A Reduced Folate Carrier Mutation Produces Substrate-dependent Alterations in Carrier Mobility in Murine Leukemia Cells and Methotrexate Resistance with Conservation of Growth in 5-Formyltetrahydrofolate*

(Received for publication, December 31, 1997, and in revised form, January 21, 1998)

Rongbao Zhao, Yehuda G. Assaraf‡, and I. David Goldman§

From the Departments of Medicine and Molecular Pharmacology and the Albert Einstein Comprehensive Cancer Center, Albert Einstein College of Medicine, Bronx, New York 10461

With 5-formyltetrahydrofolate (5-CHO-THF) as the folate source a methotrexate (MTX) transport-deficient murine leukemia cell line, L1210-G1a, was isolated after chemical mutagenesis and MTX selection. This cell line was 10-fold resistant to MTX in comparison to parental L1210 cells, yet the EC₅₀ for 5-CHO-THF was increased by a factor of only 2. The initial uptake of MTX, at a concentration of 1 μM, was decreased by a factor of 40, whereas influx of 5-CHO-THF dropped by a factor of only 8. This difference in initial uptake rates was attributed solely to changes in influx Vₘₐₓ without a significant change in Kₘₐₓ. Whereas the RFC1 mRNA level in L1210-G1a cells was indistinguishable from that of parental L1210 cells, a serine to asparagine substitution was identified at amino acid 46 within the first predicted transmembrane domain. This was a result of a homozygous mutation of G→A in the genome. Transfection of the mutated RFC1 cDNA into MTX²A cells, which lack functional endogenous carrier, resulted in a clonal derivative MTX²A-S46N. The increase in influx of 5-CHO-THF and 5-CH₃THF was 5 and 13 times greater than that for MTX in the transfectant, consistent with the influx ratio in the L1210-G1a line. The functional expression of the mutated RFC1 reduced the growth requirement for 5-CHO-THF by a factor of 30, compared with only a 3-fold decrease in the MTX IC₅₀. This represents the first reported RFC1 mutation that confers resistance to MTX due to a markedly impaired influx with relative conservation of reduced folate transport. The kinetic changes are consistent with a substrate-dependent alteration in carrier mobility that favors reduced folates over MTX. These changes may account for the development of MTX resistance due to impaired drug transport in vivo, allowing tumor cells to meet their folate requirement with 5-CH₃THF, the predominant blood folate.

Folate cofactors are required for the de novo synthesis of purines, pyrimidines, and some amino acids. Mammalian cells meet their requirement for folates by uptake from exogenous sources via several transport systems that have been described

* This work was supported by Grant CA-38807 from the National Cancer Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Department of Biology, The Technion, Israel Institute of Technology, Haifa 32000, Israel.

§ To whom correspondence should be addressed: Albert Einstein Comprehensive Cancer Center, Albert Einstein College of Medicine, Chanin Two, 1300 Morris Park Ave., Bronx, NY 10461.

1 The abbreviations used are: RFC1, reduced folate carrier; MTX, methotrexate; 5-CHO-THF, 5-formyltetrahydrofolate; 5-CH₃THF, 5-methyltetrahydrofolate; HBS, HEPES-buffered saline (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, and 5 mM glucose, pH 7.4); bp, base pair(s); PCR, polymerase chain reaction.
Davis, Ann Arbor, MI. All other reagents were obtained in the highest purity available from various commercial sources.

Cell Culture Conditions and Growth Studies—L1210 leukemia cells were grown in RPMI 1640 medium containing 2.3 μM folic acid supplemented with 5% bovine calf serum (HyClone), 2 mM glutamine, 20 μM 2-mercaptoethanol, penicillin (100 units/ml), and streptomycin (10 μM thymidine). For analyses of growth requirement, cells were grown in 96-well plates (10,000 cells/well) and then exposed to concentrations of MTX, folic acid, or 5-CHO-THF for 72 h, following methods described previously (27), with minor modifications. Briefly, cells were incubated at 37 °C for 25 min, following which, cell numbers were determined by hemocytometer count, and viability was assessed by trypan blue exclusion. G418 was omitted from medium in growth studies with transfecants (see below).

