Restoration of Transport Activity by Co-expression of Human Reduced Folate Carrier Half-molecules in Transport-impaired K562 Cells

LOCALIZATION OF A SUBSTRATE BINDING DOMAIN TO TRANSMEMBRANE DOMAINS 7–12

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Reduced folates such as 5-methyl tetrahydrofolate and classical antifolates such as methotrexate are actively transported into mammalian cells by the reduced folate carrier (RFC). RFC is characterized by 12 stretches of mostly hydrophobic, α-helix-promoting amino acids, internally oriented N and C termini, and a large central linker connecting transmembrane domains (TMDs) 1–6 and 7–12. Previous studies showed that deletion of the majority of the central loop domain between TMDs 6 and 7 abolished transport, but this segment could be replaced with mostly non-homologous sequence from the SLC19A2 thiamine transporter to restore transport function. In this report, we expressed RFC from separate TMD1–6 and TMD7–12 RFC half-molecule constructs, each with a unique epitope tag, in RFC-null K562 cells to restore transport activity. Restored transport exhibited characteristic transport kinet

Reduced folates such as 3-[(hydroxymethyl)amino]propanesulfonate (homocysteine), 3-[(hydroxymethyl)amino]propanesulfonate (homocysteine), and classical antifolates such as methotrexate are actively transported into mammalian cells by the reduced folate carrier (RFC). RFC is characterized by 12 stretches of mostly hydrophobic, α-helix-promoting amino acids, internally oriented N and C termini, and a large central linker connecting transmembrane domains (TMDs) 1–6 and 7–12. Previous studies showed that deletion of the majority of the central loop domain between TMDs 6 and 7 abolished transport, but this segment could be replaced with mostly non-homologous sequence from the SLC19A2 thiamine transporter to restore transport function. In this report, we expressed RFC from separate TMD1–6 and TMD7–12 RFC half-molecule constructs, each with a unique epitope tag, in RFC-null K562 cells to restore transport activity. Restored transport exhibited characteristic transport kinet

The murine and hamster RFCs were cloned in 1994 (6, 7) and in 1995 human RFC (hRFC) was cloned and characterized (9–12). For both the rodent and human carriers, hydropathy analysis of amino acid character predicts 12 stretches of mostly hydrophobic, α-helix-promoting amino acids, internally oriented N and C termini, and a large central linker connecting transmembrane domains (TMDs) 1–6 and 7–12 (1, 2, 7) (Fig. 1A). For hRFC, the single N-glycosylation consensus site at asparagine 58 was predicted to be extracellular. This 12 TMD topology model has been experimentally confirmed by a combination of approaches including scanning hemagglutinin (HA) epitope insertion and glycosylation insertion mutagenesis (13, 14) and, most recently, by scanning cysteine insertional mutagenesis and accessibility methods (15, 16).

The amino acids located in the TMDs are, in general, highly conserved (70.2%) between species with somewhat greater homology concentrated in TMDs 1–6 than 7–12 (75.9 and 64.7%, respectively). Potentially important amino acids in the RFC protein were identified by site-directed mutagenesis of conserved charged amino acids (e.g. Asp88, Arg233, Arg273, Lys411; all numbers correspond to hRFC) (17–20) or selection of structurally modified RFC forms in the presence of elevated levels of antifolate inhibitors (e.g. Gly44, Glu46, Ser46, Ile48, Val105, Ser127, Ala132, Ser309) (21–27). Unfortunately, it is nearly impossible to distinguish direct functional effects of amino acid replacements on substrate binding and membrane translocation from indirect effects mediated by charge interactions and altered protein conformations. Nonetheless, recent studies with cysteine scanning accessibility methods have corroborated findings of mutant studies that implicated amino acids flanking TMD1 (e.g. Gly44, Ile48) as functionally important (15, 28).

