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Role of the Amino Acid 45 Residue in Reduced Folate Carrier Function and Ion-Dependent Transport as Characterized by Site-Directed Mutagenesis

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ABSTRACT

In previous reports, an E45K mutation in reduced folate carrier (RFC1) resulted in marked substrate-specific changes in folate binding and the induction of an obligatory inorganic anion requirement for carrier function. In this study, site-directed mutagenesis was employed to further characterize the role of glutamate-45 in carrier function by replacement with glutamine, arginine, aspartate, leucine, or tryptophan followed by tranfection of the mutated cDNAs into the MTX^{r}A line, which lacks a functional endogenous carrier. Alterations in transport function with amino acid substitutions at this residue were not charge related. Hence, E45Q, E45R, and E45K all 1) increased carrier affinity for 5-formyltetrahydrofolate ~4-fold, 2) increased affinity for folic acid ~6- to 10-fold, 3) did not change affinity for 5-methyltetrahydrofolate, and 4) except for E45R decreased affinity for methotrexate (2- to 3-fold). In contrast, mutations E45D, E45L, and E45W generally reduced affinity for all these folates except for folic acid. Finally, chloride-dependent influx was only noted in the E45R mutant. These data further substantiate the important role that glutamate-45 plays in the selectivity of binding of folates to RFC1 and establish that it is the addition of a positive charge at this site and not the loss of a negative charge that results in the induced anion dependence. These and other studies indicate that mutations in the first transmembrane domain can have a markedly selective impact on the affinity of RFC1 for folate compounds and in particularly a highly salutary effect on binding of the oxidized folate, folic acid.

The reduced folate carrier (RFC1), a member of the major facilitator superfamily of transport carriers, delivers folates into cells that are essential for one-carbon-requiring biosynthetic reactions (Pao et al., 1998). The RFC1 gene from various species was recently cloned (Dixon et al., 1994; Williams et al., 1994; Moscow et al., 1995; Prasad et al., 1995; Williams and Flintoff, 1995; Wong et al., 1995) and its murine and human genomic organizations characterized (Brigle et al., 1997; Tolner et al., 1997, 1998). Information is now evolving on RFC1 domains, in general, and specific amino acids, in particular, that plays key roles in function and substrate specificity. This has been based largely, to this point, on the characterization of mutant carriers that have been identified under antifolate selective pressure. The first transmembrane domain has been identified as one important site for mutation under these conditions (Jansen et al., 1998; Tse et al., 1998; Zhao et al., 1998a, 1998b).

In an initial report from this laboratory, the substitution of lysine for glutamate at amino acid 45 in murine RFC1 resulted in a transport phenotype that displayed several interesting features (Zhao et al., 1998b). The influx K_i for methotrexate (MTX) was increased and the K_i for folic acid and 5-formyltetrahydrofolate (5-CHO-THF) was decreased (Zhao et al., 1998b). Wild-type RFC1 in L1210 cells is competitively inhibited by a variety of inorganic and organic anions (Goldman, 1971; Henderson and Zevely, 1983). However, the E45K mutation resulted in the induction of an obligatory requirement for small inorganic anions for carrier function as indicated by an anion-dependent augmentation of influx V_{max}. Recently, this same mutation in RFC1 was also identified in a MTX-resistant human leukemia cell line (CCRF-CEM/MTX) in which there was a similar change in the spectrum of affinities for different folate substrates as well as the induction of chloride-dependent MTX influx (Jansen et al., 1998).

In this paper, we describe the application of site-directed mutagenesis to further characterize the role that the glutamate-45 residue plays in the selectivity of binding of folate compounds and establish that the anion requirement observed for function of the lysine-substituted mutated carrier

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ABBREVIATIONS: RFC1, reduced folate carrier; MTX, methotrexate; 5-CHO-THF, 5-formyltetrahydrofolate; 5-CH_3-THF, 5-methyltetrahydrofolate; HBS, HEPES-buffered saline.

is due to the addition of the positive charge at this site rather than the loss of the negatively charged glutamate residue.

Materials and Methods

Chemicals. [3′,5′,7-^{3}H](6*S*)-5-CHO-THF and [3′, 5′,7-^{3}H](6*S*)-5-methyltetrahydrofolate (5-CH_3THF), obtained from Moravek Biochemicals (Brea, CA), and [3′,5′,7-^{3}H]MTX, obtained from Amersham (Arlington Heights, IL), were purified by HPLC. Unlabeled MTX and 5-CHO-THF, provided by Lederle (Carolina, Puerto Rico), as well as folic acid and 5-CH_3-THF, obtained from Sigma (St. Louis, MO), were also purified by HPLC. Silicon and mineral oil used for the specific surface binding assay were purchased from Aldrich Chemical (Milwaukee, WI). All other reagents were of the highest purity available from commercial sources.

