Localization of a Substrate Binding Domain of the Human Reduced Folate Carrier to Transmembrane Domain 11 by Radioaffinity Labeling and Cysteine-substituted Accessibility Methods

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The human reduced folate carrier (hRFC) mediates the membrane transport of reduced folates and classical anti-folates into mammalian cells. RFC is characterized by 12 transmembrane domains (TMDs), internally oriented N and C termini, and a large central linker connecting TMDs 1–6 and 7–12. By co-expression and N-hydroxysuccinimide methotrexate (Mtx) radioaffinity labeling of hRFC TMD 1–6 and TMD 7–12 half-molecules, combined with endoproteinase GluC digestion, a substrate binding domain was previously localized to within TMDs 8–12 (Witt, T. L., Stapels, S. E., and Matherly, L. H. (2004) J. Biol. Chem. 279, 46755–46763). In this report, this region was further refined to TMDs 11–12 by digestion with 2-nitro-5-thiocyanatobenzonic acid. A transport-competent cysteine-less hRFC was used as a template to prepare single cysteine-replacement mutant constructs in which each residue from Glu-394 to Asp-420 of TMD 11 and Tyr-435 to His-457 of TMD 12 was replaced individually by a cysteine. The mutant constructs were transfected into hRFC-null HeLa cells. Most of the 50 single cysteine-substituted constructs were expressed at high levels on Western blots. With the exception of G401C hRFC, all mutants were active for Mtx transport. Treatment with sodium (2-sulfonatoethyl) methanethiosulfonate (MTSES) had no effect on hRFC activity for all of the cysteine mutants within TMD 12 and for the majority of the cysteine mutants within TMD 11. However, MTSES inhibited Mtx uptake by the T404C, A407C, T408C, T412C, F416C, I417C, V418C, and S419C mutants by 25–65%. Losses of activity by MTSES treatment for T404C, A407C, T412C, and I417C hRFCs were appreciably reversed in the presence of excess leucovorin, a RFC substrate. Our results strongly suggest that residues within TMD 11 are likely critical structural and/or functional components of the putative hRFC transmembrane channel for anionic folate and anti-folate substrates.

The natural folates are water-soluble members of the B-class of vitamins. They act as one-carbon carriers in a series of metabolic reactions leading to the biosynthesis of purines, thymidylate, methionine, histidine, and serine (1). Thus, folates are essential for cell proliferation and tissue regeneration (1). Because folates cannot be synthesized de novo in mammalian cells, external dietary sources are required. Several physiological states are associated with folate deficiency, ranging from cardiovascular disease to neural tube defects and cancer (2, 3).

As hydrophilic anionic molecules, natural folates show only minimal capacities to cross biological membranes by diffusion alone. Accordingly, sophisticated membrane transport systems have evolved to facilitate membrane translocation of these essential cofactors (4). The reduced folate carrier (RFC) is expressed ubiquitously in tissues and tumors (5) and has long been recognized as the major membrane transporter for uptake of reduced folates in mammalian cells and tissues (4). RFC levels are also critical determinants of the anti-tumor activities of anti-folate drugs such as methotrexate (Mtx) and Pemetrexed (Alimta), and impaired transport is a frequent mechanism of anti-folate resistance (6, 7).

RFC cDNAs were first cloned in 1994 and 1995 (8–13). Hydrophyt analyses of the amino acid sequences for both the rodent and human RFCs predict an integral membrane protein with up to 12 transmembrane domains (TMDs), internally oriented N and C termini, and a large central linker connecting TMDs 1–6 and 7–12 (see Fig. 1). Much of this topology has now been experimentally confirmed (14–16).

Despite extensive studies to characterize the functional properties of the RFC protein, remarkably little is known about the amino acid residues or domains that are important to binding and/or membrane translocation of anionic folate and anti-folate substrates. However, from mutant studies, several amino acids including Gly-44, Glu-45, Ser-46, Ile-48, Asp-88, Val-106, Trp-107, Ser-127, Ala-132, Arg-133, Ser-313, Arg-373, and Lys-411 (Fig. 1; numbers refer to the human RFC (hRFC)) have been implicated as functionally or structurally important, including several located in the TMDs (17–27). We recently used co-expression and N-hydroxysuccinimide (NHS) Mtx radioaffinity labeling of hRFC TMD 1–6 and TMD 7–12 half-molecules, combined with endoproteinase GluC digestions, to localize a substrate binding domain to within TMDs 8–12 (28).

