Functional interactions between arginine-133 and aspartate-88 in the human reduced folate carrier: evidence for a charge-pair association

Xiang Y. LIU* and Larry H. MATHERLY*†‡¹

*Cancer Biology Graduate Program, Wayne State University School of Medicine, 110 E. Warren Avenue, Detroit, MI 48201, U.S.A., †Experimental and Clinical Therapeutics Program, Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, 110 E. Warren Avenue, Detroit, MI 48201, U.S.A., and ‡Department of Pharmacology, Wayne State University School of Medicine, 110 E. Warren Avenue, Detroit, MI 48201, U.S.A.

The human reduced folate carrier (hRFC) is an integral membrane protein that mediates cellular uptake of reduced folates and antifolates. hRFC contains several highly conserved charged residues predicted to lie in the transmembrane domains (TMDs). To explore the possible roles of the conserved arginine-133, located in TMD 4, in hRFC structure and function, this residue was systematically mutagenized to histidine, leucine, lysine and glutamate. When transfected into transport-impaired K562 cells, the mutant hRFC constructs were expressed at high levels; however, only lysine-133 hRFC was able to transport methotrexate and (6S)-5-formyl tetrahydrofolate. Substitution of aspartate-453 (in hRFC TMD 12) by valine largely preserved transport activity for both substrates. Although mutagenesis of aspartate-88 (in TMD 2) to leucine completely abolished trans-

port activity in transfected cells, substitution with a glutamate preserved low levels ($\approx 12\%$) of transport. To assess the possibility that arginine-133 and aspartate-88 may form a charge-pair to stabilize hRFC tertiary structure, both charges were neutralized (by substituting leucine and valine, respectively) in the same construct. In contrast to the singly mutated hRFCs, the double mutant exhibited high levels of transport with both methotrexate and 5-formyl tetrahydrofolate. These results strongly suggest that arginine-133 and aspartate-88 form a charge-pair and that TMD 4 lies next to TMD 2 in the hRFC tertiary structure.

Key words: methotrexate, mutagenesis, tetrahydrofolate.

INTRODUCTION

Folates are essential cofactors required for the biosynthesis of purine nucleotides, thymidylate, serine and methionine, for which they act as the one-carbon carriers [1]. There is growing evidence that folate deficiency contributes to chromosomal instability, malignant transformation [2] and fetal abnormalities [3], and that it also plays an important role in the development of cardiovascular disease [4]. Unlike bacteria, eukaryotic cells cannot synthesize folates and therefore must obtain these cofactors from extracellular sources. Absorption of dietary folates occurs primarily in the small intestine, from which they enter the blood stream. Reflecting their hydrophilic dianionic character and low capacities for passive diffusion across biological membranes, sophisticated uptake systems have evolved for the uptake of the folates into mammalian cells [5,6]. From the low level of plasma 5-methyl tetrahydrofolate ($H_4PteGlu$) presented *in vivo*, efficient transport through the plasma membrane becomes critical for cell proliferation. Membrane transport is also an important determinant of anti-tumour activity of antifolate therapeutics [methotrexate (MTX); Tomudex] used in cancer chemotherapy, and impaired cellular uptake is a frequent mode of drug resistance [7–9].

The reduced folate carrier (RFC) is an integral plasma-membrane protein that provides the majority of transmembrane transport of reduced folates and classical antifolates in mammalian cells [6–9]. RFC cDNAs have been cloned from mouse [10], hamster [11] and human [12–15] sources. Hydropathy

analysis of the predicted amino acid sequence of the human RFC (hRFC) conforms to a model expected for an integral membrane protein with up to 12 stretches of 17–25, mostly hydrophobic, α -helix-promoting amino acids, an internally oriented C-terminus and a single outwardly facing N-glycosylation consensus site at position 58 (Figure 1A). Portions of this topological model have been confirmed experimentally [16,17]. hRFC shares 64–66% homology with the rodent carriers [6,12]. There is a high degree of sequence conservation of the primary amino acids in several of the putative transmembrane domains (TMDs), including TMDs 1–5, 7 and 8, and there is nearly a complete lack of homology in the large central loop connecting TMDs 6 and 7, and in the N- and C-terminal regions (Figure 1). Furthermore, hRFC contains 72–79 more amino acids than the homologous rodent proteins.