Cell Culture Conditions. L1210 leukemia cells and the MTX^{r}A line, which lacks endogenous RFC1 function (Schuetz et al., 1989; Brigle et al., 1995), 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, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 5% CO_2.

Site-Directed Mutagenesis of RFC1. Site-directed mutagenesis was carried out according to the QuickChange protocol from Strategene (La Jolla, CA). Wild-type RFC1 cDNA was isolated from L1210 cells and directionally cloned into the mammalian expression vector pcDNA3.1(+) (Invitrogen, San Diego, CA) in the same way as reported for the mutated RFC1-E45K (Zhao et al., 1998b). The resulting vector was used as the template, and two completely complementary 24-mer oligonucleotides, which carry the targeted nucleotide changes in the middle region, were used as the primers for the QuickChange protocol. Sense primers containing the degenerate codons for amino acid 45 are listed in Table 1. The codons for the amino acids introduced were chosen so that the single *Hin*dIII restriction site in the RFC1 coding region was eliminated to facilitate screening for positive clones. The RFC1 expression vector (30 ng), a pair of primers (each 150 ng), dNTP (each 10 ng), and *Pfu* polymerase (2.5 U) were dissolved in 50 μl of reaction buffer. The temperature was cycled 17 times for 30 s at 95°C, 1 min at 55°C, and 14 min at 68°C. *Dpn*I (10 U) was added and incubated at 37°C for 1 h to digest the parental supercoiled dsDNA. The mixture (1 μl) was transformed into 50 μl DH5α competent cells (Life Technologies, Rockville, MD). Plasmids harboring the mutations were identified by restriction analysis with *Hin*dIII and then confirmed by DNA automated sequencing, in the Albert Einstein Comprehensive Cancer Center DNA Sequencing Facility, to be free of any mutations in the RFC1 coding region that might be introduced by *Pfu* polymerase. The plasmids were then ready for transfection.

Transfections. MTX^{r}A (1×10^7 cells) were electroporated (250 V, 200 μf) 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. Cells were then diluted in 20 ml of complete RPMI 1640 medium, allowed to recover for 48 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 $\sim 4 \times 10^4$ 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} was chosen for each mutation and subjected to further study. The transfectants were maintained in RPMI 1640 medium containing 750 μg/ml of G418.

Northern Analyses. Total RNA was isolated from the transfectants, MTX^{r}A, and L1210 cells using the 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 (Zhao et al., 1997). Transcripts were quantitated by PhosphorImager analysis of the hybridization signals and normalized to β-actin.

Analysis of Specific 5-CH_3-THF Binding to the Cell Surface as a Measure of RFC1 Membrane Expression. Cells were harvested, washed twice with, and resuspended in, HEPES (190 mM HEPES, 5 mM KCl, 2 mM $MgCl_2$, 5 mM glucose, pH 7.4) to a density of 5×10^7 cells/ml. Portions of 200 μl were incubated with 1 μM [^{3}H]5-CH_3-THF (specific activity of 800 dpm/pmol) at 0°C for 10 min in the presence or absence of a 10-fold excess of unlabeled 5-CH_3-THF. Bound and free ligands were then separated by centrifugation of a 180-μl aliquot through 150 μl of a mixture of silicon and mineral oil (9:1) in a 5.8 × 47.5-mm plastic (0.4 ml) microfuge tube at 14,000 rpm for 15 s. The tip of the tube, which contained the cell pellet, was cut off, dropped into a glass scintillation vial, and the contents digested with 0.5 ml 1 N KOH following which scintillation fluid was added and radioactivity 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 expressed as nmol/g dry wt. of cells.

For 5-CH_3-THF binding kinetics, six substrate concentrations, 0.05, 0.1, 0.2, 0.5, 0.75, and 1.0 μM, were used in the absence and presence of 10 μM unlabeled 5-CH_3-THF. Under these conditions, the binding constant (K_b) for 5-CH_3-THF in MTX^{r}A-R16 cells, which overexpress wild-type RFC1 (Brigle et al., 1995), was 0.11 ± 0.023 μM ($n = 3$), one-ninth the substrate concentration used in the assay, a value similar to what had been reported previously (Henderson et al., 1980). The binding assay provides an accurate comparison of RFC1 expression in MTX^{r}A-E45K, MTX^{r}A-E45Q, and MTX^{r}A-E45R cells in which the influx K_i, a reflection of substrate binding, is unchanged (see result sections). For E45L, the binding K_d is 0.39 ± 0.06 μM (n = 3). Hence, the assay underestimates carrier expression by ~30%.

