Restoration of high-level transport activity by human reduced folate carrier/ThTr1 thiamine transporter chimaeras: role of the transmembrane domain 6/7 linker region in reduced folate carrier function

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The reduced folate carrier (RFC; SLC19A1) is closely related to the thiamine transporter, SLC19A2 (ThTr1). Hydropathy models for these homologous transporters predict up to 12 transmembrane domains (TMDs), with internally oriented N- and C-termini and a large central loop between TMDs 6 and 7. The homologies are localized mostly in the TMDs. However, there is little similarity in their N- and C-terminal domains and the central peptide linkers connecting putative TMDs 1–6 and TMDs 7–12. To explore the functional role of the 61-amino acid central linker in the human RFC (hRFC), we introduced deletions of 49 and 60 amino acids into this region, differing by the presence of a stretch of 11 highly conserved amino acids between the human and rodent RFCs (positions 204–214). An additional hRFC construct was prepared in which only the 11 conserved amino acids were deleted. The resulting hRFC^{D215–R263Δ}, hRFC^{K204–R263Δ} and hRFC^{K204–R214Δ} proteins were transfected into transport-impaired K562 cells. The deletion constructs were all expressed

in plasma membranes; however, they were completely inactive for methotrexate and (6S)5-formyl tetrahydrofolate transport. Insertion of non-homologous 73- and 84-amino acid fragments from the structurally analogous ThTr1 linker region into position 204 of hRFC^{K204–R263Δ} restored low levels of transport (16–21 % of the wild type). Insertion of the ThTr1 linkers into hRFC^{D215–R263Δ} at position 215 restored 60–80 % of wild-type levels of transport. Collectively, our results suggest that the role of the hRFC linker peptide is to provide the proper spatial orientation between the two halves of the hRFC protein for optimal function, and that this is largely independent of amino acid sequence. Our results also demonstrate a critical transport role for the stretch of 11 conserved amino acids starting at position 204 of hRFC.

Key words: methotrexate, mutagenesis, thiamine transporter.

INTRODUCTION

The reduced folate carrier (RFC; SLC19A1) is the major mechanism of membrane transport for folate cofactors in mammalian cells [1,2]. RFC is also an important determinant of anti-tumour activity of antifolate therapeutics [methotrexate (MTX) and Tomudex] used in cancer chemotherapy, and the loss of expression or impaired function of RFC protein is associated with drug resistance [2,3].

RFC cDNAs were originally cloned from mouse [4] and hamster [5] cells in 1994, and from human cells in 1995 [6–9]. Shortly thereafter, human RFC (hRFC) homologues were described (SLC19A2 and SLC19A3; 40 and 39 % amino acid identities, respectively, to hRFC) [10–15]. In spite of their remarkable structural similarities, there appears to be no functional overlap between hRFC and the SLC19A2 and SLC19A3 proteins, in that the preferred substrate for the SLC19A2 and SLC19A3 is thiamine and not folates [11,16,17]. Accordingly, these thiamine transporters are designated ThTr1 and ThTr2, respectively.

Hydropathy analysis for all these homologous transporters predicts up to 12 transmembrane domains (TMDs) composed of stretches of mostly hydrophobic, α -helix-promoting amino acids, internally oriented N- and C-terminal domains, and a large central linker connecting putative TMDs 1–6 and 7–12 (Figure 1A). Portions of this topological model have been experimentally confirmed [18,19]. The homologies are localized mostly in the

TMDs; however, there is little similarity in either the N- or C-terminal regions, or in the peptide linkers between TMDs 6 and 7. For RFCs from different species, this homology for the TMD6/7 linker is limited to a stretch of 11 highly conserved amino acids starting at position 204 of hRFC. Only four of these residues are conserved in ThTr1 and ThTr2.

In this report, we explore the structural and functional roles of the 61-amino acid peptide linker connecting the N- and C-terminal halves of hRFC. We describe herein results with deletion-mutant hRFC constructs in which portions of the peptide linker have been either removed or replaced with the structurally analogous domain of ThTr1. Our results strongly suggest that the role of the hRFC linker peptide is simply to provide the proper spatial orientation between the two halves of the carrier for optimal transport function and that, outside of 11 conserved amino acids beginning at position 204, this is independent of sequence. Our results also suggest a critical transport role for the 204–214 peptide of hRFC.

