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Discrimination among Reduced Folates and Methotrexate as Transport Substrates by a Phenylalanine Substitution for Serine within the Predicted Eighth Transmembrane Domain of the Reduced Folate Carrier

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ABSTRACT. A phenylalanine substitution for serine in the reduced folate carrier at residue 309 (RFC1-S309F) was identified in a methotrexate (MTX)-resistant cell line selected with 5-formyltetrahydrofolate (5-CHO-THF) as the sole folate source. The transport characteristics of the mutated carrier were studied by transfection into the MTX^{r}A line, which lacks endogenous RFC1 function. The level of expression of carrier in the cell lines studied was determined by specific surface binding of 5-methyltetrahydrofolate (5-CH_3-THF). Influx of 5-CH_3-THF and 5-CHO-THF mediated by RFC1-S309F was 20- and 7-fold greater than that of MTX, respectively. Consistent with the influx difference between 5-CHO-THF and MTX, the growth requirement (EC_{50}) for 5-CHO-THF in MTX^{r}A-S309F cells was decreased by a factor of 9, while the MTX IC_{50} was reduced by a factor of only ~2 as compared with the recipient MTX^{r}A cells. The decrease in 5-CH_3-THF influx mediated by the mutated carrier was attributed to a decrease in the mobility of the 5-CH_3-THF–carrier complex, since the influx K_t was essentially unchanged. However, the reduction in 5-CHO-THF and MTX influx was attributed to decreases in both carrier affinity and V_{max}, although the decline in the MTX influx V_{max} appeared to be much greater than for 5-CHO-THF. The inhibitory effect of chloride on 5-CHO-THF influx observed for L1210 cells was eliminated in the MTX^{r}A-S309F line. This study represents another example of a single mutation in RFC1 that markedly impairs MTX influx but partially preserves transport of reduced folates when cells are selected with 5-CHO-THF as the available folate substrate. The data indicate that residues in the predicted eighth transmembrane domain of RFC1 can play an important role in the selectivity of folate binding and the mobility of the carrier–substrate complex. BIOCHEM PHARMACOL **58**;10:1615–1624, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. reduced folate carrier; carrier-mediated transport; Major Facilitator Superfamily; anticancer drug resistance; folates; antifolates

The recently cloned RFC1† [1–6] mediates transport of 5-CH_3-THF into mammalian cells and is therefore a required element in the provision of one-carbon substrates for the biosynthesis of purines, thymidine, and methionine and for the methylation of nucleic acids. This carrier also transports a variety of antifolates that include MTX and new generation agents that act in their polyglutamate forms as direct, potent inhibitors of glycinamide ribonucleotide (GAR) transformylase, thymidylate synthase, or both of these enzymes [7–9]. RFC1 is a member of the Major Facilitator Superfamily of transport carriers predicted to consist of twelve transmembrane domains with the N- and C-termini, and the large loop between the sixth and seventh transmembrane domains, directed to the cytoplasm [1, 10]. RFC1 is an equilibrating carrier that achieves uphill transport into cells through an exchange mechanism with organic anions that are concentrated within the intracellular compartment [11–13]. The impact of this carrier on concentrative transport is opposed by a distinct energy-requiring unidirectional efflux process(es) present in most mammalian cells [14–16].

This laboratory has undertaken studies to identify regions and residues that are important determinants of RFC1 function. One approach has employed chemical mutagenesis under MTX selective pressure, utilizing growth media in which the folate substrate is either folic acid (for which

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† *Abbreviations*: RFC1, reduced folate carrier; MTX, methotrexate; 5-CHO-THF, 5-formyltetrahydrofolate; 5-CH_3-THF, 5-methyltetrahydrofolate; TMQ, trimetrexate; IC_{50}, concentration required to achieve 50% inhibition of cell growth; EC_{50}, folate concentration required to achieve 50% of the maximum cell growth rate; RT–PCR, reverse transcription–polymerase chain reaction; HBS buffer, 20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM glucose, pH 7.4; HEPES–sucrose–MgO buffer, 20 mM HEPES, 235 mM sucrose, pH 7.4, with MgO; and DHFR, dihydrofolate reductase.

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transport is largely independent of RFC1) or the reduced folate, 5-CHO-THF, which has transport characteristics similar to those of 5-CH_3-THF [17, 18]. With the latter substrate, survival of tumor cells requires sufficient residual transport capacity for reduced folates to permit their growth and replication.

We have reported on MTX-resistant cell lines selected under these conditions with mutations that produce marked changes in the spectrum of affinities for oxidized and reduced folates [17, 18]. One cell line, selected with 5-CHO-THF as the sole folate source, with an asparagine for serine substitution at amino acid 46, manifested highly selective changes in maximum carrier translocation rates in which the mobility of the MTX–carrier complex was reduced markedly in comparison to the decline in mobility of the carrier complexed to reduced folates [17]. We now report on another MTX-resistant cell line, isolated by chemical mutagenesis in the presence of 5-CHO-THF, with a single point mutation in RFC1 resulting in a phenylalanine for serine substitution at amino acid 309 within the eighth transmembrane domain. This mutation resulted in marked, but selective, loss in carrier mobility along with marked changes in the relative affinities of carrier for 5-CHO-THF and 5-CH_3-THF, suggesting that the 309 residue may be in the region of the binding pocket that accommodates the one-carbon moiety at the N5 position of the pteridine ring. In addition, there was the loss of chloride inhibition of transport mediated by the mutated carrier that is observed with wild-type RFC1.

MATERIALS AND METHODS

Chemicals

[3′, 5′, 7-^{3}H]-(6*S*)-5-CHO-THF and [3′, 5′, 7-^{3}H]-(6*S*)-5-CH_3-THF were obtained from Moravek Biochemicals, and [3′, 5′, 7-^{3}H]MTX was obtained from the Amersham Corp. These agents, as well as unlabeled MTX and 5-CHO-THF (Lederle) and folic acid and 5-CH_3-THF (Sigma), were purified by high performance liquid chromatography. All other reagents were of the highest purity available from various commercial sources.

