Impaired Membrane Transport in Methotrexate-resistant CCRF-CEM Cells Involves Early Translation Termination and Increased Turnover of a Mutant Reduced Folate Carrier*

(Received for publication, August 24, 1998, and in revised form, January 5, 1999)

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The basis for impaired reduced folate carrier (RFC) activity in methotrexate-resistant CCRF-CEM (CEM/Mtx-1) cells was examined. Parental and CEM/Mtx-1 cells expressed identical levels of the 3.1-kilobase RFC transcript. A ~85-kDa RFC protein was detected in parental cells by photoaffinity labeling and on Western blots with RFC-specific antiserum. In CEM/Mtx-1 cells, RFC protein was undetectable. By reverse transcriptase-polymerase chain reaction and sequence analysis, G to A point mutations were identified in CEM/Mtx-1 transcripts at positions 130 (P1; changes glycine 44 → arginine) and 380 (P2; changes serine 127 → asparagine). A 4-base pair (CATG) insertion detected at position 191 (in 19–30% of cDNA clones) resulted in a frameshift and early translation termination. Wild-type RFC was also detected (0–9% of clones). Wild-type RFC and double-mutated RFC (RFCP1+P2) cDNAs were transfected into transport-impaired K562 and Chinese hamster ovary cells. Although RFC transcripts paralleled wild-type protein, for the RFCP1+P2 transfectants, disproportionately low RFCP1+P2 protein was detected. This reflected an increased turnover of RFCP1+P2 over wild-type RFC. RFCP1+P2 did not restore methotrexate transport; however, uptake was partially restored by constructs with single mutations at the P1 or P2 loci. Cumulatively, our results show that loss of transport function in CEM/Mtx-1 cells results from complete loss of RFC protein due to early translation termination and increased turnover of a mutant RFC protein.

Despite the availability of newer antifolates, methotrexate (Mtx) continues to play an important role as an antineoplastic agent. To reach its intracellular target, dihydrofolate reductase, the preferred route of Mtx entry involves the reduced folate carrier (RFC; 1, 2). RFC transport of Mtx is critical to drug action because of its role in generating sufficient unbound intracellular folate to sustain maximal enzyme inhibition (1). Furthermore, high levels of Mtx are also necessary for the synthesis of Mtx polyglutamates (1).

Defective membrane transport of Mtx by RFC has been identified as a major mechanism of Mtx resistance (1–11). Transport alterations can manifest as reduced rates of carrier translocation (reduced V max), decreased affinities for transport substrates (increased K m), or both, and may involve decreased levels of normal RFC (6) or the expression of structurally altered RFC proteins (7–11). For instance, in Mtx-resistant K562 (K500E) cells, impaired Mtx transport is accompanied by decreased RFC transcripts and protein (6). A G to A transition at position 890 of the murine RFC cDNA resulted in a substitution of serine 297 by asparagine and a selective decrease in Mtx binding affinity (~4-fold) without effects on other antifolate analogs (aminopterin, 10-ethyl-10-deazaaminopterin; Ref. 9). Likewise, replacement of serine 46 by asparagine (10) or glutamate 45 by lysine (11) in murine RFC resulted in greater impairment of uptake for Mtx than (6S)-5-formyl tetrahydrofolate. In severely transport defective L1210 cells (MtxA), loss of transport activity appeared to reflect a single (G to C) point mutation at nucleotide 429 of the murine RFC cDNA sequence which resulted in the substitution of proline 130 by alanine (7). However, these cells also contained a wild-type RFC allele that was not transcribed. A silent wild-type RFC allele was described for Mtx-resistant MOLT-3 cells (MOLT-3/Mtx10,000; Ref. 8). Moreover, two mutations in the RFC coding region were detected which resulted in the creation of new stop codons and synthesis of truncated nonfunctional RFCs (8).

In this report, the molecular mechanisms responsible for the transport-impaired phenotype (~3% of wild-type) of Mtx-resistant (~243-fold) CCRF-CEM (CEM/Mtx-1;12) cells were examined. We show that although the levels of RFC transcripts are essentially unchanged from wild-type cells, there is a complete loss of RFC protein due to early translation termination and increased turnover of a double mutant RFC protein. The residual transport activity previously described in this transport-impaired line (12) presumably reflects extremely low levels of wild-type RFC and/or, possibly, non-RFC modes of Mtx uptake (13–15).

