Transmembrane Domains 4, 5, 7, 8, and 10 of the Human Reduced Folate Carrier Are Important Structural or Functional Components of the Transmembrane Channel for Folate Substrates

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The human reduced folate carrier (hRFC) facilitates membrane transport of folates and antifolates. hRFC is characterized by 12 transmembrane domains (TMDs). To identify residues or domains involved in folate binding, we used substituted cysteine (Cys) accessibility methods (SCAM) with sodium (2-sulfonatoethyl)methanethiosulfonate (MTSES). We previously showed that residues in TMD11 of hRFC were involved in substrate binding, whereas those in TMD12 were not (Hou, Z., Stapels, S. E., Haska, C. L., and Matherly, L. H. (2005) J. Biol. Chem. 280, 36206–36213). In this study, 232 Cys-substituted mutants spanning TMDs 1–10 and conserved stretches within the TMD6–7 (residues 204–217) and TMD10–11 connecting loop domains were transiently expressed in hRFC-null HeLa cells. All Cys-substituted mutants showed moderate to high levels of expression on Western blots, and only nine mutants including R133C, I134C, A135C, Y136C, S138C, G163C, Y281C, R373C, and S313C were inactive for methotrexate transport. MTSES did not inhibit transport by any of the mutants in TMDs 1, 3, 6, and 9 or for positions 204–217. Whereas most of the mutants in TMDs 2, 4, 5, 7, 8, and 10, and in the TMD10–11 connecting loop were insensitive to MTSES, this reagent inhibited methotrexate transport (25–75%) by 26 mutants in these TMDs. For 13 of these (Y126C, S137C, V160C, S168C, I134C, A135C, Y136C, S138C, G163C, Y281C, R373C, and S313C) inhibition was prevented by leucovorin, (6,7)-5-CHO-H4PteGlu; MTSES, sodium (2-sulfonatoethyl)methanethiosulfonate; MFS, major facilitator superfamily; LacY, lactose/proton symporter; GlpT, inorganic phosphate/glycerol 3-phosphate antiporter.

Folic acid is the fully oxidized monoglutamyl form of the water-soluble vitamin that is used in dietary supplements and in fortified foods. In cells and tissues, folic acid is converted to coenzyme forms required in one-carbon transfer reactions involved in the biosynthesis of nucleotides and the amino acids, serine and methionine (1). Thus, folates are critical for cell proliferation and tissue regeneration. Further, folic acid supplementation has been credited with providing a beneficial role in preventing a range of disorders, including cardiovascular disease, neural tube defects, and cancer (2, 3).

Because folates cannot be synthesized de novo in mammalian cells, external dietary sources are essential. Reflecting their anionic character, the natural folates show only a minimal capacity to cross biological membranes by diffusion alone. Accordingly, sophisticated membrane transport systems have evolved to facilitate uptake of folate cofactors by mammalian cells and tissues, of which the ubiquitously expressed reduced folate carrier (RFC) (4) appears to predominate (5).

RFC levels are also important determinants of the anti-tumor activities of chemotherapy drugs such as methotrexate (Mtx) and the newer antifolates, pemetrexed (Alimta) and raltitrexed (Tomudex). Moreover, loss of RFC activity is a common mechanism of antifolate resistance both in vitro and in vivo (6, 7). In clinical studies, low levels of human RFC (hRFC) transcripts accompanied impaired Mtx transport and a poor prognosis in primary osteosarcomas (8). Moreover, in a recent case-control study of primary specimens from children diagnosed with B-precursor acute lymphoblastic leukemia, low levels of hRFC correlated with treatment failure (9).