In striking contrast to the TMD regions, the 61 amino acids comprising the central connecting loop between TMDs 6 and 7 of hRFC are less conserved (44.3% homology between hRFC and rodent RFCs). Indeed, large deletions in this region of the murine (31 of 66 amino acids) and hamster (45 of 67 amino acids) ha

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acids) RFCs preserved membrane targeting and transport activity, as long as a highly conserved stretch of 11 amino acids (corresponding to positions 204–214 in hRFC) was present (29, 30). In hRFC, deletions of 49 or 60 amino acids from the TMD6/7 linker (amino acids 215–263 and 204–263, respectively) completely abolished transport activity for both Mtx and 5-formyl tetrahydrofolate (5-CHO-H4PteGlu) (31), suggesting that some minimal length for the linker is necessary. Further, replacement of the deleted segments with non-homologous 73 or 84 amino acid segments of the structurally analogous thiamine transporter SLC19A2 (18% homologous to hRFC for the TMD6/7 linker) completely restored transport (31). Thus, with the exception of the conserved 204–214 amino acid segment, it appears that the primary role of the connecting loop between TMDs 6 and 7 is to ensure the proper spacing between the two halves of hRFC protein for optimal function, and that this is virtually independent of amino acid sequence.

In this report, we further explore the role of the central connecting loop and the relationship between the N- and C-terminal membrane-spanning domains by expressing hRFC.
from separate TMD1–6 and TMD7–12 half-molecule constructs, each with a unique epitope tag, in our well-established hRFC-null K562 (K500E) model (32). Our results establish an absolute requirement for both TMD1–6 and TMD7–12 half-molecules for high-level surface expression and restoration of transport activity. Using transfected cells expressing both TMDs 1–6 and 7–12 as separate polypeptides, we covalently labeled the TMD7–12 region with N-hydroxy succinimide (NHS) [3H]Mtx, a documented radioaffinity ligand for RFC (33–35), to directly demonstrate an important role for this region in transport substrate binding.

### EXPERIMENTAL PROCEDURES

**Reagents—** [3',5',7'-H]Mtx (28 Ci/mmol) and [3',5',7'-H]-5-CHO-H,PteGlu (17 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). Unlabeled Mtx and (6R,S)-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 HPLC prior to use (36). Restriction and modifying enzymes were obtained from Promega (Madison, WI) or New England Biolabs (Beverly, MA). Synthetic oligonucleotides were obtained from MWG Biotech (Brea, CA). Unlabeled Mtx and (6R,S)-5-CHO-H,PteGlu (17 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). Unlabeled Mtx and (6R,S)-5-CHO-H,PteGlu (17 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA).

**Cell Culture—** The Mtx transport-deficient K562 subline, designated K500E, was selected from wild-type K562 cells (American Type Culture Collection) and maintained in complete RPMI 1640 medium containing 10% iron-supplemented calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin, and 0.5 μg/ml of Mtx (32). K500E cells were transfected with the wild-type hRFC in pCDNA3 (designated pC43) to generate the K43-6 subline (32), or with a hRFC construct including a C-terminal HA insertion in pCDNA3 (designated pC43) to generate the K43-6 subline (32). These cells and all other hRFC transfectants (see below) were cultured in complete RPMI 1640 medium with 10% serum and antibiotics, plus 1 mg/ml G418 in a humidified atmosphere at 37 °C in the presence of 5% CO2.

For experiments in which hRFC-transfected cells were treated with tunicamycin to inhibit N-glycosylation, cells were continuously maintained in 3 μg/ml tunicamycin for a minimum of 2 weeks prior to experiment.

**Preparation and Expression of the hRFCMyc-His10, hRFC-TMD1–6HA, hRFC-TMD7–12Myc-His10 Constructs—** The hRFCMyc-His10 construct was prepared by PCR from the hRFCMyc-His6 construct in pCDNA3.1/Myc-HisA plasmid (Invitrogen) (28), using the P6 primer (5'-CCTGGTCTTCCGGGTTCAACA3') and the antisense hRFCMyc-His10 primer (5'-CGCTCGAGTCTAGAGGGCAACTCAATGATGATGATGATGATGATGATGATGGTCGACGGCGCTATTCAGATC3'). PCR conditions were 1 min at 94 °C for 1 cycle, followed by 35 cycles at 94 °C for 30 s, 61 °C for 45 s, and 72 °C for 1 min, followed by a 7-min extension at 72 °C. The amplicon was digested with SfiI and XbaI and subcloned into SfiI/XbaI-digested pC43.

