0026-895X/99/010068-09$3.00/0

MOLECULAR PHARMACOLOGY, **56:**68–76 (1999).

Pattern of Mutations that Results in Loss of Reduced Folate Carrier Function under Antifolate Selective Pressure Augmented by Chemical Mutagenesis

RONGBAO ZHAO, IRAIDA G. SHARINA, and I. DAVID GOLDMAN

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

Received December 18, 1998; accepted April 5, 1999

This paper is available online at http://www.molpharm.org

ABSTRACT

Chemical mutagenesis with *N*-methyl-*N*-nitrosourea was employed to study the pattern of mutations in the reduced folate carrier (RFC1) that results in transport-related methotrexate resistance and to identify amino acid residues that are critical to carrier structure and/or function. Thirty-four methotrexate transport-defective L1210 leukemia cell lines were isolated with folic acid as the sole folate source under antifolate selective pressure. The RFC1 mRNA levels were comparable with, or not substantially decreased, in most of these cell lines relative to wild-type L1210 cells. The molecular basis for the transport defects was investigated by sequencing multiple RFC1 cDNA clones isolated from these mutants by reverse transcription-polymerase chain reaction, which encompassed the entire coding region. The mutations identified were further confirmed either by direct sequencing or, when applicable, by restriction analysis of total reverse transcription-polymerase chain reaction products. The majority of mutations (21) led to single amino acid substitutions that were in, or near, 9 of 12 predicted transmembrane domains, with the highest frequencies in the first, fifth, and eighth. There were no mutations in the sixth, ninth, and twelfth transmembrane domains. Glycine, serine, and arginine were the most frequently mutated residues. These data suggest that several transmembrane domains, rather than the amino- and carboxyl-termini, and the large intracellular loop between the sixth and seventh transmembrane domains play key roles as sites for RFC1 inactivation because of single point mutations. This panel of mutated cell lines offers an important resource for studies on RFC1 structure-function and for the evaluation of transport-related cross-resistance patterns with new-generation antifolate inhibitors of tetrahydrofolate cofactor-dependent enzymes.

The reduced folate carrier (RFC1) mediates transport of the folic acid family of compounds, which are critical for de novo synthesis of purines and thymidine in mammalian cells. RFC1-mediated transport is essential for the activity of most folate-based chemotherapeutic drugs, such as methotrexate (MTX), along with new-generation inhibitors of tetrahydrofolate cofactor-dependent enzymes that undergo polyglutamylation at the γ-carboxyl position of the glutamate moiety (Jackman and Calvert, 1995; Mendelsohn et al., 1996; Shih et al., 1998). Impaired membrane transport is a common mechanism of acquired resistance to these drugs in both murine and human model tumor systems and in human neoplasms after treatment of patients with MTX (Rosowsky et al., 1980; Sirotnak et al., 1981, 1985; Cowan and Jolivet, 1984; Schuetz et al., 1988; Underhill and Flintoff, 1989; Pui and Evans, 1998). This has been studied in detail as an important factor in treatment failure in patients with acute lymphoblastic leukemia (Trippett et al., 1992; Matherly et al., 1995; Gorlick et al., 1997; Zhang et al., 1998)

With the recent cloning of the RFC1 gene from a variety of species, an understanding of the molecular basis for transport-related drug resistance has now become possible. RFC1 has a predicted secondary structure that consists of 12 transmembrane domains divided in half by a large internal loop, which, along with the amino and carboxyl termini, resides in the cytoplasm. (Dixon et al., 1994; Williams et al., 1994; Williams and Flintoff, 1995; Moscow et al., 1995; Prasad et al., 1995; Wong et al., 1995). Consistent with other major facilitator superfamily carriers, there is no nucleotide binding pocket in RFC1, and carrier activity is not dependent upon ATP hydrolysis (Pao et al., 1998). Rather, RFC1 mediates uphill folate transport through a countertransport with organic anions that are concentrated within cells (Goldman, 1971; Henderson and Zevely, 1983; Yang et al., 1984).

Researchers at our laboratory have recently undertaken studies, using chemical mutagenesis, to characterize RFC1

This work was supported by Grant CA39807 from the National Cancer Institute.

ABBREVIATIONS: RFC1, reduced folate carrier; MTX, methotrexate; 5-CHO-THF, 5-formyltetrahydrofolate; 5-CH_3-THF, 5-methyltetrahydrofolate; TMQ, trimetrexate; RT, reverse transcription; PCR, polymerase chain reaction; HBS, HEPES buffer saline.

amino acid residues that are important determinants of substrate binding and carrier mobility. In initial reports, carriers with mutations within the first predicted transmembrane domain have been identified that produce highly selective transport changes (Zhao et al., 1998a,b). An asparagine substitution for serine at amino acid 46 in the first transmembrane domain produced a marked decrease in the mobility of carrier loaded with MTX and conserved, in part, the mobility of carrier loaded with 5-formyltetrahydrofolate (5-CHO-THF) or 5-methyltetrahydrofolate (5-CH_3-THF) (Zhao et al., 1998a). A lysine substitution for glutamate at amino acid 45 resulted in a marked decreased in carrier mobility and the introduction of an obligatory requirement for univalent anions for carrier function. This was accompanied by selective alterations in binding to the carrier with 3- and 7-fold increases in affinity for 5-CHO-THF and folic acid, respectively, but a 7-fold decrease in affinity for MTX (Zhao et al., 1998b). Reports are emerging from other laboratories that further define the important role of the first transmembrane domain, as well as other carrier sites, in binding and translocation of folate substrates (Roy et al., 1998; Tse et al., 1998).

This article provides an overview of the spectrum of mutations that occurred in RFC1 when murine L1210 leukemia cells were subjected to chemical mutagenesis followed by MTX selective pressure with folic acid as the sole folate substrate. The data further demonstrate the high frequency of mutations in amino acids within transmembrane domains as a basis for antifolate resistance caused by impaired transport.

Materials and Methods

Chemicals. [3′,5′,7-^{3}H]MTX (specific radioactivity, 5–20 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL) and purified by HPLC before use (Zhao et al., 1998a). Unlabeled MTX and 5-CHO-THF provided by Lederle (Carolina, Puerto Rico) and folic acid purchased from Sigma (St. Louis, MO) were also purified before use by HPLC. Trimetrexate (TMQ), was a gift from Warner-Lambert company (Ann Arbor, MI). All other reagents were of the highest purity available from various commercial sources.

Cell Culture Conditions. L1210 murine leukemia cells were grown in RPMI-1640 medium containing 2.3 μM folic acid, supplemented with 5% bovine calf serum (HyClone, Logan, UT), 2 mM glutamine, 20 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. All MTX-resistant cell lines were maintained in MTX-free RPMI-1640 medium supplemented as above.