The characterization of the molecular alterations in RFC accompanying resistance has begun to shed light on the amino acid residues or domains that are critically important to transport substrate binding and/or membrane translocation. Most of these studies have focused on the murine RFC. Thus conserved amino acids located in hRFC TMD 1 (glycine-44 [19], glutamate-45 [20,21], serine-46 [22] and isoleucine-48 [23]), TMD 4 (alanine-132 [24] and serine-127 [19]) and TMD 8 (serine-313 [25]) have all been implicated as important to carrier function. From the anionic character of folate substrates and the role of the 4-amino moiety in substrate binding to RFC, it seems likely that charged or otherwise electron-rich amino acids in the TMDs are, likewise, important. By analogy with other membrane proteins [26–28],

Abbreviations used: RFC, reduced folate carrier; MTX, methotrexate; hRFC, human RFC; $H_4PteGlu$, tetrahydrofolate; TMD, transmembrane domain; 5-CHO- $H_4PteGlu$, (6S)-5-formyl tetrahydrofolate.

¹ To whom correspondence should be addressed, at the Experimental and Clinical Therapeutics Program (e-mail matherly@kci.wayne.edu).

A **B**

Human	71	VLSYSYLAVLVPVFLLT <u>D</u> YL	
Mouse		M.P..H.....	TMD2
Hamster		..P..H.....I.....	
Rat		-MP..H.....I.....	
Human	124	LFYSVTMAAR <u>I</u> AYSSYIFSLV	
Mouse		V.....	TMD4
Hamster		V.....	
Rat		V...I.....	
Human	437	VYFLILSIIYFLGAML <u>D</u> GL	
Mouse		I...M...TC.AW.G....	TMD12
Hamster		...MV..V.C.V..V...V	
Rat		M...T....CLAW.G....	

Figure 1 Topological structure of the hRFC

(A) A topological model for hRFC (using the TMPRED program, see [18]) is shown depicting 12 TMDs, internally oriented N- and C-termini, and an externally oriented N-glycosylation site at asparagine 58. The numbers (1–12) in the lower bar designate the TMDs and the black circles indicate residues that are completely conserved among species including human, mouse, hamster and rat. The locations of the conserved arginine-133, aspartate-88 and aspartate-453 residues are also noted. (B) Sequence homologies for TMDs 2, 4 and 12 between the human and rodent RFCs. V71, L124 and V437 are indicated. D88, R133 and D453 are shown underlined in bold. The dots designate conserved residues between species.

interactions between charged residues localized within hydrophobic domains can also serve to stabilize protein conformation.

In this report, we directly consider these possibilities by exploring the functional and structural roles of highly conserved arginine (position 133) and aspartate (positions 88 and 453) residues in hRFC (located in TMDs 4, 2 and 12 respectively; Figure 1). By systematic site-directed mutagenesis, we show that arginine-133 and aspartate-88 probably interact to form a charge-pair that contributes to conformational stability required for transport activity.