EXPERIMENTAL PROCEDURES

Materials—[α-32P]dCTP (300 Ci/mmol) and [α-3H]thiol]ATP (1400 Ci/mmol) were obtained from NEN Life Science Products Inc. [3',5',7',9'-H]Mtx (20 Ci/mmol) and [4,5'-H]leucine (120 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). Unlabeled Mtx was provided by the Drug Development Branch, NCI, National Institutes of Health, Bethesda, MD. Both labeled and unlabeled Mtx were purified by high-performance liquid chromatography prior to use (16). GW1843U89 (17) was obtained from Glaxo-Wellcome Pharmaceuticals (Research Trian...
deoxyribonucleotide sequence from S. U. Biochemical Corp. (Cleveland, OH). Restriction and modifying enzymes were obtained from Promega (Madison, WI). Synthetic oligonucleotides were obtained from Genosys Biotech. Inc. (The Woodlands, TX).

**Cell Culture**—Wild-type CCRF/CEM and transport-impaired CEM/Mtx (18) lymphoblastic leukemia lines were gifts of Dr. Andre Rosowsky (Boston, MA). Cells were grown in soft agar (6, 19) and clonal lines (designated CEM-4 and CEM/Mtx-1 for the parental and Mtx-resistant cells, respectively) were used for all experiments. Transport-deficient K500E cells were selected from wild-type K562 cells by cloning in soft agar with 500 nM Mtx (6). K500E cells containing the RFC (KS43) to generate the K43-1 and K43-6 sublines, as described previously (6). The cell lines were maintained in RPMI 1640 medium as described previously (6, 12).

Transport-defective Mtx-resistant Chinese hamster ovary (CHO) cells, MtxRIIOua R2-4 (20), were a gift of Dr. Wayne Flentiff (London, Ontario, Canada). Cells were grown in α-minimal essential medium with 10% iron-supplemented bovine calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). The pC4/10 CHO line was derived from MtxRIIOua R2-4 cells by transfection with the full-length human RFC cDNA (KS43; Ref. 21). CHO cells were grown as monolayers for transfection and general maintenance; for transport experiments, cells were grown in suspension in spinner flasks.

**Preparation of Recombinant RFC Antiserum**—The complete coding sequence of the KS43 RFC cDNA (21) was subcloned into a pGEX glutathione S-transferase (GST) fusion vector (Pharmacia Biotech, Piscataway, NJ). Following transformation of Escherichia coli (BL21) cells and induction by isopropyl-β-D-thiogalactoside (0.5 mM) for 4 h at room temperature, GST-RFC fusion proteins were purified from bacterial lysates by affinity chromatography using glutathione-Sepharose 4B (Pharmacia Biotech), as recommended by the manufacturer. Authenticity and purity of the purified RFC fusion protein were confirmed by Coomassie Blue staining and Western analysis with anti-GST (Pharmacia Biotech) and RFC peptide-specific (RFC/p5; Ref. 26) antibodies. Anti-GST-RFC antiserum was raised in rabbits with purified GST-RFC fusion protein as antigen (Pocono Rabbit Farms and Laboratories, Canadensis, PA). Both immune and preimmune sera were purified on protein A-agarose columns prior to use (27).

**Preparation of Plasma Membranes and Western Analysis**—Plasma membranes were prepared by differential centrifugation (19, 28). Where noted, particulate membrane fractions were additionally purified on discontinuous sucrose gradients (19, 28). Plasma membrane contamination of crude particulate and sucrose density gradient-purified membranes was established by 5′-nucleotidase (29) and NADPH-cytochrome c reductase (30) assays, respectively.

Membrane proteins were electrophoresed on 7.5% gels in the presence of SDS (31) and electroblotted onto polyvinylidene difluoride membranes (DuPont) for detection with protein A-purified GST-RFC antibody and enhanced chemiluminescence (Pierce, Rockford, IL). A few experiments employed RFC peptide-specific (RFC/ps) antibody (26). Light emission was recorded on x-ray film with various exposure times, and the signal was analyzed with a computing densitometer and ImageQuant software ( Molecular Dynamics, Sunnyvale, CA). For some experiments, the heterogeneously glycosylated RFCs were enzymatically deglycosylated with N-glycosidase F (Boehringer-Mannheim), as described previously (6, 28).