"Scanning cysteine accessibility methods" or SCAM is now the method of choice for characterizing membrane topologies and substrate binding sites in polytopic membrane proteins (29–33). Typically cysteines are inserted into a "cysteine-less" template and functional cysteine mutants are expressed in a suitable cell model. Substrate binding domains within the aqueous accessible membrane "channel" can be identified from the loss of activity upon treatment with membrane impermeable methods; NTCB, 2-nitro-5-thiocyanatobenzoic acid; MTSES, sodium (2-sulphonatoethyl) methanethiosulfonate; NHS, N-hydroxysuccinimide; leucovorin, (6R,5S)-5-CHO-H4PteGlu; BM, biotin maleimide; SM, stilbenedisulphonate maleimide; PBS, phosphate-buffered saline; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; wt, wild type; CHO, Chinese hamster ovary.
measurable hydrophilic alkylthiosulfonates (32), and surface accessibilities can be established by reactivities with impermeable maleimides (34). Recent hRFC studies with SCAM have corroborated findings of mutant studies that implicated amino acids flanking TMD 1 (e.g. Gly-44, Ile-48) as functionally important and established the membrane topology for TMDs 9–12 (16, 35, 36).

In this report, we expand upon our recent studies of hRFC structure and function (16, 28, 35) and use radioactivity labeling of hRFC half-molecules with 2-nitro-5-thiocyanatobenzoic acid (NTCB) digestions, and SCAM with sodium (2-sulfonatoethyl) methanethiosulfonate (MTSES) treatments, to localize a critical substrate binding domain to within TMD 11. This is the first report to definitively localize a substrate-binding region within a specific TMD segment of the hRFC molecule.

MATERIALS AND METHODS

Reagents—[3′,5′,7′-3H]Mtx (20 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). Unlabeled Mtx and (6-R,5S)-5-CHO-H,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 (37). Synthetic oligonucleotides were obtained from Toronto Research Chemicals (Toronto, ON, Canada).

Mutagenesis—Single cysteine-substituted hRFC mutants were generated by site-directed mutagenesis using the QuikChange™ kit (Stratagene). Primers for generating Cys substitutions were designed on the Stratagene web site. Sequences for the mutation primers are available upon request. hRFCmyc-his6 in pcDNA3.1 (35) was used as template to generate the single cysteine-replacement mutant constructs in which each residue from Glu-394 to Asp-420 of TMD 11 and Tyr-435 to His-457 of TMD 12 was replaced individually by a cysteine residue. All mutations were confirmed by DNA sequencing.

Cell Culture—hRFC-null K562 cells, expressing hRFC half-molecule constructs encoding TMDs 1–6 (designated hRFC-TMD1–6Cysless), composed of 238 amino acids spanning TMDs 1–6 and a 13 amino acid HA epitope insertion at Glu-226) and TMDs 7–12 (hRFC-TMD7–12Cysless), composed of 308 amino acids from Gly-230 to Leu-537 of hRFC and including an identical 5′-untranslated region and 18 N-terminal amino acids to the full-length hRFC and with a Myc-His10 epitope at the C terminus, were generated as previously reported (28). Cells were maintained in complete RPMI 1640 medium (Sigma-Aldrich), containing 10% iron-supplemented calf serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin, plus 1 mg/ml G418, in a humidified atmosphere at 37 °C in the presence of 5% CO2. Transport-defective Mtx-resistant HeLa cells, designated R5 (38), were a gift of Dr. I. David Goldman (Bronx, New York). R5 cells were maintained in RPMI 1640, supplemented with 10% fetal bovine serum, 2 mM l-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) in a humidified atmosphere at 37 °C in the presence of 5% CO2.

hRFCmyc-his6 constructs (see below) were transfected into transport-defective R5 cells with Lipofectamine Plus reagent (Invitrogen). Typically, R5 cells (2.2 × 105) were plated in 100-mm dishes, in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum 24 h prior to transfection. Cells were transfected with 15.6 μg of plasmid DNA, per the manufacturer’s instruction. 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 membranes were prepared by differential centrifugation (39). For standard Western blotting, membrane proteins were electrophoresed on 7.5% polyacrylamide gels in the presence of SDS (40) and electroblotted onto polyvinylidene difluoride membranes (Pierce) (41). hRFC proteins were detected with Myc monoclonal antibody (Covance, Berkeley, CA) and IRDye®800 conjugated goat anti-mouse IgG (Rockland, Gilbertsville,
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### TABLE ONE

| Position | Peptide | Length (amino acids) | Peptide mass (Da) |
|----------|---------|----------------------|------------------|
| 32 (245) | MVPSSPAVEKQVPVEPGNPGPQKGLHSAVRVA | 32 | 3176.684 |
| 151 (364) | CGDGLAMLREGLDLSPLQQLMLWLNWFNASAGYLVVYIVHNLWNEVDPTTNSARVNYNGAADASATLIGATSFAAGVXKRWAKSSKLLIAVGTAQAGLVLFPILAHTRPSSWNL | 119 | 13338.444 |
| 182 (395) | CYAAPPVFLGQSYQPVLPIATFQIASSLKEL | 31 | 3471.074 |
| 244 (457) | CALVFNGVNTTAVKTVTTTFIVSDVRGLLPPRQFPQLYSVFLILSITFVFLGAMLGLDLRH | 62 | 7000.451 |
| 361 | CQRGHRPQPAPPQGLRASAEKAAQALSVKDGKLGLQPAQSPPLSPEDSLGAVGPAISLQEQFLSHPYLAQAPAPQAEDLQPGSEGLTKLGGEQKLTSEELDSAVDHHHHHHHHHH | 117 | 12427.614 |