Selection and Isolation of Clonal MTX-Resistant L1210 Cells. L1210 cells were grown in complete RPMI medium and treated with 0.4 mM *N*-methyl-*N*-nitrosourea for 12 h to achieve about 10% cell survival (Lee et al., 1992). Cells were washed to remove the mutagen, placed in 24-well plates at a density of 2×10^5 cells/ml, and allowed to grow for 3 days. The cells from each well were then seeded in fresh 24-well plates containing complete RPMI medium, supplemented with 200 nM MTX, and grown for 2 additional weeks. One surviving clone was picked from each well and maintained in this selection medium for another 3 weeks, after which they were plated in complete RPMI-1640 containing 0.5% soft agar in the absence of MTX. After an additional 2 weeks, a single clone was picked up from each soft agar plate. Two separate selections were performed under these conditions, and the MTX-resistant cells obtained were identified as C and D lines.

Transport Studies. Influx measurements were performed by methods described previously (Zhao et al., 1997). Briefly, exponentially growing cells were harvested, washed twice, and resuspended in HEPES-buffered saline (HBS; 20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, and 5 mM glucose, pH 7.4) to a density of 1.5×10^7 cells/ml. Cell suspensions were incubated at 37°C for 25 min, after which uptake was initiated by the addition of radiolabeled MTX, and samples were removed at different times. Uptake was terminated by injection of 1 ml of the cell suspension into 9 ml of ice-cold HBS. Cells were collected by centrifugation, washed twice with ice-cold HBS, and processed for measurement of intracellular radioactivity by liquid scintillation counting (Zhao et al., 1997).

Cell Growth Studies. Before assessment of folate growth requirement, cells were grown for 1 to 2 weeks in folate-free RPMI-1640 medium supplemented with 5% dialyzed bovine calf serum, 200 μM glycine, 100 μM adenosine, and 10 μM thymidine to deplete endogenous folates. For analyses of growth requirement and antifolate IC_{50}, cells were grown in 96-well plates (1×10^5 cells/ml), exposed continuously to the appropriate concentrations of MTX, TMQ, folic acid, or 5-CHO-THF for 72 h, after which cell numbers were determined by hemocytometer count and viability was assessed by trypan blue exclusion.

Northern Analyses. Total RNA was isolated using the TRIzol reagent (Life Technologies, Gaithersburg, MD) and 20 μg were resolved by electrophoresis on 1% agarose gels containing formaldehyde. Transfer and hybridization were performed as described previously (Zhao et al., 1997). Transcripts were quantified by PhosphorImager analysis of the hybridization signals and normalized to β-actin.

Cloning and Sequencing the Mutated Reduced Folate Carrier. Poly(A)$^+$ mRNA was purified using Dynabeads mRNA DIRECT kit (Dynal, Oslo, Norway). The first DNA strand synthesis was carried out with Superscript Reverse Transcriptase according to the manufacturer's protocol (Life Technologies). The RFC1 protein-coding sequence was amplified with Pfu polymerase (Stratagene, La Jolla, CA) with the use of oligonucleotide primers that flank the coding region of RFC1 (upstream primer: at nucleotide −46 from the translation start codon 5′-GCGGATCCTGGAGTGTCATCTTGG-3′ and downstream primer at nucleotide +82 from translation stop codon 5′-GCCTCGAGCTGGTTCAGGTGGAGT-3′). Two 8-basepair (bp) linkers (underlined) were introduced into the primers so that both *Bam*HI and *Xho*I restriction sites were created in the polymerase chain reaction (PCR) products to facilitate directional recloning. The PCR 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 predicted PCR product was purified from an agarose gel (Qiagen, Valencia, CA) and cloned into a pCR-Blunt vector (Invitrogen, Carlsbad, CA). Full sequencing of the coding region of RFC1 was achieved using three primers: the upstream primer of the reverse transcription (RT)-PCR reaction, 5′-^{562}CTGGGCTTCATCCTCTTCAGCC^{583}-3′ and 5′-^{968}TGAGCATCCGCTGGACTCTGTG^{989}-3′. Automated sequencing was performed on ABI 373A and ABI 373 Sequencers (Perkin Elmer, Norwalk, CT), in the Nucleic Acid Sequencing Shared Resource of the Albert Einstein College of Medicine Comprehensive Cancer Center.

Restriction Analysis of RT-PCR Products. Whenever a mutation was identified in RFC1 cDNA clones, a segment (30 bp) containing the mutation was analyzed by DNASIS (Hitachi Software Engineering American, San Francisco, CA) to determine whether the base change added or removed a restriction site. If the restriction pattern was altered, the RT-PCR products (see above) from mutants and L1210 cells were treated with appropriate restriction enzymes and resolved on an 1.0% agarose gel.

Results

Thirty-four MTX-resistant L1210 leukemia cell clones were isolated under 200 nM MTX selective pressure with *N*-methyl-*N*-nitrosourea as mutagen and folic acid as the sole folate source in two separate selections (C and D). All MTX-resistant cell lines exhibited markedly impaired MTX influx.

RFC1 mRNA levels were evaluated in all MTX-transport defective cell lines by Northern blot analysis (Fig. 1) and the normalized values, obtained by quantitative analyses on a PhosphorImager, are listed in Tables 1, 2, and 3. The amount of RFC1 transcript was decreased by a factor of about four in C5, C6, C7, and D5 cells. The level was either unchanged or within half that of wild-type L1210 cells in all the other cell lines.

To analyze for mutations in the carrier, entire RFC1 cDNAs were isolated from MTX-transport defective cell lines by RT-PCR and cloned. At least three randomly picked cDNA clones from each mutant were initially fully sequenced and compared with the published RFC1 sequence in L1210 cells (Dixon et al., 1994; Brigle et al., 1995). In six cell lines, there were mutations in the open reading frame that resulted in no protein, truncated proteins, or carriers with deletions (Table 1). In D10 cells, there was a G-to-A mutation in the initiation codon. A single nucleotide change introduced premature stop codons in RFC1 genes isolated from C6, D15, and D2 cells, so that RFC1 peptides ended at position 24, 104, or 451, respectively. Apart from the premature stop mutation found in D15 cells, wild-type RFC1 was also isolated from this cell line (one out of three clones). Deletions in the RFC1 coding region, suggested by the lower molecular weights of the RT-PCR products in D8 and D12 cells were generated from alternative splicing of the RFC1 gene (data not shown). It is noteworthy that the RFC1 peptide in D2 cells contains the 12 predicted transmembrane domains; however, the cytoplasmic carboxyl terminus (61 amino acid residues) had been deleted.