**Photoaffinity Labeling of Cell Surface RFC Proteins**—Cell surface RFC proteins in wild-type CCRF/CEM and CEM/Mtx-1 cells (1 × 10⁸ cells per labeling condition) were photoaffinity labeled using N-[4-amino-4-deoxy-10-methylpteroyl]-N′-[4-azido-5-[(32)iodosalicylicyl]-1-lysine (APA-41151IAA-Lys), as described previously (6, 21). Specificity of labeling was established by performing identical incubations in the presence of 100 μM aminopterin. Equal aliquots of labeled proteins were electroblotted to a 4–10% gradient gel in the presence of SDS (31). The gel was dried and exposed to x-ray film.

**Transport of [3H]Mtx in Transfected Cells**—Initial [3H]Mtx uptake rates were determined over 180 s using 1–2 × 10⁶ cells/ml (6, 12, 19, 21) and a Mtx concentration of 0.5 μM. The levels of intracellular radioactive Mtx were expressed as picomoles/mg of protein, calculated from direct measurements of radioactivity and protein contents of the cell homogenates. Protein assays were expressed by the method of Lowry et al. (32). Kinetic constants (Kt and Vmax) were calculated from the Lineeweaver-Burk plots.

**RESULTS**

**Impaired Mtx Transport in CEM/Mtx-1 Cells Is Independent of Changes in RFC Transcripts or Gene Structure**—Northern analysis of total RNAs from parental CCRF/CEM (CEM-4) and Mtx-resistant CEM/Mtx-1 cells showed that essentially identical levels of a major 3.1-kb Mtx RNA transcript were expressed (Fig. 1) despite a ∼33-fold difference in relative Mtx transport (12). Although a 1-kb RNA species hybridized with the RFC cDNA in parental cells and a unique 9.5-kb band was detected in CEM/Mtx-1 cells (Fig. 1), the significance of these forms is not clear. These forms were still present even when poly(A) RNAs were used for Northern analysis (data not shown). Restriction analysis (BamHI or HindIII) of genomic DNA from CEM-4 and CEM/Mtx-1 cells did not reveal any major alterations in RFC gene organization or copy number between the lines (data not shown).

**Analysis of RFC Coding Region and Genomic Sequence**—RFc cDNAs from parental and CEM/Mtx-1 cells were synthesized from total RNA with random hexamers using a RT-PCR kit from Perkin-Elmer. Four sets of PCR primers were used to generate overlapping partial cDNAs spanning the entire RFC coding region. The PCR primers for RFC cDNA amplification are shown in Table I. PCR conditions were 94 °C for 30 s, 63 °C for 45 s, and 72 °C for 1 min (35 cycles), and 72 °C for 7 min (1 cycle). PCR products were subcloned into the pCRII-1 plasmid using the TA cloning kit (Invitrogen) and the nucleotide sequences were determined by dideoxynucleotide sequencing (23). RT-PCR reactions were repeated 2–3 times for regions containing the P1 and P2 mutations and for each primer set, multiple cDNA clones were sequenced.

Genomic fragments containing point mutations identified in the CEM/Mtx-1 cDNAs were PCR amplified and the PCR products subcloned and sequenced as described above. Primers for genomic amplification were based on the human RFC cDNA (21) and gene (24, 25) nucleotide sequences. For amplifying the fragment containing the P1 mutation, a nested PCR approach was used. In the primary PCR reaction, two RFC intron-specific primers, RFC-IP1 (5′-ctgcagaccatcttccaaggtgccctga; downstream of the splice donor site at 189) and RFC-IP2 (5′-gacagctcctcaggtgccctga; downstream of the splice donor site at 189), were used. For the secondary nested PCR reaction, the primers used were the exonic-specific primer P8 (Table I) and another intron-specific primer RFC-IP3 (5′-actactggtgctgcctgc; downstream of the splice donor site at 189). The fragment containing the P2 mutation was amplified with intron-specific RFC-IP4 (5′-gacagctcctcaggtgccctga; upstream of the splice acceptor site at 190) and exon-specific primer P7 (Table I) primers. PCR conditions for amplifying genomic DNA were 94 °C for 10 s, 63 °C for 60 s, and 72 °C for 60 s (35 cycles), and 1 cycle of 72 °C for 7 min.