hRFC belongs to the major facilitator superfamily (MFS), the largest group of ion-coupled transporters (10). MFS proteins generally contain 400–600 amino acids and a symmetrical structure composed of two halves, each including six transmembrane segments connected by a large hydrophilic loop with cytosolic N and C termini (Fig. 1 is a schematic of hRFC topology that conforms to this structure). Structural insights into this family of transporters have been fostered by reports in...
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In this study, we considered the tertiary structure of hRFC. We continued our SCAM studies of hRFC TMDs 1–10, drawing from our previous SCAM results with TMDs 11 and 12 (30) and the solved structures for the LacY and GlpT MFS homologs (11, 12). Our results strongly support a role for amino acids localized to TMDs 4, 5, 7, 8, and 10, in addition to those previously identified in TMD11 (30), in forming the putative substrate-binding pocket of hRFC. Our proposed model for hRFC tertiary structure appears to be in excellent agreement with mutant data for the murine and human carriers, validating the notion that transporter tertiary structure is highly conserved among MFS family members, including those with divergent substrates and origins (39).

MATERIALS AND METHODS

Reagents—[3',5',7'-3H]Mtx (20 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). Unlabeled Mtx and (6-R,S)-5-CHO-H$_2$PteGlu (leucovorin) were provided by the Drug Development Branch, NCI, National Institutes of Health (Bethesda, MD). Both labeled and unlabeled Mtx were purified by high pressure liquid chromatography prior to use (40). Synthetic oligonucleotides were obtained from Invitrogen. Tissue culture reagents and supplies were purchased from assorted vendors with the exception of fetal bovine serum, which was purchased from Hyclone Technologies (Logan, UT). MTSES was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada).

Mutagenesis—Single cysteine-substituted hRFC mutants were generated by site-directed mutagenesis using the QuikChange$^\text{TM}$ kit (Stratagene, La Jolla, CA). Primers for generating Cys substitutions were designed according to the manufacturer’s instructions. Sequences for the mutation primers are available upon request. hRFCmyc-his$^\text{Cys-less}$ constructs (see below) were transfected into R5 cells with Lipofectamine Plus reagent (Invitrogen) (30). Cells were harvested after 48 h for the preparation of plasma membranes and Western blotting. For other experiments (MTSES treatments, transport assays), cultures were split 24 h after transfection and assayed after an additional 24 h.

Western analysis of Mutant hRFC Transfectants—Plasma membrane preparation, SDS gel electrophoresis, electrophoresis to polyvinylidene difluoride membranes, and fluorescence detection of immunoreactive proteins were performed exactly as reported previously (30).

Membrane Transport Assay, MTSES Treatment, and Leucovorin Protection—The uptake of $^{3}$H]Mtx (0.5 μM) was measured over a span of 2 min at 37°C in 60-mm dishes in Hepes-Sucrose-Mg$^{2+}$ buffer (HSM; 20 mM Hepes, 235 mM sucrose, pH adjusted to 7.3 with MgO) as described previously (30). Levels of intracellular radioactivity were expressed as pmol/mg protein calculated from direct measurements of radioactivity and protein contents of cell homogenates. Protein assays were based on the method of Lowry et al. (42). MTSES treatments of cells were performed as in our previous report (30) followed by assays of $^{3}$H]Mtx uptake. To assess the protective effects of a hRFC substrate from MTSES inhibition, leucovorin (300 μM final concentration) was added 5 min before adding the MTSES reagent.

Modeling of hRFC—The sequences of GlpT and LacY (Protein Data Bank codes 1PW4 and 1PV6, respectively) were aligned on the basis of their structures using the Dali server (43). The putative transmembrane-spanning helices of hRFC were then aligned with the respective α-helices of GlpT and LacY. This initial alignment was optimized by fine-tuning some regions using computed helical wheel plots and experimental data. Modeling was performed with the “auto” mode for Mod-
RESULTS AND DISCUSSION

