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Mutational Analysis of the Functional Role of Conserved Arginine and Lysine Residues in Transmembrane Domains of the Murine Reduced Folate Carrier

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ABSTRACT

The reduced folate carrier (RFC1) plays a major role in the delivery of folates into mammalian cells. RFC1 is an anion exchanger with seven conserved positively charged amino acid residues within 12 predicted transmembrane domains. This article explores the role of these residues in transport function by the development of cell lines in which arginines and lysines in RFC1 were replaced with leucine by site-directed mutagenesis. Three cell lines transfected with R131L, R155L, or R366L all lacked activity, despite high levels of protein expression in the plasma membrane, suggesting the crucial role of these amino acid residues in RFC1 function. In several mutant carriers, R26L, R42L, and K332L, there was little or no change in the influx K_t value for MTX or influx K_i value for folic acid. However, the R26L, R42L, and K332L carriers had decreased affinity for reduced folates. This was most prominent for K404L, which had 11- and 4-fold increases in influx K_i for 5-methyl-THF and 5-formyl-THF, respectively, compared with L1210 cells. The marked influx stimulation observed with wild-type carrier when extracellular chloride was decreased was significantly diminished when influx was mediated by the K404L carrier, but was only slightly decreased with the R26L, R42L, and K332L mutants. This suggested that the K404 residue may be a major site of inhibition by chloride in the wild-type carrier. These studies indicate the important role that some positively charged residues within transmembrane domains of RFC1 play in RFC1 function.

The reduced folate carrier (RFC1) mediates the membrane transport of the major plasma folate, 5-methyltetrahydrofolate (5-methyl-THF), and hence is crucial to the delivery of one-carbon units required for nucleic acid and methionine biosynthesis (Dixon et al., 1994). RFC1 is a member of the major facilitator superfamily of carriers that transport a variety of diverse inorganic and organic solutes in prokaryotic and eukaryotic cells (Pao et al., 1998). RFC1 is predicted to have 12 transmembrane domains bisected by a long cytoplasmic loop, which, along with the N and C termini, are directed to the cytoplasm (Dixon et al., 1994; Ferguson and Flintoff, 1999). RFC1 generates uphill transport of folates through an exchange mechanism linked to organic anions that are concentrated within the intracellular compartment (Goldman, 1971; Henderson and Zevely, 1983; Yang et al., 1984).

Recent studies from this laboratory employing chemical mutagenesis with antifolate selective pressure, along with studies from other laboratories, have identified residues, particularly within the first transmembrane domain of RFC1, that, when mutated, produce marked changes in the spectrum of carrier affinities for folate and antifolate substrates as well as selective changes in the mobility of the carrier-folate complex (Zhao et al., 1998a,b, 1999a; Jansen et al., 1998; Tse et al., 1998).

There are 12 charged amino acids within the predicted transmembrane domains of RFC1; of these, seven are conserved among the cloned mammalian carriers and carry a positive charge. Charged amino acids within transmembrane domains of other facilitative carriers have been shown to take part in substrate recognition and binding, maintenance of tertiary carrier structure, and changes in carrier conformation that are associated with the bidirectional movements of carrier and substrate (Jarolim et al., 1995; Muller et al., 1996; Steiner-Mordoch et al., 1996; Fei et al., 1997; Kavanaugh et al., 1997; Merickel et al., 1997; Karbach et al., 1998; Lanz and Erni, 1998). Because folates are bivalent anions and RFC1 is an anion exchanger that is inhibited by a broad spectrum of organic and inorganic anions (Goldman,

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ABBREVIATIONS: RFC1, the reduced folate carrier; 5-formyl-THF, 5-formyltetrahydrofolate; 5-methyl-THF, 5-methyltetrahydrofolate; MTX, methotrexate; PCR, polymerase chain reaction; HBS, HEPES buffered saline.

1971; Henderson and Zevely, 1983), it seemed plausible that interactions between folates and this carrier would involve, and require the integrity of, positively charged residues. This possibility was explored in the present studies by the application of site-directed mutagenesis to substitute each of the conserved lysines and arginines located in transmembrane domains of RFC1 with the neutral leucine. The data demonstrate the importance of these amino acid residues in the maintenance of carrier function; in particular, the binding of reduced folates.

Materials and Methods

Chemicals. [3′,5′,7-^{3}H]-(6*S*)-5-formyl-THF and [3′,5′,7-^{3}H]-(6*S*)-5-methyl-THF were obtained from Moravek Biochemicals (Brea, CA); [3′,5′,7-^{3}H]-MTX was obtained from Amersham Pharamcia Biotech (Piscataway, NJ). Folates were purified by high performance liquid chromatography before use (Fry et al., 1982). Nonlabeled reduced folates were racemic. All other reagents were obtained in the highest purity available from various commercial sources.