Northern Analyses—Total RNA was isolated using the TRizol reagent (Life Technologies, Inc.). RNA (20 μg) was fractionated by electrophoresis on 1.0% formaldehyde-agarose gels. Transfer and hybridization were performed as described previously (27). Transcripts were quantitated by PhosphorImager analysis of the hybridization signals and normalized to β-actin.

Transport Studies—Influx measurements were performed by methods described previously (27), with minor modifications. Briefly, cells were electroporated (250 V, 200 microfarads) with 50 μg of nonlinearized pCDNA3.1(+) harboring the mutated cDNA from RFC1-S46N in a final volume of 800 μl of serum-free RPMI 1640 medium. Cells were then diluted to 200,000 cells/ml of RPMI 1640 medium, allowed to recover for 45 h, adjusted to 2 × 10^5 cells/ml in medium containing G418 (750 μg/ml of active drug), and then distributed into 96-well plates at approximately 4 × 10^4 cells/well. G418-resistant clones were isolated by limiting dilution and then expanded for further studies. One of the clones, MTX-A-S46N, which exhibited the highest level of RFC1 mRNA, was chosen for detailed studies.

Genomic Amplification and Restriction Analysis—An RFCl-specific 221-bp fragment was amplified from genomic DNA using the primers that flanked the mutation identified in L1210-G1a cells: 5'-CTACGTTGCGCCCAAGTCGTTCTCTTG-3' and 5'-GGTGGAGTTCGTCGCTTTGATTTAAGTC-3' (based on GenBank accession no. U32469). Genomic DNA was isolated with the Easy-DNA kit (Invitrogen), and 0.5 μg was used for amplification. Following an initial melt for 2 min at 95 °C, amplification was carried out for 35 cycles of 45 s at 95 °C, 1 min at 55 °C, and 1 min at 72 °C. DNA was then precipitated with ethanol and subjected to restriction analysis with HindIII without further purification.

RESULTS

MTX Sensitivity and 5-CHO-THF Growth Requirement in L1210-G1a Cells—The stable, MTX-resistant L1210-G1a line was isolated from L1210 cells after chemical mutagenesis with ethylmethanesulfonate and subsequent selection in 100 μM MTX with 5-CHO-THF as the sole folate source. As indicated in Fig. 1A, L1210-G1a cells were 10-fold resistant to MTX in the presence of 25 μM 5-CHO-THF, the concentration used in the selection medium. The IC_{50} for MTX was further decreased slightly in both lines (parental > mutant) when 2.3 μM folic acid was the folate source, resulting in a net increase in the difference in drug sensitivities between L1210 and L1210-G1a cells (Fig. 1B). In contrast, sensitivity to the lipophilic antifolate trimetrexate in either folic acid or 5-CHO-THF was increased by a factor of 4 in L1210-G1a cells, ruling out a change at the level of dihydrofolate reductase (not shown). Finally, the 5-CHO-THF EC_{50} was increased by a factor of only 2 in L1210-G1a cells (Fig. 1C). Hence, a cell line was developed in which sensitivity to MTX decreased by an order of magnitude, whereas the growth requirement for the 5-CHO-THF substrate was minimally increased.

Transport of MTX and 5-CHO-THF in L1210-G1a Cells—MTX resistance could not be attributed to down-regulation of transcription of RFC1. Indeed, RFC1 mRNA levels in L1210-G1a and L1210 cells were comparable as shown in Fig. 2 (lanes labeled MTX-A-S46N and MTX-A will be discussed below). There were, however, important differences in influx of MTX and 5-CHO-THF in these cell lines, as illustrated in Fig. 3. Influx of 5-CHO-THF was only ~25% greater than that of MTX in parental L1210 cells. In contrast, MTX influx was markedly decreased in L1210-G1a cells, with barely measurable residual transport activity. Influx of 5-CHO-THF was also markedly decreased in L1210-G1a cells, but residual transport was 12% that of L1210 cells. Hence, the decrease in transport of MTX above was observed in drug-free RPMI medium containing 0.5% soft agar. After an additional 2 weeks, individual clones were picked up and expanded, and MTX influx, 5-CHO-THF growth requirement, and dihydrofolate reductase level were evaluated. One clone, L1210-G1a, exhibiting both markedly impaired MTX transport and the lowest 5-CHO-THF growth requirement, was selected for further study. The L1210-G1a clone line has been maintained in drug-free complete RPMI 1640 medium and displayed a stable phenotype for more than 9 months.