**Preparation of Mutant RFC Constructs and Transfection of Transport-defective K562 and CHO Cells**—Constructs containing the P1 (RFC P1-19) and P2 (RFC P2-7) mutations were digested with BamHI and XhoI and the −2 kb fragments subcloned into a BamHI/XhoI-digested pCDNA3 expression vector. The construct containing both P1 and P2 mutations was prepared by ligating the EcoRI/BamHI digested P1 fragment and EcoRI/XhoI digested P2 fragment into pCDNA3. Mutant constructs were transfected into transport defective MtxRIIOua R2-4 and K500E cells as described previously (6, 21). G418-resistant clones were expanded and screened for RFC transcripts (Northern), immunoreactive RFC protein (Western), and [3H]Mtx uptake.
Mtx-1 cells was analyzed by Western blotting using antibody to recombinant RFC fusion protein (GST-RFC) and chemiluminescence detection, and by photoaffinity labeling with APA-[\(^{125}\)I]ASA-Lys (6, 21). For both methods, a broadly migrating RFC band centered at ~85 kDa was identified in parental cells (Fig. 2, left panel, and Fig. 3, respectively). Identical results were obtained on Western blots with peptide-specific (RFC/ps) antiserum (not shown). Slight differences were seen in the relative migrations for RFC, reflecting the different gel systems used for separation (7.5% for the Western versus 4–10% for the photoprobe experiments). By both approaches, the major bands identified as RFC were converted to a single ~65-kDa deglycosylated form by treatment with N-glycosidase F (shown for the immunoblotted RFC in parental CCRF-CEM cells; Fig. 2, right panel). This is the size predicted from the RFC cDNA sequence (21, 33, 34). By contrast, in CEM/Mtx-1 cells none of the ~85-kDa RFC protein was detected either by Western blotting with anti-GST-RFC (Fig. 2) or peptide-specific antiserum (not shown), or by photoaffinity labeling with APA-[\(^{125}\)I]ASA-Lys (Fig. 3). However, an unidentified 42-kDa protein was specifically labeled with the photoprobe (Fig. 3). Although there were no changes in the background staining on Western blots following treatment of CEM/Mtx-1 proteins with N-glycosidase F, the 42-kDa photolabeled band was converted to ~37 kDa by this treatment (not shown).

Identification of Mutations in the RFC Coding Sequence in CEM/Mtx-1 Cells—The RFC coding sequences from parental CCRF-CEM and CEM/Mtx-1 cells were examined by RT-PCR and dideoxynucleotide sequencing of the PCR products. Four primer sets were used to amplify the entire RFC coding sequence from parental CCRF-CEM cells by primer set P7/P8 and encoding the RFC amino terminus. These include two G to A point mutations at positions 120 (designated P1; nucleotide position 1 is the translation start) and 380 (P2) in all of the 16 CEM/Mtx-1 clones sequenced, and a 4-bp (CATG) insertion at position 191 in 3 of the clones. By contrast, none of the 9 cDNA clones amplified with P7/P8 from parental CCRF-CEM cells contained any alterations from wild-type RFC sequence (21, 33, 34).

Analogous results were obtained by amplification of a fragment containing the P2 locus (positions 141–549) with the P4/P7 primer set (21/23 with a P2 mutation, including 7 with insertion at position 191). However, 2 of 23 clones derived from CEM/Mtx-1 also contained wild-type sequence at this position. All of the 15 clones amplified from parental CCRF-CEM cells with P4/P7 primers contained wild-type sequence at the P2

**TABLE I**

| Primer | Sequence | Position |
|--------|----------|----------|
| P1     | 5’–tcgcttatcctctctacatct | 401 – 421 |
| P3     | 5’–gtatgggctgctgctcttg | 1572 – 1552 |
| P4     | 5’–gatagccagggagagacct | 120 – 140 |
| P7     | 5’–gagcagcagatggataggaaggct | 572 – 550 |
| P8     | 5’–aggagagaaagggcgcagccggca | 46 – 24 |
| P9     | 5’–ccgaggggaagggcgcagccggca | 1427 – 1449 |
| P10    | 5’–acccacacctttccccagcaaaaa | 2024 – 2002 |
locus; however, for 3 of these wild-type clones, the 4-bp CATG insertion was detected.

PCR amplification of CEM/Mtx-1 genomic DNA with both intron- and exon-specific primers, and sequencing of the PCR products confirmed both P1 and P2 mutations at the genomic level. Again, neither of the mutations was detected in parental cells. Notably, the 4-bp (CATG) insertion could not be found in any of the genomic DNAs amplified with the P8 and RFC-IP3 primers, suggesting that this probably arose from alternative splicing of intron sequence at the splice donor junction at position 189 (24, 25). Not surprisingly, a number of CEM/Mtx-1 clones (1 of 8 for P1 and 3 of 4 for P2, amplified with separate primer sets) exhibited wild-type genomic sequence.