MATERIALS AND METHODS

Reagents

[3',5',7-³H]MTX (20 Ci/mmol) and [3',5',7,9-³H](6S)5-formyl tetrahydrofolate (5-CHO-H₄PteGlu; 17 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA, U.S.A.). Unlabelled MTX and (6R,S)5-CHO-H₄PteGlu were provided

Abbreviations used: 5-CHO-H₄PteGlu, (6S)5-formyl tetrahydrofolate; MTX, methotrexate; RFC, reduced folate carrier; hRFC, human RFC; TMD, transmembrane domain.

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Figure 1 Schematic diagram of hRFC topology and hRFC/ThTr1 deletion and chimaeric constructs

(A) Depicts a computer predicted topology model for hRFC with putative 12 TMDs (numbered 1–12), internally oriented N- and C-termini, and an externally oriented N-glycosylation site at Asn-58. The solid circles designate positions of key amino acids in the deletion constructs, as described in the text. Portions of this model have been experimentally confirmed [18,19]. In (B), the deletion constructs (hRFC^{D215-R263Δ}, hRFC^{K204-R263Δ} and hRFC^{K204-R214Δ}) and hRFC-ThTr1 chimaeric constructs (hRFC^{K204-R263Δ}+TT1, hRFC^{D215-R263Δ}+TT1, hRFC^{K204-R263Δ}+TT2 and hRFC^{D215-R263Δ}+TT2) are depicted, as described in the text. Shaded circles indicate the TT1 and TT2 fragments (see Figure 2) from the ThTr1 transporter. Two extra amino acids (Arg and Pro) that were added to the chimaeric protein from cloning are also indicated.

A

Human	204	<u>KRPKRS</u> LFFNRDDRGRCETSASELERMNP GPG---GKLGHALRVACGDSVLARMLRELGD SLRR	264
Mouse	202	STAL.-GALPC..DQ.H...DRPETR..DRM.-GT.R..F.V...S..VENA.Q	264
Hamster	204	H.....SALVH-KALPC..DQ.H...RPEP...ERV.-GS.RN.F.VC...S..VGN..Q	266
Rat	201	R.....SALVQ-GALPC..DQ.H...RPEPR..ERM.-GT.RD.F.V...S..VKNV.Q	263

B

hRFC	204	KRPKRS	LFFNRDDRGRCETSASELERMNP	G-----PG-----GKLGHALR---VA	245	
ThT1	209	PMPQKS	LFFHHIP-STCQR-VNGIKVQNGGIVTDTPASNHLPGWEDIESKIPLNMEEP	PVE	267	
		TT1				
		TT2				
hRFC	246	CGDSVLARM--LRELGD	SL-----RRPQLRLW	SLWVFN	SAGYYLVVYYVHILWNEVDPT	298
ThT1	268	EPEPKPDRLLVLKVLWNDFLMCYSSR	PLL-CWSV	WWALSTCGYFQVVNYTQGLWEK	VMPS	326
		TT1				
		TT2				

Figure 2 Homology comparison of the central linkers from the human and rodent RFCs and ThTr1

In (A) is shown a comparison of homologies for the TMD6/TMD7 linker region for the human, mouse, hamster and rat RFCs. The conserved 11 amino acid sequence in position 204–214 of hRFC is underlined. In (B) is shown a comparison of the TMD6/TMD7 linkers between hRFC and the human ThTr1. Also shown are the TT1 and TT2 peptides from ThTr1 used to construct the chimaeric hRFC/ThTr1 transporters, as described in the text. The putative TMD7 for hRFC and ThTr1 is shown in italics.

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 [20]. Restriction and modifying enzymes were obtained from Promega (Madison, WI, U.S.A.) or Roche (Indianapolis, IN, U.S.A.). Synthetic oligonucleotides were obtained from Invitrogen (Carlsbad, CA, 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 MTX-transport-deficient K500E subline was selected from wild-type K562 cells (American Type Culture Collection) and maintained in complete RPMI 1640 medium (Life Technologies) containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.5 µM MTX [21,22]. K500E cells were transfected with the wild-type (KS43) hRFC in pCDNA3 (designated pC43) to generate the K43-6 subline [21]. These cells and all other mutant hRFC transfectants (see below) were cultured in complete RPMI 1640 medium plus 1 mg/ml G418 in a humidified atmosphere at 37 °C in the presence of 5% CO₂.

hRFC deletion constructs and hRFC-ThTr1 chimaeric constructs

To generate deletions of 49 and 60 amino acids in the 61-amino acid linker (residues Lys-204–Arg-264) of hRFC, two deletion constructs were generated by PCR using the pC43 wild-type hRFC construct as a template and the K204-R263Δ (5'-AAGGAAAAAAGCGGCCGAGGAAGAGGGCGAGGACCA-C-3') and D215-R263Δ (5'-AAGGAAAAAAGCGGCCGAGGCGGTTGAAGAAGAGGCTGCG-3') antisense primers (the *NotI* restriction site is underlined), each with the P8 sense primer

(5'-CAGTGTCACCTTCGTCCCCTCCG-3'; positions -46 to -24). PCR conditions were 94 °C for 30 s, 56 °C for 30 s and 72 °C for 60 s for 35 cycles. The amplicons were digested with *BbrPI* and *NotI* and subcloned into the pC43 vector, digested with the same enzymes.