MTSES treatments were performed as described previously (35). Briefly, transfected R5 cells in 60-mm dishes were washed (3×) with PBS and then treated with 10 mM MTSES at 37 °C for 15 min. Reactions were quenched by a quick wash with 2-mercaptoethanol (14 mM in ice-cold PBS), followed by two additional PBS washes and a single wash with anion-free buffer (see above). [3H]Mtx uptake was assayed as described above. 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.

**Detection of the TMD 11-Loop Boundary with BM/SM**—Thiol-reactive reagents were used for mapping the TMD 11-loop boundary region facing the extracellular side. R5 cells, grown in 60-mm dishes expressing hRFC<sup>myc-his<sub>6</sub></sup>Cys-less and the F416C, I417C, V418C, S419C, D420C, G421C, R422C, and G423C single cysteine-substituted hRFCmyc mutants were treated with 200 μM SM (in PBS) at room temperature (25 °C) for 30 min followed by treatment with 200 μM BM (in PBS), or BM alone at room temperature for 30 min. The cells were briefly treated with 14 mM 2-mercaptoethanol to remove excess reagent and then washed three times with PBS. Cells were harvested, and the plasma membranes were prepared, as described above. For immunoprecipitations, membrane proteins were solubilized in 1 ml of cell lysis buffer A (50 mM Tris (pH 7.5), 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate). The insoluble material was pelletted (12,000 rpm, 10 min) and the supernatant was precleared for 4 h with protein G-plus-agarose beads. The beads were pelletted (12,000 rpm, 30 s) and the supernatant was incubated with anti-myc antibody and fresh protein G-plus-agarose beads overnight at 4 °C. The beads were washed twice with lysis buffer A, twice with lysis buffer B (50 mM Tris (pH 7.5), 500 mM NaCl, 0.1% Nonidet P40, 0.05% sodium deoxycholate), and once with lysis buffer C (50 mM Tris (pH 7.5), 0.1% Nonidet P40, 0.05% sodium deoxycholate). The immunoprecipitated proteins were eluted with 70 μl of 3× Laemmli sample buffer (containing 2% (w/v) SDS and 2 μl 2-mercaptoethanol). Immunodetection of biotinylated hRFC was carried out using peroxidase-linked streptavidin and Lumi-LightPLUS substrate (Roche Diagnostics), whereas total immunoprecipitated hRFC was detected using hRFC-specific antibody (21) and standard Lumi-Light substrate. Images were recorded on x-ray film with multiple exposures.

**Affinity Labeling of hRFC with NHS-Mtx Ester**—Radiolabeled NHS-Mtx was prepared as described previously (28, 39, 43). The radiospecific activity of the NHS-[3H]Mtx was 28 Ci/mmol. Cells were treated with NHS-[3H]Mtx (700 nm) and membrane proteins were prepared, and membrane pellets were solubilized in 1% SDS. Aliquots of the radioaffinity-labeled membrane proteins (150 μg) were treated with 7.5 μl guanidine HCl for 2 h and then digested with 10 mM of NTCB (Sigma) at pH 9 (pH adjusted with 1M Tris-HCl, pH 11) for 72 h at 37 °C. The NTCB-treated samples were dialyzed against 1 M Tris-HCl, pH 8, overnight at 4 °C.

Undigested and digested NTCB-treated proteins were fractionated for 28 h on 18% polyacrylamide gels with Tris-Tricine buffer (44). The gels were sliced into 2-mm segments, and the radioactivity was extracted and directly counted. Positions of molecular mass standards (in kDa), along with the sizes of the major radioactive proteins are indicated. The dye front migrates at fraction 45.

**Confocal Microscopy**—Confocal microscopy was performed as described previously (28, 35). Briefly, cells were fixed with 3% paraformaldehyde/PBS, permeabilized with 0.1% Triton X-100, and
stained with anti-myc antibody, followed by goat anti-mouse IgG conjugated with Alexa Fluor 488 (Molecular Probes). Fluorescence staining was observed with a Zeiss laser-scanning microscope 310 using a 63× water immersion lens.