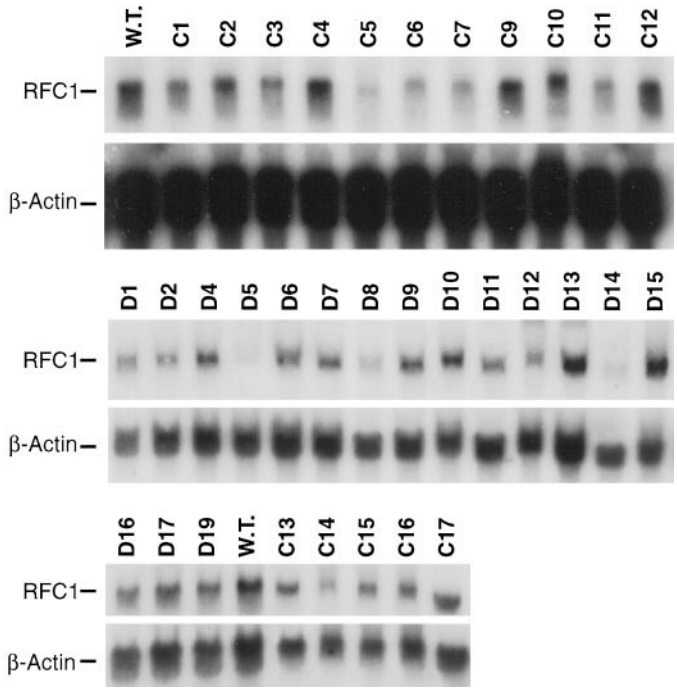

Fig. 1. Autoradiogram of Northern blot analysis of total RNA isolated from MTX-resistant cell lines. Total RNA was probed successively with the full-length murine RFC1 and β-actin cDNAs. The radioactive blots were directly quantified by PhosphorImager analysis. RFC1 message levels so obtained for each mutant, normalized to β-actin and to an L1210 mRNA level of 1, are listed in Tables 1–3. The upper panel and lower two panels were derived from two separate blots, each with wild-type (W.T.) L1210 RFC1 mRNA as controls.

Table 2 summarizes properties of the 23 cell lines in which amino acid substitutions in RFC1 were identified. The mutations were divided into four groups according to the amino acid residues mutated. Within the same group, the mutations are listed in order of the position of the change. In cases where two mutated RFC1 alleles were detected in the same cell line, the more frequent mutation was used for the grouping. As shown, also in Table 1, the nucleotide changes were limited to G-to-A or C-to-T transitions; the latter change occurred at a lower frequency consistent with the expected pattern reported for the mutagen (Lee et al., 1992). In group 1, glycine was the residue most frequently mutated and was always replaced with a negatively charged aspartate, glutamate, or with a positively charged arginine. Only 1 of 21 proline residues in RFC1 was mutated, and the same mutation to serine at amino acid 68 was identified in three different cell lines. In group 2, positively charged arginine was substituted at four positions, twice by histidine and in one case each with glutamine or cysteine. Replacement of negatively charged glutamate with a positively charged lysine, identified in C8 cells (Zhao et al., 1998b) was also present in D13 cells. Apart from the serine-to-asparagine mutation at amino acid 46 reported earlier (Zhao et al., 1998a), a change from serine to phenylalanine occurred at three different positions in four different mutants in group 3. Other amino acid substitutions, Ala to Val and Ala to Thr, were also identified. In four lines, two mutated RFC1 alleles with different frequencies were isolated. In one case, two mutations were found in the same allele, Met120Ile and Arg353Cys, in D19 cells. Six mutations were found in more than one cell line. Pro68Ser was identified three times in different cell lines, and Gly44Asp, Glu45Lys, Gly161Arg, Ser166Phe, and Gly303Asp were each detected in two mutant lines.

When nucleotide substitutions led to elimination or addition of one restriction site, whole RT-PCR products were subjected to restriction analysis to assess their homogeneity or percentage in the whole cDNA population as illustrated in Fig. 2. Figure 2A shows the single nucleotide substitutions that remove a restriction site in the RFC1 coding sequence, so that the RT-PCR products either could not be cut or resulted in a larger restriction fragment, compared with wild-type RFC1. Thus, all RFC1 cDNAs isolated from C3 cells harbor the nucleotide change that leads to Gly303Asp, but only the major component of those from C4 cells have the same mutation. The rest of the cDNAs in this cell line contain another mutation, Gly161Arg (Table 2). Likewise, *Nae*I identified Gly321Asp as a homogeneous mutation in C10 cells. The G-to-A change identified in D10 cells was also homogeneous and removes one of three *Ban*I-restriction sites in RT-PCR products (Table 1). Fig. 2B shows that the nucleotide changes that create a restriction site in the RT-PCR product result in smaller restriction fragments compared with wild-type RFC1. *Taq*I revealed that Pro68Ser occurred in 60, 100, and 100% of cDNAs isolated from C9, C13, and D16 cells, respectively. The restriction pattern with *Nae*I shows that the premature stop codon in D2 cells was present in almost all cDNAs. The Arg366His mutation in D11 cells was present in about 70% of the cDNA population. Likewise, *Xmn*I revealed that Glu45Lys is present only in about 50% of the cDNA population in both C8 and D13 cells. In addition, the use of *Xmn*I permitted identification of another mutation, Gly111Asp, in C8 cells.

Whenever the point mutations identified by initial sequencing could not be verified by restriction analyses as shown above, the total products of a separate RT-PCR reaction were sequenced with appropriate primers to confirm the mutation found in these cell lines. Thus Gly44Glu, Gly169Asp, Gly280Asp, Arg131His, and Arg155Gln were homogeneous in the cDNA population from D17, C7, D7, C1, and C2 cells, respectively, because the nucleotides present in wild-type RFC1 were not visible at the mutated positions in the sequencing chromatograms. In D9, C15, and C11 cells, Ser166Phe, Ser385Phe, and Ala376Thr, respectively, occurred in more than half of the cDNA population. However, more accurate quantification was not possible because of the varied ratios between peaks associated with the nature of the sequencing reactions. Similarly, Ser166Phe and Ala400Val were identified as the major and minor mutations, respec-

TABLE 1

Mutations that resulted in no expression of RFC1 or expression of incomplete RFC1 polypeptides

Cell Line	MTX IC_{50} (Fold Increase Relative to L1210 Cells)	MTX Influx (% of L1210 Cells)	RFC1 mRNA Levels Relative to L1210 Cells	Nucleotide Change	RFC1 Polypeptide
D10	32	1.5	1.0	3 ATG→ATA	No polypeptide
C6	220	1.0	0.27	75 TGG→TGA	First 24 aa
D15	12	12	1.51	315TGG→TGA	First 104 aa
D2	73	2.0	0.62	1354CAG→TAG	First 451 aa
D8	290	0.5	0.50	Deletion	Deletion
D12	230	0.9	0.89	Deletion	Deletion

aa, amino acids.