hRFC-TMD1–6HA was prepared by PCR from the full-length hRFCHIS6 construct in pCDNA3 (14), using the P8 primer (5'-AGGGTACCATCGTCGCAAGCGGCCCCAGGCGGGAAGCTCGTGGAACCTGGG-3') and H6 Stop (rev) (5'-CGTCTAGATCAAGCTCCTCCGCCGGCGGATCTCAGGC-3') primers. PCR conditions were 1 min at 94 °C for 1 cycle, 35 cycles at 94 °C for 30 s, 61 °C for 45 s, and 72 °C for 1 min, followed by a 7-min extension at 72 °C. The amplicon was digested with XhoI and XbaI and ligated into XhoI/XbaI-digested pCDNA3.1/Neo (Invitrogen).

hRFC-TMD7–12Myc-His10 was prepared by amplifying from pC43 with sense (SEDC89:5'-ATGGTGCAATCAGCCGCGGGAAGCTCGTGGAACCTGGG-3') and antisense (RFC-Out12: 5'-GCAAGATGTCGCAAGGCGGCGGGAAGCTCGTGGAACCTGGG-3') primers. The amplicon was cloned into pGEM-T Easy Vector (Promega) and reamplified with Kpn-RFC-UTR (5'-AGACCGACCGTTGGAGAAGTGCTCCCTCCGGAGCTGACCTGTTCTCCGCCGGGATCTCAGGC-3') and RFC-Out12 primers. For both amplifications, PCR conditions were 1 min at 94 °C for 1 cycle, 35 cycles at 94 °C for 30 s, 61 °C for 45 s, and 72 °C for 1 min, followed by a 7-min extension at 72 °C. The amplicon was digested with KpnI and SfiI and ligated into KpnI/SfiI-digested pCDNA3.1/Neo (Invitrogen).

Constructs were transfected individually (hRFCMyc-His10, hRFC-TMD1–6HA, and hRFC-TMD7–12Myc-His10) or in combination (for hRFC-TMD1–6HA and hRFC-TMD7–12Myc-His10) into K500E cells with lipofectin (Invitrogen) and transfectants selected with 1 mg/ml G418.

Individual clones were isolated, expanded, and screened on Western blots with HA-, Myc-, and hRFC-specific antibodies (see below). High expressing clones were selected for further study.

**Western Analysis of Mutant hRFC Transfectants—** Plasma membranes were prepared by differential centrifugation (35). For standard Western blotting, membrane proteins were electrophoresed on 10% or 15% polyacrylamide gels in the presence of SDS (38) and electropholated onto polyvinylidene difluoride (PVDF) membranes (Pierce) (39). hRFC...
proteins were detected with HA- or Myc-specific mouse antibodies (BABCO), or protein A-purified hRFC-specific antibody (prepared in rabbits) (25) and enhanced chemiluminescence (Pierce).

For N-glycosidase F (New England Biolabs) digestions of plasma membrane proteins, samples were incubated at 37°C overnight following denaturation for 10 min with 0.5% SDS and 1% 2-mercaptoethanol, followed by addition of 50 mM sodium phosphate (pH 7.5), 1% Nonidet P-40, and N-glycosidase F (1 unit). Samples were diluted with 3X Laemmli sample buffer, fractionated on 10% polyacrylamide gels, and analyzed by Western blotting.

**Confocal Microscopy**—For confocal microscopy, cells were fixed with 3.3% paraformaldehyde/DPBS, permeabilized with 0.1% Triton X-100, and analyzed with a Zeiss laser scanning microscope 310 using a 63× water immersion lens.

**Fig. 2.** Expression and transport activity of co-expressed hRFC-TMD1-6HA and hRFC-TMD7-12Myc-His10 half-molecules. A, Western blot results are shown for plasma membranes prepared from hRFC-null K500E cells (lane 2, 20 μg), and K500E cells transfected with hRFC-HA12 (lane 1, 5 μg), or co-transfected with the hRFC-TMD1-6HA and hRFC-TMD7-12Myc-His10 half-molecule constructs (lanes 3 and 4 show results for 2 clones, designated Clone 3 and Clone 5; 20 μg each). The upper panel shows a blot probed with HA-specific antibody whereas the lower panel shows the identical blot, following stripping, probed with Myc antibody. B, results are shown for uptake of [3H]Mtx (0.5 μM) and 5-CHO-[3H]H4PteGlu (1 μM) after 180 s as described under "Experimental Procedures." Transport results are the mean values ± S.E. from 3 to 5 separate experiments.