Cell Culture Conditions and Growth Studies

L1210 murine leukemia cells and the MTX^{r}A line, which lacks endogenous RFC1 function due to a proline for alanine substitution at amino acid 130 [19, 20], 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 U/mL), and streptomycin (100 μg/mL) at 37° in a humidified atmosphere of 5% CO_2. Prior to assessment of folate growth requirement, cells were grown for 1–2 weeks in folate-free RPMI-1640 medium supplemented with 5% dialyzed bovine calf serum, 200 μM glycine, 100 μM adenosine, and 10 μM thymidine (GAT) to deplete endogenous folates. For analyses of growth requirement or MTX inhibition, cells were grown in 96-well plates (1×10^5 cells/mL), exposed continuously to appropriate concentrations of MTX, or to 5-CHO-THF for 72 hr, following which cell numbers were determined by hemocytometer count and viability was assessed by trypan blue exclusion.

Isolation of the MTX-Transport Deficient L1210-G5 Cell Line

L1210 cells were maintained in folate-free, GAT-containing medium for 1 week to deplete endogenous folate pools prior to treatment with 1.6 mM ethylmethanesulfonate for 12 hr, a concentration that produced 10% cell survival. After cells were washed to remove the mutagen, they were grown in GAT medium for an additional 2 days, transferred to folate-free medium supplemented with dialyzed calf serum, 25 nM 5-CHO-THF and 200 nM MTX, and plated on 24-well clusters. This concentration of MTX is 20 times greater than the IC_{50} under these conditions and suppresses growth of parental L1210 cells completely. The surviving cells were maintained in this selection medium for another 3 weeks, after which they were plated in complete 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 DHFR level were evaluated. One clone, L1210-G5, exhibiting markedly impaired MTX influx, is the subject of this study. The L1210-G5 cell line has been maintained in drug-free complete RPMI-1640 medium, and has displayed a stable level of MTX resistance, for more than 1 year.

Cloning of the Mutated Reduced Folate Carrier

Poly(A)$^+$ mRNA was purified from L1210-G5 and L1210 cells, using a Dynabeads mRNA DIRECT kit (Dynal). 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) by utilizing oligonucleotide primers that flank the coding region of RFC1 (upstream primer: at nt −46 from the translation start codon 5′-GCGGATCCTGGAGTGTCATCTTGG-3′ and downstream primer at nt +82 from translation stop codon 5′-GCCTCGAGCTGGTTCAGGTGGAGT-3′). Two 8-bp linkers were introduced into the primers so that both *Bam*HI and *Xho*I restriction sites were created in the PCR products to facilitate directional cloning. The PCR amplifications were performed for 35 cycles of 45 sec at 95°, 45 sec at 60°, and 3 min at 72°. The 1682-bp-long predicted PCR product was purified on an agarose gel (Qiagen), cloned into a pCR-Blunt vector (Invitrogen), and the sequence determined on automated sequencer models ABI 373A and ABI 373 from the Perkin Elmer Corp. in the DNA Sequencing Shared Resource of the Albert Einstein College of Medicine Comprehensive Cancer Center.

Transfections

RFC1-S309F cDNA was excised from the pCR-blunt vector (see above) by a double restriction with *Bam*HI and *Xho*I and recloned into pCDNA3.1 (+) (Invitrogen) with the same restriction sites. MTX^{r}A cells (1×10^7) were electroporated (250 V, 330 microfarads) with 50 μg of nonlinearized pcDNA3.1 (+) harboring the mutated RFC1 cDNA in a final volume of 800 μL of serum-free RPMI-1640 medium. Then cells were diluted in 20 mL of complete RPMI-1640 medium, allowed to recover for 48 hr, adjusted to 2×10^5 cells/mL in medium containing G418 (750 μg/mL of active drug), and distributed into 96-well plates at approximately 4×10^4 cells/well. About 20 surviving clones were picked for each transfection and expanded in the presence of G418. After initial screening for MTX growth inhibition, one clone with the lowest MTX IC_{50}, MTX^{r}A-S309F, was chosen for further study. This cell line was maintained in RPMI-1640 medium containing 750 μg/mL of G418.

Northern Analyses

Total RNA was isolated from L1210-G5, MTX^{r}A-S309F, MTX^{r}A, and L1210 cells using TRIzol reagent (Life Technologies). RNA (20 μg) was resolved by electrophoresis on 1% agarose gels containing formaldehyde. Transfers and hybridizations were performed as described previously [21]. Transcripts were quantitated by PhosphorImager analysis of the hybridization signals and normalized to β-actin.

Quantitation of Specific 5-CH$_3$-THF Binding to RFC1 at the Cell Surface

Cells were harvested, washed twice with, and resuspended in, HBS buffer to a density of 5×10^7 cells/mL. Portions of 200 μL were incubated with 2 μM [^{3}H]-5-CH$_3$-THF (specific activity of 800 dpm/pmol) at 0° for 10 min in the presence or absence of a 10-fold excess of unlabeled 5-CH$_3$-THF. Then bound and free ligands were separated by centrifugation of an 180-μL portion through 150 μL of a mixture of silicon and mineral oil (8:2), in a 5.8×47.5 mm plastic (0.4 mL) microfuge tube, at 14,000 rpm for 15 sec. The tip of the tube, which contained the cell pellet, was cut off and dropped into a glass scintillation vial; the contents were digested with 0.5 mL of 1 N KOH, following which scintillation fluid was added and radioactivity was determined. Other tared tubes, containing pellets with an equal number of cells, were weighed after drying to determine dry weight so that surface binding could be determined as nanomoles per gram dry weight of cells.