The P2 mutation would result in a change of glycine 44 to arginine and, by computer prediction (Garnier-Robson-Osguthorpe; 35), introduce an altered secondary structure in the region immediately upstream from this locus. The P2 mutation results in a substitution of serine 127 by asparagine in a conserved putative transmembrane domain (residues 124 to 144), yet secondary structure is seemingly unaffected. The CATG insertion at position 191 of the RFC coding sequence generates a frameshift and early translation termination at position 1176, resulting in a truncated RFC protein (48 kDa) with only 11% of recognizable primary sequence. However, this would appear to account for no more than 30% of the loss of the full size RFC protein.

Characterization of Mutant RFC P1

The lack of detectable RFC protein in CEM/Mtx-1 cells may reflect its inefficient synthesis, membrane targeting, or decreased stability of the double-mutated RFC. To evaluate these possibilities, wild-type RFC and a mutant construct containing G to A mutations at both positions 130 and 380 (designated RFCP1P2) were transfected into transport-defective K562 (K500E) and CHO (pC43/10 and RP11-2F3) transfectants, and untransfected cells (K500E and MtxRIIOuaR2-4) were analyzed on Northern blots probed with a 32P-labeled human RFC cDNA (KS43; Ref. 20). Equal loading was established by staining with ethidium bromide (not shown). Size markers (18 S and 28 S, or molecular standards) are noted. Panels A and D, equal amounts of total RNAs (15 μg) from K500E (K43–6, K43P1P2, and K43P1P2/22) and CHO (pC43/10 and RP11-2F3) transfectants, and untransfected cells (K500E and MtxRIIOuaR2-4) were analyzed on Northern blots probed with a 32P-labeled human RFC cDNA (KS43; Ref. 20). Equal loading was established by staining with ethidium bromide (not shown). Size markers (18 S and 28 S, or molecular standards) are noted. Panels B and E, particulate membrane fractions from the K500E and CHO transfectants were analyzed on Western blots with untransfected cells. The protein amounts (in μg) analyzed for each of the sublines are noted. Detection was with anti-GST-RFC antibody and enhanced chemiluminescence. Migrations of wild-type RFC (70 kDa) and RFCP1P2 (70 kDa) are noted. In the wild-type RFC transfectants, a 70-kDa band comigrating with RFCP1P2 is seen at some exposures, likely reflecting less glycosylated variants of wild-type RFC.

Panels C and F, initial uptake rates for [3H]Mtx (0.5 μM) were assayed over 180 s as described under “Experimental Procedures.” Mean data are shown for duplicate incubations from a single representative experiment.

FIG. 4. Expression and transport of wild-type RFC and RFCP1P2 cDNA constructs in K500E (left panels) and CHO (right panels) transfectants. Panels A and D, equal amounts of total RNAs (15 μg) from K500E (K43–6, K43P1P2, and K43P1P2/22) and CHO (pC43/10 and RP11-2F3) transfectants, and untransfected cells (K500E and MtxRIIOuaR2-4) were analyzed on Northern blots probed with a 32P-labeled human RFC cDNA (KS43; Ref. 20). Equal loading was established by staining with ethidium bromide (not shown). Size markers (18 S and 28 S, or molecular standards) are noted. Panels B and E, particulate membrane fractions from the K500E and CHO transfectants were analyzed on Western blots with untransfected cells. The protein amounts (in μg) analyzed for each of the sublines are noted. Detection was with anti-GST-RFC antibody and enhanced chemiluminescence. Migrations of wild-type RFC (70 kDa) and RFCP1P2 (70 kDa) are noted. In the wild-type RFC transfectants, a 70-kDa band comigrating with RFCP1P2 is seen at some exposures, likely reflecting less glycosylated variants of wild-type RFC.
RFCP1-P2 transcripts were, likewise, detected on Northern blots for the majority of both K500E (8 of 12) and MtxRII-Oua62-4 (11 of 18) transfectants (not shown), invariably as multiple hybridizing bands (Fig. 4, A and D, shows representative data). The smallest band approximated the size (~2 kb) of the RFCP1-P2 cDNA so that all transcript forms were of sufficient size to encode the RFCP1-P2 protein. For only two K500E (K43P1-P2/4 and K43P1-P2/22) clones and one MtxRII-Oua62-4 (RIIP1-P2/13) clone was RFCP1-P2 protein detected on Western blots (shown in Fig. 4, panels B and E). For these, the levels of mutant protein were exceedingly low (estimated by densitometry as 4–8% of the wild-type value relative to levels of total RFC or RFCP1-P2 transcripts). Furthermore, RFCP1-P2 protein was distinctly smaller (~70 kDa) than the wild-type carrier (Fig. 4). Both wild-type RFC and RFCP1-P2 completely reverted to 65-kDa deglycosylated forms upon treatment with N-glycosidase F (Fig. 5), establishing that these differences in carrier size reflected their extents of N-glycosylation.