MATERIALS AND METHODS

Reagents

3',5',7-³H-Labelled (20 Ci/mmol) MTX and 3',5',7,9-³H-labelled (17 Ci/mmol) (6S)-5-formyl tetrahydrofolate (5-CHO-H₄PteGlu) were purchased from Moravsek Biochemicals (Brea, CA, U.S.A.). Unlabelled MTX and (6R,S)-5-CHO-H₄PteGlu were provided by the Drug Development Branch, National Cancer Institute, Bethesda, MD, U.S.A. Both labelled and unlabelled MTX were purified by HPLC prior to use [29]. Restriction

Table 1 PCR primers for preparing hRFC mutant constructs

Mutation	Forward mutation primer	Reverse mutation primer
D88V	5'-GTGTTCTGCTACCGTCTACCTGCGCTACACG-3'	5'-CGTGTAGCGCAGGTGACGGTGAGCAGGAACAC-3'
D88E	5'-GTGTTCTGCTACCGAGTACCTGCGCTACACG-3'	5'-CGTGTAGCGCAGGTACTCGGTGAGCAGGAACAC-3'
R133E	5'-GTCACCATGGCCGCGGAGATCGCCTATTCCTCC-3'	5'-GGAGGAATAGGCGATCTCCGCGGCCATGGTGAC-3'
R133H	5'-GTCACCATGGCCGCGGACATCGCCTATTCCTCC-3'	5'-GGAGGAATAGGCGATGTGCGCGGCCATGGTGAC-3'
R133K	5'-GTCACCATGGCCGCGAAGATCGCCTATTCCTCC-3'	5'-GGAGGAATAGGCGATCTTCGCGGCCATGGTGAC-3'
R133L	5'-GTCACCATGGCCGCGGCTCATCGCCTATTCCTCC-3'	5'-GGAGGAATAGGCGATGAGCGCGGCCATGGTGAC-3'
D453V	5'-CGTCACGGCGTCAGGTTGGTGTACCGGGGGTT-3'	5'-AACCCCGGTACGACCAACCTGACGCCGTGACG-3'

and modifying enzymes were obtained from Promega (Madison, WI, U.S.A.) or Roche (Indianapolis, IN, U.S.A.). Synthetic oligonucleotides were obtained from Genosys Biotechnologies (The Woodlands, TX, U.S.A.). Tissue-culture reagents and supplies were purchased from assorted vendors with the exception of iron-supplemented calf serum, which was from Hyclone Laboratories (Logan, UT, U.S.A.).

Cell culture

The transport-deficient K500E sub-line was selected from wild-type K562 cells as described previously [30]. Cells were maintained in complete medium containing RPMI 1640 medium, 10% iron-supplemented calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin, in a humidified atmosphere at 37 °C in the presence of 5% CO₂. Cells were maintained routinely in 0.5 µM MTX; however, prior to transport assays, MTX was removed from the medium. The K43-6 cell line was derived from K500E cells by transfection with full-length wild-type hRFC cDNA in pCDNA3 expression vector [30]. All the hRFC transfectants including K43-6 were cultured in complete medium in the presence of 1 mg/ml G418.

Mutagenesis and stable transfections

Site-directed mutagenesis with 'SOE' (splicing by overlap extension) PCR [31] was used to generate all mutations, using the complementary mutagenesis primers shown in Table 1. To generate mutations of residues 88 and 133, two PCR steps were performed to amplify two separate products, using the appropriate forward mutation primer (see Table 1) and the rHA-7 primer (5'-CTCGTTCCACAGGATGTGCAC-3'; positions 882–862 relative to the start codon ATG, where A is number 1), or the appropriate reverse mutation primer (see Table 1) and p8 primer (5'-CAGTGTCACCTTCGTCCCTCCG-3'; positions –46 to –24). PCR conditions for the primary PCR were 94 °C for 30 s, 56 °C for 30 s and 72 °C for 60 s. Secondary PCR was performed with the p8/rHA-7 primers and primary PCR products as template, using the same conditions as for the primary PCR.