Construction of Plasmids. Mutations were generated using overlapping PCR (Ho et al., 1989) with *Pfu* DNA polymerase (Stratagene, La Jolla, CA). In all the PCR reactions, pSRC was used as the template. pSRC plasmid was produced by subcloning the RFC1 coding sequence into the pcDNA3.1 mammalian expression vector (Invitrogen, San Diego, CA). The RFC1 coding sequence was obtained from the template pPGK-RFC1 plasmid (Brigle et al., 1995) by PCR with the following primers: upstream GCGGATCCACC**ATG**GTGCCCACTGGCCAGGTG and downstream GCCTCGAG**TCA**CAGCCCCGCCCAGGCAAAGCAG containing the indicated *Bam*HI and *Xho*I restriction sites (underlined), respectively. The *Bam*HI-primer contained the start codon and the *Xho*I-primer contained the stop codon (marked in bold). The PCR fragment was digested with *Bam*HI/*Xho*I restriction enzymes and subcloned into the pcDNA3.1 expression vector. To generate the site-directed mutants, two overlapping upstream and downstream fragments containing the desired mutations were generated and then used for PCR-fusion [for details see Ho et al. (1989)]. *Bam*HI- and *Xho*I- containing primers described above were used as flanking primers. To create specific substitutions, the nucleotide pairs represented in Table 1 were used as internal primers that carried the mutations. PCR-fused fragments harboring the specific mutations within the full-length RFC1 cDNA were subcloned into the pcDNA3.1 vector using *Bam*HI/*Xho*I restriction sites. Single mutations in all constructs were verified by sequencing the entire RFC1 coding region on automated sequencer models ABI 373A and ABI 377 (PerkinElmer Corporation, Norwalk, CT) in the DNA-Sequencing Facility of the Albert Einstein Comprehensive Cancer Center.

Cell Culture Conditions and Generation of Cell Lines. Cells were grown in RPMI 1640 medium containing 2.3 μM folic acid, supplemented with 10% bovine calf serum (HyClone, Logan, UT), 2 mM glutamine, 20 μM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cell lines expressing mutant RFC1s were obtained by transfection of the MTX^{r}A line, which lacks functional carrier, with DNA plasmids containing site-directed RFC1 mutants as reported previously (Brigle et al., 1995). Briefly, 1×10^7 MTX^{r}A cells were electroporated (300 V, 800 μF) with 40 μg of circular plasmid and selected in RPMI 1640 medium containing 750 μg/ml G418. Lines were isolated by subsequent cloning on soft agar (Kuroki, 1973).

Northern Analyses. Total RNA was isolated using TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD) and 30 μg of RNA was fractionated by electrophoresis on 1% formaldehyde-agarose gels. Transfer and hybridization was performed as described (Brigle et al., 1995). RFCI mRNA was quantified by analysis on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Western Analysis. A polyclonal antibody, AE390, to the distal C terminus of murine RFC1 (Met^{499} through Ala^{512}) was used to probe both total cell lysate and plasma membranes, as reported previously (Zhao et al., 2000). For total lysate preparation, cells (3×10^7) were harvested, washed twice with HBS (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM glucose, pH 7.4 at 0°C) and suspended in 100 μl of the same solution containing 10 μl of protease inhibitor (P-8340; Sigma Chemical, St. Louis, MO). The cell suspension was sonicated for 20 s in a tube submerged in ice water. Plasma membranes were extracted as reported, except a protease inhibitor cocktail (P8340) at a dilution of 1 to 1000 was used instead of 1 mM phenylmethylsulfonyl fluoride (Henderson and Zevely, 1984). Protein concentrations of the total lysate and plasma membranes were determined with the BCA Protein Assay Kit (Pierce, Rockford, IL). The proteins were dissolved in a SDS-polyacrylamide gel electrophoresis loading buffer [60 mM Tris, 10% glycerol (v/v), 2% SDS, and trace bromphenol blue, pH 6.8] without heating and resolved on a 12% SDS-polyacrylamide gel. The proteins were transferred to polyvinylidene difluoride transfer membranes and processed by the enhanced chemiluminescence ECL Plus Western blotting detection system, both obtained from Amersham.

Transport Studies. Influx measurements were performed by the method described previously (Zhao et al., 1997). Cells were harvested, washed twice with HBS (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, and 5 mM glucose, pH 7.4) and resuspended in HBS to 1.5×10^7 cells/ml. Cell suspensions were incubated at 37°C for 25 min, then uptake was initiated by the addition of [^{3}H]MTX, [^{3}H]5-formyl-THF, or [^{3}H]5-methyl-THF and samples were taken at the indicated times. Uptake was terminated by injection of 1 ml of cell suspension into 9 ml of ice-cold HBS buffer. Cells were collected by centrifugation, washed twice with ice-cold HBS and

TABLE 1
Internal primers used for generating mutations in RFC1
Underlined nucleotides represent the codons that were changed to introduce a corresponding mutation.