Transport Studies

Influx measurements were performed by methods described previously [21] with minor modifications, in HBS buffer, HEPES–sucrose–MgO buffer, or mixtures of both. Briefly, cells were harvested, and washed twice with, and resuspended in, the appropriate buffer to a density of 1.5×10^7 cells/mL. Cell suspensions were incubated at 37° for 25 min following which uptake was initiated by the addition of radiolabeled folate and samples were taken at the indicated times. Uptake was terminated by the injection of 1 mL of the cell suspension into 10 mL of ice-cold HBS. Cells were collected by centrifugation, washed twice with ice-cold HBS, and processed for measurement of intracellular radioactivity [21]. For influx determinations, uptake intervals were adjusted so that cell MTX did not exceed the DHFR binding capacity assuring that unidirectional uptake conditions were sustained. For other folates studied, initial rates were established over an interval in which cell folate uptake was linear as a function of time with an extrapolated ordinate intercept at time zero near the point of origin. Prior to transport studies with the transfectant, MTX^{r}A-S309 cells were expanded for no longer than 5 days without G418 to ensure that the expression of the mutated RFC1 was maintained.

Occurrence of the C→T Mutation in cDNA and Genomic DNA Populations Isolated from L1210-G5 Cells

The cDNA was prepared as described above, and genomic DNA was isolated with the Eazy-DNA kit (Invitrogen). Both cDNA and genomic DNA were amplified by *Pfu* Turbo (Stratagene) with primers 5′-^{562}CTGGGCTTCATCCTCTTCAGCC^{583}-3′ and 5′-^{988}ACAGAGTCCAGCGGATGCTCAG^{967}-3′ for 35 cycles each of 45 sec at 95°, 45 sec at 60°, and 2 min at 72°. The DNA fragments were purified by agrose gel extraction (Qiagen) and sequenced with the antisense primer used for the PCR.

RESULTS

Folate Influx, MTX Growth Inhibition, and 5-CHO-THF Growth Requirement in the L1210-G5 MTX-Resistant Cell Line

A clonal MTX-resistant L1210 leukemia cell line (L1210-G5) was isolated in the presence of 200 nM MTX with 25 nM 5-CHO-THF as the sole folate source after cells were treated with ethylmethanesulfonate. This cell line displayed a 44-fold increase in MTX IC_{50}, but a lesser, 15-fold, increase in 5-CHO-THF EC_{50} as compared with L1210 cells (Table 1). The IC_{50} for TMQ growth inhibition for this cell line was decreased slightly as compared with that of L1210 cells, excluding the possibility that DHFR was over-expressed or altered. Influx of MTX, 5-CHO-THF, and 5-CH$_3$-THF in L1210-G5 cells was reduced markedly as compared with the parental cells: the residual influx activity for 5-CH$_3$-THF (6%) was only slightly higher than that for 5-CHO-THF (3.5%) and for MTX (2.6%). The growth rate of the mutant cells in RPMI-1640 medium or folate-free medium supplemented with 25 nM 5-CHO-THF was similar to that of L1210 cells.

TABLE 1. Comparison of folate influx, 5-CHO-THF growth requirement, and MTX growth inhibition in L1210 and MTX-resistant L1210-G5 cells

FOLATE	L1210	L1210-G5	L1210-G5/L1210
MTX growth inhibition IC_{50} (nM)	10.3 ± 1.7	457 ± 30	44
TMQ growth inhibition IC_{50} (nM)	3.8 ± 0.3	2.3 ± 0.4	0.6
5-CHO-THF growth requirement EC_{50} (nM)	1.35 ± 0.31	19.7 ± 3.7	15
Initial uptake rate (nmol/g dry wt/min)			
MTX	1.32 ± 0.06	0.034 ± 0.006	0.026
5-CHO-THF	2.26 ± 0.02	0.078 ± 0.004	0.035
5-CH_3-THF	2.50 ± 0.02	0.149 ± 0.011	0.060

Results are the means ± SEM of at least three experiments.

Identification of a Mutation in RFC1 cDNA and Genomic DNA

The RFC1 mRNA level in the L1210-G5 line was increased by 50% as compared with that of L1210 cells, as shown in Fig. 1A. Thus, a mutation in RFC1 was considered likely to be the basis for the low folate influx observed with this cell line. To assess this possibility, cDNA encompassing the entire RFC1 coding region was isolated from L1210-G5 cells by RT–PCR and then cloned and sequenced. A single mutation, C→T, at position 926 (counting from the initiation codon) was identified in the entire coding region of one cDNA clone, resulting in the substitution of phenylalanine for serine at amino acid residue 309. This single base change was verified by sequencing two additional randomly picked cDNA clones.

To assess the homogeneity of this mutation in the cDNA population and to verify it further at the genomic level, PCR fragments were generated from both cDNA and genomic DNA isolated from this cell line, as shown in Fig. 1B. Because the mutation is located close to the 3′ end of exon 4, the intron between exon 4 and 5 is also amplified in genomic PCR products [22]. As a result, the genomic