Expression and Function of Single Cysteine-substituted hRFC Mutants for TMDs 1–10, Amino Acids 204–217, and the TMD10–11 Connecting Loop—A goal of our study was to use SCAM and published structures for the LacY and GlpT proteins (11, 12) to generate a workable model of hRFC tertiary structure. As an extension of our previously published results with affinity labeling and SCAM that identified TMD11 of hRFC as important to substrate binding (30), we focused our effort toward establishing the functional roles of amino acids spanning TMDs 1–10 of hRFC. Accordingly, we used the hRFCmyc-his6 Cys-less construct (36) as a template to prepare 204 single Cys-substituted mutants for amino acids located in TMDs 1–10 (supplemental Table 1). In addition, we generated 28 mutants spanning the highly conserved stretches from Lys-204 to Arg-217 flanking TMD6 and from Val-380 to Lys-393 in the loop domain between TMDs 10 and 11 (Fig. 1) to explore the possibility that these highly conserved segments might be functionally important.

The single Cys-substituted mutant hRFC constructs were transiently transfected into hRFC-null R5 HeLa cells. Representative Western blotting results of plasma membrane proteins from the 232 mutant transfectants, along with membrane proteins from hRFCmyc-his6 Cys-less transfected and untransfected R5 cells are included in supplemental Fig. S1, and densitometry results are summarized in supplemental Table 1. With only a few exceptions (i.e., R133C, G163C, S168C, D216C, L316C, I319C, F322C, A323C, A324C), the single Cys-substituted constructs were expressed in R5 cells within a 2–3-fold range. Although the migrations and apparent molecular masses for the 232 hRFC mutants were nearly identical to that for hRFCmyc-his6 Cys-less and spanned a broad range (~65 to ~80 kDa), for the S360C, S361C, I362C, W363C, and L364C mutants of TMD10 (supplemental Fig. S1), additional higher molecular mass bands, presumably corresponding to aggregated hRFC, were reproducibly seen. The basis for this anomalous migration is not clear.

To establish the functional consequences of the cysteine substitutions, [3H]Mtx uptake was measured over a 2-min span for each hRFCmyc-his6 Cys mutant. The uptake results were expressed as a percentage of that for hRFCmyc-his6 Cys-less and compared with those for R5 cells (supplemental Table 1). For only nine residues, including Arg-133, Ile-134, Ala-135, Tyr-136, and Ser-138 in TMD4, Gly-163 in TMD5, Tyr-281 in TMD7, Ser-313 in TMD8, and Arg-373 in TMD10, did cysteine substitution abolish transport (equal or less than 2-fold over the residual level in R5 cells; highlighted in supplemental Table 1). Loss of transport activity was not associated with low levels
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The significant and disproportionate losses of transport activity for G36C, G171C, G317C, and G374C mutants further support the uniquely important roles of these conserved glycine residues in hRFC function as facilitators of conformational changes required for substrate translocation (46). In our previous report, G401C was functionally inert (30). Although deletion of the segment between 204 and 214 was previously found to abolish transport by hRFC (14), the only Cys mutant from 204–217 that showed an appreciable loss of transport activity was L210C (supplemental Table 1). Similarly, for the conserved TMD10–11 loop domain, previous amino acid replacements (i.e. I387N) abolished activity (47); however, we found that cysteine substitutions in this stretch were reasonably well tolerated.

Replacement of Arg-133 in hRFC (with Glu, His, or Leu) or Arg-373 in hamster RFC (with His, Gln, Asn, or Ala) or substitution of leucine at the homologous positions in murine RFC (Arg-131 and Arg-373, respectively) significantly inhibited Mtx transport activity (20, 23, 26). However, replacement of arginine at these positions with a positively charged lysine was reasonably well tolerated (20, 26). For hRFC, Arg-133 appeared to form a charge-pair with Asp-88 in TMD2 (20). Characterization of Mtx-resistant L1210 murine leukemia cells identified S309L (corresponding to Ser-313 in hRFC), suggesting that this position may play an important role in substrate selectivity involving the one-carbon moiety at the N-5 position of reduced folate forms such as 5-methyl and 5-formyl tetrahydrofolate (25). Although Ile-134, Ala-135, Tyr-136, Ser-138, and Tyr-281 have not previously been implicated in carrier function, these residues are highly conserved between the human and rodent transporters (Fig. 1), suggesting their possible structural or functional importance.