Mutation	Primers (upstream/downstream)
R26L	5′-GAGATAGAACACCAGGCATAGCCAGGATTTCAGTTCGTG-3′/
	5′-CACGAACTGAAATCCTGGCTATGCCTGGTGTTCTATCTC-3′
R42L	5′-CTTCGGCTTCATGGCCCAGTTGCTTCCTGGGGAAAGCTTCATCAC-3′/
	5′-GTGATGAAGCTTTCCCCAGGAAGCAACTGGGCCATGAAGCCGAAG-3′
R131L	5′-GTCACCATGGCGGCCCTCATCGCCTACTCCTCC-3′/
	5′-GGAGGAGTAGGCGATGAGGGCCGCCATGGTGAC-3′
R155L	5′-ATGGCCAGCTACTCACTGGCGGCAGTACTGCTG-3′/
	5′-CAGCAGTACTGCCGCCAGTGAGTAGCTGGCCATG-3′
K332L	5′-CGCTGGACTCTGTGGTCCCTGCTGGTCATCGCAGGTGTG-3′/
	5′-CACACCTGCGATGACCAGCAGGGACCACAGAGTCCAGCG-3′
R366L	5′-ACCTTTGTGCTTTTCCTTGGGGCCTACCAGTTC-3′/
	5′-GAACTGGTAGGCCCCAAGGAAAAGCACAAAGG-3′
K404L	5′-CTTTCCTAGCTACTGCGCTTCTGACCTGTATCACACTTGTG-3′/
	5′-CACAAGTGTGATACAGGTCAGAAGCGCAGTAGCTAGGAAAG-3′

processed to determine intracellular tritium (Fry and Goldman, 1982). For all influx measurements, uptake intervals were adjusted to ensure that initial rates of MTX, 5-formyl-THF, and 5-methyl-THF uptake were sustained. For studies that assessed the effect of extracellular chloride on MTX influx, HBS was replaced with a HEPES buffer containing 190 mM HEPES, 5 mM glucose, 5 mM KCl, and 2 mM $MgCl_2$, pH 7.4. In some studies, HEPES-sucrose-MgO buffer (20 mM HEPES and 235 mM sucrose, pH 7.4, with MgO) or a mixture with HBS were used. All buffers were adjusted to an osmolarity of 290 mmol/kg.

Results

Mutagenesis and Cell Line Generation. Conserved positively charged amino acids predicted to reside in the transmembrane domains of RFC1 were identified by comparison of protein sequences from different species aligned by the Clustal method (Higgins and Sharp, 1988). Arginines at positions 26, 42, 131, 155, and 366, and lysines at positions 322 and 404, are conserved in the mammalian RFC1 genes cloned to date; among them, arginines 26, 42, 131, and 366 were invariant in these species as well as in *Caenorhabditis elegans* (Fig. 1). Each of these amino acid residues was replaced with leucine by site-directed PCR mutagenesis. Constructs containing a single mutated carrier were transfected into the MTX^{r}A line, which is deficient in endogenous RFC1 function because of the substitution of a proline for alanine at position 130 in the 3rd transmembrane domain (Brigle et al., 1995).

The transfected plasmids, designated pR26L, pR42L, pR131L, pR155L, pK332L, pR366L, and pK404L, produced stable clonal cell lines designated MTX^{r}A-R26L, MTX^{r}A-R42L, MTX^{r}A-R131L, MTX^{r}A-R155L, MTX^{r}A-K332L, MTX^{r}A-R366L, and MTX^{r}A-K404L. Figure 2A illustrates message expression by Northern analyses. All transfectants except K404L overexpressed mutated message by 1.5- to 6.7-fold compared with L1210 cells (Table 2). Expression in the MTX^{r}A-K404L line was similar to that of L1210 cells. Fig. 2B is a Western analysis of total lysate. It can be seen that, in general, the highest levels of mRNA are associated with the highest levels of protein and the lines with the lowest levels of RFCI mRNA have the lowest levels of protein. Fig. 2C is a Western blot analysis of the plasma membrane fraction from the cell lines that had essentially no transport function (see below). Protein expression in the cell membranes of these lines was present at levels comparable with, or greater than, that of L1210 cells, indicating that there was no trafficking defect.

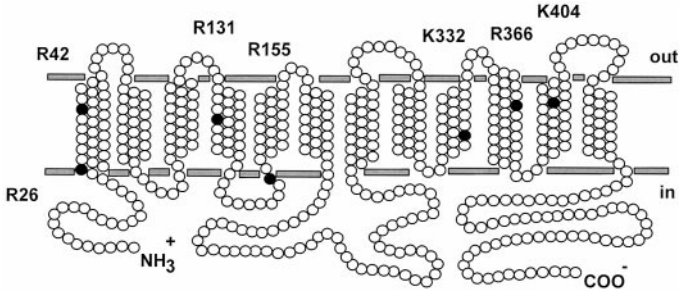

Fig. 1. Predicted topology of murine RFC1 within the cell membrane with locations of mutated residues denoted by ● and C termini along with the long loop between the 6th and 7th transmembrane domains predicted to be directed into the cytosol. The cell membrane is enclosed by the shaded horizontal bars.