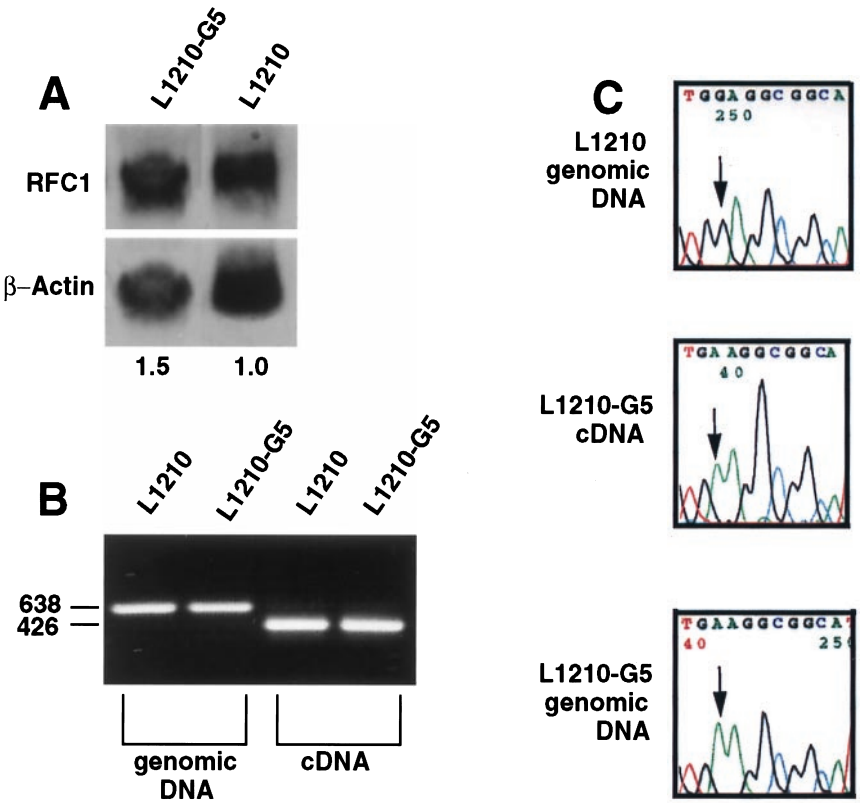

FIG. 1. (A) Representative northern blot of total RNA from L1210 and L1210-G5 cells. Total RNA was probed successively first with the full-length murine RFC1 and then with β-actin cDNA. The numbers below the lanes, averaged from two separate experiments, are RFC1 mRNA levels relative to that of L1210 cells. (B) Amplification of cDNA and genomic fragments harboring the mutation. The PCR reaction was conducted under the same conditions with the same primers as indicated in Materials and Methods. The genomic fragments (638 bp) were larger than those of the cDNA (426 bp) due to co-amplification of intron 4 [22]. (C) Sequencing chromatograms of PCR fragments from L1210 and L1210-G5 cells. The fragments were sequenced with the antisense primers used for PCR; the arrows indicate the position of the base change.

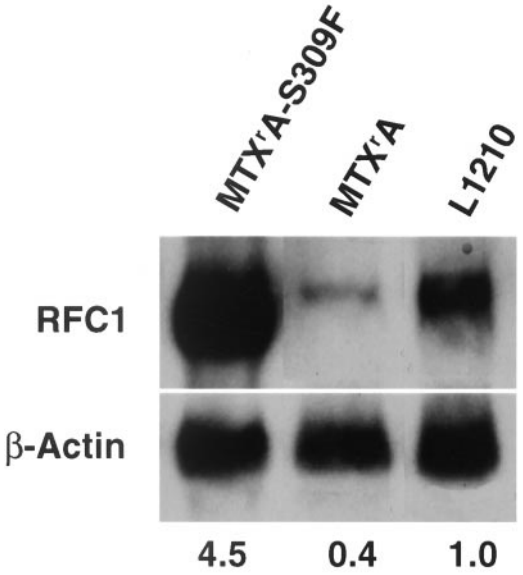

FIG. 2. Northern blot of total RNA from MTX^{r}A-S309, MTX^{r}A, and L1210 cells. Total RNA was probed successively first with the full-length murine RFC1 and then with β-actin cDNAs. The molecular weight of the endogenous RFC1 transcript in L1210 and MTX^{r}A cells (2.3 kb) was slightly greater than that of the transfectants (1.9 kb), which was derived from the expression vector. The numbers below the lanes are RFC1 mRNA levels, determined by PhosphorImager analysis, relative to that of L1210 cells. Data are the averages of two separate experiments.

PCR fragment was about 200 bp larger than that of the cDNA RT–PCR product. Because sequencing with a sense primer yielded a weak C signal in the wild-type RFC1, whole fragment populations were sequenced with an antisense primer, and the results are shown in Fig. 1C. As indicated in the chromatograms, the only change noted in the genomic DNA was the complete G →A substitution detected in the cDNA population isolated from L1210-G5. Thus, this mutation is homogeneous at both the cDNA and genomic levels; the wild-type RFC1 allele is not present in the genome.

Transfection of RFC1-S309F cDNAs into MTX^{r}A Cells and Assessment of the Transport Phenotype

To assess the function of the mutated carrier, RFC1-S309F cDNA was transfected into MTX^{r}A cells, which lack endogenous carrier function [19, 20]. The RFC1 mRNA level in the MTX^{r}A-S309F transfectant was 4 times higher than that of L1210 cells when the level of endogenous RFC1 message present in the recipient MTX^{r}A cells was considered (Fig. 2). Specific 5-CH_3-THF binding to the cell surface was also determined to assess levels of carrier protein expressed. MTX^{r}A-R16 cells were used as a positive control. These cells have been transfected with wild-type RFC1 cDNA, overexpress RFC1 mRNA by a factor of 7, and have a 9-fold increase in MTX influx relative to L1210 cells [21]. As shown in Fig. 3, surface binding to MTX^{r}A-R16 cells was 4.5 times greater than binding to L1210 cells,

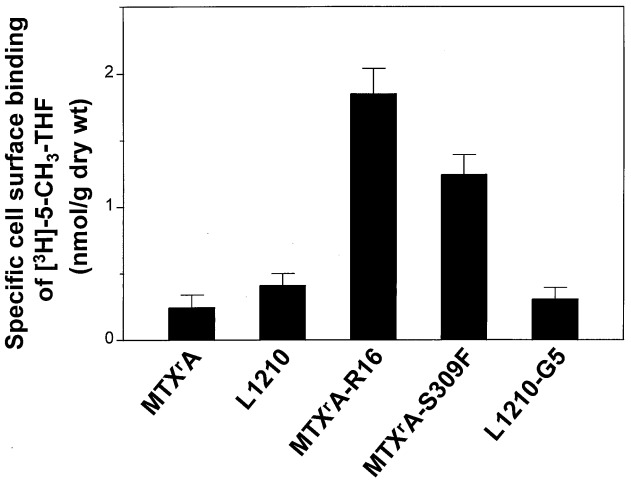

FIG. 3. Specific 5-CH_3-THF surface binding in MTX^{r}A, L1210, MTX^{r}A-R16, MTX^{r}A-S309F, and L1210-G5 cells. The binding experiments were performed in HBS with 2 μM [^{3}H]-5-CH_3-THF as the radioactive ligand. The difference between radioactivity bound to cell surface in the absence and presence of a 20 μM concentration of unlabeled 5-CH_3-THF was normalized to the dry weight of cell pellets. Data are the means ± SEM of five separate experiments.

and binding to MTX^{r}A cells was about half that of L1210 cells, consistent with results reported previously [20]. Surface binding in L1210-G5 cells was slightly lower than binding to L1210 cells; binding to MTX^{r}A-S309F cells was 5 and 3 times greater than to MTX^{r}A and L1210 cells, respectively. Thus, the RFC1 protein level in the transfectant determined by the binding assay was roughly comparable to the increase in mRNA as determined by PhosphorImager analysis.