Deletion of the 11 conserved amino acids (from Lys-204 to Arg-214) was generated by the splice-overlap-extension ('SOE') PCR method, as described previously [23,24]. Primary PCRs used the S11del forward deletion primer (5'-CTCGCCCTCTT-CCTGGACGACCGGGGACGGTGCGAA-3'), with the rHA7 primer (5'-CTCGTTCCACAGGATGTGCAC-3'; positions 882–862), and the AS11del reverse deletion primer (5'-CCG-TCCCCGGTCGTCCAGGAAGAGGGCGAGGACCAC-3') with the P8 primer. PCR conditions were 94 °C for 30 s, 56 °C for 30 s and 72 °C for 60 s for 35 cycles. Secondary PCR was performed with the rHA7/P8 primers and primary PCR products as templates, using the same conditions as for the primary PCRs. The secondary PCR products were digested with *BbrPI* and *NotI*, purified, and ligated into *BbrPI/NotI*-digested pC43 vector.

To prepare the hRFC-ThTr1 chimaeric constructs, two DNA fragments (TT1 and TT2; see Figure 2) were amplified from the ThTr1 cDNA (provided by Ellis Neufeld, Boston, MA, U.S.A.) with the tt1 (5'-AAGGAAAAAAGCGGCCGCTATGCC-ACAGAAGAGCCTCTTC-3') and tt2 (5'-AAGGAAAAAAGCGGCCGCTTCTACCTGCCAGAGAGTGAAT-3') sense primers, each with same *rett* antisense primer (5'-AAGGAAAAAAGCGGCCGAGGCGAGAGGAGTAGCACATC-3'); *NotI* sites are underlined. The amplicons were digested with *NotI* and subcloned into *NotI*-digested hRFC^{K204-R263Δ} or hRFC^{D215-R263Δ} constructs in pCDNA3.

The mutant hRFC constructs were transfected into transport-impaired K500E cells with lipofectin, as described previously [21]. Cells were cloned in 0.35% noble agar in complete medium

with G418 (1 mg/ml) and G418-resistant clones isolated and expanded for further analysis.

Western analysis of mutant hRFC transfectants

Plasma membranes were prepared by differential centrifugation [25]. Membrane proteins were electrophoresed on 7.5% polyacrylamide gels in the presence of SDS [26] and electroblotted on to PVDF membranes (DuPont) [27]. Immunoreactive hRFC proteins were detected with Protein A-purified hRFC specific antibody [28] and enhanced chemiluminescence (Roche). Densitometry was performed on a Kodak Digital Science Image Station 440CF.

Membrane transport assays

Initial rates of [^3H]MTX or [^3H]5-CHO- H_4PteGlu uptake were measured over 180 s, as described previously [6,21,24]. Levels of intracellular radioactivity are expressed as pmol/mg of protein, calculated from direct measurements of radioactivity and protein contents of the cell homogenates. Protein assays were done by the method of Lowry et al. [29]. Kinetic constants (K_t , V_{max}) were calculated from Lineweaver–Burk plots.

RESULTS AND DISCUSSION

Deletion mutants in the hRFC TMD6-7 peptide linker

hRFC is characterized by a 61-amino acid central linker between TMDs 6 and 7 that joins the two halves of the protein (Figure 1A). With the exception of a stretch of 11 conserved amino acids (positions 204–214 in hRFC), the linker shows little homology

with RFCs from other species, including mouse, rat and hamster (Figure 2A) [1,2].

To explore the functional role of the hRFC linker domain, we introduced 49- and 60-amino acid deletions into this region, differing by the presence or absence of the conserved peptide from positions 204–214 (Figure 1B). The resulting constructs were designated hRFC^{D215–R263Δ} and hRFC^{K204–R263Δ}. An additional construct (hRFC^{K204–R214Δ}) was prepared in which only the 11 conserved amino acids were deleted (Figure 1B). The hRFC^{K204–R214Δ}, hRFC^{D215–R263Δ} and hRFC^{K204–R263Δ} constructs were all transfected into MTX-resistant K562 (K500E) cells, characterized by nearly undetectable levels of hRFC transcripts and protein [21].