Effects on Transport Activity of Cys-Substituted hRFC Mutants of TMDs 1–10, Amino Acids 204–217, and the TMD10–11 Connecting Loop by MTSES—The underlying principle behind our SCAM experiments was to establish aqueous accessibility of residues predicted to lie within the lipid bilayer (reflecting their participation in forming the putative aqueous “channel” for hydrophilic folate and antifolate substrates) through replacement with cysteines and their reactivity with the small, water-soluble thiol-reactive alkylthiosulfonate reagent MTSES (30, 34–36, 45). For the hRFC<sup>cmyc-his<sup>6</sup></sup> cysteine mutants, reactivity with MTSES is reflected in the loss of hRFC-mediated <sup>[3]H</sup>Mtx transport.

Fig. 2 shows the effects of treating the 223 active single Cys-substituted hRFC<sup>cmyc-his<sup>6</sup></sup> transfectants for TMDs 1–10 and the 204–217 and 380–393 peptides with 10 mM MTSES on <sup>[3]H</sup>Mtx uptake. Results were compared with those for hRFC<sup>cmyc-his<sup>6</sup></sup>Cys-less. Of the 187 active Cys mutants predicted to lie within the TMDs by topology models, 25, including those

of hRFC membrane proteins on Western blots (supplemental Fig. S1 and supplemental Table 1). Although activity was low (only 2–3-fold over R5 cells) for other mutants (e.g. G36C, Y74C, P82C, F141C, S142C, S167C, G171C, L210C, D310C, G317C, G325C, G374C, Y376C, A388C), in general, cysteine substitutions in the TMDs were extremely well tolerated, given that Mtx uptake far exceeded the residual low level in the hRFC-null R5 subline.

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III, VI, IX, and XII appear to serve mostly structural roles in the LacY and GlpT proteins.

Based on this analogy and on our SCAM results for hRFC, we present folding models of hRFC tertiary structure consisting of /H9251-helices of helix-forming amino acids localized to the TMDs (Figs. 4 and 5). For the two-dimensional model in Fig. 4, all of the MTSES-sensitive positions are predicted to lie on one (aqueous-accessible) face of each TMD helix (shown as numbered red and yellow circles) with the exception of Leu-283 in TMD7. Protection from MTSES inhibition by leucovorin (Fig. 4, red circles) identifies

FIGURE 2. Effects of MTSES treatment on the Mtx uptake by single cysteine-substituted hRFC\textsuperscript{myc-his\textsubscript{6}} mutants of TMDs 1–10, positions 204–217, and the TMD10–11 connecting loop. RS cells expressing hRFC\textsuperscript{myc-his\textsubscript{6}}Cys-less and single cysteine-substituted hRFC\textsuperscript{myc-his\textsubscript{6}} mutants were preincubated with and without 10 mM MTSES for 15 min at 37 °C. Cells were washed, and 0.5 μM \textsuperscript{3}H Mtx uptake was assayed at 37 °C for 2 min. For each mutant, uptake is presented as a percentage of the level measured in the absence of MTSES. All transport results are expressed as the average values ± range for two separate experiments. Inhibitions of at least 25% are noted with an asterisk. NA, not active.
positions that directly abut the bound folate within the putative transmembrane channel; however, this may or may not be coincident with amino acids directly involved in substrate binding.

Leu-283 is preceded by Gly (at position 280), a well established “helix breaker.” Glycine was implied to facilitate the bending of helices in the LacY and GlpT structures. This supports the notion that such “irregular” helices provide the structural flexibility needed to assume the conformations required for transport (46). In hRFC, Gly-280 may serve a similar purpose and may render position 283 aqueous-accessible for reaction with MTSES. Similar functional roles were previously implied for Gly-401 in TMD11 (30) and in the present study for Gly-36 (TMD1), Gly-171 (TMD5), Gly-317 (TMD8), and Gly-374 (TMD10) for which replacement with cysteines resulted in significant losses of transport activity.