TABLE 2

Point mutations in RFC1 isolated from MTX transport-defective L1210 variants

Cell Lines	MTX IC_{50} (Fold Increase[a])	MTX Influx (% of L1210 Cells)	RFC1 mRNA Levels[b]	Nucleotide Change	Amino Acid Change
Glycine and proline					
D17	150	1	0.90	131GGG→GAG	44 Gly→Glu
C12	210	1.1	0.67	131GGG→GAG	44 Gly→Glu
				508CAG→TAG	170 Gln→Stop
C17	37	8.9	0.98	481GGA→AGA	161 Gly→Arg
C7	170	0.8	0.24	506GGC→GAC	169 Gly→Asp
D7	77	1.2	0.56	839GGC→GAC	280 Gly→Asp
C3	500	0.4	0.72	908GGC→GAC	303 Gly→Asp
C4	280	1.2	1.1	908GGC→GAC	303 Gly→Asp
				481GGA→AGA	161 Gly→Arg
C10	170	0.7	0.78	962GGC→GAC	321 Gly→Asp
D6	350	0.6	0.57	1181GGG→GAG	394 Gly→Glu
C9	26	2.2	0.77	202CCG→TCG	68 Pro→Ser
C13	42	3.6	0.88	202CCG→TCG	68 Pro→Ser
D16	15	3.8	0.77	202CCG→TCG	68 Pro→Ser
Arginine and glutamate					
C2	87	1.5	0.66	392CGC→CAC	131 Arg→His
C1	190	0.9	0.86	464CGG→CAG	155 Arg→Gln
D19	18	4	0.98	757CGC→TGC &	253 Arg→Cys
				360ATG→ATA	120 Met→Ile
D11	270	0.8	0.48	1097CGT→CAT	366 Arg→His
C8	19	8	1.2	133GAA→AAA	45 Glu→Lys
				332GGC→GAC	111 Gly→Asp
D13	230	3.8	0.58	133GAA→AAA	45 Glu→Lys
Serine and alanine					
D9	330	0.7	0.82	497TCT→TTT	166 Ser→Phe
D1	53	2.1	0.90	497TCT→TTT	166 Ser→Phe
				1199GCT→GTT	400 Ala→Val
D4	270	0.6	0.67	950TCC→TTC	317 Ser→Phe
				919GCC→GTC	307 Ala→Val
C15	47	2.1	0.93	1154TCT→TTT	385 Ser→Phe
C11	39	5.7	0.42	1126GCC→ACC	376 Ala→Thr

[a] Compared with L1210 cells.
[b] Relative to L1210 cells.

TABLE 3

Cell lines in which RFC1 cDNA was altered but no mutation data could be obtained

Cell Line	MTX IC_{50} (Fold Increase Relative to L1210 Cells)	MTX Influx (% of L1210 Cells)	RFC1 mRNA Level Relative to L1210 Cells	Reason
C5	260	0.7	0.21	Failed to isolate cDNA
D5	330	0.5	0.26	Failed to isolate cDNA
C16	21	7.2	0.77	Lower yield of RT-PCR reaction and no mutation in RFC1 cDNA
D14	320	0.4	0.40	Multiple RT-PCR products
C14	320	0.4	0.56	Multiple mutations in RFC1 cDNA

tively, in D1 cells, whereas Ser317Phe and Ala307Val were found to be the major and minor mutations in D4 cells, respectively.

No specific RFC1 mutation was identified in the remaining five cell lines, although RFC1 cDNAs were clearly altered in these mutants (Table 3). RFC1 cDNA could not be isolated from C5 and D5 cells despite the presence of RFC1 mRNA at a level 20% that of L1210 cells in the both mutants. No mutation was found in RFC1 cDNA isolated from C16; however, an exceptionally low yield of the RT-PCR reaction was obtained. The cDNAs isolated from D14 cells by RT-PCR consisted of fragments with different molecular weights, visible from the broadness of the band on agarose gel. Because of this obvious heterogeneity in the cDNA population, no further cloning effort was made. Finally, cDNAs isolated from C14 cells were cloned and sequenced, but multiple mutations were found in part of the RFC1 coding sequence. These mutations could not be verified by sequencing another cDNA clone, and no further attempt was made to define the RFC1 mutation in this cell line.

Cell lines were screened for changes in transport among the folate compounds studied and for potential resistance related to alterations in DHFR activity. This information was obtained by measuring MTX influx and growth inhibition, along with 5-CHO-THF and folic acid growth requirements, as indictors of folate delivery into cells. TMQ, a lipophilic antifolate, enters cells by passive diffusion and does not form polyglutamate derivatives; it was used to detect resistance associated with changes in DHFR activity. When these data suggested that a mutation was associated with highly specific or selective changes in transport, influx characteristics of all folates (MTX, folic acid, 5-CHO-THF, and 5-CH_3-THF) were assessed. One such cell line from the panel (C8) has already been described (Zhao et al., 1998b).

The MTX IC_{50} value in the mutant cell lines was increased from 12-fold in L1210-D15a cells to 500-fold in L1210-C3a cells compared with L1210 cells. MTX influx was profoundly decreased to a few percentage of that of parent L1210 cells in most of the lines and even less in some cell lines (Tables 1–3). Figure 3, top, illustrates the correlation between the decline in MTX influx and the increase in MTX IC_{50} values. A reduction in MTX influx to ~10% that of wild-type L1210 cells was associated with a 10-fold increase in the MTX IC_{50} value (the lowest level graphed). As influx was decreased further, resistance increased, approaching an IC_{50} value nearly 800 times that of parent L1210 cells. In particular, small decreases in residual transport (<2% of L1210 cells) produced marked increases in resistance. There was no correlation between MTX and TMQ IC_{50} values (data not shown). Thirty-two of the cell lines were at least as sensitive to TMQ as the L1210 cells, ruling out the possibility of altered DHFR properties in the MTX transport-defective cell lines. The remaining two cell lines, C8 and D13, exhibited only a slight increase in TMQ IC_{50} value (<2 fold). Hence, resistance to MTX in these cell lines could be attributed solely to the decline in transport.

The growth requirement for 5-CHO-THF increased with MTX-resistance as shown in Fig. 3, center. The growth requirement for folic acid was increased in all but two cell lines by an average factor of 2 to 3 and was independent of sensitivity to MTX (Fig. 3, bottom). This change in folic acid growth requirement was surprising, because only a small portion of folic acid transport in L1210 cells is mediated by RFC1 (Yang et al., 1983). However, cells were folate-depleted under the conditions of the growth requirement assay, a factor that might magnify the requirement for delivery of exogenous folic acid into cells. The two cases in which an MTX-influx defect was associated with a decrease in the folic acid growth requirement were in the C8 and D13 lines, as depicted by the arrows. The C8 line, with a glutamate-to-lysine substitution, was the subject of an initial report (Zhao et al., 1998b) and the same mutation was identified in D13 cells (Table 2).