Cloning of the Reduced Folate Carrier—Poly(A)^+ mRNA was purified from 4 × 10^6 cells using Dynabeads mRNA DIRECT kit (Dynal) and dissolved in 20 μl of diethyl pyrocarbonate-treated water. The first strand synthesis was carried out with a Superscript reverse transcriptase (Life Technologies, Inc.) using 10 μl of poly (A)^+ RNA. The protein coding sequence was isolated from the products of the first strand synthesis by amplification utilizing oligonucleotide primers that flanked the coding region of RFCl (5′ primer at nucleotide ~46 from the translation start codon 5′-GGGATCCGTTAGGCTGATCTTG-3′ and 3′ primer at nucleotide +82 from stop codon 5′-GGTGGAGTTCGTCGCTTTGATTTAAGTC-3′). Two 8-bp linkers were introduced into the PCR products at the XbaI restriction site of the PCR products to facilitate directional subcloning. Pfu Polymerase (Stratagene) was employed to ensure accuracy of the amplified PCR fragment. The amplifications were performed for 35 cycles of 45 s at 95 °C, 45 s at 65 °C, and 3 min at 72 °C. The 1682-bp-long predicted PCR product was purified from agarose gel (Qiagen) and cloned into a pCR-Blunt vector (Invitrogen). The nucleotide sequences of cloned cDNAs were determined on automated sequencer models ABI 373A and ABI 373 from Perkin-Elmer Corp. in the sequencing facility of the Albert Einstein College of Medicine Comprehensive Cancer Center.

Transfections—RFC1-S46N cDNA with a serine to asparagine substitution at amino acid 46 was excised from the pCR-blunt vector (see above) by a double restriction with BamHI and XhoI and recloned into pCDNA3.1(+) (Invitrogen) with the same restriction sites. MTX-A cells (1 × 10^7 cells), which lack functional endogenous RFC1 (37), were electroporated (250 V, 200 microfarads) with 50 μg of nonlinearized pCDNA3.1(+) harboring the mutated cDNA from RFC1-S46N in a final volume of 800 μl of serum-free RPMI 1640 medium. Cells were then diluted to 200,000 cells/ml of RPMI 1640 medium, allowed to recover for 45 h, adjusted to 2 × 10^5 cells/ml in medium containing G418 (750 μg/ml of active drug), and then distributed into 96-well plates at approximately 4 × 10^4 cells/well. G418-resistant clones were isolated by limiting dilution and then expanded for further studies. One of the clones, MTX-A-S46N, which exhibited the highest level of RFC1 mRNA, was chosen for detailed studies.

Folate Influx Kinetic Parameters in L1210-G1a Cells—To characterize the mechanism that underlies the differences in 5-CHO-THF and MTX transport activities in L1210-G1a cells, influx kinetic parameters for these folates, together with 5-CHO-THF, the physiological reduced folate, were determined.
in L1210 and L1210-G1a cells. As shown in Fig. 4, influx of all these folates follows Michaelis-Menten kinetics. The kinetic parameters as derived from nonlinear regression to the Michaelis-Menten equation and those obtained from the double reciprocal plots are almost identical. As indicated in Table I, the influx $K_m$ for MTX and 5-CHO-THF is the same in L1210 cells, whereas the $K_m$ for 5-CH$_3$THF influx is about 2–3-fold lower. These values are essentially unchanged in L1210-G1a cells. In contrast, whereas the influx $V_{max}$ is the same for all folates in L1210 cells, there are substantial differences in the extent to which the influx $V_{max}$ decreased in L1210-G1a cells.

The influx $V_{max}$ in L1210-G1a cells decreased by a factor of 44 for MTX but declined by a factor of only 8 and 6.6 for 5-CHO-THF and 5-CH$_3$THF, respectively, relative to L1210 cells. Hence, differences in the rates of translocation of reduced folates and MTX, rather than carrier affinity for folate substrates, appeared to be the basis for the preferential transport of reduced folates observed in the L1210-G1a line.