RESULTS

NTCB Digestion of Affinity-labeled hRFC TMD7–12Myc-His10 with NHS-[3H]Mtx—We previously expressed hRFC from separate TMD1–6 and TMD7–12 hRFC half-molecule constructs, each with a unique epitope tag, in hRFC-null K562 cells to restore transport activity (28). Restored transport exhibited characteristic transport kinetics for Mtx, a capacity for trans-stimulation by pretreatment with leucovorin, and inhibition by NHS-Mtx, a documented affinity inhibitor of RFC. By co-expression and NHS-[3H]Mtx radioaffinity labeling of hRFC TMD1–6 and TMD7–12 half-molecules, combined with GluC digestions, a critical substrate binding domain was localized to within TMDs 8–12 (28).

To further refine the region covalently modified with NHS-[3H]Mtx, plasma membranes from hRFC-TMD1–6/TMD7–12Myc-His10 transfected cells were digested with NTCB, followed by electrophoresis on Tris-Tricine gels. NTCB is a cysteine cutter, which selectively cleaves peptide bonds N-terminal to cysteine residues. For the TMD7–12 half-molecule, up to five theoretical cuts are predicted, generating a range of peptide bonds N-terminal to cysteine residues. For the TMD7–12 half-molecule, combined with GluC digestions, a critical substrate binding domain was localized to within TMDs 8–12 (28).

hRFC Cys-less exhibit normal membrane targeting and transport function in hRFC-null R5 HeLa Cells—A functional hRFC Cys-less construct was previously prepared and found to restore transport to RFC-null MTXRII Oua1b2–4 Chinese hamster ovary (CHO) cells (35). However, the CHO model was restrictive in that it was necessary to isolate stable transfectants over several weeks and, even then, net hRFC expression levels and transport activities were modest (35). For the present study, wild type hRFC Cys-less constructs were transiently expressed in hRFC-null R5 HeLa cells (38), whereupon they were detected at high levels and were efficiently targeted to the cell surface to restore transport activity (Fig. 3). On Western blots, the expressed proteins migrated at ~58–80 kDa, similar to results with ectopically expressed hRFC Cys-less and hRF-Cys-less proteins in CHO cells (35). Expression of the hRFC Cys-less protein was ~60% of that for the wild type protein; transport was 51% of the wild type. Thus, R5 HeLa cells represent a new high throughput cell model that affords us a means to efficiently expand the scope of our SCAM experiments to probe hRFC ligand binding domains.

Expression and Function of Single Cysteine-substituted hRFC Mutants of TMDs 11 and 12—Based on the finding that NTCB cleavage of the radioaffinity-labeled hRFC-TMD7–12Myc-His10 half-molecule localized substrate binding to within TMDs 11 and 12 (Fig. 2), we used the hRFC Cys-less construct as a template to prepare consecutive single Cys-substituted mutants from Glu-394 to Asp-420 in TMD 11 and from Tyr-435 to His-457 in TMD 12. The single Cys-substituted mutant hRFC Cys-less constructs were transiently transfected into transport-impaired R5 cells. A representative Western blot of plasma membrane proteins from the 50 mutant transfectants, along with proteins from hRFC Cys-less transfected and untransfected R5 cells, is shown in Fig. 4, A and B. With only a few exceptions (e.g. F416C), all of the single Cys-substituted constructs of hRFC TMDs 11 and 12 were expressed in R5 cells within a 2–3-fold range. Although the migrations of 49 of the hRFC mutants were nearly identical to that for hRFC Cys-less, for D420C, high molecular mass bands, presumably corresponding to aggregated hRFC, were reproducibly seen.

To assess the effects of the cysteine substitutions in TMDs 11 and 12 on hRFC function, [3H]Mtx uptake was measured for 2 min for each hRFC Cys-less mutant. Results are shown in Fig. 4, C and D and are expressed as a percentage of the uptake for hRFC Cys-less, for comparison with R5 cells. Most of the cysteine substitutions were remarkably well tolerated, as reflected by the significantly increased Mtx uptake over the low residual level for the R5 subline. Indeed, of the 27