A three-dimensional model for hRFC based on the solved MFS structures and the biochemical data for hRFC is shown in Fig. 5, which depicts TMDs 1, 2, 4, 5, 7, 8, 10, and 11 as components of the putative membrane-spanning channel flanked by TMDs 3, 6, 9, and 12 (although amino acids within TMDs 1 and 2 were not directly implicated as aqueous-accessible in the present study, these regions contributed to the aqueous transmembrane channel in the LacY and GlpT structures, as noted above). From the nearly complete ablation of transport activity upon cysteine substitution, a number of amino acids would seem to be structurally or functionally important in hRFC, including Arg-133, Ile-134, Ala-135, Tyr-136, and Ser-138 in TMD4, Tyr-281 in TMD7, Ser-313 in TMD8, and Arg-373 in TMD10. Moreover, our previous results imply that Lys-411 in TMD11 is important for binding certain transport substrates (28) and, most recently, that it is a likely target for covalent modification by the activated ester N-hydroxysuccinimide Mtx.4 This suggests that Lys-411 participates in binding to the glutamate

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Compelling evidence from mutant studies establishes that Ser-313 and Arg-373 are functionally important and contribute to substrate binding specificity (25, 26). From these results, we suggest that Lys-411, Ser-313, and Arg-373 comprise a hydrophilic binding pocket for anionic folate substrates (Fig. 5). Given its close juxtaposition to both Ser-313 and Arg-373 in this model, the conserved Tyr-281 can easily be envisaged to participate in substrate binding as well. Our finding that cysteine substitutions of Arg-133, Ile-134, Ala-135, Tyr-136, and Ser-138 abolished transport is, likewise, consistent with earlier suggestions of functionally important residues localized to TMD4 (Ser-127, Ala-132, Arg-133) (20, 23, 24).

Clearly, an important step toward better understanding the molecular mechanism of concentrative (anti)folate transport by hRFC will involve identification of key determinants of substrate recognition and membrane translocation through the
FIGURE 5. Proposed three-dimensional models of hRFC, based on solved crystal structures of LacY and GlpT and SCAM analysis, and the hypothesized substrate binding site of hRFC. A three-dimensional hypothetical model for hRFC is presented based on structure alignments between hRFC and LacY and GlpT and fine-tuned based on experimental data. Modeling was performed with the Modeler 8v1 auto mode (44). All models were drawn using PyMOL (50). A, a side view of the hRFC for which the extended C-terminal segment is truncated at Lys-479. TMDs 1, 2, 4, and 5 of the N-terminal region and TMDs 7, 8, 10, and 11 of the C-terminal region are hypothesized to be involved in formation of the hydrophilic cavity for anionic substrate binding (colored sky blue). TMDs 3, 6, 9, and 12 are likely buried in the lipid bilayer and do not directly participate in substrate binding (colored green). Panel A also depicts key amino acids (shown in assorted colors) that may contribute to the binding pocket for anionic folate substrates, as described under “Results and Discussion.” B, a cytosolic view of only the TMD segments (numbered 1–12 as in Fig. 4) of the hRFC molecule so that the order of helix packing can be seen easily. TMD coloring is the same as described in A and in the two-dimensional model of hRFC in Fig. 4. C, enhanced view of the hypothetical substrate binding site comprising the same key amino acids depicted in A, including Lys-411, Ser-313, Tyr-281, and Arg-373, as described in the text. Other residues that may contribute to the substrate-binding pocket are also shown and include Arg-133, Ile-134, Ala-135, Tyr-136, and Ser-138. The physical distances between the carboxyl groups of Lys-411, Ser-313, Tyr-281, and Arg-373 are given in angstroms.
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use of systematic site-directed mutagenesis of these potentially critical amino acids.

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