**Fig. 3.** Co-immunoprecipitation of hRFC-TMD1-6HA and hRFC-TMD7-12Myc-His10 half-proteins. Membrane pellets from K500E transfectants expressing the hRFC-TMD1-6HA and hRFC-TMD7-12Myc-His10 half-molecules (clone 5) were immunoprecipitated with anti-Myc antibody (lane 1) or IgG (lane 2) and analyzed on Western blots as described under "Experimental Procedures" with anti-HA (upper panel) and anti-Myc (lower panel) antibodies. hRFCMyc-His10-transfected cells immunoprecipitated with anti-Myc antibody were included as a positive control (lane 3).

**Fig. 4.** Confocal microscopy of hRFC-TMD1-6HA/TMD7-12Myc-His10, hRFCHA12, and hRFCMyc-His10 transfectants and hRFC-null K500E cells, showing localization to the plasma membrane. Cells were fixed with 3.3% paraformaldehyde, permeabilized with 0.1% Triton X-100, incubated with mouse anti-HA (hRFC-TMD1-6HA/TMD7-12Myc-His10 and hRFCHA12 transfectants, and K500E cells) or Myc (hRFC-TMD1-6HA/TMD7-12Myc-His10 and hRFCMyc-His10 transfectants, and K500E cells) primary antibodies followed by anti-mouse IgG-Alexa Fluor 488-conjugated secondary antibody (Ab), and spun onto microscope slides. Slides were visualized with a Zeiss laser scanning microscope 310 using a 63× water immersion lens.
and stained with anti-HA and anti-Myc antibodies (as above), followed by a secondary goat anti-mouse IgG conjugated with Alexa Fluor 488 (Molecular Probes), as previously described (28). Detection was performed with Zeiss laser scanning microscope 310 using a 63× water immersion lens.

**Immunoprecipitation Protocol**—For immunoprecipitations, membrane pellets (typically from 3 × 10⁶ cells) were suspended in 500 μl radioimmune precipitation assay buffer (500 μl; 50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1%SDS (pH 8)) (40), including proteolytic inhibitors (35). Samples were precleared with 25 μl of protein G beads (EZView Red Protein G Affinity Gel; Sigma), then treated with 5 μg of Myc (Invitrogen) antibody for 1 h. Samples were immunoprecipitated with protein G beads, centrifuged, and the beads washed 3× with radioimmune precipitation assay buffer. Samples were eluted with 1× Laemmli sample buffer and fractionated on a 10% polyacrylamide gel in the presence of SDS for Western blotting.

**Membrane Transport Assays**—Initial rates of [3H]Mtx or (6-[3H]H4PteGlu uptake were measured over 180 s, as previously described (9, 32). Levels of intracellular radioactivity were expressed as pmol/mg protein, calculated from direct measurements of radioactivity and protein contents of the cell homogenates. Protein assays were performed by the method of Lowry et al. (41). Kinetic constants (Kc, Vmax) were calculated from Lineweaver-Burk plots. To assess the capacity of leucovorin to trans-stimulate Mtx influx via hRFC (32, 42), we continuously maintained in the presence and absence of 3 μg/ml tunicamycin (TN) (Fig. 5) could be replaced with non-homologous segments (73 or 84 amino acids, respectively) of the structurally analogous SLC19A2 protein to preserve transport function. The approximate molecular masses (in kDa) for the immunoreactive hRFC proteins are indicated.

**RESULTS**

**Restoration of Transport Function by Co-expression of hRFC**—For hRFC, major portions of the TMD6/7 linker (amino acids 215–263 and 204–263) (Fig. 1) could be replaced with non-homologous segments (73 or 84 amino acids, respectively) of the structurally analogous SLC19A2 protein to preserve transport function (31). Because this suggested that the primary role of the linker region was to ensure optimal spacing between the TMD1–6 and TMD7–12 regions and was largely independent of amino acid sequence, we hypothesized that TMDs 1–6 and TMDs 7–12 could be expressed as separate polypeptides to restore transport function.