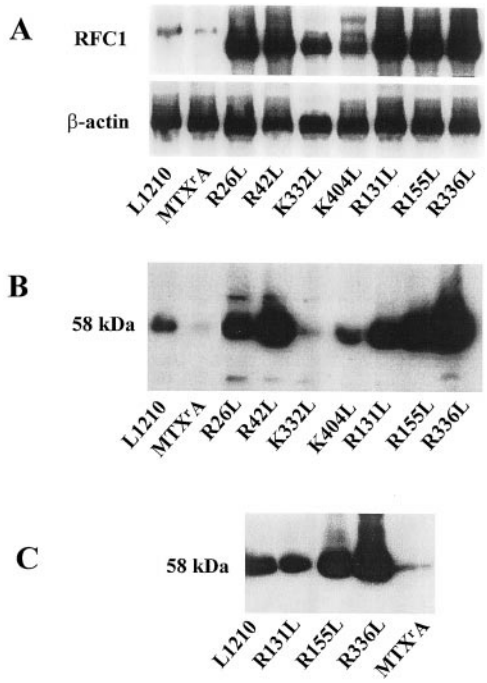

Fig. 2. Northern blot analyses of total RNA and Western blot analyses of RFC1 proteins in L1210, MTX^{r}A and tranfectants. A, Northern blot analysis of RFC1 transcripts. RNA (30 μg) resolved on 1% formaldehyde-agarose gels was probed with the full-length RFC1 (position 137 to 1675 of GenBank clone U66103) then reprobed with β-actin cDNA as indicated under *Materials and Methods*. The radioactive blots were quantified by PhosphorImager analysis (results are shown in Table 2) and exposed to X-ray film. The blot shown is representative of two such analyses. B, Western blot analysis of total cell lysates. Cells were sonicated and mixed with the sample loading buffer and resolved on a 12% SDS-polyacrylamide gel. The blotting and subsequent processing were performed with the ECL Plus Western blotting detection system. The data are representative of two separate analyses. Three micrograms of protein were loaded for L1210, MTX^{r}A, and R155L cells; 4.5 μg of protein was loaded for R26L, R42L, K404L, and R131L cells; and 6 μg was loaded for K332L and R366L cells. C, Western analysis of plasma membranes from L1210, R131L, R155L, R366L, and MTX^{r}A cells. The plasma membranes were prepared according to a published protocol (Henderson and Zevely, 1984) with only minor modification (see details under *Materials and Methods*). The data are representative of two separate Western analyses. The amount of plasma membrane protein loaded was 20 μg for L1210, R131L, and R155L, 30 μg for R336L and MTX^{r}A cells.

MTX Influx Properties in Transfected Lines. Transfectants were assessed for their capacity to transport MTX. Four among seven mutants demonstrated MTX transport

TABLE 2
MTX influx in L1210, MTX^{r}A and lines transfected with mutated carriers
RFC1 mRNA was quantified by PhosphorImager analysis. Influx was determined at $[MTX]_e$ = 1 μM. Results are the mean ± SEM of three experiments.

Cell line	Initial uptake rate	Influx in L1210 cells	RFC1 mRNA relative to L1210 cells
	nmol/g of dry wt/min	%	
L1210	0.91 ± 0.1	100	1
MTX^{r}A	0.02 ± 0.01	3	0.3
R26L	1.76 ± 0.2	193	3.6
R42L	0.53 ± 0.06	58	3.2
K332L	0.40 ± 0.08	44	1.5
K404L	0.89 ± 0.07	97	0.9
R131L	0.03 ± 0.01	4	4.8
R155L	0.05 ± 0.02	7	4.7
R366L	0.03 ± 0.01	3	6.7

activity (Fig. 3, Table 2). There was no detectable increase in MTX influx in the MTX^{r}A-R131L, MTX^{r}A-R155L, and MTX^{r}A-R366L transfectants compared with influx in the MTX^{r}A line, despite the high level of mutated proteins present in the plasma membrane (see above and Fig. 2C).

The kinetic basis for the changes in MTX influx mediated by the mutated carriers was determined in the lines in which there was sufficient residual transport activity to permit accurate measurements. Initial rates in all the cell lines followed Michaelis-Menten kinetics. As indicated in Table 3, there was no significant difference in the MTX influx K_t value for R26L and R42L mutant lines. The influx K_t values for K332L and K404L were within 40% of the value in L1210 cells. The influx V_{max} rates were comparable among the lines but no attempt was made to normalize the values to either the level of message or protein. However, in view of the small or absent change in influx K_t value for the mutant carriers, any changes in influx mediated by R26L, R42L, and K332 seem to be accounted for largely by changes in influx V_{max}.