The point mutations found in this study are depicted in Fig. 4. The top portion shows the topology of RFC1 predicted based on a hydropathy analysis (Dixon et al., 1994) with 12 transmembrane domains and both amino and carboxyl termini in a cytoplasmic orientation. The bottom portion of the

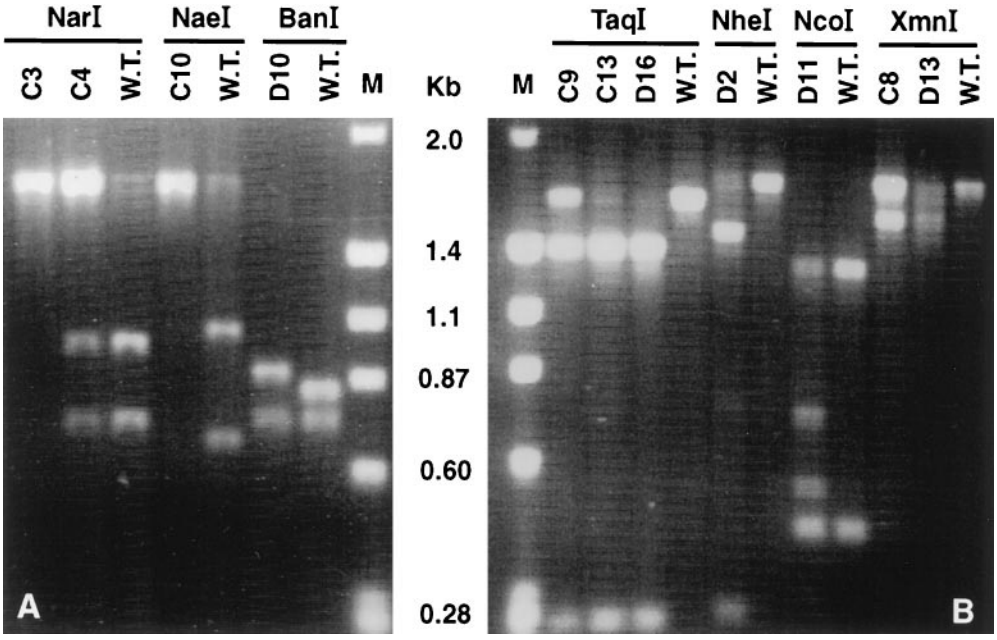

Fig. 2. Restriction analysis of total RT-PCR product isolated from L1210 (W.T.) and mutant cells with *Nar*I, *Nae*I, *Ban*I, *Taq*I, *Nhe*I, *Nco*I, and *Xmn*I, respectively. A, mutations in cell lines in which a restriction site in the RT-PCR products (1.7 kilobase pairs) was removed, resulting in an uncut or larger fragment. B, mutations that added a restriction site in the RT-PCR products leading to a smaller fragment compared with those isolated from wild-type (W.T.) L1210 cells. All restrictions were performed with total RT-PCR product isolated from the mutant and L1210 cells.

figure indicates the position and nature of amino acid substitutions in a linear peptide chain. It is clear that the mutations identified in this study were found exclusively in or near the predicted transmembrane domains (except Arg250Cys, which is positioned in the large intracellular loop between the sixth and seventh transmembrane domains). No mutations were identified in the amino or carboxyl termini nor in the sixth, ninth, and twelfth transmembrane domains. Four mutations were identified in the first, fifth, and eighth transmembrane domains. Three mutations were found in the fourth and two mutations found in the third, ninth, and tenth, and only one mutation was found in the second and seventh transmembrane domains.

Discussion

With the cloning of RFC1, the major route of folate and antifolate transport into mammalian cells, it has become possible to understand the structural properties of RFC1 that determine function and specificity of the carrier and the molecular changes that result in impaired transport and antifolate resistance. In this report, we document the pattern of RFC1 mutations that occurred in MTX transport-defective L1210 murine leukemia cells exposed to chemical mutagens when folic acid was used as the folate source under MTX selective pressure. Under these conditions, mutations that result in impaired MTX transport cannot significantly compromise folate utilization because folic acid is transported primarily by a mechanism distinct from RFC1 (Yang et al., 1983). Using a strategy of single-step selection with continuous exposure to MTX, all resistant cell lines had a transport defect and the degree of resistance to MTX correlated with the magnitude of the decline in transport.

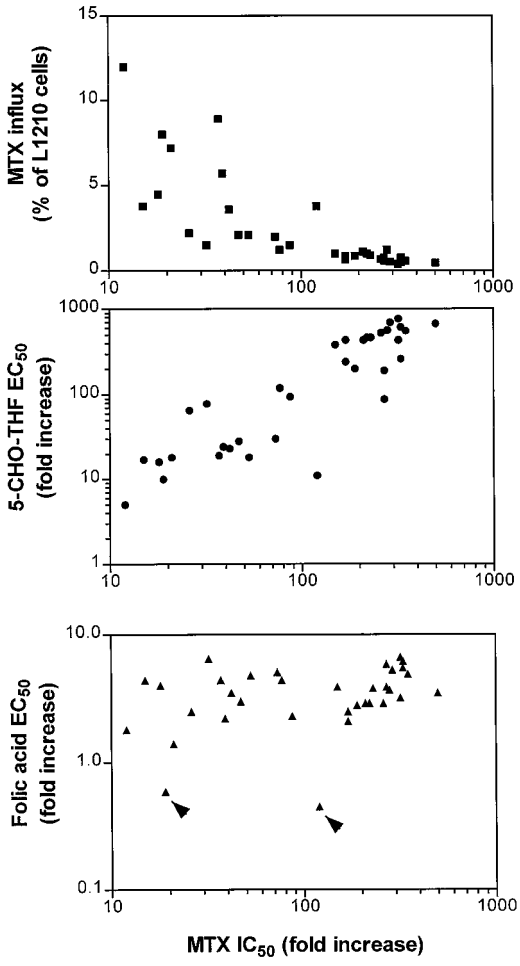

Fig. 3. Relationships among MTX influx (top), 5-CHO-THF growth requirement (middle), and folic acid growth requirement (bottom) as a function of MTX growth inhibition in MTX-transport defective cell lines. MTX growth inhibition, 5-CHO-THF, and folic acid growth requirements are expressed in fold increase relative to L1210 cells and are plotted logarithmically, whereas MTX influx is the percentage of L1210 cells shown in linear scale.

Of the 21 point mutations in the RFC1 open-reading frame identified in this study, all but one were found in or adjacent to transmembrane domains. The one, R253C, predicted to be in the large intracellular loop that separates the six α-helical transmembrane domains on either side, is unlikely to account for inactivation of RFC1. First, it was accompanied by another mutation in the same RFC1 cDNA, which results in a methionine-to-isoleucine substitution at amino acid 120 in the predicted fourth transmembrane domain and probably produces a significant alteration in function. Second, this residue is not conserved; in fact, cysteine is found in this region in the hamster RFC1 at the equivalent position of the murine arginine 253 (Williams et al., 1994).

No mutations were located in the predicted cytoplasmic carboxyl or amino termini. However, deletion of the complete carboxyl terminus resulted in complete loss of function in D2 cells (Table 1). It is unclear whether carrier lacking this region is not functional or is not correctly processed and integrated into the cell membrane. In the case of *chlorella*, the glucose/H^+ symporter, the carboxyl-terminal 27, but not the 42 carboxyl-terminal amino acids, could be deleted without affecting transport activity. Both truncated proteins were made, but the larger deletion might have prevented targeting of the protein to the plasma membrane (Caspari et al., 1994). For the glucose transporter (Glut1), at least, loss of the carboxyl terminus has been suggested to lock the mutated transporter in an inward-facing conformation, with low affinity for exofacial ligands, which results in a large reduction in sugar transport activity (Oka et al., 1990).