Transport Studies. Influx measurements for all the folate compounds were performed by methods described in detail previously in HEPES-buffered saline (HBS) (20mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM glucose, pH 7.4) or HEPES buffer (190 mM HEPES, 5 mM KCl, 2 mM $MgCl_2$, 5 mM glucose, pH 7.4) (Zhao et al., 1997). K^+-HEPES-sucrose buffer (20 mM HEPES, 235 mM sucrose, pH 7.4) and Mg^{2+}-free HBS buffer (20 mM HEPES, 140 mM NaCl, 5 mM glucose, pH 7.4) were also used in some experiments. Before transport studies with the transfectants, cells were expanded for only three to seven doublings without further addition of G418 to ensure that expression of the mutated RFC1 was maintained.

TABLE 1

Sense primers used to generate site-directed mutants

The wild type and degenerate codons for amino acid 45 are underlined. The only *Hin*dIII restriction site in the RFC1 coding sequence is highlighted.

Amino Acid Residue at Position 45	Carrier Designation	Sense Primer for Site-Directed Mutagenesis
Glutamate (wild type)	RFC1	5′-^{123}GCGTCCTGGGGAAAGCTTCATCAC^{146}-3′
Glutamine	E45Q	5′-^{123}GCGTCCTGGGCAGAGCTTCATCAC^{146}-3′
Arginine	E45R	5′-^{123}GCGTCCTGGGCGTAGCTTCATCAC^{146}-3′
Aspartate	E45D	5′-^{123}GCGTCCTGGGCACAGCTTCATCAC^{146}-3′
Leucine	E45L	5′-^{123}GCGTCCTGGGCTCAGCTTCATCAC^{146}-3′
Tryptophan	E45W	5′-^{123}GCGTCCTGGGTGGAGCTTCATCAC^{146}-3′

Results

Expression of E45 Site-Directed Mutants in MTX^{r}A Cells. The cDNAs of the mutated RFC1s were transfected into the murine L1210 leukemia cell line, MTX^{r}A, which lacks endogenous RFC1 function due to a proline for alanine substitution at amino acid 130 within the fourth transmembrane domain (Schuetz et al., 1989; Brigle et al., 1995). One transfected clone, with the greatest restoration of sensitivity to MTX for each mutant, was identified and used for further detailed studies. The nomenclature for these cell lines is MTX^{r}A followed by the specific amino acid substitution. Figure 1 shows a representative Northern blot analysis. The radioactive blots were quantitated directly by PhorphorImage analysis, and RFC1 mRNA levels were normalized to β-actin and a wild-type L1210 cell level of 1. The amount of RFC1 transcript in the transfectants derived from the expression vector was higher than that of L1210 cells by factors of 2.6 to 8.0. Consistent with previous determinations, RFC1 mRNA in recipient MTX^{r}A cells was about half that of L1210 cells (Brigle et al., 1995; Zhao et al., 1998a, 1998b).

Specific 5-CH_3-THF binding to membrane RFC1 of L1210, MTX^{r}A, and transfected cells was assessed in HEPES buffer along with MTX^{r}A-R16 cells. The latter, transfected with wild-type RFC1 cDNA, overexpresses carrier (Zhao et al., 1997). 5-CH_3-THF specifically bound to MTX^{r}A-R16 cells was nearly 3.5 times greater than that of L1210 cells (Fig. 2); binding to MTX^{r}A cells (0.19 $\pm$0.06 nmol/g dry wt.) was half that of L1210 cells, consistent with the results reported previously (Brigle et al., 1995). Specific binding was detected in all lines transfected with mutated carriers in excess of the level in the recipient MTX^{r}A line. When the background level in MTX^{r}A cells is subtracted from the level of specific binding in the transfectants as indicated in Fig. 2, the expression of mutated carriers ranged between levels about one-half to nearly equal that of L1210 cells.