As shown in the upper panel of Fig. 4, influx of all folates was enhanced by the mutated carrier as compared with the recipient MTX^{r}A cells (5-CH_3-THF > 5-CHO-THF > MTX). Influx of folates in MTX^{r}A-S309F cells, less the residual contribution by MTX^{r}A, is analyzed in the left lower panel of Fig. 4; this represents the specific flux contributed by the mutated carrier alone. It can be seen that the mutated carrier has virtually no transport activity for MTX. Influx of 5-CH_3-THF and 5-CHO-THF mediated solely by RFC1-S309F exceeded that of MTX by factors of 20 and 7, respectively. Influx of these folates in L1210 cells is indicated in the right-hand side of the lower panel. In L1210 cells, the initial uptake rates of 5-CH_3-THF and 5-CHO-THF were comparable and about twice that of MTX. Hence, the absolute rates of transport mediated by RFC1-S309F were quite low as compared with L1210 cells, especially when the high level of protein expression is considered, and the mutated carrier had a marked change in the spectrum of transport activity for the different folates.

MTX Growth Inhibition and 5-CHO-THF Growth Requirement in the RFC1-S309 Transfectant

Another aspect of the impact of RFC1-S309F expression in MTX^{r}A cells was assessed by comparing MTX growth

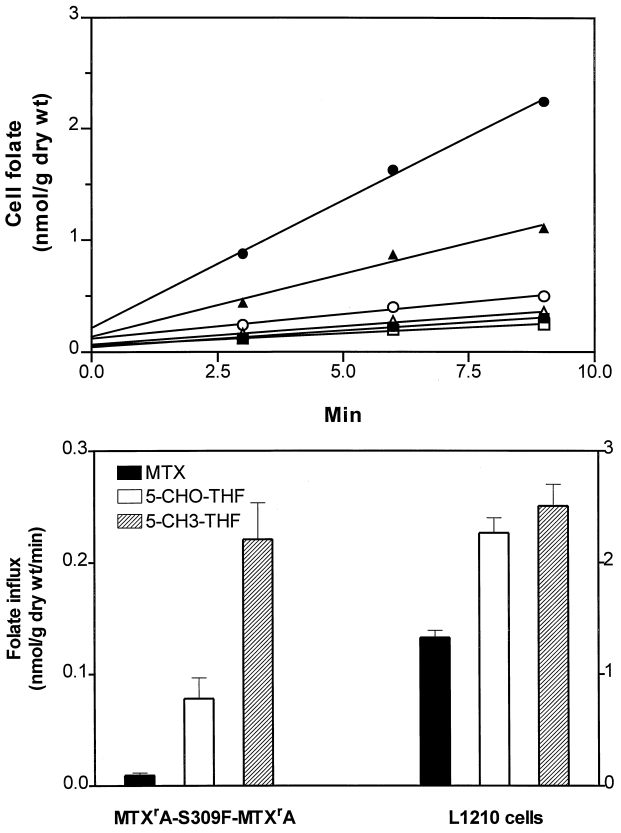

FIG. 4. Influx of MTX, 5-CHO-THF, and 5-CH_3-THF in the MTX^{r}A, MTX^{r}A-S309F, and L1210 cells. Upper panel: Following a 25-min incubation in HBS at 37°, MTX^{r}A (open symbols) and MTX^{r}A-S309F (closed symbols) cells were exposed to a 1 μM concentration of tritiated MTX (squares), 5-CHO-THF (triangles), or 5-CH_3-THF (circles), and the time course of uptake was monitored. Data are representative of three separate experiments. Lower panel: The initial uptake rates in MTX^{r}A-S309F cells, less those in the recipient MTX^{r}A cells, are plotted in the left side of the lower panel and those in L1210 cells are in the right side. Data are the means ± SEM of at least three experiments.

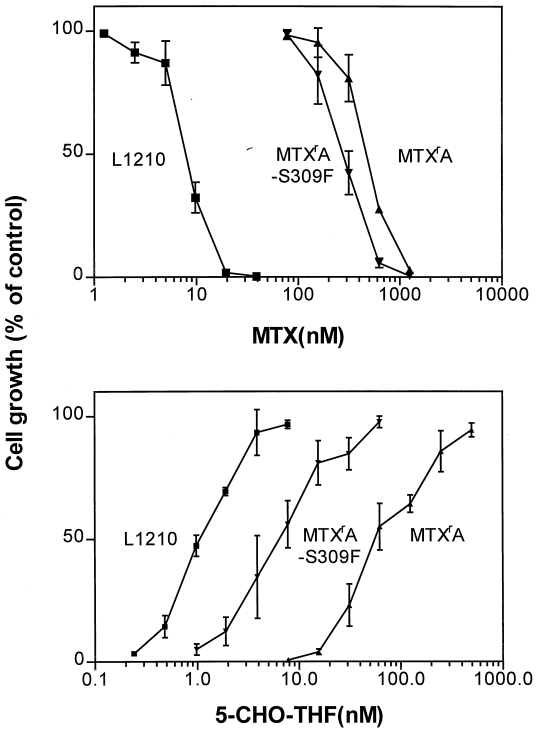

FIG. 5. Growth inhibition by MTX (upper panel) and growth requirement for 5-CHO-THF (lower panel). Cells were transferred from regular RPMI-1640 medium to folate-free RPMI-1640 medium containing GAT and supplemented with 5% dialyzed calf bovine serum and grown for 1–2 weeks to deplete endogenous folate pools prior to the determination of growth requirement. Cells (10^5/mL) were exposed continuously to different concentrations of MTX or 5-CHO-THF in a 96-well plate for 3 days before they were counted. Data are the means ± SEM of three separate experiments.

inhibition and 5-CHO-THF growth requirement between the MTX^{r}A and MTX^{r}A-S309F lines (Fig. 5). The MTX IC_{50} in MTX^{r}A-S309 cells (upper panel) was reduced negligibly (by a factor of 2); however, the 5-CHO-THF EC_{50} (lower panel) was decreased by a factor of nearly 10 as compared with MTX^{r}A cells, consistent with the difference in influx of these folates mediated by RFC1-S309F. Hence, MTX^{r}A-S309F cells were 35-fold resistant to MTX as compared with L1210 cells but needed only 6 times more 5-CHO-THF (to 6 nM) to support their growth.