Identification of a Mutation in the RFC1 Coding Region—Because the level of RFC1 transcript was unchanged in the L1210-G1a line, studies were undertaken to determine whether a mutation in RFC1 was the basis for the transport differences observed. Utilizing primers based on the published murine RFC1 sequence, cDNAs encompassing the entire coding region of RFC1 were isolated from L1210-G1a cells by reverse transcription-PCR and then cloned and fully sequenced. The nucleotide sequence of the RFC1 open reading frame in both parental and L1210-G1a lines was identical except for a single G$\rightarrow$A mutation at nucleotide position 260 (based on GenBank accession no. U32469). This change resulted in the substitution of asparagine for serine at amino acid residue 46. Fortuitously, this single purine nucleotide change eliminates a single Hin$\text{d}$III restriction site in the RFC1 coding region, thus allowing for the determination of whether all mRNAs in L1210-G1a cells harbored the same mutation. As shown in Fig. 5A, the reverse transcription-PCR products derived from L1210-G1a cells were not cut by Hin$\text{d}$III, in contrast to those derived from the parental cells, demonstrating that this mutation was present in all cDNAs isolated from the L1210-G1a line.

To access whether the single G$\rightarrow$A nucleotide change occurred in both RFC1 alleles, a 222-bp genomic fragment from RFC1 was amplified by PCR and subjected to restriction analysis. As illustrated in Fig. 5B, Hin$\text{d}$III failed to cut the genomic
PCR fragment derived from this line, thus establishing that this mutation is homozygous in the genome.

Transfection of the Mutated Carrier into the Transport-deficient MTX-A L1210 Derivative—The cDNA of the mutated carrier, designated RFC1-S46N, was electroporated into MTX-A cells, and a clonal derivative, MTX-A-S46N was established to characterize the properties of this carrier variant. The transcript generated by the pcDNA3.1 expression vector was diagnostically shorter than that encoding endogenous RFC1 in L1210 and MTX-A cells (Fig. 2), with a level 50% higher than that of RFC1 in L1210 cells based on two separate Northern blot analyses. The initial uptake rates of MTX, 5-CHO-THF, and 5-CH₃THF in MTX-A-S46N were all increased as compared with the MTX-A line from which it was derived (Fig. 6). However, the magnitude of the increase in influx was quite different among these folates, as quantitated in Table II. Influx was augmented by a factor of 4 and 10 for 5-CHO-THF and 5-CH₃THF relative to MTX in MTX-A-S46N cells (Table II, line 4), respectively. The impact of expression of RFC1-S46N was better seen when background transport in the MTX-A cells, (Table II, line 3), was subtracted from that of the MTX-A-S46N transfectant line (line 4). This yielded net increases in 5-CHO-THF and 5-CH₃THF influx that were 5 and 13 times higher, respectively, than that of MTX (Table II, line 5). These values are all consistent with the differences observed in L1210-G1a cells (Table II, line 2).

Kinetic parameters for the influx of MTX, 5-CHO-THF, and 5-CH₃THF in L1210 and L1210-G1a cells

|       | L1210 | L1210-G1a | L1210/L1210-G1a |
|-------|-------|-----------|-----------------|
| MTX   | V_max | 11.5 ± 0.7| 0.26 ± 0.02     | 44               |
|       | Kᵣ    | 6.9 ± 0.4 | 5.4 ± 0.9       | 1.3              |
| 5-CHO-THF | V_max | 13.5 ± 0.33 | 1.65 ± 0.15 | 8.0 |
|       | Kᵣ    | 5.07 ± 0.11 | 7.20 ± 0.78 | 0.70            |
| 5-CH₃THF | V_max | 10.2 ± 0.61 | 1.54 ± 0.07 | 6.6 |
|       | Kᵣ    | 1.97 ± 0.11 | 2.68 ± 0.08 | 0.73            |

Fig. 5. Restriction analysis of reverse transcription-PCR (A) and genomic PCR fragments (B) with HindIII. Fragments from L1210 and L1210-G1a cells were amplified as described under “Materials and Methods,” restricted with HindIII, and fractionated on 1.5 and 2% agarose gels, respectively. The molecular weights (bp) of PCR products and the larger restriction fragments are indicated by the arrows. Molecular markers are mixes of Lambda DNA-HindIII and φX174 DNA-HaeIII digests (Promega) or the latter alone.