![FIGURE 3. Expression and transport of hRFC Cys-less and hRFC Cys-less Cys-less in R5 cells. A, hRFC expression on a Western blot of membrane proteins (2.5 μg) from R5 cells, and from R5 transfecants expressing hRFC Cys-less and hRFC Cys-less Cys-less proteins is shown. Detection involved anti-myc antibody and IRDye800 conjugated secondary antibody with a Li-COR Odyssey infrared imaging system. B, results are shown for levels of [3H]Mtx uptake in R5 cells and in R5 transfecants expressing hRFC Cys-less and hRFC Cys-less Cys-less. (B) [3H]Mtx uptake were measured for 2 min at 37 °C. Transport results are expressed as the averages ± range for 2 duplicate experiments. C, R5 cells and R5 transfecants expressing hRFC Cys-less and hRFC Cys-less Cys-less were fixed with 3.3% paraformaldehyde, permeabilized with 0.1% Triton X-100, incubated with mouse anti-myc primary antibody followed by anti-mouse IgG-Alexa Fluor 488-conjugated secondary antibody, and spun onto microscope slides. Slides were visualized with a Zeiss laser-scanning microscope 310 using a 63× water immersion lens.](http://www.jbc.org/Downloaded from http://www.jbc.org/Downloaded from http://www.jbc.org/Downloaded from http://www.jbc.org/Downloaded from http://www.jbc.org/Downloaded from http://www.jbc.org/Downloaded from http://www.jbc.org/Downloaded from http://www.jbc.org/Downloaded from http://www.jbc.org/Downloaded from http://www.jbc.org/Downloaded from)
mutant constructs for TMD 11 and the 23 mutants for TMD 12, only G401C-hRFCmyc-his6 was completely inactive despite a high level of expression on Western blots. This implies that Gly-401 may be structurally or functionally important to hRFC transport. Interestingly, our data also suggest that Cys substitutions may be somewhat better tolerated in TMD 12 than in TMD 11, as reflected in the relative transport

FIGURE 4. Expression and transport function of single cysteine-substituted hRFCmyc-his6 mutants of TMDs 11 and 12. A and B, hRFC expression is shown on a Western blot of plasma membrane proteins (2.5 μg) from R5 cells, R5 transfectants expressing hRFCmyc-his6Cys-less, and single Cys-substituted hRFCmyc-his6 mutants spanning TMD 11 (A) and TMD 12 (B). Detection of immunoreactive hRFC was with anti-myc antibody and IRDye800 conjugated secondary antibody with an Odyssey® Infrared Imaging System. Densitometry was done with the Odyssey software (v 1.2) and results are reported as the numbers listed below each lane relative to the value for hRFCmyc-his6Cys-less. C and D, results are shown for levels of [3H]Mtx uptake in R5 cells and R5 transfectants expressing hRFCmyc-his6Cys-less, and single Cys-substituted hRFCmyc-his6 mutants for TMD 11 (C) and TMD 12 (D). [3H]Mtx (0.5 μM) uptakes were measured for 2 min at 37 °C. Transport results are expressed relative to the level for hRFCmyc-his6Cys-less as the averages ± range for two separate experiments.
activities of the Cys mutants compared with that of the Cys-less hRFC for each of these regions (i.e. nearly half of the TMD 11 mutants were no more than 50% as active as hRFC^{cys-less}).

Modification of Transport Activity of Cys-substituted hRFC Mutants of TMDs 11 and 12 by MTSES—The effects of the small, water-soluble thiol-reactive alkylthiosulfonate agent, MTSES, on Mtx transport by the hRFC^{cys-less} cysteine mutants should provide an excellent gauge of the aqueous accessibilities of the cysteine substitutions in TMDs 11 and 12. (32–35). Fig. 5 shows the effects of treating the 50 single Cys-substituted hRFC^{cys-less} transfectants for TMDs 11 and 12 with MTSES under optimized conditions (37 °C, 15 min, 10 mM MTSES) on 0.5 μM [3H]Mtx uptake over 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. ND, not detected. A, results are shown for single cysteine-substituted hRFC^{cys-less} mutants spanning TMD 11. B, results are shown for single cysteine-substituted hRFC^{cys-less} mutants spanning TMD 12.

Although MTSES inhibition establishes aqueous accessibilities of reactive thiols, evidence for participation of this region in substrate binding involves protection from MTSES inhibition by excess transport substrate. Accordingly, for the T404C, A407C, T408C, T412C, F416C, I417C, V418C, S419C-hRFCs) showed appreciable (25–65%) losses of transport activity compared with that of the hRFC^{cys-less} mutants (Fig. 5A). Conversely, there were no adverse effects from treating the 23 single Cys-substituted hRFC^{cys-less} transfectants in TMD 12 with MTSES (Fig. 5B).

Mapping the TMD 11 Extracellular Boundary with BM and SM—R5 cells expressing hRFC^{cys-less} Cys-less and cysteine-substituted hRFC^{cys-less} proteins spanning the predicted TMD 11 extracellular boundary (i.e. F416C, I417C, V418C, S419C, D420C, V421C, R422C, and G423C, Fig. 1) were treated with the surface-labeling reagent BM, with and without the membrane-impermeable blocking agent SM (45), to map the extracellular boundary for TMD 11. S301C, in which the cysteine substitution is located in the connecting loop between TMDs 7 and 8 (16), was included as a positive control. After the treatments, BM-reactive hRFC immunoreactivity in Fig. 7A). Only the S419C, D420C, V421C, R422C, G423C, and S301C mutants were effectively immunoprecipitated (as reflected in the patterns of BM-reactivity and protection by SM, the TMD 11 extracellular boundary lies between positions 418 and 419.