The distribution of mutations within RFC1 could not be attributed to the frequency of occurrence of nucleotides coding for these regions and the mutagen *N*-methyl-*N*-nitrosourea employed in these studies. This reagent produced selective changes, either G to A (70%) or C to T (30%), consistent with its reported mutation patterns (Lee et al., 1992). The percentage of G and C in the coding region for the carboxyl terminus, 33 and 28%, respectively, is not different from the 26 and 30%, respectively, found in the entire RFC1 coding region. This is true also for the large loop between the sixth and seventh transmembrane domains, which consists of almost the same number of amino acid residues as the carboxyl terminus based upon the predicted model (30% of G and 30% of C). In the coding region for the predicted eighth transmembrane domain, which harbors the most mutations (four), the content of nucleotides (23% of G and 42% of C) is not favorable for attack by these mutagens. This is consistent with an important role for this transmembrane domain in carrier function.

Many of the amino-acid substitutions detected produce structural changes that might disrupt the tertiary structure of the carrier without representing a specific site critical for

folate binding or translocation across the cell membrane. Both proline and glycine residues play important structural and dynamic roles in transmembrane α helices (Williams and Deber, 1991; Yan and Sun, 1997). Replacement of seven different glycine residues within transmembrane domains each led to inactivation of the carrier. Furthermore, the glycine residues were always replaced with positively charged arginine or negatively charged glutamate or aspartate residues, changes that should have a profound impact on the hydrophobic transmembrane milieu. Serine-to-phenylalanine substitutions were also frequent, which represents a large change in both size and polarity that would result in steric constraints and disruption of hydrogen bonds. Some of these mutations may produce structural changes so profound that the mutated carriers cannot properly fold or insert into the cell membrane, a possibility not excluded in these studies.

Folates and most hydrophilic antifolates carry two negative charges at physiological pH. Translocation of folates by RFC1 presumably involves interactions between positively charged amino acid residues and the negatively charged folates. Hence, it is not surprising that three of the five conserved arginine residues (at positions 131, 155, and 366) within or near the predicted transmembrane domains were mutated. However, function was lost in two cell lines when arginine was replaced at position 131 with the positively charged histidine, which indicates a requirement beyond charge alone. Arginine mutations play an important functional role in other anion transporters. For instance, when each of five arginine residues in the yeast ADP/ATP mitochondrial carrier was replaced by other residues including histidine, ADP/ATP exchange activity was suppressed (Heidkamper et al., 1996). Substitution of arginine 509 and 748 in mouse band 3 protein with noncharged or even positively charged lysine abolished chloride equilibrium exchange (Karbach et al., 1998). A threonine for arginine substitution at amino acid 479 in the Na^+-dependent glutamate transporter was found to eliminate glutamate transport without affecting targeting to, and integration of, the mutant protein to the plasma membrane (Conradt and Stoffel, 1995).

Although small concurrent alterations in dihydrofolate reductase (DHFR) or other enzyme activities cannot be excluded in these mutant cell lines, it is clear from these studies that loss of transport activity is the major factor in the acquired resistance to MTX, based upon the clear relationship between the decrease in influx and the level of resistance. Although the use of mutagens would be expected to produce mutations in DHFR, under the conditions of these experiments, survival was achieved by selection for cell lines with mutations in the carrier that result in markedly impaired transport. Transport loss is attributed to the mutations detected in RFC1, because this is the sole route of MTX transport in wild-type L1210 cells. Further studies that encompass transfection of mutated carrier RFC1 cDNAs into transport-deficient cells are required to define unambiguously the basis for the loss of transport activity (i.e., alter-