Folate Influx Kinetic Parameters

The kinetic basis for the difference in influx of MTX, 5-CHO-THF, and 5-CH_3-THF was explored. Figure 6 shows 5-CH_3-THF influx in the S309F transfectant as a function of substrate concentration along with a double-reciprocal analysis of the data in the inset. The kinetic parameters derived from a nonlinear regression to the Michaelis–Menten equation and from the double-reciprocal analysis were essentially the same. As indicated in Table 2, the 5-CH_3-THF influx V_{max} was 7% that of L1210 cells, while the K_t was not decreased significantly. Since expression of mutated RFC1, as assessed by specific 5-CH_3-THF binding to the cell surface, in the transfectant was 2.5-fold

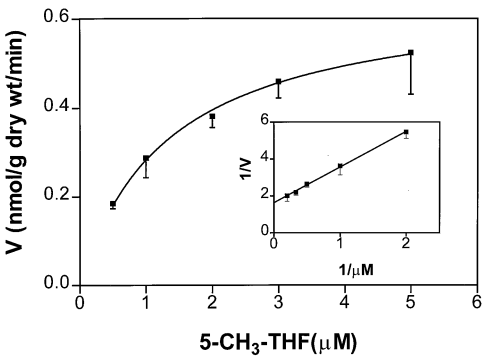

FIG. 6. Determination of 5-CH_3-THF influx kinetic parameters. L1210-G5 cells were suspended in HBS buffer and incubated at 37° for 25 min. Then tritiated 5-CH_3-THF was added over the concentration range indicated, and influx was measured. Results are the means ± SEM of three experiments. Inset: Lineweaver–Burk representation of the data.

TABLE 2. folate influx kinetics in L1210 cells and the MTX^r A-S309F transfectant

	L1210	MTX^r AS309F	MTX^r A-S309F/L1210
5-CH_3-THF			
K_t (μM)	1.85 ± 0.13	1.30 ± 0.25	0.70*
V_{max} (nmol/g dry wt/min)	8.9 ± 1.3	0.66 ± 0.13	0.074
MTX			
K_i (μM)	4.55 ± 0.35	22.3 ± 2.6	4.9
V_{max} (nmol/g dry wt/min)	11.5 ± 0.7†	0.21‡	0.018
5-CHO-THF			
K_i (μM)	3.15 ± 0.65	13.7 ± 2.0	4.3
V_{max} (nmol/g dry wt/min)	13.5 ± 0.3†	1.1‡	0.08
Folic acid			
K_i (μM)	243 ± 38	367 ± 70	1.5*

5-CH_3-THF influx kinetic parameters were obtained from nonlinear regression to the Michaelis-Menten equation. The K_i values for MTX and 5-CHO-THF were determined from Dixon analyses based upon inhibition of 5-CH_3-THF influx. Data are the means ± SEM of three separate experiments.

*P = ~0.2.

†Reported previously [17].

‡Calculated from the Michaelis-Menten equation: influx V_{max} = v (K_t + S)/S, where v is influx obtained from the data of Fig. 4, K_t is assumed to be equal to K_i, and S is the extracellular folate concentration of 1 μM.

greater than that of L1210 cells, as noted in Fig. 3, the influx V_{max}, normalized to the level of expression, must be much lower.

Influx K_i values for MTX, 5-CHO-THF, and folic acid in MTX^{r}A-S309F and L1210 cells were determined from Dixon analyses based upon inhibition of 5-CH_3-THF influx, the folate substrate with the highest initial rate of transport. The influx K_i values for MTX and 5-CHO-THF mediated by the mutated carrier were both increased by a factor of 4–5 as compared with the influx K_i for wild-type RFC1 (Table 2). Hence, the much lower influx of MTX relative to 5-CHO-THF mediated by RFC1-S309F (Fig. 4) must be due to a greater decrease in influx V_{max} for the antifolate. Indeed, the calculated influx V_{max} for 5-CHO-THF, based upon the computed influx at an extracellular concentration of 1 μM and the measured influx K_i, was 5-fold greater than that calculated for MTX. There was a small (but not statistically significant) increase in influx K_i for folic acid in the transfectant as compared with L1210 cells.

Impact of Chloride on 5-CHO-THF Influx in MTX^{r}A-S309F Cells

Chloride is a competitive inhibitor of the influx of folates mediated by wild-type RFC1 [11, 23]. As indicated in Fig. 7, 5-CHO-THF influx in L1210 cells increased gradually as the chloride concentration in the buffer decreased. The initial uptake rate in HEPES–sucrose–MgO buffer (no Cl^-) was about 3 times greater than that in HBS (140 mM Cl^-). In contrast, 5-CHO-THF influx in MTX^{r}A-S309F cells was essentially unchanged as the chloride concentration was decreased from 140 to 50 mM, and then fell by ~30% as chloride was eliminated from the buffer. Hence, the inhibitory effect of chloride on RFC1-mediated influx was abolished by the S309F substitution.