FIGURE 5. Effects of MTSES treatment on the Mtx uptake by single cysteine-substituted hRFC^{cys-less} mutants of TMDs 11 and 12. R5 cells expressing hRFC^{cys-less} Cys-less and single cysteine-substituted hRFC^{cys-less} mutants were preincubated with and without 10 μM MTSES for 15 min at 37 °C. Cells were washed, and 0.5 μM [3H]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. ND, not detected. A, results are shown for single cysteine-substituted hRFC^{cys-less} mutants spanning TMD 11. B, results are shown for single cysteine-substituted hRFC^{cys-less} mutants spanning TMD 12.

FIGURE 6. Protection by leucovorin of T404C-, A407C-, T408C-, T412C-, F416C-, I417C-, V418C- and S419C-hRFC^{cys-less} mutants from MTSES inhibition. R5 cells expressing hRFC^{cys-less} Cys-less and MTSES-sensitive Cys-substituted mutants from TMD 11 (Fig. 5) were treated with 0.5 mM leucovorin before the transport assay. Controls were incubated under identical conditions without MTSES. Cells were washed and [3H]Mtx (0.5 μM) uptake was assayed at 37 °C for 2 min. For each mutant, the uptake is presented as a percentage of the level measured in the absence of MTSES (± leucovorin). Results are expressed as average values ± range for two separate experiments. Protection of at least 1.6-fold by leucovorin is noted with an asterisk.
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FIGURE 7. Mapping the TMD 11 junction with biotin maleimide and stibenedisulfonate maleimide. R5 cells expressing single cysteine-substituted hRFCmyc-fusion and hRFCmyc-fusion Cys-less proteins were treated with 200 μM BM with or without pretreatment with 200 μM SM. Membrane proteins were immunoprecipitated by anti-myc antibody and Protein G-plus-agarose beads, followed by Western blotting. Detection of immunoprecipitated proteins was done with streptavidin peroxidase conjugate (A) and hRFC-specific antibody (B), after stripping the polyvinylidene difluoride membrane with 0.2 N NaOH.

DISCUSSION

The present report continues our systematic characterization of the structure and function of hRFC, a physiologically important transporter and a member of the Major Facilitator Superfamily of transporters (46). Major Facilitator Superfamily proteins are represented in animals, plants, fungi, lower eukaryotes, bacteria, and eukaryotic organelles and transport a diverse range of substrates in a uniport, symport, or antiport fashion, including amino acids, neurotransmitters, sugars, vitamins, nucleosides, and organic phosphates (46). They typically contain 400–600 amino acids and a structural motif composed of two halves, each including of six transmembrane α-helices connected by a large hydrophilic loop, with cytosolic N and C termini.

X-ray crystallographic structures of the Major Facilitator Superfamily proteins, lactose/proton symporter (LacY) (47) and the inorganic phosphate/glycerol 3-phosphate antiporter (GlpT) (48), were recently reported at resolutions of 3.5 and 3.3 Å, respectively. Comparison of LacY and GlpT identified highly similar structural features, although the sequence identity between GlpT and LacY is only 21% (49). By comparative modeling, this structure is highly conserved among other Major Facilitator Superfamily family members (49). In both the GlpT and LacY structures, hydrophilic cavities accommodate the substrate binding sites formed by helices I, IV, V of the N-terminal domain, and helices VII and XI of the C-terminal domain (47, 48).

Although amino acids localized to TMDs 1, 2, 3, 4, 8, 10, and 11 have been implicated as functionally or structurally important from mutant studies (17–27), it is difficult to separate direct effects of amino acid substitutions on substrate binding and membrane translocation from indirect effects of charge interactions and altered protein conformation. Most recently, by co-expression and NHS-Mtx radioaffinity labeling of hRFC TMD1–6 and TMD7–12 half-molecules, a substrate binding domain was definitively localized to TMDs 7–12 (28). With endoproteinase GluC digestions, it was possible to localize NHS-Mtx labeling to a ~20-kDa region, corresponding to amino acids 295–477, including TMDs 8–12.

In this report, we further refined this labeled region to between amino acids 394 and 457, corresponding to TMDs 11 and 12, by cleaving the radioaffinity labeled TMD7–12 polypeptide adjacent to cysteines by treatment with NTCB. To confirm the roles of TMDs 11 and 12 in substrate binding and/or translocation, we expressed single cysteine-substituted hRFC mutants, from Glu-394 to Asp-420 with the PHD (Profile network prediction Heidelberg) program (52, 53). Abbreviations are: H, helix; E, random coil. Lower panel, the helical wheel for amino acids Val-402 to Thr-415 was drawn with a web-based program.

and S419C) was markedly inhibited by MTSES. With the T404C, A407C, T412C, and I417C mutants, excess leucovorin afforded appreciable protection from the inhibitory effects of MTSES.