Fig. 6. Initial uptake of MTX, 5-CHO-THF, and 5-CH₃THF in the MTX-A-S46N transfectant and MTX-A cells. After 25 min incubation in HBS at 37 °C MTX-A (open symbols) and MTX-A-S46N (closed symbols) cells were exposed to 1 μM of radiolabeled MTX (circles), 5-CHO-THF (triangles), or 5-CH₃THF (squares). The graph is representative of three experiments.
line. The \( K_r \) values for 5-CHO-THF and 5-CH\(_3\)THF influx were 5.5 and 2.4 \( \mu M \), respectively, virtually identical to the those in L1210 and L1210-G1a cells.

**MTX Sensitivity and 5-CHO-THF Growth Requirement in MTX\(^r\)A-S46N Cells**—The small increase in MTX influx in MTX\(^r\)A-S46N cells (Table II) resulted in only a small increase in sensitivity of these cells to the drug, as illustrated in Fig. 7A. The IC\(_{50}\) for MTX in MTX\(^r\)A-S46N still remained about 20 times greater than that of L1210 cells. In contrast, a 7-fold increase in 5-CHO-THF influx in MTX\(^r\)A-S46N relative to MTX\(^r\)A cells (Table II) resulted in a 30-fold decrease in the 5-CHO-THF growth requirement (Fig. 7B), close to the EC\(_{50}\) of L1210 cells, although influx was still \( \frac{1}{8} \) that of the latter line.

**DISCUSSION**

In prior studies, MTX transport-deficient cell lines have been characterized that were selected in vitro with folic acid as the sole folate source (7, 19, 28–32). In these cell lines, transport of the reduced folates, 5-CHO-THF and 5-CH\(_3\)THF, was decreased as well, and the growth requirement for 5-CHO-THF was substantially increased (7, 18, 19, 33). Because the physiological folate cofactor in the circulation of humans and rodents is 5-CH\(_3\)THF, these MTX resistant cells would not be expected to survive in vivo. Consistent with this is the observation that a MTX-resistant L1210 variant derived in vitro in the presence of folic acid, with a substantial increase in the EC\(_{50}\) for 5-CHO-THF, grew upon implantation in mice only if folic acid was co-administered to augment the levels of its reduced folate derivatives (19). Hence, the mechanism of MTX resistance in these cells is unlikely to be relevant to the in vivo setting in which tumor cell survival requires the transport and utilization of 5-CH\(_3\)THF.

Folic acid is a viable folate source when RFC1 function is lost in L1210 cells because this agent is transported to a large extent by another mechanism (17). Folic acid is, however, a trivial component of folates found in blood and tissues and hence cannot meet one-carbon requirements in humans when RFC1 is not functional. How then do tumor cells survive when resistance to MTX due to loss of transport develops in patients treated with related antifolates? To address this question, MTX resistance was developed, in vitro, under conditions in which the available folate source was the reduced cofactor 5-CHO-THF, an excellent substrate for RFC1. Although 5-CH\(_3\)THF, the physiological reduced folate, is an even better substrate for the carrier, its poor stability renders it an unreliable folate source in cell culture. A chemical mutagen ethylmethanesulfonate was employed to enhance the mutation rate, and a single step selection protocol was chosen to minimize the possibility of dihydrofolate reductase amplification (13). This design is not unlike the clinical situation in which MTX is often administrated in regimens that include DNA-damaging agents.

Using this strategy, a cell line, L1210-G1a, was isolated that displayed a 10-fold resistance to MTX but only a 2-fold increase in the growth requirement for 5-CHO-THF. The relatively small change in 5-CHO-THF growth requirement was based, in part, upon the observation that influx of MTX in L1210-G1a cells was decreased by factor of more than 40, yet influx of 5-CHO-THF was decreased by a factor of only 8. Hence, there was preferential conservation of transport of the tetrahydrofolate cofactor, a phenomenon shown to be relevant for 5-CH\(_3\)THF as well. Beyond this, the increase in 5-CHO-THF growth requirement was only \( \frac{1}{4} \) the decline in influx. This is probably attributable to the lack of proportionality between the decline in influx and steady-state folate levels mediated by RFC1, as characterized for MTX. In prior studies from this laboratory, changes in RFC1 activity in L1210 cells primarily influence the bidirectional fluxes with much smaller changes in the steady-state levels achieved (27). Consistent with this is the observation that although MTX influx in L1210-G1a cells decreased by a factor of more than 40, the IC\(_{50}\) for MTX decreased by a factor of only 10. Because it is free folate that is the substrate for polyglutamylation and subsequent one-carbon transfer.
transfer reactions within the cell (influx is not rate-limiting), it is this parameter that will determine the folate growth requirement.