Folate Influx in Transfectants. Table 2 summarizes initial uptake rates for MTX, 5-CH_3-THF, and 5-CHO-THF in all cell lines studied. The most active of the mutated carriers was E45Q. In fact, transport of 5-CHO-THF and 5-CH_3-THF in MTX^{r}A-E45Q cells was nearly three times faster than that in L1210 cells and far exceeded the rate of MTX transport. For the E45L carrier, influx of the reduced folates was comparable with that mediated by wild-type RFC1; MTX influx was ~50% less. RFC1 expression in both E45Q and E45L transfectants was comparable with the wild-type level in L1210 cells. For other mutated carriers, influx was decreased and residual activity of each of the folate substrates among the mutants varied considerably. The lowest activities were observed for the asparate and tryptophan substitutions. Although the membrane binding assay underestimates the level of E45W expression due to its high influx K_i (see Table 3 below), residual transport activity measured was <10% that mediated by wild-type RFC1

The Inhibitory Effect of 5-CHO-THF, 5-CH_3-THF and Folic Acid on MTX Influx Mediated by the Mutated Carriers. Inhibition of MTX influx by reduced folates and folic acid was assessed as a prelude to the determination of the affinity of the mutated carriers for these substrates. Three different concentrations of each inhibitor were used: the wild-type influx K_t, one-fifth the influx K_t, and five times the influx K_t, along with a control in which no inhibitor was added. As shown in the upper panel of Fig. 3, the potency of inhibition of MTX influx by 5-CHO-THF was markedly altered in the transfectants as compared with L1210 cells. Inhibition of MTX influx was 1) not abolished even at a 5-CHO-THF concentration of 25 μM in MTX^{r}A-E45W cells, 2) markedly decreased in MTX^{r}A-E45D cells, 3) markedly increased in MTX^{r}A-E45Q and MTX^{r}A-E45R cells whereas 4) unchanged in MTX^{r}A-E45L cells. The pattern of inhibition of MTX influx by 5-CH_3-THF resembled that of 5-CHO-THF, but 5-CH_3THF inhibition appeared greater in MTX^{r}A-E45W and weaker in MTX^{r}A-E45R relative to L1210 cells (middle panel of Fig. 3). Folic acid inhibition of MTX influx in the transfectants differed from that observed with either 5-CHO-THF or 5-CH_3-THF (lower panel). At all concentrations, inhibition was markedly increased in MTX^{r}A-E45Q, MTX^{r}A-E45R, and MTX^{r}A-E45L cells as compared with that of L1210 cells, whereas it was roughly comparable in MTX^{r}A-

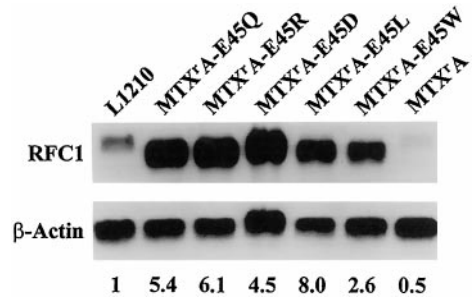

Fig. 1. A representative Northern blot of total RNA from L1210, MTX^{r}A, and the transfectants. Total RNA was probed successively first with the full-length murine RFC1 and then with β-actin cDNAs. The molecular weight of the endogenous RFC1 transcript in L1210 and MTX^{r}A cells (2.3 kb) was slightly greater than that of the transfectants (1.9 kb), which was derived from the expression vector. The radioactive blots were quantitated directly by PhorporImager anaysis, and the results from two separate experiments, normalized to β-actin and a message level of L1210 cells of 1, are indicated below each lane.

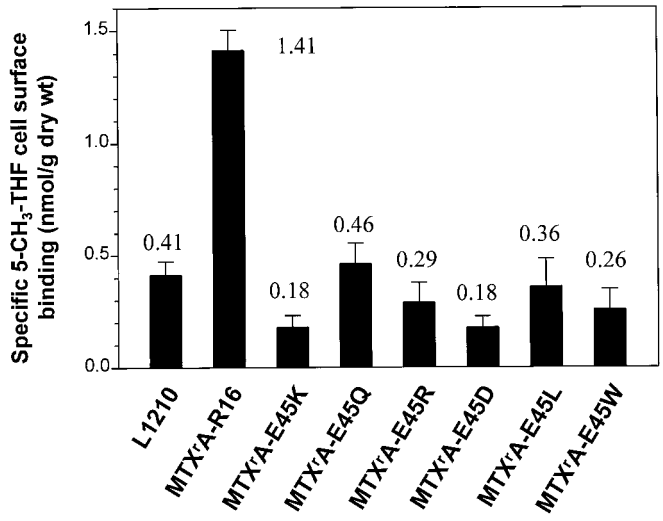

Fig. 2. Specific 5-CH_3-THF binding to membrane RFC1 in L1210, MTX^{r}A-R16 cells, and transfectants. The binding experiments were performed at 4°C in HEPES buffer (9 mM Cl^-) with 1 μM [^{3}H]5-CH_3-THF as the radioactive ligand. The difference between [^{3}H]5-CH_3-THF bound to the cell surface in the absence and presence of 10 μM unlabeled 5-CH_3-THF was normalized to the dry weight of cell pellets. The data indicate the level specifically bound to transfectants including the MTX^{r}A-R16 line less the amount bound to the recipient MTX^{r}A cells. No correction was made for L1210 cells. The data are the mean $\pm$ S.E. of six separate experiments.

