.g. transport $K_m$ values for folic acid in the millimolar range) efflux transporters of the ABC superfamily (53) (Figs. 1B and 6). However, in an attempt to provide a comparative quantification of the intracellular folate-depleting effect of RFC with those of ATP-driven folate exporters, we devised a novel mathematical biomodeling (Fig. 6). The in silico experiments showed 10- and 100-fold decreases in the intracellular folate pool within 8.7 and 17.1 h under folate-free growth conditions, respectively, as a result of the estimated cellular efflux activity of RFC (i.e. 0.6 μM/min as was calculated under “Experimental Procedures”; Fig. 6). Furthermore, the activity of RFC resulted in a dramatic contraction in the intracellular folate pool within days (i.e. 10- and 100-fold decreases in the intracellular folate pool within 2.2 and 4.3 days, respectively) if only 10% of the cellular efflux capacity (i.e. 0.1 μM/min) was attributed to this transporter (Fig. 6). In contrast to RFC, the folate efflux activity of MRPs and MRPs resulted in the retention of the vast majority of the intracellular folate pool (i.e. 96 and 89% retention, respectively) after 7 days of incubation in folate-free medium (Fig. 6). This lack of a substantial folate-depleting effect was observed even when the complete cellular folate efflux capacity (i.e. 1 μM/min) was attributed to each of the two ABC transporters (Fig. 6). Hence these results provide a mechanistic basis for the highly specific down-regulation of the cytotoxic efflux activity of the RFC but not of folate exporters of the ABC superfamily (Fig. 3) that failed to cause any major decrease in the intracellular folate pool (Fig. 6) upon short term (up to 7 days) folate deprivation. However, the medium term and long term (i.e. weeks and months, respectively) folate-depleting effect of several transporters of the ABC superfamily has been suggested previously to be detrimental to cells under folate-deficient conditions (18, 21, 23). Indeed our mathematical biomodeling reveals that the folate efflux activity of MRPs, as a representative of folate exporters of the ABC superfamily, may be responsible for a 30% decrease in the intracellular folate pool within 3 weeks of exposure to folate-free growth conditions; this moderate depleting effect of this ABC transporter may contribute to the detrimental effect of medium term and long term folate deprivation. Collectively our findings strongly suggest that the bidirectional folate transport activity of RFC is responsible for its double-edged sword impact on folate homeostasis. Moreover it is possible that the ability to down-regulate RFC and GGH gene expression under states of severe folate deprivation stems from evolutionary roots originating in unicellular organisms and perhaps metazoic ancestral organisms undergoing transient yet frequent states of starvation and severe folate deprivation. One important emerging question from the current study is what physiological-pathological conditions and syndromes could match the transient folate deprivation conditions used in the current study. The first pathological syndrome, hereditary folate malabsorption (56), is caused by loss of function mutations in the proton-coupled folate transporter (PCFT/HCPI/SLC46A1) normally responsible for the high affinity intestinal influx of naturally occurring folates in the acid microclimate of the upper intestinal mucosal epithelium. Hence hereditary folate malabsorption patients suffer from extremely low folate levels ≤0.2 nm in the blood and cerebrospinal fluid (40, 56). Another physiological-pathological condition that may match such a severe folate deprivation state is nutritional folate deficiency or insufficiency. Indeed folate deficiency affects ~10% of the population as well as more than 50% of the children and elderly that live in poverty in the United States, Thailand, and rural areas of India (38, 57, 58). Hence under such physiological-pathological conditions of severe folate deficiency, RFC and GGH may possibly undergo a significant down-regulation in gene expression and activity to protect cells from further loss of intracellular folates due to folate efflux via the RFC.

The current study may have profound implications relating to various disciplines in biology and medicine including the following. (a) In regard to developmental biology and embryonic development, the central developmental role that mammalian RFC plays has been revealed in RFC knock-out mice studies (41). In contrast to this vital role of the RFC under conditions in which folates are available for the pregnant dams, our findings suggest that under conditions of severe nutritional folate deficiency or insufficiency of mammalian pregnant dams the RFC may in fact exacerbate the pathological status of the embryos caused by the folate deficiency by further extruding folates. Thus, RFC may play a key role in inducing early abortions during early stages of mammalian pregnancy when folates are severely limited in the growth environment and thereby may confer an evolutionary advantage by eliminating embryos that are certain to fail at normal cell proliferation, differentiation, and proper development. (b) In regard to molecular medicine, RFC is an important route for the uptake of various anti-
folates including for example MTX and pemetrexed (Alimta) (59, 60). Hence our findings suggest that the down-regulation of the RFC as a result of folate-deficient conditions may decrease antifolate uptake and thus compromise the pharmacological efficacy of antifolates currently used in chemotherapy of various cancers. Moreover down-regulation and mutational inactivation of the RFC serve as major mechanisms of antifolate drug resistance exhibited by cancer cells (47, 59, 61, 62). Hence antifolate-resistant cancer cells may readily survive not only the drug resistance exhibited by cancer cells (47, 59, 60). Hence our findings suggest that the down-regulation of folates including for example MTX and pemetrexed (Alimta) (59, 60). Hence our findings suggest that the down-regulation of folates including for example MTX and pemetrexed (Alimta).

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