For the LacY and GlpT models, the eight TMDs that form the hydrophilic cavity contain a disproportionate number of glycine and proline residues, well recognized “helix breakers” that result in “irregular” or bent α helices. These structures are believed to provide the necessary flexibilities to assume the different conformations required for substrate translocation (50). A similar consideration applies to TMD 11 of hRFC. Thus, the peptide from Val-402 to Thr-415 is predicted to assume a classical α helical secondary structure, with 3.6 residues/turn (Fig. 8).

From a helical wheel model for these 14 residues, the MTSES reactive positions 404, 407, 408, and 412 are all predicted to lie on one (aqueous-accessible) face of a putative helix (Fig. 8). However, position 411, also predicted to lie on the same side of the α helix and implicated as functionally important (27), was apparently unreactive with MTSES. From the patterns of BM reactivity and SM protection, the TMD 11 extracellular boundary lies between Val-418 and Ser-419. Although positions 416–420 are predicted to exist as a random coil within the plasma membrane, the modifications of F416C, I417C, and V418C by MTSES nonetheless establish their aqueous accessibilities and contributions as structural components of the predicted hRFC transmembrane channel. Because Ser-419 is also accessible to BM and SM, the modification of S419C by MTSES must reflect its localization to the TMD 11 exofacial loop.

Based on their protection by leucovorin, positions 404, 407, 412, and 417 should participate or be spatially juxtaposed to residues involved in
substrate binding. Although this could also reflect indirect effects of substrate-induced conformational changes at distal binding sites, from our radioaffinity labeling results that localized binding to TMDs 11 or 12, a more likely possibility is that these four residues in TMD 11 directly or indirectly participate in substrate binding, as noted above. Of course, from the lack of leucovorin protection, it would seem that positions 408, 416, 418, and 419 do not directly participate in substrate binding and that the loss of transport activity by MTSES treatment is due to indirect (conformational) effects. However, this result could also partly reflect a loss of reduced folate binding to these mutants.

Of particular interest is Gly-401 which flanks the 402–415 helical stretch of residues and seems to function as a helix breaker (Fig. 8). By analogy with the LacY and GlpT proteins, we propose a critical transport role for Gly-401 through its capacity to facilitate dynamic conformation changes within TMD 11, a notion entirely consistent with our finding that cysteine substitution at position 401 completely abolishes hRFC transport.

In conclusion, our affinity labeling and SCAM results establish an important functional role for TMD 11 of hRFC. As noted above, Lys-411 has been implicated as functionally or structurally important in hRFC transport (27). By random chemical mutagenesis of the murine RFC, mutations of Gly-394 and Ala-400, corresponding to Gly-401 and Ala-407 in hRFC, were associated with anti-folate resistance (51). Our future studies will continue to focus on identification of critical determinants of substrate recognition and translocation for hRFC, an absolute prerequisite to understanding the molecular mechanism of folate and anti-folate membrane transport by this physiologically important transport system.