Figure 4 illustrates the inhibitory effects of a variety of folates, over a spectrum of concentrations, on MTX influx. It can be seen that the pattern of inhibition by tetrahydrofolic acid, 5-formyl-THF, and 5-methyl-THF among the different cell lines was similar. Inhibition of MTX influx by all the

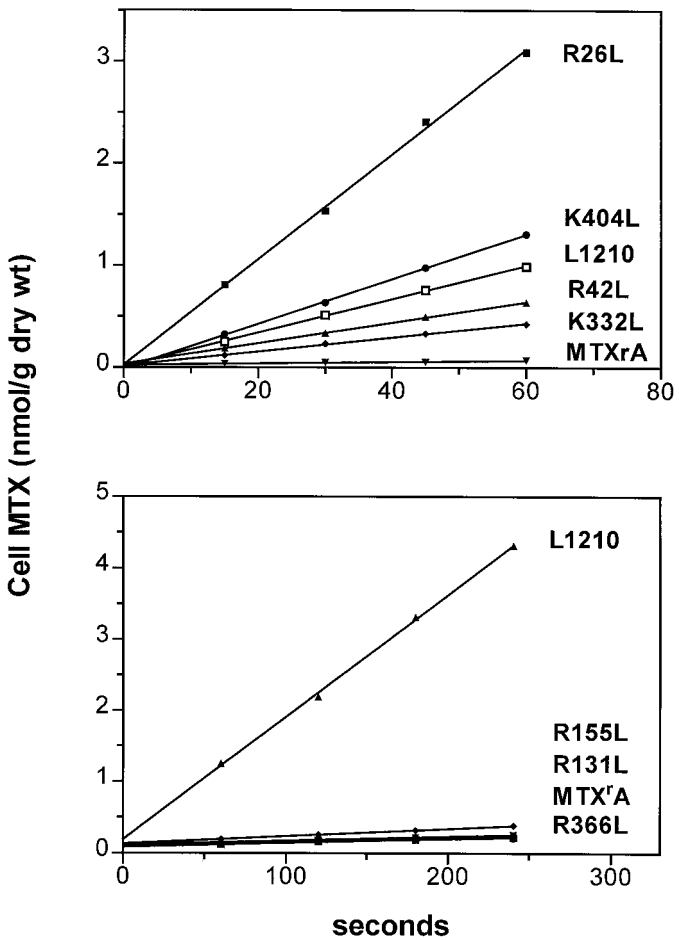

Fig. 3. MTX influx in L1210 cells and the MTX^{r}A-derived lines transfected with mutated carriers. After 25-min incubation in HBS buffer at 37°C, uptake was initiated by addition to the cell suspension of [^{3}H]MTX to achieve a final concentration of 1 μM. The results are representative of three experiments.

TABLE 3
Kinetic parameters for MTX influx
K_t and V_{max} values are the average ± SEM from three separate experiments determined from nonlinear regression to the Michaelis-Menten equation. Statistical analyses were performed using the Wilcoxon rank sum test, SAS system, version 6.12 (SAS Institute Inc., Cary, NC).

Cell line	K_t	V_{max}
	μM	*nmol/g of dry wt/min*
L1210	7.5 ± 1.1	10.3 ± 1.1
R26L	5.2 ± 0.4[a]	13.9 ± 1.6
R42L	8.3 ± 0.9	9.4 ± 3.0
K332L	10.6 ± 1.3[b]	8.2 ± 1.7
K404L	10.1 ± 0.4[a]	9.1 ± 1.0

[a] p = ~0.2 compared with L1210 cells.
[b] p = 0.08 compared with L1210 cells.

reduced folates studied was similar in the MTX^{r}A-R26L, MTX^{r}A-R42L, and MTX^{r}A-K332L lines with an IC_{50} value under these conditions 1.5- to 2.5-fold greater than that observed for inhibition of MTX influx in L1210 cells. However, inhibition of MTX influx in MTX^{r}A-K404L by the reduced folates was markedly decreased, with an IC_{50} value of more than 4 to 6 times that of L1210 cells. On the other hand, the range of inhibition of MTX influx by folic acid was much narrower among all the cell lines.

The transport properties of MTX^{r}A-K404L were assessed in more detail. As indicated in Fig. 5, although influx of MTX was comparable in L1210 and MTX^{r}A-K404L lines, influx of 5-formyl-THF and 5-methyl-THF was much slower than that of MTX. The apparent influx K_t values for the reduced folates were so high that accurate influx kinetics could not be obtained. Instead, K_i values were assessed based upon Dixon analyses of inhibition of MTX influx and compared with that of L1210 cells (Table 4). It can be seen that there were profound changes in influx K_i value. Although the K_i value for 5-methyl-THF was lower than that of 5-formyl-THF in L1210 cells, both were increased to the same level of ~30 μM in the MTX^{r}A-K404L line because of an 11- and 4-fold rise in K_i values, respectively. Hence, not only was the affinity of RFC1 for these reduced folates decreased, but also the difference in affinity observed for these two substrates in the wild-type carrier was eliminated in the K404L mutant. There was no significant change in the influx K_i value for folic acid.