Finally, because the EC_{50} for 5-CHO-THF increased from ~2.5 to 5.0 nM but the concentration of this folate in the medium was 25 nM, the loss of RFC1 activity would have little functional impact on folate metabolism. This is not surprising because to meet the need for folates during cell growth, sufficient transport is necessary to increase folate pools by a factor of only 2 over the doubling time of the cells. Because reduced folates are retained within the intracellular compartment as polyglutamyl derivatives, their levels are apparently sustained even at a slow rate of uptake.

Of considerable interest is the functional basis for the different loss of transport activities among the folate compounds in the L1210-G1a line. These differences were not due to an alteration in the substrate binding pocket within RFC1 that influences affinity for the folates because wild type influx \( K'_t \) values were preserved. Rather, the fall in influx was attributed almost entirely to a decline in \( V_{\text{max}} \). Within the context of the usual carrier paradigm, the influx \( V_{\text{max}} \) is determined by the total number of carriers and the rate-limiting step in the carrier cycle (34). For transport of folates in L1210 cells, the rate-limiting step in influx is the translocation of the unloaded carrier from the intracellular to extracellular membrane interfaces, a process that is accelerated when cells are loaded with carrier substrate (35). Carrier mobility has not been thought to be influenced by the substrate transported, and in fact, the influx \( V_{\text{max}} \) for all the folates was the same in L1210 cells. The data suggest that this is not the case with the L1210-G1a mutant. Here, the influx \( V_{\text{max}} \) is determined by the transport substrate, suggesting that in this cell line, entry of the loaded carrier into the cell is rate-limiting and that the velocity of this step in the entry process varies with the different folate species. The basis for this difference in \( V_{\text{max}} \) is not clear but it may be that the 4-amino group in the MTX pteridine ring results in an allosteric change in the mutated carrier upon substrate binding that restricts its mobility in comparison to the rate of translocation of the 5-CHO-THF- and 5-CH_3-THF-carrier complex.

The molecular basis for the changes in carrier cycling observed in the L1210-G1a line was a single mutation in RFC1 that resulted in a serine to asparagine substitution at amino acid 46. There have been no reported differences in RFC1 mutations in tumor cells with impaired MTX transport. However, in one study, RFC1 mRNA levels were normal or slightly decreased in human leukemic lymphoblasts, making it likely that there were mutations in RFC1 and that reduced folate transport must have been relatively preserved (16). There have been four documented mutations in RFC1 identified in tumor cells developed under MTX selective pressure in vitro that result in altered transport of folates. Introduction of premature stop codons in RFC1 cDNAs in MOLT3/MTX_10,000 produced a truncated protein with a decreased influx \( V_{\text{max}} \), without a change in \( K'_t \) (36), a change that would not result in a selective loss of function. An alanine to proline substitution in the fourth transmembrane domain of the murine RFC1 with resultant induction of a rigid kink within the \( \alpha \)-helical peptide backbone led to a decrease in the MTX influx \( V_{\text{max}} \) of 2 orders of magnitude without a change in the extent or affinity of specific binding to carrier at the cell surface (37). A serine to asparagine mutation at amino acid 127 associated with a low frequency truncation of RFC1 in the second allele was associated with a decrease in folate binding affinities in CCRF-CEM cells (38).

In a preliminary report, two mutations in the murine RFC1, one of which is at amino acid 48, were each shown to increase carrier affinity for folic acid, thereby augmenting folate accumulation and rendering cells resistant to the glycaminide ribonucleotide transformylase inhibitor DDATHF (39). The RFC1 mutations identified in the L1210-G1a cells occurred just two amino acids away in the first putative transmembrane domain, which is conserved in all mammalian species studied (20–25). The lack of change in the affinity of the mutated carrier for reduced folates and MTX suggested that this amino acid residue is not involved in the binding of substrates. Replacement of the serine with other amino acids or substitutions at adjacent sites will further define the importance of this residue and region in carrier cycling and substrate binding.