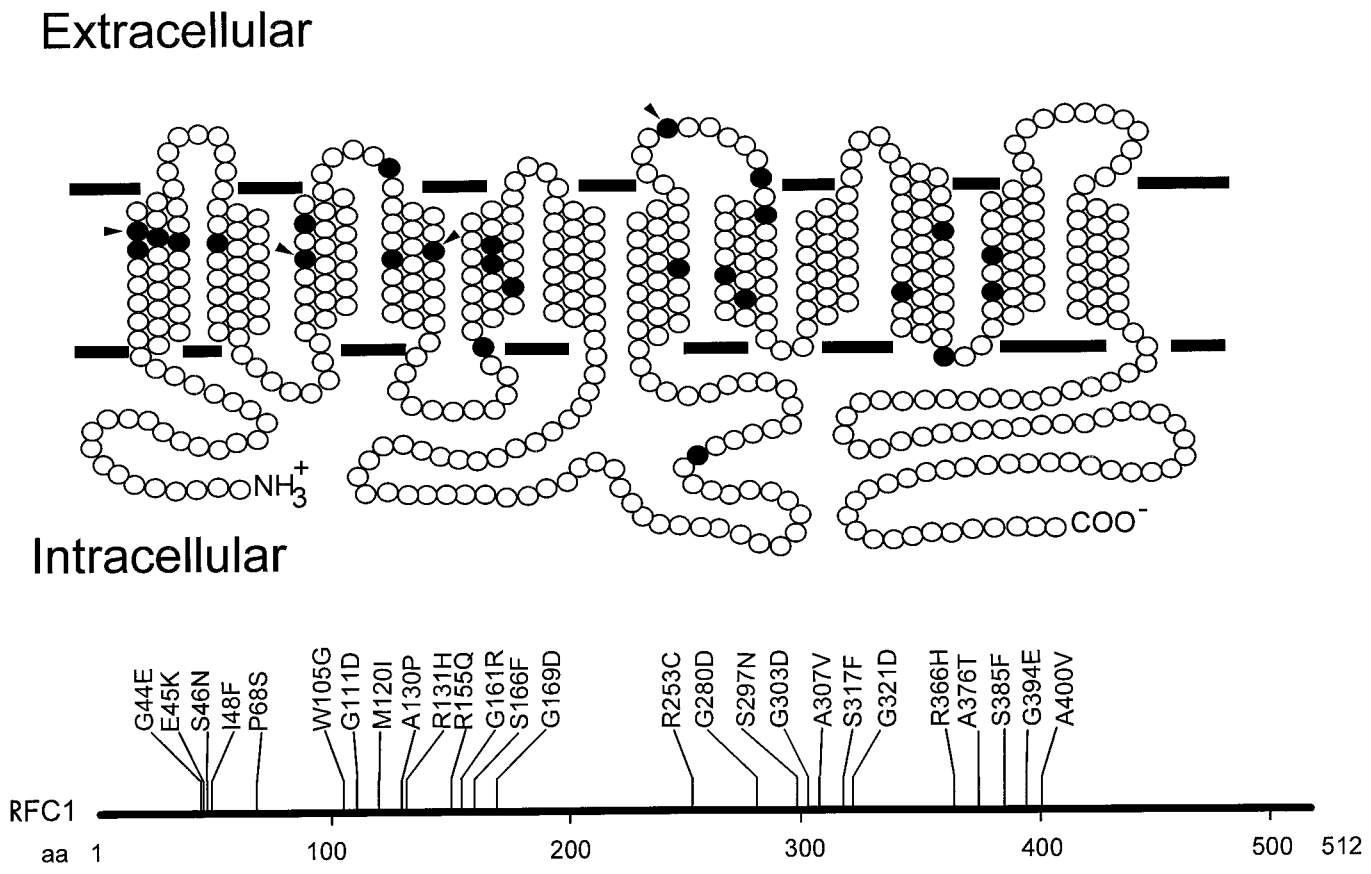

Fig. 4. Distribution of point mutations in the predicted secondary structure of murine RFC1 within the plasma membrane. The predicted topology was based upon the hydropathy analysis of RFC1 (Dixon et al., 1994). ●, positions of all mutations found in murine RFC1; arrows, those mutations previously reported by researchers in this and other laboratories (Brigle et al., 1995; Roy et al., 1998; Tse et al., 1998; Zhao et al., 1998a). The nature and position of the amino acid substitutions are depicted in the linear peptide chain.

ations in binding and/or carrier mobility), as obtained for other mutations (see below). For instance, researchers in this laboratory have replaced charged residues in the putative transmembrane domains of RFC1 with leucine by site-directed mutagenesis; remarkably, substitutions of arginine residues 131, 155, and 366 all resulted in loss of carrier activity (I.G.S., R.Z., S. Babani and I.D.G., unpublished observations).

The detection of multiple mutations in several transmembrane domains suggests that these regions play an important role in the maintenance of carrier integrity and interaction with folate substrates. There were four mutations clustered in the first transmembrane domain. Among them, E45K and S46N have been characterized in detail by researchers in this laboratory (Zhao et al., 1998a,b). E45K increased RFC1 affinity for 5-CHO-THF, along with a 7-fold increase in affinity for folic acid with a comparable decrease in affinity for MTX. This mutation also adds a univalent anion requirement for carrier function (Zhao et al., 1998b). S46N did not alter binding of folates but produced a highly selective change in the translocation rate of carrier loaded with folate substrate such that the mobility of the MTX-carrier complex was one eighth and one seventh that of the 5-CH_3-THF- and 5-CHO-THF-carrier complexes, respectively (Zhao et al., 1998a). A mutation at amino acid 48, I48F, produced a marked and selective increase in carrier affinity for folic acid with little change in transport of MTX (Tse et al., 1998). Four point mutations were identified in the eighth predicted helix, but an analysis of folate growth requirements did not indicate transport specificity, which suggests that this region is broadly critical to carrier function. Interestingly, another mutation, S297N, located in the adjacent external loop, produced a 3-fold increase in MTX influx without a change in V_{max} (Roy et al., 1998).

Identification of mutations selected under in vitro conditions not only provides information on carrier structure-function relationships but may also help to further elucidate the molecular basis for transport-related drug resistance in clinical samples. Impaired transport has been associated with normal RFC1 mRNA levels, so that functional changes caused by mutations in human RFC1 are likely but have not as yet been reported (Gorlick et al., 1997; Zhang et al., 1998). Identification of the E45K mutation in the human leukemia cell line CEM/MTX in vitro suggests that the pattern of mutations in human RFC1 may be similar to that in murine RFC1 (Jansen et al., 1998). Finally, this panel of cell lines with defined mutations can offer a very valuable resource for screening cross-resistance patterns with new generation antifolates to further assess the impact of these mutations on RFC1 substrate specificity and function.

References

Brigle KE, Spinella MJ, Sierra EE and Goldman ID (1995) Characterization of a mutation in the reduced folate carrier in a transport defective L1210 murine leukemia cell line. *J Biol Chem* **270:**22974–22979.

Caspari T, Stadler R, Sauer N and Tanner W (1994) Structure/function relationship of the Chlorella glucose/H+ symporter. *J Biol Chem* **269:**3498–3502.

Conradt M and Stoffel W (1995) Functional analysis of the high affinity, Na(+)-dependent glutamate transporter GLAST-1 by site-directed mutagenesis. *J Biol Chem* **270:**25207–25212.

Cowan KH and Jolivet J (1984) A methotrexate-resistant human breast cancer cell line with multiple defects, including diminished formation of methotrexate polyglutamates. *J Biol Chem* **259:**10793–10800.

Dixon KH, Lanpher BC, Chiu J, Kelley K and Cowan KH (1994) A novel cDNA restores reduced folate carrier activity and methotrexate sensitivity to transport deficient cells. *J Biol Chem* **269:**17–20.

Goldman ID (1971) The characteristics of the membrane transport of amethopterin and the naturally occurring folates. *Ann NY Acad Sci* **186:**400–422.

Gorlick R, Goker E, Trippett T, Steinherz P, Elisseyeff Y, Mazumdar M, Flintoff WF and Bertino JR (1997) Defective transport is a common mechanism of acquired methotrexate resistance in acute lymphocytic leukemia and is associated with decreased reduced folate carrier expression. *Blood* **89:**1013–1018.

Heidkamper D, Muller V, Nelson DR and Klingenberg M (1996) Probing the role of positive residues in the ADP/ATP carrier from yeast. The effect of six arginine mutations on transport and the four ATP versus ADP exchange modes. *Biochemistry* **35:**16144–16152.

Henderson GB and Zevely EM (1983) Structural requirements for anion substrates of the methotrexate transport system of L1210 cells. *Arch Biochem Biophys* **221:** 438–446.

Jackman AL and Calvert AH (1995) Folate-based thymidylate synthase inhibitors as anticancer drugs. *Ann Oncol* **6:**871–881.

Jansen G, Mauritz R, Drori S, Sprecher H, Kathmann I, Bunni M, Priest DG, Noordhuis P, Schornagel JH, Pinedo HM, Peters GJ and Assaraf YG (1998) A structurally altered human reduced folate carrier with increased folic acid transport mediates a novel mechanism of antifolate resistance. *J Biol Chem* **273:**30189–30198.

Karbach D, Staub M, Wood PG and Passow H (1998) Effect of site-directed mutagenesis of the arginine residues 509 and 748 on mouse band 3 protein-mediated anion transport. *Biochim Biophys Acta* **1371:**114–122.

Lee GS, Blonsky KS, Van On DL, Savage EA, Morgan AR and von Borstel RC (1992) Base alterations in yeast induced by alkylating agents with differing Swain-Scott substrate constants. *J Mol Biol* **223:**617–626.