E45D and MTX^{r}A-E45W cells. These data were then used to identify appropriate concentrations for determination of inhibitory constants for these folates as indicated below.

Influx Kinetics. As indicated in Table 3, the influx K_t for MTX was increased in all mutated carriers except E45R; the greatest increase in K_t was observed with E45D. Influx kinetics could not be determined for E45W due to the very low level of transport activity. The influx V_{max} was increased by a factor of 2 in E45Q, but influx was minimally decreased (Table 2), attributed to the accompanying 2-fold increase in influx K_t. The V_{max} was unchanged in E45L and was decreased by 50–60% in the E45R and E45D transfectants as compared with that of L1210 cells; the latter can be explained, at least in part, by a comparable decrease in carrier protein expression (Fig. 2, Table 2).

Influx K_i for the other folates were then assessed by Dixon analysis and compared with wild-type RFC1 (Table 3). The influx K_i for 5-CH_3-THF in E45K was also determined, and together with K_t for 5-CHO-THF influx and K_i for folic acid, which were reported previously (Zhao et al., 1998b), are listed for the purpose of comparison. As observed earlier for the E45K, the K_i for 5-CHO-THF influx was decreased by a factor of ~4, the 5-CH_3-THF influx K_i was essentially unchanged, and the folic acid K_i was decreased by a factor of 6 to 10 in the E45Q and E45R. The E45L mutation resulted in a 2- and 4-fold decrease in carrier affinity for 5-CHO-THF and 5-CH_3-THF, respectively. For the remaining mutant carriers, E45D and E45W, the influx K_i was increased for both reduced folates, although the loss of affinity was much greater for 5-CHO-THF (9- and 29-fold, respectively) than 5-CH_3-THF (3- and 10-fold, respectively). The K_i for folic acid was greater for E45D than wild-type RFC1 but was approximately one-half the wild-type K_i for the E45W and E45L mutants. Hence, amino acid substitutions at the 45 position are associated with profound changes in binding of the natural folates to RFC1.

Although the high affinity of E45Q is associated with high influx rates for 5-CHO-THF and 5-CH_3-THF, influx mediated by E45R is less than that of L1210 cells (Table 2) despite the fact that the influx K_t is comparable with E45Q, suggesting that the mobility of the mutant carrier-substrate complex is less than that for the wild-type carrier complex. In contrast, despite a 2- to 4-fold increase in K_t for 5-CHO-THF and 5-CH_3-THF for the E45L mutant, rates of transport are comparable with L1210 cells (Table 2), consistent with an increase in mobility of the mutated carrier-substrate complex.

The Anion Dependence of MTX Influx in E45 Mutants. Folate transport in L1210 cells is inhibited by virtually all inorganic and organic anions and influx is stimulated by the removal of chloride (Goldman, 1971; Henderson and Zevely, 1988). In contrast, activity of the E45K mutant was markedly reduced in the absence of chloride (Zhao et al., 1998b). To determine the effect of the other amino acid substitutions on the anion-dependence of transport, all but 9 mM extracellular chloride was replaced by HEPES and MTX influx was assessed. MTX influx in HEPES buffer was increased ~150% in L1210 cells and all other transfectants except for E45Q in which the increase was ~50% and E45R in which the effect was reversed with a 60% decrease in influx (Fig. 4). The latter was qualitatively the same as observed for E45K, and the increase in MTX influx in MTX^{r}A-E45R as extracellular chloride was increased, similar to what was observed for 5-CHO-THF influx in MTX^{r}A-E45K cells (Fig. 5) (Zhao et al., 1998b).

TABLE 2

Influx of MTX, 5-CHO-THF, and 5-CH_3-THF in L1210 cells, MTX^{r}A cells and transfectants

The data are the mean ± S.E. of three experiments.