Biomedical and biophysical analysis of mutated membrane transporters has been the primary approach to understanding the structure-function relationships for many carriers because crystallization of membrane proteins is extremely difficult. Hence, characterization of the bacterial lactose permease (40) and human glucose transporters (GLUT) (41), among others, have been based upon this approach. Extensive site-directed mutation analyses have revealed that amino acid residues critical for glucose transport activity are rare. For instance, sequential replacement of every residue with alanine within the conserved helix of transmembrane VIII in GLUT3 produced only one mutant with a small increase in influx \( K'_t \) (42). Substitution of all six cysteines with serine in GLUT1 resulted in a mutated carrier that retained all kinetic features of the wild type allele, thus excluding involvement of these residues in GLUT1 function or oligomer formation (43).

The serine to asparagine substitution in RFC1 was found to affect both alleles. This is in contrast to other mutations in which the wild type RFC1 allele was present but not transcribed (37) or the normal RFC1 allele was present and transcribed along with the mutant alleles (36). Two different transcripts were also recently identified in CEM/MTX(38). It is possible that only one allele was present in the L1210 cells at the time that it was mutated or that the second allele might have been lost during the selection of L1210-G1a cells. The homozygosity of RFC1 is an important factor contributing to the stability of the phenotype of this cell line.

REFERENCES
1. Goldman, I. D., and Matherly, L. H. (1986) in Membrane Transport of Antineoplastic Agents (Goldman, I. D., ed) pp. 283–308, Pergamon Press, Oxford, England
2. Sirotanok, F. M. (1986) in Membrane Transport of Antineoplastic Agents (Goldman, I. D., ed) pp. 241–281, Pergamon Press, Oxford, England
3. Henderson, G. B. (1990) Annu. Rev. Nutr. 10, 319–335
4. Wang, X., Shen, F., Freisheim, J. H., Gentry, L. E., and Ratnam, M. (1992) Biochem. Pharmacol. 44, 1988–1991
5. Antony, A. C. (1992) Blood 76, 2807–2820
6. Brigg, K. E., Spinella, M. J., Westin, E. H., and Goldman, I. D. (1994) Biochem. Pharmacol. 47, 337–345
7. Henderson, G. B., and Strauss, B. P. (1990) Cancer Res. 50, 1709–1714
8. Sierra, E. E., Brigg, K. E., Spinella, M. J., and Goldman, I. D. (1997) Biochem. Pharmacol. 53, 223–231
9. Kumar, C. K., Moyer, M. P., Deuda, P. K., and Said, H. M. (1997) J. Biol. Chem. 272, 6226–6231
10. Chiao, J. H., Roy, K., Tolner, B., Yang, C.-H., and Sirotanok, F. M. (1997) J. Biol. Chem. 272, 11165–11170
11. Jolivet, J., Cowan, K. H., Curt, G. A., Clendenninn, N. J., and Chabner, B. A. (1983) N. Engl. J. Med. 309, 1094–1094
12. Gorlick, R., Goker, E., Tripett, T., Walsham, M., Banerjee, D., and Bertino, J. R. (1996) N. Engl. J. Med. 335, 1041–1048
13. Sirotanok, F. M., Mocci, D. M., Kelleher, L. E., and Goutas, L. J. (1981) Cancer Res. 41, 4447–4452
14. Tripett, T., Schlenmer, S., Elsiseyfi, Y., Goker, E., Wachter, M., Steinherz, P., Tan, C., Berman, E., Wright, J. E., Rosowsky, A., Schweitzer, B., and Bertino, J. R. (1992) Blood 80, 1158–1162
15. Matherly, L. H., Tao, J. W., Ravindranath, Y., Proefke, S. A., Wong, S. C., Gimotty, P., Buck, S., Wright, J. E., and Rosowsky, A. (1995) Blood 85, 500–509
16. Gorlick, R., Goker, E., Tripett, T., Steinherz, P., Elsiseyfi, Y., Mazumdar, M., Flintoff, W. F., and Bertino, J. R. (1997) Blood 80, 1013–1018
17. Yang, C.-H., Dembo, M., and Sirotanok, F. M. (1983) J. Membr. Biol. 75, 11–20
18. Spinella, M. J., Brigg, K. E., Sierra, E. E., and Goldman, I. D. (1995) J. Biol. Chem. 270, 7842–7849
RFC1 Mutation and Reduced Folate Transport