The Impact of RFC1 Mutations on the Inhibitory Effects of Anions on MTX Influx. A variety of inorganic and organic anions inhibit RFC1-mediated transport and influx is stimulated when chloride is removed from the assay buffer (Goldman, 1971; Henderson and Zevely, 1983). Additional studies were undertaken to determine whether neutralization of the positive charge of amino acids in transmembrane domains might alter these ionic effects. The degree of inhibition of MTX influx by 10 mM ATP was not different among the transfectants compared with L1210 cells (Fig. 6, top). MTX influx was increased by 250% in anion-deficient HEPES in L1210 cells; influx in the MTX^{r}A-R26L, MTX^{r}A-R42L, and MTX^{r}A-K332L lines was increased ~200% under these conditions. However, MTX influx was increased by only ~25% in HEPES buffer in the MTX^{r}A-K404L line (Fig. 6, top). Thus, the K404L mutant carrier seems to be only minimally sensitive to the presence of chloride in the buffer.

The effects of chloride on MTX influx mediated by the K404L carrier was further explored over a broad range of chloride concentrations, as illustrated in Fig. 6, bottom. As the chloride concentration was progressively reduced from

140 to 0 mM, MTX influx in L1210 cells was increased. On the other hand, as the chloride level was decreased from 140 to 55 mM in the MTX^{r}A-K404L line, there was a gradual increase in MTX influx but to a much lesser extent (~30%) than in L1210 cells. Then, as the chloride concentration approached zero, influx in the mutant line decreased to a level ~50% of the maximal rate. Hence, elimination of the charge at position 404 markedly changed the sensitivity of RFC1 to chloride.

Discussion

For most facilitative carriers, charged amino acid residues within transmembrane domains can play a crucial role in substrate recognition, binding, and translocation across the cell membrane (Jarolim et al., 1995; Muller et al., 1996; Steiner-Mordoch et al., 1996; Fei et al., 1997; Kavanaugh et al., 1997; Merickel et al., 1997; Lanz and Erni, 1998), as well as in the maintenance of tertiary carrier structure (Kaback and Wu, 1997; Karbach et al., 1998). RFC1 contains 12 charged amino acid residues within its 12 predicted transmembrane domains. These residues include four arginines, two lysines, three histidines, two glutamates, and one aspartate. RFC1 has optimal activity at neutral pH; arginine and lysine are positively charged at neutral pH, and the histidine charge varies depending on the surrounding amino acid residues.

Based upon the protein sequence alignment of RFC1 cDNA from all species cloned to date (human, hamster, mouse, rat, and *C. elegans*), arginines R26, R42, R155, and R366, all of which reside in transmembrane domains, are fully conserved. Two of these, R26 and R155, are positioned in or are in immediate proximity to the origin of the predicted first and fifth transmembrane domains, respectively (Fig. 1). R131 is conserved in mammalian RFC1 but not in nematode, where it is replaced by the oppositely charged glutamate. However, its predicted location in the middle of the fourth transmembrane domain, in a highly conserved region, suggests functional importance. Neither K332 nor K404 are fully conserved; amino acid substitutions at corresponding positions in the nematode do not retain a positive charge because they are replaced with glutamine and aspartate, respectively. However, because these amino acids are invariant in all known mammalian RFC1s, their functional importance was studied.

No MTX transport activity at all was detected in three of the seven mutant lines: MTX^{r}A-R131L, MTX^{r}A-R155L, and MTX^{r}A-R366L. The presence of carrier protein within the cell membrane indicates that this was not caused by a failure of protein trafficking or insertion. Hence, these residues seem to be essential for folate substrate binding and/or translocation. This is consistent with the mutation and loss of function of these residues (R131H, R155Q, and R366H) under antifolate selective pressure with chemical mutagenesis (Zhao et al., 1999b). This loss of function may be caused by the loss of critical interactions between the carboxyl group of the folates and the positively charged arginine residues within the carrier (Gutknecht et al., 1998; Lampinen et al., 1998; Passoja et al., 1998). Substitution of leucine for arginine should not result in a major distortion of the α-helical structure. However, loss of intramolecular bonds created by oppositely charged amino acids within transmembrane domains could disrupt the tertiary structure and function of the carrier, as has been demonstrated for paired charged amino acid residues in transmembrane domains of the lactose permease and the vesicular monoamine transporter (VMAT2) (Kaback and Wu, 1997; Merickel et al., 1997). Pairs of charged amino acid residues could also participate directly in conformational changes associated with the translocation of substrates. A scenario in which arginine/glutamate and glutamate/histidine pair between helixes VIII and V has been proposed as the basis for the mechanism of energy coupling in lactose permease (Kaback and Wu, 1997).