By Western blotting, the hRFC^{K204–R214Δ}, hRFC^{D215–R263Δ} and hRFC^{K204–R263Δ} proteins were all expressed at high levels in plasma membranes (Figure 3). hRFC^{K204–R263Δ} migrated as an ≈ 58.4 kDa species, similar to the size predicted for this clone and suggesting that the 60-amino acid deletion resulted in an altered conformation and loss of glycosylation at Asn-58. However, both the hRFC^{D215–R263Δ} and hRFC^{K204–R214Δ} proteins migrated as broad bands (centred at ≈ 75 –85 kDa), resembling the pattern of the glycosylated wild-type hRFC in K43-6 cells [21,22]. Thus, while deletion of most, or all, of the middle loop of hRFC clearly influences patterns of N-glycosylation, the mutated proteins appear to be adequately targeted to the plasma membrane.

However, transport assays with the hRFC^{D215–R263Δ}, hRFC^{K204–R263Δ} and hRFC^{K204–R214Δ} transfectants and [^3H]MTX or [^3H]5-CHO- H_4PteGlu confirmed that none of these mutant carriers was functional (Figure 4). Thus the linker domain between TMDs 6 and 7 of hRFC seems to play an important structural or functional role in transport. Our results also suggest

Figure 3 Western blot of wild-type and mutant hRFCs expressed in K500E cells

Proteins were fractionated on a 7.5% gel in the presence of SDS, electroblotted on to a PVDF membrane, and detected with anti-hRFC antibody and an enhanced chemiluminescence kit. Left-hand panel: Western blot results are shown for plasma membrane proteins from hRFC-null K500E cells and for K500E transfectants expressing wild-type hRFC (K43-6) and mutant hRFCs as shown. Right-hand panel: Western blot results are shown for a separate gel for plasma membrane proteins from the hRFC^{K204–R214Δ} transfectant, K43-6, K500E and parental K562 cells. Molecular-mass standards (in kDa) are indicated. Protein loadings were 100 μg for parental K562 cells and 20 μg for the K500E and the transfected sublines. The relative densities for the immunoreactive hRFC bands, as measured by densitometry, are indicated.

Figure 4 Rates of [³H]MTX and [³H]5-CHO-H₄PteGlu in K562 transfectants expressing wild-type and mutant hRFCs

[³H]MTX and [³H]5-CHO-H₄PteGlu (LCV) uptakes were measured for 180 s at 37 °C (both at 0.5 μM). Data are shown for the wild-type (K43-6) and mutant hRFC transfectants, as described in the text, for comparison with parental K562 and transport-impaired K500E cells. All transport results are means ± S.E.M. from four or five separate experiments.

Table 1 Kinetic constants for MTX and 5-CHO-H₄PteGlu

Kinetic constants for MTX and 5-CHO-H₄PteGlu were determined from Lineweaver–Burk plots for the transfectants expressing the wild-type hRFC (K43-6), and the hRFC^{K204-R263Δ+TT1} and hRFC^{D215-R263Δ+TT1} proteins, as described in the text. V_{max} (normalized) is the calculated V_{max} normalized to levels of hRFC protein on Western blots (Figure 3). The data shown are means ± S.E.M. from four or five independent experiments.

Construct	MTX			5-CHO-H ₄ PteGlu		
	V_{max} (pmol/mg per 3 min)	V_{max} (normalized)	K_t (μM)	V_{max} (pmol/mg per 3 min)	V_{max} (normalized)	K_t (μM)
Wild type	22.36 ± 4.53	22.36	1.41 ± 0.46	18.24 ± 4.55	18.24	2.99 ± 0.66
hRFC ^{K204-R263Δ+TT1}	9.60 ± 3.68	10.67	2.75 ± 0.74	5.19 ± 1.13	5.78	2.05 ± 0.31
hRFC ^{D215-R263Δ+TT1}	16.89 ± 3.27	28.15	1.50 ± 0.27	14.98 ± 3.01	24.97	2.66 ± 0.66

an essential transport role for the stretch of 11 conserved amino acids starting at position 204.