19. Sirotnak, F. M., Goutas, L. J., and Mines, L. S. (1985) Cancer Res. 45, 4732–4734
20. Dixon, K. H., Lanpher, B. C., Chiu, J., Kelley, K., and Cowan, K. H. (1994) J. Biol. Chem. 269, 17–20
21. Williams, F. M. R., Murray, R. C., Underhill, T. M., and Flintoff, W. F. (1994) J. Biol. Chem. 269, 5810–5816
22. Williams, F. M. R., and Flintoff, W. F. (1995) J. Biol. Chem. 270, 2987–2992
23. Prasad, P. D., Ramamoorthi, S., Leibach, F. H., and Ganapathy, V. (1995) Biochem. Biophys. Res. Commun. 206, 681–687
24. Moscow, J. A., Gong, M. K., He, R., Sgagias, M. K., Dixon, K. H., Anzick, S. L., Meltzer, P. S., and Cowan, K. H. (1995) Cancer Res. 55, 3790–3794
25. Wong, S. C., Proefke, S. A., Bhushan, A., and Matherly, L. H. (1995) J. Biol. Chem. 270, 17468–17475
26. Fry, D. W., Yalowich, J. C., and Goldman, I. D. (1982) J. Biol. Chem. 257, 1890–1896
27. Zhao, R., Seither, R., Bringle, K. E., Sharina, I. G., Wang, P. J., and Goldman, I. D. (1997) J. Biol. Chem. 272, 21207–21212
28. Schuetz, J. D., Matherly, L. H., Westin, E. H., and Goldman, I. D. (1988) J. Biol. Chem. 263, 9840–9847
29. Rosowsky, A., Lazarus, H., Yuan, G. C., Beltz, W. R., Mangini, L., Abelson, H. T., Modest, E. J., and Frei, E. (1980) Biochim. Biophys. Acta 29, 648–652
30. Cowan, K. H., and Jolivet, J. (1984) J. Biol. Chem. 259, 10793–10800
31. Underhill, T. M., and Flintoff, W. F. (1988) Mol. Cell. Biol. 8, 1784–1758
32. Assaraf, Y. G., and Schimke, R. T. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 7154–7158
33. Dixon, K. H., Trepel, J. B., Eng, S. C., and Cowan, K. H. (1991) Cancer Commun. 3, 357–365
34. Stein, W. D. (1990) in Channels, Carriers, and Pumps: An Introduction to Membrane Transport (Stein, W. D., ed) p. 127, Academic Press, San Diego
35. Goldman, I. D. (1971) Biochim. Biophys. Acta 233, 624–634
36. Gong, M., Yess, J., Connolly, T., Ivy, S. P., Ohnuma, T., Cowan, K. H., and Moscow, J. A. (1997) Blood 89, 2494–2499
37. Bringle, K. E., Spinella, M. J., Sierra, E. E., and Goldman, I. D. (1995) J. Biol. Chem. 270, 22974–22979
38. Wong, S. C., Proefke, S. A., Bhushan, A., and Matherly, L. H. (1997) Proc. Am. Assoc. Cancer Res. 38, 162
39. Tse, A., Bringle, K. E., and Moran, R. G. (1997) Proc. Am. Assoc. Cancer Res. 38, 162
40. Kaback, H. R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 5539–5543
41. Saravolac, E. G., and Holman, G. D. (1997) in Facilitative Glucose Transporters (Gould, G. W., ed) pp. 39–66, R. G. Landers and Co., Georgetown, TX
42. Seatter, M. J., Kane, S., Porter, L. M., Arbuckle, M. I., Melvin, D. R., and Gould, G. W. (1997) Biochemistry 36, 6401–6407
43. Due, A. D., Cook, J. A., Fletcher, S. J., Quinn, Z. C., Powers, A. C., and May, J. M. (1995) Biochem. Biophys. Res. Commun. 208, 590–596
A Reduced Folate Carrier Mutation Produces Substrate-dependent Alterations in Carrier Mobility in Murine Leukemia Cells and Methotrexate Resistance with Conservation of Growth in 5-Formyltetrahydrofolate

Rongbao Zhao, Yehuda G. Assaraf and I. David Goldman

J. Biol. Chem. 1998, 273:7873-7879.
doi: 10.1074/jbc.273.14.7873

Access the most updated version of this article at http://www.jbc.org/content/273/14/7873

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 39 references, 24 of which can be accessed free at http://www.jbc.org/content/273/14/7873.full.html#ref-list-1