Accordingly, a series of hRFC constructs were prepared including: (i) hRFC-TMD1–6HA, composed of 238 amino acids spanning TMDs1–6 and a 13 amino acid HA epitope insertion buffer (43). The gels were sliced and processed for radioactivity measurements, as described above.

**TN**

**FIG. 5. Deglycosylation of hRFC proteins with N-glycosidase F.** Membrane proteins were either untreated or deglycosylated with N-glycosidase F as described under “Experimental Procedures” (20 and 30 μg, respectively, for hRFC-TMD1–6HA/TMD7–12Myc-His10; 10 and 20 μg for K43-6; 2.5 and 5 μg for hRFC-TMD1–6HA/TMD7–12Myc-His10). Membrane proteins were either untreated or deglycosylated with N-glycosidase F as described under “Experimental Procedures” (20 and 30 μg, respectively, for hRFC-TMD1–6HA/TMD7–12Myc-His10; 10 and 20 μg for K43-6; 2.5 and 5 μg for hRFC-TMD1–6HA/TMD7–12Myc-His10)
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Kinetic properties of hRFC transfectants

Kinetic constants for Mtx were determined by Lineweaver Burk plots for transfectants expressing wild-type hRFC (K43-6), hRFC, hRFC<sup>CHA12</sup>, hRFC<sup>Cys-His<sub>10</sub></sup>, and hRFC-TMD1–6<sup>CHA12</sup>/TMD7–12<sup>Cys-His<sub>10</sub></sup> proteins, as described in the text. The data shown are the mean values ± S.E. from three separate experiments.

| Cell line                              | K<sub>i</sub> (µM) | V<sub>max</sub> (pmol/mg/3 min) | V<sub>max</sub>/K<sub>i</sub> |
|----------------------------------------|-------------------|-------------------------------|-----------------------------|
| K43-6                                  | 1.18 ± 0.24       | 24.51 ± 2.95                  | 20.77                       |
| hRFC<sup>CHA12</sup>                   | 1.16 ± 0.23       | 23.60 ± 5.73                  | 20.81                       |
| hRFC<sup>Cys-His<sub>10</sub></sup>    | 0.91 ± 0.08       | 27.38 ± 5.96                  | 30.09                       |
| hRFC-TMD1–6<sup>CHA12</sup>/TMD7–12<sup>Cys-His<sub>10</sub></sup> | 1.20 ± 0.25       | 14.44 ± 1.45                  | 12.03                       |

at Glu<sup>226</sup> (Fig. 1B), and (ii) hRFC-TMD1–6<sup>CHA12</sup>/TMD7–12<sup>Cys-His<sub>10</sub></sup> composed of 307 amino acids from Asn<sup>231</sup> to Leu<sup>537</sup> of hRFC and including an identical 5′-untranslated region and 18 N-terminal amino acids to the full-length hRFC (K543) and hRFC-TMD1–6<sup>CHA12</sup> and with a Myc-His<sub>10</sub> epitope at the C terminus (Fig. 1C). Additional control constructs were designed to encompass TMDs 1–12 in their entirety along with specific epitope tags and included (iii) hRFC<sup>CHA12</sup> in which a HA epitope was inserted into the C terminus at Gln<sup>537</sup> (14, 37), and (iv) hRFC<sup>Cys-His<sub>10</sub></sup>, in which a Myc-His<sub>10</sub> epitope was inserted after Leu<sup>537</sup>.