Studies from our laboratory and others have identified mutations in cell lines under antifolate selection that markedly alter RFC1 transport activity and specificity. One cluster of mutations in the first transmembrane domain at amino

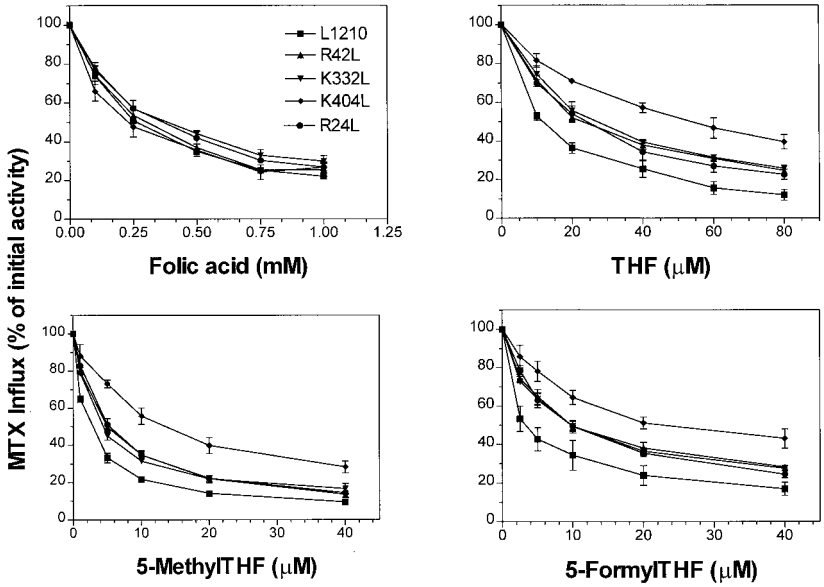

Fig. 4. The inhibitory effects of folates on influx of MTX in L1210 cells and cell lines transfected with mutated RFC1. MTX influx was determined as described in the legend to Fig. 3. The points are the average of three separate experiments ± SEM.

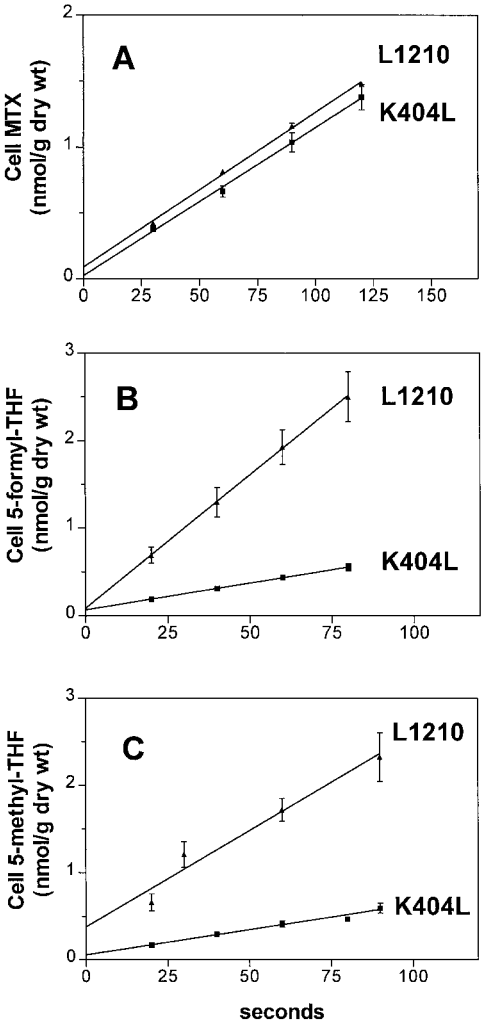

Fig. 5. Influx of MTX, 5-formyl-THF or 5-methyl-THF in L1210 cells and the MTX^{r}A-K404L transfectant. Uptake was determined as described in the legend to Fig. 3. The data is the mean ± SEM from three separate experiments.