**Turnover of Wild-type RFC and RFCP1-P2 Proteins**—The decreased levels of mutant RFCP1-P2 compared with wild-type RFC in transfected cells (and by extension, CEM/Mtx-1) may, in part, reflect differential rates of carrier degradation. To explore this possibility, K43-6 and K43P1-P2/22 transfectants were treated with 0.2 mg/ml cycloheximide (results in ~40% inhibition of protein synthesis, as reflected in trichloroacetic acid-precipitable [3H]leucine). Rates of exponential decline of wild-type RFC and RFCP1-P2 were assayed over 24 h on Western blots (Fig. 6). By this analysis, the level of wild-type RFC decreased by approximately 50% over 24 h. In contrast, RFCP1-P2 protein exhibited a rapid turnover (Fig. 6). The half-life of RFCP1-P2 was calculated as 2.0 ± 0.56 h (mean ± S.E.; n = 3) and no RFCP1-P2 protein could be detected after 8 h following addition of cycloheximide.

**Functional Properties of the RFCP1-P2, RFCP1, and RFCP2 Proteins**—The effects of the P1 and P2 mutations on [3H]Mtx uptake for MtxRII-Oua62-4 and K500E transfectants expressing wild-type RFC and RFCP1-P2. By this analysis, clones expressing the double mutant RFCP1-P2 (RIIP1-P2/13, K43P1-P2/4, and K43P1-P2/22, respectively) were completely devoid of [3H]Mtx transport activity (Fig. 4, panels C and F); i.e., initial rates of [3H]Mtx uptake over 180 s were identical for the transfected cells and the untransfected lines from which they were derived.

[3H]Mtx transport was partially restored for the single mutant RFCP1 and RFCP2 constructs, expressed in MtxRII-Oua62-4 cells (i.e., RIIP1-P2, and RIIP-P2/15A for RFCP1 and RFCP2, respectively; Fig. 7). For the RIIP-P2/15A cells, expressing the highest levels of RFCP2 (8-fold greater than wild-type RFC in pC43/10), uptake of [3H]Mtx (0.5 μM) was ~50% of that for wild-type RFC-expressing cells. Although we were able to identify stably expressed RFCP1 (in RIIP-P2 cells) by screening over 20 G418-resistant colonies, expression was low (Fig. 7, inset). However, both RIIP-P2 and RFCP2/15A exhibited sufficient transport activity to calculate kinetic constants for Mtx uptake (Table II). With both RFCP1 (RIIP1-P2) and RFCP2 (RIIP-P2/15A), the K<sub>i</sub> values for Mtx were increased from that for wild-type RFC (~11 and ~5-fold, respectively; Table II). The absolute V<sub>max</sub> values for Mtx for both RFC mutant constructs were at least 70% of that for wild-type carrier (i.e., pC43/10 cells; Table II). When normalized to levels of immunoreactive RFC protein on Western blots (measured by densitometry; Fig. 7, inset), the relative V<sub>max</sub> for RFCP1 (RIIP1-P2 subline) exceeded that for the wild-type carrier by 3-fold, whereas the relative V<sub>max</sub> for RFCP2 (RIIP-P2/15A subline) was only 16% of the wild-type value (Table II). The V<sub>max</sub> (normalized)/K<sub>i</sub> values were 27 and 2.7%, respectively, of that for wild-type RFC. In contrast to RFCP1-P2 (see above), the substitution of arginine for glycine 44 in the single-mutated RFCP1 construct and asparagine for serine 127 in RFCP2 had no obvious effect on the processing to mature, glycosylated (~85 kDa) RFC proteins (Fig. 7, inset).