Cell Lines	MTX		5-CHO-THF		5-CH_3-THF		RFC1 Expression Relative to L1210 Cells[c]
	Influx[a]	Ratio[b]	Influx[a]	Ratio[b]	Influx[a]	Ratio[b]	
L1210	1.31 ± 0.10	1.0	1.99 ± 0.16	1.0	2.48 ± 0.04	1.0	1.0
MTX^{r}A	0.017 ± 0.003	0.013	0.037 ± 0.005	0.018	0.061 ± 0.009	0.024	0.43
MTX^{r}A-E45Q	1.09 ± 0.19	0.83	5.35 ± 0.42	2.7	6.91 ± 1.02	2.8	1.1
MTX^{r}A-E45R	0.43 ± 0.03	0.33	0.49 ± 0.10	0.25	0.98 ± 0.10	0.40	0.70
MTX^{r}A-E45D	0.20 ± 0.03	0.15	0.13 ± 0.01	0.065	0.26 ± 0.01	0.10	0.43
MTX^{r}A-E45L	0.53 ± 0.10	0.40	1.94 ± 0.19	0.97	2.75 ± 0.43	1.11	0.87
MTX^{r}A-E45W	0.125 ± 0.024	0.095	0.043 ± 0.011	0.022	0.094 ± 0.020	0.038	0.62

[a] Initial uptake rates expressed in nmol/g dry wt/min.
[b] As compared to L1210 cells.
[c] RFC1 expression relative to L1210 cells assessed by specific 5-CH_3THF membrane binding.

TABLE 3

MTX influx kinetic parameters and inhibition constants for 5-CHO-THF, 5-CH_3-THF, and folic acid in the transfectants and L1210 cells

MTX influx K_t and V_{max} (nmol/g of dry wt/min) values are the average of two experiments determined from nonlinear regression to the Michaelis-Menten equation. Folate influx K_i values (μM) are the average of two experiments obtained from Dixon plot analysis based on inhibition of MTX influx. The numbers in parentheses are the ratio of parameters in transfectants to L1210 cells.

Cell Lines	MTX			5-CHO-THF	5-CH_3-THF	Folic Acid
	K_t	V_{max}	Relative RFC1 Expression	K_i	K_i	K_i
	μM				μM	
L1210	7.0 (1)	12 (1)	1	5.6 (1)	1.5 (1)	260 (1)
MTX^{r}A-E45K			0.43	1.5 (K_t) (0.27)	1.3 (0.87)	34 (0.13)
MTX^{r}A-E45Q	15.8 (2.3)	23 (1.9)	1.1	1.6 (0.28)	0.6 (0.4)	42 (0.16)
MTX^{r}A-E45R	8.0 (1.1)	5.3 (0.44)	0.70	1.2 (0.21)	1.4 (0.93)	27 (0.1)
MTX^{r}A-E45D	25 (3.6)	4.5 (0.38)	0.43	50 (8.9)	4.3 (2.9)	400 (1.5)
MTX^{r}A-E45L	13 (1.8)	15 (1.3)	0.87	11.4 (2.0)	6.5 (4.3)	117 (0.45)
MTX^{r}A-E45W			0.62	163 (29)	15 (10)	130 (0.5)

Discussion

Previous studies from this and other laboratories have begun to elucidate some of the structure-function properties of the reduced folate carrier. These insights have derived largely from analyses of the functional consequences of mutations in RFC1 in cell lines obtained under antifolate selective pressure augmented by chemical mutagenesis (Zhao et al., 1999). The first transmembrane domain has been identified as one important site for mutations that result in antifolate drug resistance with highly selective changes in the spectrum of carrier affinities for folate substrates and/or mobilities of the carrier-substrate complex (Tse et al., 1998; Zhao et al., 1998a, 1998b). The current study was undertaken to further clarify the structural basis for the functional changes that occurred when lysine was substituted for glutamate at amino acid 45—increased affinity for folic acid and 5-CHO-THF, decreased affinity for MTX, and the induction of an obligatory requirement for small inorganic anions to allow carrier function (Zhao et al., 1998b).

Based on the substitution of glutamate with amino acids of different charge, polarity, and size, the data indicate that preservation of the negative charge at amino acid 45 is not an essential element in RFC1 function. In fact, replacement of glutamate with the uncharged glutamine, or positive-charged arginine, resulted in increased affinity for 5-CHO-