The same strategy and PCR conditions were used to generate the D453V mutation but with different sets of primers. KS2 (5'-CGCAGCCTCTTCTTCAACCGC-3'; positions 622–642) and RFCout12 (5'-GACAACCCCTTCCCCCTGCACTCTGT-3'; positions 1647–1619) were used with the corresponding reverse and forward mutation primers, respectively, for the primary PCR (Table 1). Secondary PCR was performed with KS2/RFCout12. For subcloning of the R133 and D88 mutant constructs, the secondary PCR products were digested with *Bbr*PI and *Not*I, purified and ligated into the *Bbr*PI- and *Not*I-digested hRFC cDNA in pCDNA3 (designated pC43) for transformation. *Not*I and *Sfi*I restriction sites were used to

subclone the D453V mutant construct. All mutant hRFC constructs were confirmed by automated sequencing.

The mutant hRFC cDNAs were transfected into transport-impaired K500E cells with lipofectin as described previously [30]. Cells were cloned in 0.35% Noble agar in complete medium and clones resistant to G418 (1 mg/ml) were isolated and expanded for further analysis.

Western-blot analysis of mutant hRFC transfectants

Plasma membranes were prepared by differential centrifugation as described earlier [32]. Membrane proteins were electrophoresed on 7.5% polyacrylamide gels in the presence of SDS and electroblotted on to PVDF membranes (DuPont). Immunoreactive hRFC proteins were detected by enhanced chemiluminescence (Roche) with protein A-purified hRFC-specific antibody generated in rabbits with a glutathione S-transferase–hRFC fusion protein [19]. Densitometry was performed on a Kodak Digital Science Image Station 440CF.

Transport assay of mutant hRFC constructs

Initial rates of [³H]MTX (0.5 µM) or [³H]5-CHO-H₄PteGlu (1 µM) uptake were measured over 180 s as described previously [12,30]. Levels of intracellular radioactivity were expressed in terms of pmol/mg of protein, calculated from direct measurements of radioactivity and protein contents of the cell homogenates. Protein assays were by the method of Lowry et al. [33]. Kinetic constants ($V_{\max}$, K_i and K_t ; where K_t is the K_m for the transporter) were calculated from Lineweaver-Burk and Dixon plots with [³H]MTX as the transport substrate.

RESULTS AND DISCUSSION

An analysis of the membrane topology model and sequence homologies between species for hRFC (Figure 1) permits the identification of amino acids that may be important for substrate binding and/or for maintaining proper carrier conformation within the phospholipid bilayer for optimal transport. Given the energetic unfavourability associated with uncompensated charged amino acids located in the TMDs, these residues are of particular interest. However, should there be a salt bridge between residues of opposite charge, TMD-localized charged residues can be stabilized substantially and participate in substrate binding or energy coupling for transport, or serve to orient adjacent TMDs for membrane insertion and/or transport function [26–28,34].

Site-directed mutagenesis of arginine-133 hRFC

By random mutagenesis with *N*-methyl-*N*-nitrosourea, Zhao et al. [25] reported that three of the four conserved arginines in the murine RFC located within the TMDs (positions 131, 155 and

Figure 2 Western blot of wild-type and mutant hRFCs expressed in K500E cells

Data are shown for a Western blot of plasma-membrane proteins from K562 (K500E) transfectants expressing wild-type hRFC (K43-6) and mutant hRFCs including D88E, D88V, R133E, R133H, R133K, R133L, R133L/D88V and D453V (the latter was run on a separate gel from the remainder of the mutant constructs). Proteins (20 μ g) were fractionated on a 7.5% gel in the presence of SDS and electroblotted on to a PVDF membrane. Detection was with anti-hRFC antibody and an enhanced chemiluminescence kit (Roche). Molecular-mass standards (in kDa) are shown. The relative levels of hRFC protein by densitometry are noted.

366; positions 133, 157 and 374 in hRFC) were mutated, resulting in a loss of transport activity. Arginine-131 (arginine-133 in hRFC), located in TMD 4 (Figure 1), was of particular interest since its replacement with a basic histidine residue was non-functional [25].