DISCUSSION

RFC1 mediates transport of reduced folates, in particular 5-CH_3-THF, the major folate cofactor in the blood, as well as antifolates. Of particular interest are the alterations in RFC1 function associated with antifolate resistance, since they provide important insights into structure–function properties of the carrier. To address this question, this laboratory has employed chemical mutagenesis to select MTX-resistant murine leukemia cell lines in the presence of a physiological level of 5-CHO-THF (25 nM) as the sole folate source to mimic as much as possible the *in vivo* conditions under which chemotherapy is administered. Although 5-CH_3-THF is the physiological folate source, it lacks sufficient chemical stability to allow its use *in vitro*. With a strategy of one-step selection and continuous exposure to MTX, cell lines with defects in MTX transport

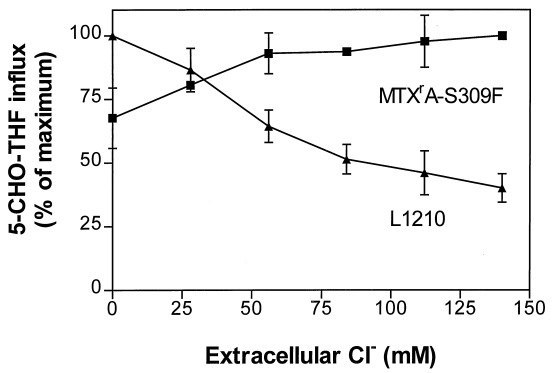

FIG. 7. Impact of extracellular chloride on 5-CHO-THF influx in L1210 and MTX^{r}A-S309F cells. After a 25-min incubation in the appropriate buffer, MTX^{r}A-S309F and L1210 cells were exposed to 1 μM 5-CHO-THF, and influx was measured. The different concentrations of chloride were obtained by mixing HEPES–sucrose–MgO buffer and HBS. The initial uptake rates are expressed as a percentage of the rate in HEPES–sucrose–MgO for L1210 cells and in HBS for MTX^{r}A-S309F cells. Data are the composite of three experiments ± SEM.

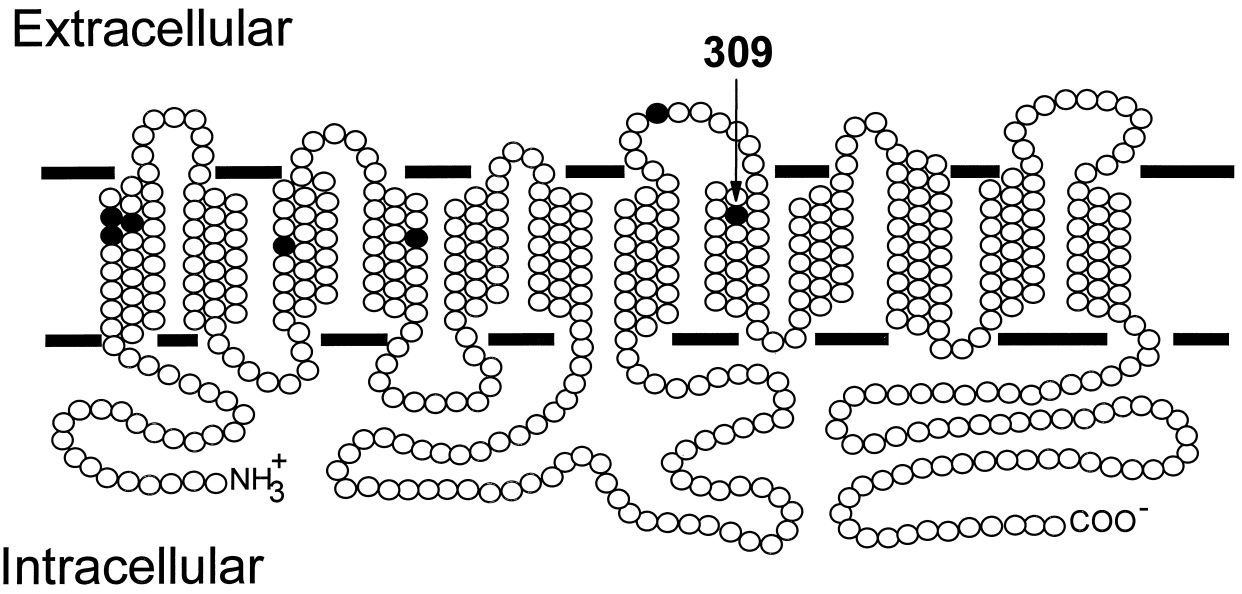

FIG. 8. Location of the S309F mutation in the predicted secondary structure of murine RFC1 within the plasma membrane. The predicted topology was based on a hydropathy analysis of RFC1 [1]. The serine at position 309 is indicated by the arrow. Other mutated residues characterized previously in murine RFC1 are also indicated and include: glutamate 45, serine 46, isoleucine 48, tryptophan 105, and serine 297 [17, 18, 31, 33]. An alanine to proline substitution at amino acid 130 is the RFC1 mutation in the MTX^{r}A line that results in loss of function [20]. This is discussed further in the text.

and the ability to grow in low concentrations of 5-CHO-THF have been selected. The characterization of one such cell line was reported recently [17]. In the present paper, we report the basis for loss of transport function in another cell line, with a mutation at amino acid residue S309 in the eighth transmembrane domain, which is associated with marked changes in the transport of MTX and the reduced folates 5-CH_3-THF and 5-CHO-THF.