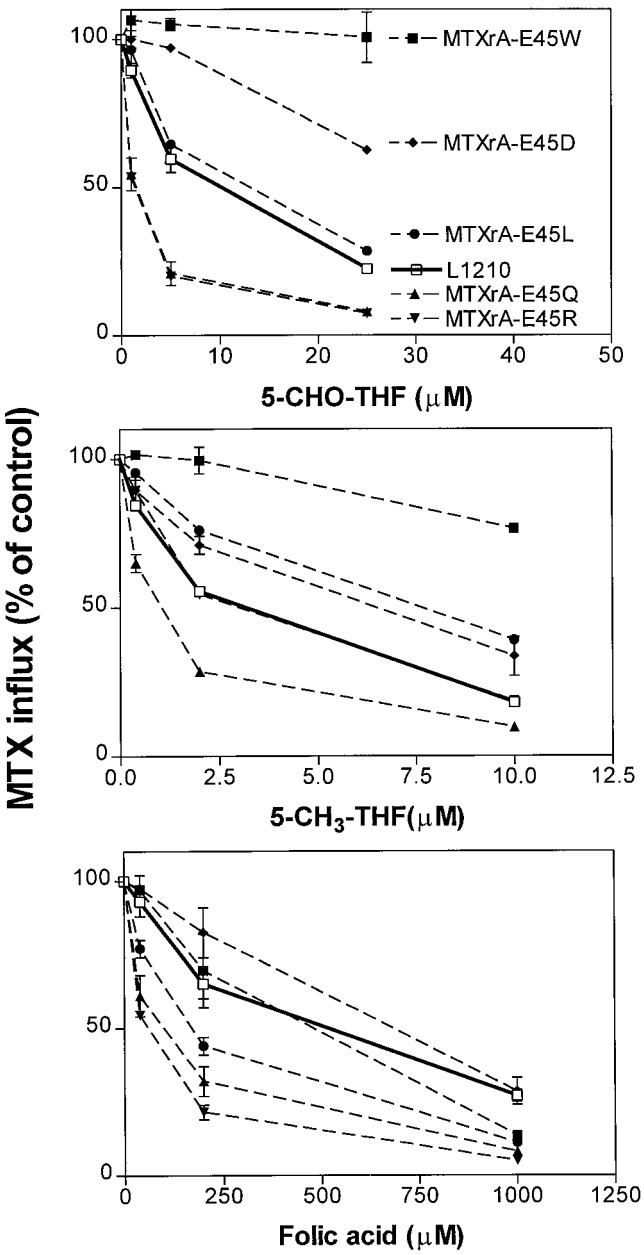

Fig. 3. Inhibition of MTX influx by 5-CHO-THF (upper panel), 5-CH_3-THF (middle panel), and folic acid (lower panel) in L1210 and transfectants. After cells were incubated in HBS at 37°C for 25 min, [^{3}H]MTX (1 μM), and appropriate concentrations of inhibitors were coadded to the cell suspension and incubation was continued over an interval during which MTX uptake was linear as a function of time. The data are presented as a percentage of influx in control cells to which no inhibitor was added. The same symbols are used to designate cell lines in all three panels. The data are the average of at least two experiments.

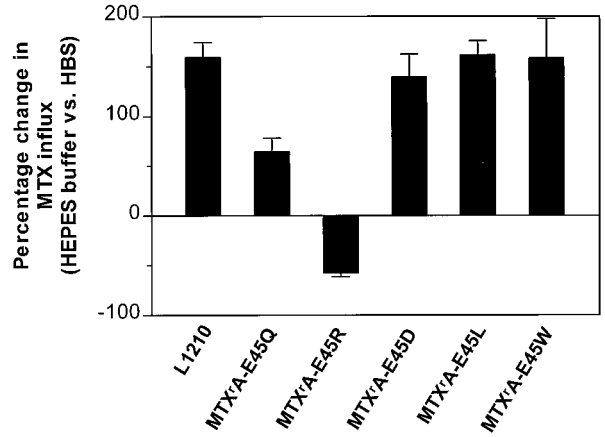

Fig. 4. MTX influx in HEPES buffer versus HBS in L1210 cells and transfectants. Cells were harvested and washed with HEPES (9 mM Cl^-) buffer or HBS (150 mM Cl^-) twice and then suspended in these buffers. After 25 min incubation at 37°C, cells were exposed to 1 μM [^{3}H]MTX and influx was measured over 1 min in L1210 and MTX^{r}A-E45Q cells, 2 min in MTX^{r}A-E45L cells, and 6 min in MTX^{r}A-E45R, MTX^{r}A-E45D, and MTX^{r}A-E45W cells. The results are the average ± S.E.M. of three experiments.