On this basis, we systematically mutagenized arginine-133 to histidine, lysine, leucine and glutamate to better assess the role of this residue in the maintenance of hRFC structure and transport function. The mutated constructs (designated R133H-, R133K-, R133L- and R133E-hRFCs, respectively) were transfected into transport-impaired K562 (K500E) cells, characterized by nearly undetectable levels of hRFC transcripts and protein [30]. G418-resistant clones were isolated and expanded, and plasma-membrane proteins prepared, followed by screening for protein expression on Western blots with hRFC-specific antibody, and for transport activity with [3 H]MTX and [3 H]5-CHO- H_4 PteGlu.

A representative Western blot for selected clonal isolates expressing the mutated hRFCs is shown in Figure 2 for comparison with K43-6 transfectants, characterized by high levels of the wild-type R133 hRFC ($\approx$ 20-fold over parental cells [30]). Similar and high levels of immunoreactive hRFC proteins were detected for all the transfected lines (Figure 2), indicating that the mutant constructs were effectively targeted for plasma-membrane insertion.

By transport assay with [3 H]MTX or [3 H]5-CHO- H_4 PteGlu, transport activity was preserved only for the R133K-hRFC construct (Figure 3). As with the murine RFC, R133H-hRFC was completely inactive, even though its level of plasma-membrane expression was $\approx$ 4-fold higher than the R133K protein and $\approx$ 2-fold higher than the wild-type R133-hRFC (in K43-6 cells; Figure 2). Other R133 mutations causing a loss of charge character (R133E, R133L) also resulted in an essentially complete loss of transport function (Figure 3). When normalized for hRFC expression (Figure 2), the initial rate of influx for MTX for R133K-hRFC was $\approx$ 85% of that for R133-hRFC expressed in K43-6 cells; uptake of [3 H]5-CHO- H_4 PteGlu for R133K-

Figure 3 Rates of [3 H]MTX and [3 H]5-CHO- H_4 PteGlu uptake in K562 transfectants expressing wild-type and mutant hRFCs

[3 H]MTX and [3 H]5-CHO- H_4 PteGlu (LCV) uptake was measured for 180 s at 37 $^{\circ}$ C, as described previously [12,30]. All transport results are means $\pm$ S.E.M. from three or four separate experiments.

hRFC was identical to wild-type levels. While the kinetic constants for MTX transport were similar between the K43-6 sub-line and the R133K-hRFC transfected cells (Table 2), there were slight differences in the K_i values between R133K-hRFC ($2.03 \pm 0.08 \mu$ M) and the wild-type carrier ($6.95 \pm 1.89 \mu$ M; $P = 0.0048$). These results establish an important structural and/or functional role for a strongly basic amino acid at position 133 of hRFC.

Identification of a charge-pair involving arginine-133 and aspartate-88 in hRFC

The formation of a charged pair between arginine-133 and either of the conserved aspartates in hRFC (aspartates-88 and -453, localized in putative TMDs 2 and 12, respectively; Figure 1) could potentially stabilize the strongly basic amino acid at position 133 and, likewise, facilitate the optimal packing of the hRFC TMDs within the plasma membrane, similar to that reported for the yeast plasma-membrane H^+ -ATPase [35], the vesicular monoamine transporter [27] and the bacterial lactose permease [36]. Both D88V- and D453V-hRFCs were expressed in K500E cells (Figure 2). However, only for aspartate-88 was preservation of a negative charge essential for maintenance of transport activity since D88V-hRFC was completely inactive, whereas D453V-hRFC was functional for both MTX and 5-CHO- H_4 PteGlu transport (Figure 3). For the former, transport could be partially preserved by substituting a glutamate for aspartate (i.e. D88E-hRFC; Figure 3). When rates of MTX and 5-CHO- H_4 PteGlu influx were normalized to levels of hRFC

Table 2 Kinetic constants for MTX transport by hRFC wild-type and mutant constructs

Kinetic constants for MTX were determined from Lineweaver–Burk plots for the transfectants expressing the wild-type arginine-133 (K43-6), lysine-133 (R133K), glutamate-88 (D88E) and double mutant leucine-133/valine-88 (R133L/D88V) hRFC proteins, as described in the text. The normalized $V_{\max}$ and $V_{\max}/K_t$ values are also shown, normalized for the hRFC expression levels on Western blots (Figure 2). The data shown are means $\pm$ S.E.M. from three–five independent experiments.