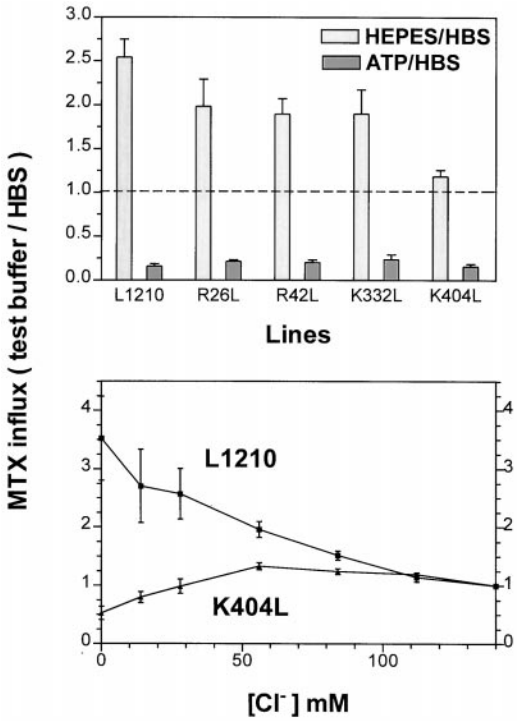

Fig. 6. The effect of addition of ATP or chloride removal on MTX influx in L1210 cells and the MTX^{r}A transfectants. Cells were incubated in HBS or Hepes buffers for 25 min after which [^{3}H]MTX was added and uptake was initiated; $[MTX]_e$ = 1 μM. Top, influx was assessed over a time course of 3 min in HBS, HBS with 10 mM ATP, or HEPES. Bottom, influx was assessed at 2 min in buffer containing a spectrum of chloride concentrations obtained by mixing HBS and Hepes-sucrose-MgO buffers. The data are the mean ± SEM from three separate experiments.

acids 45, 46, or 48 produces profound alterations in substrate binding and/or mobility of the carrier-substrate complex. Substitution of an asparagine for serine at amino acid 46 produced a substrate-specific change in the mobility of the carrier without any change in binding (Zhao et al., 1998b). A glutamate-to-lysine substitution at amino acid 45 resulted in a generalized decrease in carrier mobility, a substantial increase in folic acid binding, a lesser increase in 5-formyl-THF binding, and a marked fall in MTX binding to RFC1 (Zhao et al., 1998a). Substitution of isoleucine for phenylalanine at amino acid 48 produced a marked increase in RFC1 affinity for folic acid without significant change in MTX or dideaza-tetrahydrofolate transport (Tse et al., 1998). Interestingly, the loss of charge at position 42 located near these critical amino acids preserved some carrier function without a change in affinity for MTX (Table 3) and with a small, but comparable, loss of affinity for all the reduced folates (Fig. 4).

There were small and comparable decreases in affinities for folate substrates among all the mutated carriers except for the MTX^{r}A-K404L. In this line there was a marked fall in affinity for reduced folates, whereas the K_i value for folic acid was unchanged and there was only a small decrease in affinity for MTX (Tables 3 and 4). This discrimination between oxidized and reduced folates must be related to an adverse change in the binding pocket for the reduced folate cofactors that is influenced by the puckering of the pteridine moiety and/or possible changes in the *para*-aminobenzoic acid position that occur when the pteridine ring is reduced, as also happens in the interaction between reduced folates and dihydrofolate reductase (Bystroff et al., 1990; Reyes et al., 1995). For folic acid and MTX, the planar configuration of the pteridine ring in the oxidized state apparently preserves binding at the mutated sites. The observation that mutations involving the first, tenth, and eleventh transmembrane domains have qualitatively similar effects on binding of 5-formyl- and 5-methyl-THF and tetrahydrofolic acid suggests that all these residues in the wild-type carrier are required to create a conformation favorable for the reduced folate molecules.

Also unique to the K404L carrier was loss of the major portion of the inhibitory effect of chloride on MTX influx that

TABLE 4

Comparison of the influx K_i values for different folate substrates between L1210 and MTX^{r}A-K404L lines

Data are the mean ± SEM from three experiments determined from Dixon analyses at 1 and 2 μM $[MTX]_e$.

Substrate	L1210	K404L	Fold difference
	μM		
5-Methyl-THF	2.83 ± 0.61	29.68 ± 7.53	10.9
5-Formyl-THF	7.33 ± 2.03	29.26 ± 4.63	4.1
Folic acid	186.7 ± 31.8	206.7 ± 29.6	1.1

has been well documented for the wild-type carrier (Goldman, 1971; Henderson and Zevely, 1983; Yang et al., 1984). This raises the possibility that the interaction between the arginine 404 residue and chloride in the wild-type carrier may be involved in the usual suppression of folate binding by this anion and that the preservation of function in this mutant is caused, in part, by the loss of chloride inhibition. Interestingly, another mutation that alters the interaction between chloride and RFC1, E45K in the first transmembrane domain, has been reported for both human and mouse RFC1 (Jansen et al., 1998; Zhao et al., 1998a). For this mutant carrier, influx *falls* in the absence of chloride, but as chloride is added back to the buffer, influx *increases*—changes that are caused by an alteration in the mobility of the carrier. In the case of the E45K mutant, the stimulatory effect of chloride is attributed to neutralization of the substituted positively charged residue, permitting partial restoration of carrier mobility—an effect that was also reproduced with other, small inorganic anions (Zhao et al., 1998a). The observation that the inhibitory effect of ATP was the same in all the mutant carriers and the wild-type carrier raises the possibility that RFC1 may possess distinct binding sites for chloride and ATP (and possibly other organic anions).

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