Constructs were transfected into hRFC-null K500E cells individually and, for hRFC-TMD1–6<sup>HA</sup> and hRFC-TMD1–6<sup>CHA12</sup>/TMD7–12<sup>Cys-His<sub>10</sub></sup> proteins, as described in the text. The data shown are the mean values ± S.E. from three separate experiments.
There was no effect of N-glycosidase F treatment on the migration of the 40 kDa hRFC-TMD7–12Myc-His10 half-molecule (Fig. 5), consistent with the absence of a consensus N-glycosylation site in this region. Essentially identical results were obtained by treatment of K43-6 and hRFC-TMD1–6HA/TMD7–12Myc-His10 transfectants with tunicamycin (3 µg/ml), an inhibitor of core oligosaccharide addition to asparagine residues of nascent glycoproteins (44). As previously reported (37), tunicamycin treatments resulted in near quantitative losses of glycosylated hRFCs (including both full-length hRFC in K43-6 cells and the hRFC-TMD1–6HA half-protein in the hRFC-TMD1–6HA/TMD7–12Myc-His10 transfectants; Fig. 6). However, there was no effect of tunicamycin treatment on the migration of the hRFC-TMD7–12Myc-His10 half-molecule.

Transport of [3H]Mtx and 5-CHO-[3H]H4PteGlu was tested for the co-expressed hRFC-TMD1–6HA and hRFC-TMD7–12Myc-His10 proteins, for comparison with wild-type hRFC in K43-6 cells (32), and with hRFC-HA12 and hRFC-Myc-His10 expressed in K500E cells (Fig. 2B and Table I). With the hRFC-TMD1–6HA/TMD7–12Myc-His10 co-transfectants, uptake of 0.5 µM Mtx and 5-CHO-H4PteGlu ranged from 54–76% of the level in hRFC-HA12-transfected cells. Kinetic analysis of Mtx transport confirmed the restoration of hRFC function accompanying the co-expression of the hRFC-TMD1–6HA and hRFC-TMD7–12Myc-His10 half-carriers (Table I). There were no significant differences between the Kt values for Mtx among the K43-6, hRFC-HA12, and hRFC-Myc-His10 transfectants, and for hRFC-TMD1–6HA/TMD7–12Myc-His10 clone 5; the Vmax for hRFC-TMD1–6HA/TMD7–12Myc-His10 clone 5 was 59% of that for K43-6 cells (Table I).

In yet other experiments, trans-stimulation of Mtx uptake was assessed by preloading hRFC-TMD1–6HA/TMD7–12Myc-His10 and K43-6 transfectants for 20 min with high concentrations (50 µM) of leucovorin ((6R,5S)-5-CHO-H4PteGlu) (42). As shown in Fig. 7, for both lines, transport was stimulated (1.6- and 2.4-fold, respectively), demonstrating bidirectional transport of anionic folate substrates. Finally, [3H]Mtx transport into hRFC-TMD1–6HA/TMD7–12Myc-His10 and K43-6 transfectants was inhibited by pretreatment with NHS-Mtx (Fig. 8), a potent inhibitor of RFC-mediated transport (33–35). Similar to earlier results with this reagent in K562 cells (35), maximal inhibition of transport plateaued at ~70% at micromolar concentrations of inhibitor.

Collectively, these results demonstrate that the functional properties of hRFC can be largely restored by co-expression of the hRFC-TMD1–6HA and hRFC-TMD7–12Myc-His10 half-molecules.

Labeling of hRFC-TMD1–6HA/TMD7–12Myc-His10-expressing Cells with NHS-[3H]Mtx: Identification of a Substrate Binding Domain—Loss of RFC activity by NHS-Mtx treatment is the result of covalent modification of the carrier (33–35). Accordingly, NHS-[3H]Mtx has been used to radiolabel the RFC protein (33–35) since this reagent can be easily synthesized and exhibits a high specificity for the transporter in cultured mouse and human cells. In human (K562) cells treated with NHS-[3H]Mtx, tritium was incorporated into a broadly migrating ~65 kDa species; Fig. 5). shown for the extent of labeling of K43-6 and hRFC-TMD1–6HA/TMD7–12Myc-His10 cells (clone 5) in the presence and absence of 200 µM unlabeled aminoterin (Amt) (panels A and B) or following sustained growth in the presence and absence of 3 µg/ml tunicamycin (TN) (panels C and D). In panel E are shown results for hRFC-TMD1–6HA/TMD6–12Myc-His10-transfected cells for which plasma membranes were prepared, solubilized, and treated with endoproteinase GluC. Undigested (−GluC) and digested (+GluC) proteins were fractionated for 20 h on a 14.5% polyacrylamide gel with Tris-Tricine buffers (43). Analysis of bound radioactivity was as described above. For panels A–E, the positions of molecular mass standards (in kDa) are indicated.
TABLE II