Cell line	$V_{\max}$ (pmol/mg per 3 min)	$V_{\max}$ (normalized)	K_t (μ M)	$V_{\max}/K_t$	$V_{\max}/K_t$ (normalized)
K43-6	20.1 $\pm$ 1.22	20.1	3.15 $\pm$ 0.86	8.17 $\pm$ 3.12	8.17
R133K	7.93 $\pm$ 0.57	15.86	3.87 $\pm$ 0.96	2.21 $\pm$ 0.34	4.42
D88E	5.72 $\pm$ 0.21	3.17	2.24 $\pm$ 0.3	2.06 $\pm$ 0.24	1.14
R133L/D88V	13.03 $\pm$ 0.81	13.03	3.05 $\pm$ 0.76	4.89 $\pm$ 1.26	4.89

expression on Western blots (Figure 2), D88E-hRFC was only $\approx$ 12% as efficient as the wild-type protein. Thus the increased size of glutamate over aspartate ($\approx$ 1.5 Å), resulting from the presence of an additional methylene group, impedes hRFC transport function, suggesting that both spatial and charge considerations are important at this position. Interestingly, glutamate substitution at position 88 affects the rate of carrier translocation (i.e. $V_{\max}$) rather the substrate binding, since K_t values were nearly identical for D88E- and wild-type hRFCs (Table 2).

Of particular interest was the effect on transport activity of *simultaneously* neutralizing the charges at positions 133 and 88 in the *same* hRFC construct (i.e. R133L/D88V-hRFC). Whereas the singly mutated R133L- and D88V-hRFCs were incapable of restoring transport with both MTX and 5-CHO- H_4 PteGlu, for the doubly mutated R133L/D88V-hRFC, transport was restored to levels approaching those for the wild-type (R133/D88) hRFC. Accompanying the expression of nearly identical levels of immunoreactive hRFC protein (Figure 2), $\approx$ 80% of wild-type transport activity was restored for the doubly mutated hRFC. This was reflected in similar kinetic constants for MTX for wild-type and R133L/D88V-hRFCs (Table 2) and a similar K_t for 5-CHO- H_4 PteGlu ($4.45 \pm 0.26 \mu$ M).

These data strongly suggest that arginine-133 and aspartate-88 form a charge-pair complex that stabilizes hRFC tertiary structure. By this reasoning, a single neutral substitution is by itself incapable of restoring transport activity since this leaves an uncompensated charge that alters protein conformation.

Conclusions

In conclusion, our results strongly suggest that both arginine-133 and aspartate-88 play important roles in stabilizing the association between TMDs 2 and 4 in the hRFC tertiary structure through the formation of a charge-pair association. Disruption of the charge-pair by replacing either arginine-133 or aspartate-88, individually, with a neutral amino acid (i.e. leucine or valine, respectively) resulted in an unstable, unpaired charge in a hydrophobic TMD milieu, and was accompanied by a complete loss of hRFC transport activity. However, simultaneous neutralization of both charges resulted in the nearly complete preservation of transport of both MTX and 5-CHO- H_4 PteGlu. Although these findings suggest that neither arginine-133 nor aspartate-88 is likely to directly participate in the binding of charged transport substrates, an indirect involvement for position 133 was nonetheless inferred, since substitution of a lysine at this position slightly increased binding of 5-CHO- H_4 PteGlu over the wild-type carrier.

Our results provide the first insights into the tertiary structure of hRFC. Future studies will focus on further identifying the essential structural and functional features of this critical trans-

port system, a prerequisite to understanding the molecular mechanism of folate and antifolate membrane transport.

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