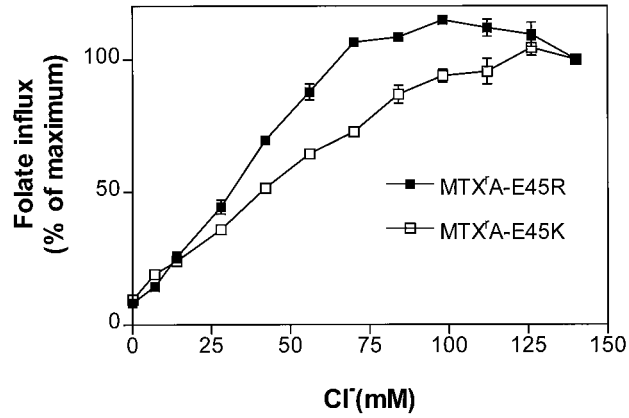

Fig. 5. Folate influx as a function of extracellular chloride concentration in MTX^{r}A-E45R and MTX^{r}A-E45K cells. Cells were suspended in buffer containing different concentration of chloride by mixing K^+-HEPES-sucrose buffer and Mg^{2+}-free HBS buffer as indicated in *Materials and Methods*. After 25 min incubation at 37°C, MTX^{r}A-E45R cells were exposed to 1 μM [^{3}H]MTX, and influx was measured over 9 min (■). The results are mean ± S.E. of three experiments. Chloride-dependent [^{3}H]5-CHO-THF influx in MTX^{r}A-E45K cells (the mean of three experiments ± S.E.) reported previously (Zhao et al., 1998b) is plotted in the same graph for the purpose of comparison (□). Maximal influx is influx in Mg^{2+}-free HBS buffer.

THF, a similar or greater affinity for 5-CH_3-THF, and a markedly increased affinity for folic acid as compared with wild-type carrier, as was observed also for the lysine-substituted RFC1 (Zhao et al., 1998b). It is of interest that the loss of the negative charge has the most profound salutary effect on folic acid binding with up to a 10-fold decrease in influx K_t. Indeed, the only substituted amino acid that produced a carrier with a slightly lower affinity for folic acid than wild-type RFC1 was aspartate, and increased binding occurred even with the tryptophan substitution despite its bulk. Another bulky substitution of phenylalanine for isoleucine at amino acid residue 48 without a change in polarity produced an even greater increase in affinity for folic acid with a 20-fold decline in influx K_t (Tse et al., 1998; Tse and Moran, 1998). Hence, it would appear that this region in the first transmembrane domain plays an important role in determining the very low affinity of wild-type carrier for this oxidized folate.

Carrier functional activity is determined by affinity for the substrate, carrier mobility, and the level of carrier protein expressed. Carrier protein levels at the cell surface were assessed using a specific binding assay for 5-CH_3-THF. All of the mutated carriers were expressed and processed to the cell membrane and the measured levels were within a factor of ~2 of the wild-type carrier. In the absence of murine RFC1 antibody, the efficacy of processing and extent to which carrier might be sequestered along trafficking route cannot be assessed. In general, the level of mutated protein present at the cell membrane (Fig. 2) was far lower than expected based on a comparison of RFC1 mRNA (Fig. 1) in the transfectants relative to L1210 cells. Discrepancies were also observed for human RFC1 transfectants in which the amount of RFC1 mRNA or protein far exceeded relative levels of RFC1 activity (Wong et al., 1997, 1998). However, these studies go beyond protein identification to the assessment of specific folate binding to RFC1 at the cell surface.

Based on the level of protein detected and the measured influx V_{max} for MTX, there were some substrate-dependent differences in the mobility of the carrier-substrate complexes, less or greater than wild-type RFC1, that accompanied changes in influx K_t. This is in contrast to the functional changes associated with the S→N mutation, at the adjacent amino acid 46, in which there was a highly selective decline in carrier mobility that allowed a much greater rate of translocation of carrier loaded with reduced folates than with MTX without any change in the spectrum of affinities for these substrates (Zhao et al., 1998a).

The further demonstration that only arginine, of all the substituted amino acids, reproduces the anion dependence of MTX influx observed with lysine clarifies the basis for this phenomenon (Zhao et al., 1998b). Induction of anion-dependent transport in the E45K mutant was associated with a decrease in influx V_{max}, consistent with the loss of carrier mobility in the absence of chloride. The data here indicate that it is the introduction of the positive charge at amino acid 45, and not the loss of the negative charge at this site, that results in the loss of function. Therefore, the impact of chloride is likely due to neutralization of the positive charge associated with the lysine or arginine substitution.

The very marked decrease in influx of MTX and the other folates associated with the aspartate substitution was of particular interest because this amino acid is of the same charge as, and is close in size to, glutamate. This resulted in a fall in affinity for all the folates. The loss of a methylene group in the side chain by this substitution relocates the carboxyl group by about 1.5 Å, a small change that is apparently sufficient to alter the interaction among this residue, its neighboring amino acids, and folates. The impact of the aspartate for glutamate substitution on carrier transport function has been well documented in other systems. For instance, the same substitution at amino acid 121 in the fourth transmembrane domain of the myo-inositol/H symporter nearly abolished transport activity (Seyfang et al., 1997). When this mutation was present in the glutamate transporter at residue 404, potassium-coupled transport was abolished, and glutamate exchange became obligatory for carrier function (Kavanaugh et al., 1997).

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