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REFERENCES
1. Stokstad, E. L. R. (1990) in Folic Acid Metabolism in Health and Disease (Picciano, M. F., Stokstad, E. L. R., and Gregory, J. F., eds) pp. 1–21, Wiley-Liss, New York
2. Lucock, M. (2000) Mol. Genet. Metabol. 71, 121–138
3. Bailey, L. B., Rampersaud G. C., and Kauwell, G. P. (2003) J. Nutr. 133, 1961–1968
4. Matherly, L. H., and Goldman, I. (2003) Vitam. Horm. 66, 403–456
5. Whetstone, J. R., Flattery, R. M., and Matherly, L. H. (2002) Biochem. J. 367, 629–640
6. Goldman, I. D., and Matherly, L. H. (1985) Pharmcol. Ther. 28, 77–102
7. Goldman, I. D., and Zhao, R. (2002) Semin. Oncol. 29, 3–17
8. Dixon, K. H., Lanphier B. C., Chiu J., Kelley K., and Cowan K. H. (1994) J. Biol. Chem. 269, 17–20
9. Prasad, P. D., Ramamoorthy, S., Leibach, F. H., and Ganapathy, V. (1995) Biochem. Biophys. Res. Commun. 206, 681–687
10. Williams, F. M., Murray, R. C., Underhill, T. M., and Flintoff, W. F. (1994) J. Biol. Chem. 269, 5810–5816
11. Moscow, J. A., Gong, M., He, R., Sgagias, M. K., Dixon, K. H., Anzick, S. L., Moltzor, P. S., and Cowan, K. H. (1995) Cancer Res. 55, 3790–3794
12. Williams, F. M., and Flintoff, W. F. (1995) J. Biol. Chem. 270, 2987–2992
13. Wong, S. C., Proefke, S. A., Bhushan, A., and Matherly, L. H. (1995) J. Biol. Chem. 270, 17468–17475
14. Ferguson, P. L., and Flintoff, W. F. (1999) J. Biol. Chem. 274, 16269–16278
15. Liu, X. Y., and Matherly, L. H. (2002) Biochem. Biophys. Acta 1564, 333–342
16. Cao, W., and Matherly, L. H. (2004) Biochem. J. 378, 201–206
17. Zhao, R., Assaraf, Y. G., and Goldman, I. D. (1998) J. Biol. Chem. 273, 7873–7879
18. Zhao, R., Assaraf, Y. G., and Goldman, I. D. (1998) J. Biol. Chem. 273, 19065–19071
19. Tse, A., Brigle, K., Taylor, S. M., and Moran, R. G. (1998) J. Biol. Chem. 273, 25953–25960
20. Wong, S. C., Zhang L., Witt T. L., Proefke S. A., Bhushan, A., and Matherly L. H. (1999) J. Biol. Chem. 274, 10388–10394
21. Zhao, R., Babani, S., Gao, F., and Goldman, I. D. (2000) Clin. Cancer Res. 6, 3304–3311
22. Liu, X. Y., and Matherly, L. H. (2001) Biochem. J. 358, 511–516
23. Sharina, I. G., Zhao, R., Wang, Y., Babani, S., and Goldman, I. D. (2001) Mol. Pharmacol. 59, 1022–1028
24. Sadlish, H., Williams, F. M., and Flintoff, W. F. (2002) J. Biol. Chem. 277, 42105–42112
25. Witt, T. L., and Matherly L. H. (2002) Biochim. Biophys. Acta 1567, 56–62
26. Witt, T. L., Stapels, S. E., and Matherly, L. H. (2004) J. Biol. Chem. 279, 46755–46763
27. Akabas, M., Stauffer, D., Xu, M., and Karlin, A. (1992) Science 258, 307–310
28. Xu, M., and Akabas, M. (1996) J. Gen. Physiol. 107, 195–205
29. Wilson, G., and Karlin, A. (1998) Neuron 20, 1269–1281
30. Karlin, A., and Akabas, M. H. (1998) Methods Enzymol. 293, 123–145
31. Brilke, K., Spinella M. J., Sierra E. E., and Goldman I. D. (1995) Mol. Pharmacol. 49, 1022–1028
32. Olofsson, S., Sahij-Toth, M., Wu, J., and Kaback, H. R. (1998) J. Biol. Chem. 273, 1281–1289
33. Loo, T. W., and Clarke, D. M. (1999) Biochim. Biophys. Acta 1461, 315–325
34. Cao, W., and Matherly, L. H. (2003) Biochem. J. 374, 27–36
35. Flintoff, W. F., Williams, F. M. R., and Sadlish, H. (2003) J. Biol. Chem. 278, 40867–40876
36. Fry, D. W., Yalowich, J. C., and Goldman, I. D. (1982) J. Biol. Chem. 257, 1890–1896
37. Zhao, R., Chattopadhyay, S., Hanscom, M., and Goldman, I. D. (2004) Clin. Cancer Res. 10, 630–639
38. Matherly, L. H., Czajkowski, C. A., and Angeles, S. M. (1991) Cancer Res. 51, 3420–3426
39. Laemmli, U. K. (1970) Nature 227, 680–685
40. Matsudaira, P. (1987) J. Biol. Chem. 262, 10035–10038
41. Lowery, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
42. Henderson, G. B., and Zevely, E. M. (1984) J. Biol. Chem. 259, 4558–4562
43. Schagger, H., and von Jagom, G. (1987) Anal. Biochem. 166, 368–379
44. Loo, T. W., and Clarke, D. M. (1995) J. Biol. Chem. 270, 843–848
45. Pao, S. S., Pauleen, T. T., and Saier, M. H. (1998) J. Biol. Chem. 273, 1–34
46. Abramson, J., Smirnova, I., Kasho, V., Verner, G., Caback, H. R., and Iwata, S. (2003) Science 301, 610–615
47. Huang, T., Lemieux, M. J., Song, J., Auer, M., and Wang, D. N. (2003) Science 301, 620–630
48. Vardy, E., Arkin, I. T., Gottschall, K. E., Caback, H. R., and Schuldiner, S. (2004) Protein Sci. 13, 1832–1840
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