**DISCUSSION**

CEM/Mtx-1 cells exhibit only 3% of normal levels of Mtx influx associated with a 4-fold increased K<sub>i</sub> and 6-fold decreased V<sub>max</sub> (12). This altered substrate binding extends to a range of folate and antifolate transport substrates (10-ethyl-10-deazaaminopterin, aminopterin, ZD1694, GW1843U89, (6R)-5,10-dideaza-5,6,7,8-tetrahydrofolate, folic acid, and leucovorin) and initially suggested to us that the synthesis of a structurally altered RFC might be responsible for the drug-resistant phenotype (12). Although the demonstration of normal levels of a major 3.1-kb transcript in CEM/Mtx-1 cells lent further credence to this notion, no significant RFC protein could be detected by Western blotting or photoaffinity labeling.
In MOLT3/Mtx\textsubscript{10,000} cells, the absence of immunoreactive RFC was previously attributed to the presence of mutations in the RFC coding sequence which resulted in early translation termination and the synthesis of severely truncated RFCs (8). Since an analogous mechanism could occur in the CEM/Mtx-1 subline, we sequenced partial cDNAs amplified from CEM/Mtx-1 transcripts. Indeed, a 4-bp (CATG) insertion was identified at position 191 of the RFC coding sequence which resulted in a frameshift and the use of a stop codon at position 1176. Although this would generate a predicted ~48-kDa protein with only 11% of recognizable RFC sequence and unlikely to react with GST-RFC antibody on Western blots, the low frequency at which the 4-bp insertion was detected (19–30% of cDNA clones) suggested, at most, its minor contribution to the lack of RFC expression in these cells.

Wild-type RFC sequence was also detected in a small number of CEM/Mtx-1 cDNAs. However, its low frequency, combined with the lack of a signal on Western blots (even at high protein loading; data not shown), indicated that an insignificant amount of wild-type RFC protein was actually synthesized in these cells. The variable frequencies at which wild-type cDNA and genomic sequences were detected in these analyses may reflect the localization of RFC to chromosome 21 (21q22.2–22.3; Ref. 33) and presence of a third (and possibly wild-type) RFC allele due to a random trisomy 21 in the CEM/Mtx-1 subline (1 of 8 karyotypes).\textsuperscript{2}

Rather, the majority of RFC transcripts in the CEM/Mtx-1 subline contained G to A substitutions at both nucleotide positions 130 and 380 (in general, without the 4-bp insertion at position 191) which result in replacements of amino acids 44 and 127. The lack of detectable mutated RFC proteins in these cells could result from impaired translation of mutant RFC transcripts, and/or an accelerated degradation or inefficient plasma membrane targeting of mutant proteins. These possibilities could not be evaluated in CEM/Mtx-1 cells. Consequently, we expressed mutant RFC cDNAs in transport-defective CHO and human cells to better correlate levels of RFC transcripts and immunoreactive protein for comparison with wild-type RFC. As with the wild-type RFC transfectants (6, 21), double-mutated RFC\textsuperscript{P1+P2} transcripts were observed on Northern blots at high frequencies for both CHO and K500E transfectants. However, only for the cells transfected with wild-type RFC constructs were significant accumulations of immunoreactive RFC protein detected. For the three clones which expressed sufficient mutant RFC\textsuperscript{P1+P2} protein for immunoblot detection, the carrier migrated as a ~70-kDa band, distinguishable from both native wild-type RFC (~85 kDa) and enzymatically deglycosylated RFC (65 kDa).

Hence, the presence of the P\textsubscript{1} and P\textsubscript{2} mutations appears to alter processing to the mature N-glycosylated carrier and, likewise, results in markedly decreased levels of membrane RFC\textsuperscript{P1+P2} protein. This, in part, reflects the dramatically accelerated turnover of the mutant carrier, likely due to altered secondary and tertiary structures, however, differences in translation efficiencies between wild-type and RFC\textsuperscript{P1+P2} cannot be discounted as a contributing factor. Increased turnover rates of mutant proteins are well established as a mechanism for maintaining cellular homeostasis (36, 37). For other integral membrane proteins, including cystic fibrosis transmembrane regulatory protein (38) and p-glycoprotein (39), increased rates of mutant protein degradation accompany incomplete glycosylation and impaired membrane targeting due to retention in the endoplasmic reticulum. However, for RFC\textsuperscript{P1+P2}, there was no evidence for endoplasmic reticulum retention and degradation since both wild-type RFC and mutant RFC\textsuperscript{P1+P2} co-localized with 5’-nucleotide activity in particulate and sucrase gradient-purified plasma membrane fractions (data not shown). Furthermore, there were no significant differences in the levels of wild-type RFC or RFC\textsuperscript{P1+P2} between sucrase gradient-purified and crude particulate membrane fractions, differing ~3-fold in NADPH-cytochrome c reductase (an endoplasmic reticulum marker enzyme) activity. Thus, both carrier.