hRFC/ThTr1 chimaeric transporters

Whereas hRFC and ThTr1 have a sequence identity of 40 %, restricted primarily to the TMDs [10,11], the TMD 6–7 peptide linkers in ThTr1 and hRFC show little sequence similarity and differ in size by 24 amino acids (Figure 2B). The percentage homology of the structurally analogous TMD6/7 loop for ThTr1 (amino acids 209–292, corresponding to residues 204–263 in hRFC) is only 18.4 %. To explore further the role of the hRFC central linker in carrier function, four hRFC/ThTr1 chimaeric constructs were generated in which the structurally analogous Pro-209–Arg-292 (designated TT1) and Ile-220–Arg-292 (TT2) domains of ThTr1 were used to replace the Lys-204–Arg-263 and Asp-215–Arg-263 domains of hRFC (Figure 1B). Since generation of the hRFC/ThTr1 chimaeras required introduction of

an additional *NotI* restriction site into the sense tt1/tt2 primers for cloning (see the Materials and methods section), the N-terminal junction for the fusion proteins contained two amino acids (Arg and Pro) not present in either hRFC or ThTr1 (Figure 1B). The fusion constructs (hRFC^{K204-R263Δ+TT1}, hRFC^{D215-R263Δ+TT1}, hRFC^{K204-R263Δ+TT2} and hRFC^{D215-R263Δ+TT2}) were transfected into K500E cells, and clonal isolates expressing high levels of membrane-localized hRFC/ThTr1 chimaeric proteins were identified on Western blots (Figure 3). All of the hRFC/ThTr1 chimaeras exhibited migration patterns similar to wild-type glycosylated hRFC.

Transport of [³H]MTX and [³H]5-CHO-H₄PteGlu was tested for the chimaeric hRFC/ThTr1 carriers, for comparison with wild-type hRFC in K43-6 cells and parental K562 cells (Figure 4). For the hRFC^{K204-R263Δ} protein, not containing the conserved 204–214 hRFC peptide but with 84- and 73-amino acid insertions from ThTr1 (hRFC^{K204-R263Δ+TT1} and hRFC^{K204-R263Δ+TT2}, respectively), uptake of both transport substrates ranged from 16

to 21 % of K43-6 levels (Figure 4). When normalized to relative expression (Figure 3), these values changed only slightly (to 12–27 % of the K43-6 levels; results not shown). For the analogous hRFC^{K215-R263Δ+TT2} and hRFC^{D215-R263Δ+TT1} carriers, differing from the hRFC^{K204-R263Δ} series only by their inclusion of the conserved 204–214 peptide, transport was significantly enhanced (to 60–80 % of K43-6). For the normalized transport data, these values were increased to 67–100 % of the levels in K43-6 cells (results not shown). There were no significant differences in relative transport between the carriers substituted with TT1 and the shorter TT2 peptide. Kinetic analyses for MTX and 5-CHO-H₄PteGlu for the hRFC^{K204-R263Δ+TT1} and hRFC^{D215-R263Δ+TT1} transfectants confirmed high-level transport activity accompanying the presence of the 204–214 peptide. The presence of the 11 amino acid stretch was manifest as an effect on $V_{\max}$ for both substrates, since differences in K_t values between the transfected sublines, and with wild-type hRFC, were nominal (Table 1).

Conclusion

In conclusion, our results establish a critical structural role for the central peptide linker between TMDs 6 and 7 in hRFC. While deletions of 49 or 60 residues from the 61-amino acid linker in hRFC completely abolished transport activity for MTX and 5-CHO-H₄PteGlu, replacement of the hRFC linker with 73- or 84-amino acid segments of non-homologous sequence from the structurally analogous region of ThTr1 restored activity. Maximal transport activity for these hRFC/ThTr1 chimaeric carriers required the presence of a stretch of 11 highly conserved amino acids in RFCs from different species.

Thus the role of the hRFC linker peptide appears to relate to a requirement that the two halves of the protein be properly spaced for optimal function. As long as some minimal length is achieved, there appears to be no deleterious effect from significantly longer insertions, since up to 86 amino acids (84 from ThTr1 plus Arg and Pro) could be inserted into hRFC^{D215-R263Δ} with minimal loss of transport. Finally, the absolute requirement for the highly conserved 204–214 peptide for maximal rates of transport strongly suggests an essential role for this region in the membrane translocation (but not binding) of anionic folate and antifolate substrates. Future studies will focus on further identifying the critical structural and functional features of hRFC, essential to understanding the molecular mechanisms of folate and antifolate membrane transport.

After submission of this article, two related reports documenting the role of the TMD6/7 connector loop on RFC function were published [30,31]. In these reports, results are described for hamster and mouse RFC constructs structurally similar to the hRFC^{K215-R263Δ} construct described herein, documenting a loss of carrier function. Activity was preserved with the rodent RFC constructs in which large portions of the TMD6/7 linker were deleted, as long as the conserved 11 amino acid domain was present.

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