A reduction of folate influx can result from a decrease in carrier expression, a decrease in carrier mobility, a decrease in carrier affinity for substrate, or a combination of these changes. Since loss of RFC1 expression is not selective, some conservation of reduced folate transport relative to MTX is likely due to mutations in RFC1 when cells must develop marked antifolate resistance in the presence of 5-CHO-THF. In a previously identified RFC1 mutation, S46N, the MTX influx V_{max} decreased by a factor of 44 as compared with reductions by factors of 8 and 6.6 in this parameter for 5-CHO-THF and 5-CH_3-THF, respectively. There was no change in carrier affinity for either of these folates or MTX [17]. The S309F mutation identified in the current study, however, was associated with a comparable 4- to 5-fold decrease in affinity for both MTX and 5-CHO-THF, but no significant change in affinity for 5-CH_3-THF. The 20-fold difference in influx between 5-CH_3-THF and MTX mediated by RFC1-S309F (Fig. 4) could be accounted for by the differences in carrier affinities and mobilities for these two substrates (Table 2). The observed comparable changes in affinities for 5-CHO-THF and MTX in RFC1-S309F cannot account for the 7-fold difference in initial rates consistent with the marked difference in influx V_{max} (5-fold). Hence, as observed for the S46N carrier, the apparent mobility of the 5-CHO-THF–and 5-CH_3-THF–carrier complex is greater than that of the MTX–carrier complex, in contrast to wild-type RFC1 in which carrier mobility is not substrate-specific [17]. In general, the decrease in mobility of the S309F carrier–folate complex was profound, especially when the high level of specific surface binding of 5-CH_3-THF in the transfectant is considered.

The difference in influx among these folates was much smaller in the resistant clone selected, L1210-G5 (Table 1), than that mediated by MTX^{r}A-S309F (Fig. 4). Since the mutation in L1210-G5 cells is homogeneous, there can be no wild-type RFC1or other mutant carrier present. It is of interest that total cellular folate cofactor accumulation in L1210-G5 cells was decreased to only ~ 25% that of L1210 cells when cells were grown in 25 nM [^{3}H]-5-CHO-THF (unpublished results). This suggests that transport and/or metabolic differences are much less pronounced at physiological folate levels. It is possible that residual transport in L1210-G5 cells is mediated by another process that masks the transport properties of the mutated carrier. There are other transporters that are distinct from RFC1 in murine leukemia L1210 cells that could contribute to this residual activity. One is a pathway with optimal activity at low pH characterized in both MTX-sensitive and MTX-resistant L1210 cells [24, 25]. This system transports 5-CHO-THF, 5-CH_3-THF, and MTX at comparable rates [25] and has been shown to be overexpressed in pyrimethamine-resistant Chinese hamster ovary cells [26]. The other is a low-affinity, high-capacity transport route for folates that has not been well defined [27]. These, and possibly other as yet unidentified, transport routes could play an important role

in drug and folate delivery when carrier-mediated transport is impaired severely.

Associated with the greater transport capacity of RFC1-S309F for 5-CHO-THF than for MTX was the very small decrease in MTX IC_{50} as compared with a nearly 10-fold decrease in the 5-CHO-THF EC_{50} in the MTX^rA-S309F transfectants as compared with MTX^rA cells. Importantly, the 5-CHO-THF EC_{50} in the L1210-G5 line (19 nM) was less than the 5-CHO-THF level in the medium and the 5-CH_3-THF concentrations usually found in the blood of humans and rodents (10–40 nM) [28]. Because 5-CH_3-THF is a much better substrate for RFC1-S309F than 5-CHO-THF (~3-fold), this mutant would survive at an even lower concentration of this folate *in vivo* than what was determined *in vitro* for 5-CHO-THF. Furthermore, the depletion of folate pools achieved by growing cells in folate-free medium prior to the assay of growth requirement probably overestimates the actual folate requirement needed to support cell growth. Hence, 5-CHO-THF influx at only a few percent of that of L1210 cells (3.5% in the case of L1210-G5 cells) is sufficient to support normal cell growth. This has important pharmacological ramifications in that the folate blood level will modulate the extent to which loss of carrier function can be tolerated by malignant cells with any specific RFC1 mutation. The greater the selectivity of the mutation and the higher the blood folate level, the greater the loss of MTX transport function that can be tolerated and the greater the degree of drug resistance achieved. Likewise, the low levels of RFC1 function that are apparently sufficient to preserve viability of tumor cells indicate that a rather substantial loss of carrier protein expression is also possible in resistant cells, consistent with the low levels of RFC1 expression detected in lymphoblasts from patients in relapse with acute lymphoblastic leukemia [29, 30].

Studies from this and other laboratories have identified the first of twelve putative transmembrane domains of RFC1 as an important region for determining the selectivity of carrier function [17, 18, 31]. As indicated in Fig. 8, the first transmembrane domain is a frequent site for RFC1 mutation under antifolate selective pressure. An E45K mutation identified in both murine [18] and human leukemia cells [32] decreased affinity for MTX and increased affinity for folic acid and 5-CHO-THF, whereas an S46N substitution [17], as discussed above, selectively decreased mobility of carrier–MTX complex. A phenylalanine substitution for isoleucine (I48F), however, markedly increases affinity for folic acid without an adverse effect on MTX transport [31]. The identification of an S309F mutation, located in the eighth transmembrane domain, demonstrates the importance of this region as a determinant of binding and translocation (Fig. 8). The role of this region is indicated further by the report of an S297N mutation, located in the external loop between the seventh and eighth transmembrane domain, which resulted in a decrease in affinity for MTX, but no change in affinity for aminopterin, 10dAMT, or edatrexate and no change in influx V_{max} [33]. This laboratory has identified four other mutations in the eighth transmembrane in MTX-resistant L1210 cell lines generated by chemical mutagenesis with folic acid as the folate substrate, the highest frequency of mutation among all transmembrane domains [34].

Chloride is a competitive inhibitor of folate influx; its replacement with sucrose, for example, results in increased affinity of RFC1 for folate substrates without a change in influx V_{max} [11–13, 23]. On the other hand, substitution of lysine for glutamate at position 45 in the first transmembrane domain resulted in the introduction of an obligatory requirement for small inorganic anions, such as chloride, nitrate, and fluoride, for carrier function associated with changes in influx V_{max}, presumably due to their neutralization of the positive charge on the substituted lysine residue [18]. The effect of chloride on 5-CHO-THF influx in MTX^rA-S309F cells was different from its effect on wild-type RFC1 or the E45K mutant. RFC1-S309F appears to have lost affinity for chloride, since alterations in chloride concentration over a 50–140 mM range did not change influx. Whereas at low chloride concentrations influx was decreased, similar to what was observed with the E45K mutant, the magnitude of the change was very small. The mechanism(s) that underlies these changes remains to be determined.

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