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**FIG. 7.** Western blotting and Mtx transport for CHO transfectants expressing wild-type RFC, and single mutated RFC\textsuperscript{P1} and RFC\textsuperscript{P2} proteins. MtxRIIOua\textsuperscript{2-4} cells were transfected with the wild-type RFC, RFC\textsuperscript{P1}, and RFC\textsuperscript{P2} constructs and positive transfectants selected with G418. The inset shows the relative levels of full size (~85 kDa) RFC protein in positive transfectants expressing wild-type human RFC (pC43/10), RFC\textsuperscript{P1} (RIIP\textsuperscript{1/2}), and RFC\textsuperscript{P2} (RIIP\textsuperscript{P2}/6A and RIIP\textsuperscript{P2}/15A). No significant immunoreactive bands were detected for wild-type CHO (Pro-3) and untransfected (or “mock” transfected; data not shown) MtxRIIOua\textsuperscript{2-4} cells. The amounts of membrane protein loaded (in micrograms) are indicated. The bar graph shows the absolute (not normalized for RFC protein on Western blots) values for the initial uptake rates of [3H]Mtx (0.5 mCi) and untransfected (or “mock” transfected; data not shown) MtxRIIOua\textsuperscript{2-4} CHO cells, and for the MtxRIIOua\textsuperscript{2-4} transfec-
tants. Mean uptake data are shown for duplicate incubations from a single representative experiment.

**TABLE II**

Kinetic constants for Mtx influx

| Cell line | Construct | $K_t$ | $V_{max}$ | $V_{max}$ | $V_{max}$ (normalized)/$K_t$ |
|-----------|-----------|-------|-----------|-----------|-----------------------------|
| pC43/10   | Wild type | 0.68 ± 0.06 | 3.78 ± 0.35 | 1.0 | 1.47 |
| RIIP\textsuperscript{1/2} | RFC\textsuperscript{P1} | 7.81 ± 4.50 | 2.80 ± 0.57 | 3.1 | 0.40 |
| RIIP\textsuperscript{P2}/15A | RFC\textsuperscript{P2} | 3.62 ± 1.89 | 2.67 ± 0.57 | 0.16 | 0.04 |

$^a$ $K_t$ and $V_{max}$ values were reported as mean ± S.E.

$^b$ $V_{max}$ (normalized) values were normalized to intensity of RFC signal/mg of protein based on the Western blot data in Fig 7.

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\textsuperscript{2} B. Hukku, unpublished observation.
forms are primarily targeted to the cell surface.

It was of interest that both point mutations identified in CEM/Mtx-1 RFC resulted in replacement of highly conserved amino acids (glycine 44 and serine 127) and, together, they completely abolished transport activity. When expressed individually in transport-impaired CHO cells, both P1 and P2 mutant constructs exhibited low levels of transport activity. Although the presence of arginine 44 in the mutant RFC{sup P1} actually increased the Mtx{sub Vmax} over wild-type RFC in RIIP{sub 1}/2 cells, this was accompanied by an increased K{sub m} so that net transport was appreciably impaired. For RFC P2, Mtx uptake in CEM/Mtx-1 cells, this was accompanied by an increased binding properties in CEM/Mtx-1 cells (12) are implicated for glutamate 45 (11) and serine 46 (10), and likely important for RFC function. Important functional roles for alanine 132 (alanine 130 in the murine RFC; Ref. 7) from synthesis of a mutated carrier with a lysine substitution for glutamate 45. These discrepancies with our results cannot be explained simply by differences in experimental methodologies or data interpretation. Rather, the most likely explanation is that the CEM/Mtx cells somehow changed during long-term culture and/or different clonal variants of CEM/Mtx-1 were studied in the different laboratories.

In our studies, the complete absence of RFC protein and the total lack of [3H]Mtx transport activity for RFC{sup P1}–{sup P2} strongly implies that the residual uptake and anomalous substrate binding properties in CEM/Mtx-1 cells (12) are not due to the RFC{sup P1}–{sup P2}. While the transport kinetics observed for this resistant subline are clearly incompatible with those of the wild-type carrier, the small amounts of wild-type RFC activity may be modulated by unknown endogenous factors (6, 21, 41, 42). Alternatively, non-RFC modes of uptake (13–15) may also contribute to the CEM/Mtx-1 transport phenotype. Studies are underway to further explore these possibilities.

Acknowledgments—We thank Dr. Wayne Flintoff for providing the Mtx transport defective MtxRHIOu#{sub 2} CHO cells and Dr. Andre Rosowsky for providing the CCRF-CEM and CEM/Mtx sublines. We thank Daryel Taliaferro for secretarial assistance in preparing this manuscript.

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