Matherly LH, Taub JW, Ravindranath Y, Proefke SA, Wong SC, Gimotty P, Buck S, Wright JE and Rosowsky A (1995) Elevated dihydrofolate reductase and impaired methotrexate transport as elements in methotrexate resistance in childhood acute lymphoblastic leukemia. *Blood* **85:**500–509.

Mendelsohn LG, Shih C, Schultz RM and Worzalla JF (1996) Biochemistry and pharmacology of glycinamide ribonucleotide formyltransferase inhibitors: LY309887 and lometrexol. *Invest New Drugs* **14:**287–294.

Moscow JA, Gong MK, He R, Sgagias MK, Dixon KH, Anzick SL, Meltzer PS and Cowan KH (1995) Isolation of a gene encoding a human reduced folate carrier (*RFC1*) and analysis of its expression in transport-deficient, methotrexate-resistant human breast cancer cells. *Cancer Res* **55:**3790–3794.

Oka Y, Asano T, Shibasaki Y, Lin JL, Tsukuda K, Katagiri H, Akanuma Y and Takaku F (1990) C-terminal truncated glucose transporter is locked into an inward-facing form without transport activity. *Nature (Lond)* **345:**550–553.

Pao SS, Paulsen IT and Saier MH, Jr (1998) Major facilitator superfamily. *Microbiol Mol Biol Rev* **62:**1–34.

Prasad PD, Ramamoorthy S, Leibach FH and Ganapathy V (1995) Molecular cloning of the human placental folate transporter. *Biochem Biophys Res Commun* **206:** 681–687.

Pui CH and Evans WE (1998) Acute lymphoblastic leukemia. *N Engl J Med* **339:** 605–615.

Rosowsky A, Lazarus H, Yuan GC, Beltz WR, Mangini L, Abelson HT, Modest EJ and Frei E 3d (1980) Effects of methotrexate esters and other lipophilic antifolates on methotrexate-resistant human leukemic lymphoblasts. *Biochem Pharmacol* **29:**648–652.

Roy K, Tolner B, Chiao JH and Sirotnak FM (1998) A single amino acid difference within the folate transporter encoded by the murine RFC-1 gene selectively alters its interaction with folate analogues. Implications for intrinsic antifolate resistance and directional orientation of the transporter within the plasma membrane of tumor cells. *J Biol Chem* **273:**2526–2531.

Schuetz JD, Matherly LH, Westin EH and Goldman ID (1988) Evidence for a functional defect in the translocation of the methotrexate transport carrier in a methotrexate-resistant murine L1210 leukemia cell line. *J Biol Chem* **263:**9840–9847.

Shih C, Habeck LL, Mendelsohn LG, Chen VJ and Schultz RM (1998) Multiple folate enzyme inhibition: Mechanism of a novel pyrrolopyrimidine-based antifolate LY231514. *Adv Enzyme Regul* **38:**135–152.

Sirotnak FM, Goutas LJ and Mines LS (1985) Extent of the requirement for folate transport by L1210 cells for growth and leukemogenesis *in vivo*. *Cancer Res* **45:**4732–4734.

Sirotnak FM, Moccio DM, Kelleher LE and Goutas LJ (1981) Relative frequency and kinetic properties of transport-defective phenotypes among methotrexate-resistant L1210 clonal cell lines derived *in vivo*. *Cancer Res* **41:**4447–4452.

Trippett T, Schlemmer S, Elisseyeff Y, Goker E, Wachter M, Steinherz P, Tan C, Berman E, Wright JE, Rosowsky A, Schweitzer B and Bertino JR (1992) Defective transport as a mechanism of acquired resistance to methotrexate in patients with acute lymphocytic leukemia. *Blood* **80:**1158–1162.

Tse A, Brigle K, Taylor SM and Moran RG (1998) Mutations in the reduced folate carrier gene which confer dominant resistance to 5,10-dideazatetrahydrofolate. *J Biol Chem* **273:**25953–25960.

Underhill TM and Flintoff WF (1989) Complementation of a methotrexate uptake defect in Chinese hamster ovary cells by DNA-mediated gene transfer. *Mol Cell Biol* **9:**1754–1758.

Williams FMR and Flintoff WF (1995) Isolation of a human cDNA that complements a mutant hamster cell defective in methotrexate uptake. *J Biol Chem* **270:**2987–2992.

Williams FMR, Murray RC, Underhill TM and Flintoff WF (1994) Isolation of a hamster cDNA clone coding for a function involved in methotrexate uptake. *J Biol Chem* **269:**5810–5816.

Williams KA and Deber CM (1991) Proline residues in transmembrane helices: Structural or dynamic role? *Biochemistry* **30**:8919–8923.

Wong SC, Proefke SA, Bhushan A and Matherly LH (1995) Isolation of human cDNAs that restore methotrexate sensitivity and reduced folate carrier activity in

methotrexate transport-defective Chinese hamster ovary cells. *J Biol Chem* **270:** 17468–17475.
Yan BX and Sun YQ (1997) Glycine residues provide flexibility for enzyme active sites. *J Biol Chem* **272:**3190–3194.
Yang C-H, Dembo M and Sirotnak FM (1983) Relationships between carrier-mediated transport of folate compounds by L1210 leukemia cells: Evidence for multiplicity of entry routes with different kinetic properties expressed in plasma membrane vesicles. *J Membr Biol* **75:**11–20.
Yang C-H, Sirotnak FM and Dembo M (1984) Interaction between anions and the reduced folate/methotrexate transport system in L1210 cell plasma membrane vesicles: Directional symmetry and anion specificity for differential mobility of loaded and unloaded carrier. *J Membr Biol* **79:**285–292.
Zhang L, Taub JW, Williamson M, Wong SC, Hukku B, Pullen J, Ravindranath Y and Matherly LH (1998) Reduced folate carrier gene expression in childhood acute lymphoblastic leukemia: Relationship to immunophenotype and ploidy. *Clin Cancer Res* **4:**2169–2177.
Zhao R, Assaraf YG and Goldman ID (1998a) A reduced carrier mutation produces substrate-dependent alterations in carrier mobility in murine leukemia cells and methotrexate resistance with conservation of growth in 5-formyltetrahydrofolate. *J Biol Chem* **373:**7873–7879.
Zhao R, Assaraf YG and Goldman ID (1998b) A mutated murine reduced folate carrier (RFC1) with increased affinity for folic acid, decreased affinity for methotrexate, and an obligatory anion requirement for transport function. *J Biol Chem* **273:**19065–19071.
Zhao R, Seither R, Brigle KE, Sharina IG, Wang PJ and Goldman ID (1997) Impact of overexpression of the reduced folate carrier (RFC1), an anion exchanger, on concentrative transport in murine L1210 leukemia cells. *J Biol Chem* **272:**21207–21212.

Send reprint requests to: Dr. I. David Goldman, Albert Einstein Comprehensive Cancer Center, Albert Einstein College of Medicine, Chanin Two, 1300 Morris Park Ave., Bronx, NY, 10461. E-mail: igoldman@aecom.yu.edu