| Position of cleavage | Peptide | Peptide length | Peptide mass (Da) |
|----------------------|---------|----------------|------------------|
| 9                    | MVPSSPAVE | 9             | 916.057          |
| 15                   | KQYVPE   | 6             | 698.817          |
| 44 (257)             | PGPNPGPGKGLHALRVCAGDSVLRMLRE | 29        | 2929.411         |
| 81 (294)             | LGDSLRRPLRLWLSLWVFNSAGGYLVLYHLWNE | 37       | 4614.334         |
| 181 (394)            | VDTPTNSARVYNGAADASTLGAIATSFAAGFKIRWARSKLLIAQVTA | 100      | 10750.454        |
| 265 (477)            | LGALYPGFVNTFATKTHIIIVDOVRGLPVRQRPQLYSVFLILSHYFL | 83       | 9389.146         |
| 266 (478)            | E        | 1             | 147.131          |
| 293 (505)            | KAAASLSVQDGLGLQLPAQSPSLPE | 27        | 2688.033         |
| 305 (517)            | DSGAVGAPSL | 12         | 1115.205         |
| 323 (535)            | QRGSPYLAQAPQAAE | 18       | 1941.087         |
| 331                  | FLQPGGSE | 8             | 833.896          |
| 339                  | LGTKLPE  | 8             | 813.949          |
| 345                  | QKLISE   | 6             | 716.833          |
| 346                  | E        | 1             | 147.131          |
| 362                  | DLSAVDHHHHHHHH | 16      | 1990.052         |

To further verify the identities of the affinity-labeled band in hRFC-TMD1–6HA/TMD7–12Myc-His10-transfected cells, cells were treated with tunicamycin (3 μg/ml). Predictably, tunicamycin treatment shifted the broadly migrating (~85 kDa) NHS-[3H]Mtx-labeled hRFC to 65 kDa, as seen on Westerns (compare the results in Figs. 6 and 9, panel A). For the hRFC-TMD1–6HA/TMD7–12Myc-His10-transfected cells treated with NHS-[3H]Mtx, there was no incorporation into a 27-kDa band, corresponding to the deglycosylated hRFC-TMD1–6HA species. Virtually identical results were obtained upon treatment of NHS-[3H]Mtx-labeled cells with N-glycosidase F, prior to electrophoresis (data not shown). Thus, the results of our radioaffinity labeling experiments with NHS-[3H]Mtx strongly suggest specific labeling of the 40 kDa hRFC-TMD7–12Myc-His10 half-molecule.

To further localize the region covalently modified with NHS-[3H]Mtx within TMDs 7–12, plasma membranes from hRFC-TMD1–6HA/TMD7–12Myc-His10-transfected cells were digested with endoproteinase GluC, prior to electrophoresis on Tris-Tricine gels. GluC is a serine proteinase, which selectively cleaves peptide bonds C-terminal to glutamic acid residues.
implicated as important to carrier activity (18–27). However, with the exception of residues flanking TMD1 (15, 28), the functional importance of these amino acids has not been independently verified.

Our hRFC-TMD1–6HA/TMD7–12Myc-His10 co-transfection model afforded a unique opportunity to shed further light on regions in the hRFC molecule that are important to transport substrate binding. By radioaffinity labeling of the hRFC-TMD1–6HA/TMD7–12Myc-His10-transfected cells with NHS-[3H]Mtx, a non-glycosylated 40 kDa protein was exclusively localized within TMDs1–6, confer distinct transport properties for (anti)folate substrates with different pteridine modifications (e.g. folic acid versus Mtx or 5,10-dideazatetrahydrofolate) (22, 24) strongly implies a role these for distal residues in substrate binding, presumably by associating with the pteridine ring.

Clearly, an important future goal will be further identification of individual domains and amino acids that directly participate in folate and antifolate binding and translocation by RFC. This will be facilitated by our ability to express hRFC as separate half-molecules, as described herein.

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