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Human K562 Transfectants Expressing High Levels of Reduced Folate Carrier but Exhibiting Low Transport Activity

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ABSTRACT. A human reduced folate carrier (hRFC) cDNA was transfected into transport-deficient K562 cells to circumvent complications that may result from carrier expression in a heterologous mammalian species. Relative to wild-type cells, hRFC transcript levels were increased 11- and 19-fold, respectively, in the K43-6 and K43-1 transfectants. Although photoaffinity labeling of hRFC protein revealed similar increases of 15- and 19-fold, respectively, only a 2-fold enhancement in methotrexate (Mtx) transport was observed. This suggests that only a small portion of the cDNA-encoded hRFC protein is actively engaged in membrane transport. Kinetic analysis of [^{3}H]Mtx transport indicated that K43-6 cells exhibited a similar affinity (K_t) but an increased V_{max} (1.7-fold) when compared with K562 cells. The restored transport was similar to that of wild-type cells in its capacity to be trans-stimulated by intracellular folates and in its sensitivity to competitive transport inhibitors (1843U89, bromosulfophthalein, folic acid, leucovorin, and ZD1694) and to irreversible inhibition by *N*-hydroxysuccinimide-methotrexate. Further, deglycosylated photoaffinity-labeled hRFC protein in both K562 and K43-6 cells migrated at ≈65–70 kDa on SDS-gels, consistent with the molecular mass from the predicted amino acid sequence. These data further establish that the expression of hRFC, alone, is sufficient to confer transport properties typical of the "classical" hRFC. However, the discrepancy between the stoichiometry of carrier expression and transport activity implies that membrane translocation of bound substrate may be regulated by additional undefined mechanisms. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;2:199–206, 1997.

KEY WORDS. folate; methotrexate; transport; cDNA; expression

Defective membrane transport of Mtx¶ via the RFC system has been identified as a major mechanism of drug resistance [1–3]. To elucidate the molecular events that result in defective Mtx transport, identification of the elements involved in RFC transport has been a major interest of many laboratories. Recently, the isolation of mouse and hamster cDNAs that restore RFC activities in transport-defective cells was reported independently [4, 5]. Subsequently, based on these sequences, homologous human cDNAs were identified [6–9].

In studies from this laboratory, human cDNAs (hRFC, GenBank accession No. U19720) that restored RFC activity in transport-defective CHO cells [6] were isolated from a library prepared from transport up-regulated K562 cells (K562.4CF; [10]). Although functional expression studies strongly suggested that the cDNAs encode the RFC, the CHO transfectants exhibited transport properties typical of both human and hamster cells. These findings imply that additional regulatory/modulatory factors may be required to express the full spectrum of transport substrate specificities and kinetics typical of the RFC, and/or that the observed hamster/human hybrid transport phenotype may be influenced by the host cells used for transfection.

Other RFC transfection studies, to date, have generally correlated Mtx transport with RFC transcripts on northern blots [5, 11], or total levels of surface [^{3}H]Mtx binding to

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¶ *Abbreviations:* Mtx, methotrexate; AMT, aminopterin; APA-[^{125}I]ASA-Lys, N^{α}-(4-amino-4-deoxy-10-methylpteroly)-N^{ϵ}-(4-azido-5-[^{125}]iodosalicylyl)-L-lysine; CHO, Chinese hamster ovary; DHFR, dihydrofolate reductase; DPBS, Dulbecco's phosphate-buffered saline; HBSS, Hanks' balanced salt solution; NHS-Mtx, *N*-hydroxysuccinimide ester of methotrexate; hRFC, human reduced folate carrier; RFC, reduced folate carrier; RFT, reduced folate transporter; RS1, regulatory subunit 1; SGLT1, Na^+-D-glucose cotransporter; and TS, thymidylate synthase.

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intact cells [5, 11]. While approximate correlations between these parameters were noted, the quantitative stoichiometries between levels of cDNA-encoded RFC protein and restored RFC function in transfected cells have not been widely reported. Moreover, there remains an unresolved discrepancy between the predicted murine RFC1 molecular mass (58 kDa) and those reported earlier by photoaffinity or radioaffinity labeling (42–48 kDa or 38 kDa) of putative murine RFCs [4, 6, 12–14]. In hamster cells transfected with human cDNAs, higher molecular weight proteins (85–94 kDa; [6]) were detected by photoaffinity labeling than were identified on western blots (56 kDa) of proteins from human breast cancer cells probed with peptide-specific antiserum based on the RFC cDNA sequence [9]. These controversies surrounding the putative hRFC cDNA-encoded proteins have been fueled by the identification of a seemingly separate 46 kDa murine transporter [15–17] or, previously, a distinct 92 kDa human transport protein [18].

In the present study, a full-length hRFC cDNA (KS43) was transfected into a transport-deficient human K562 cells to circumvent the complications that may result from the expression of the human transport protein in a heterologous mammalian system. Although our data continue to support the notion that the hRFC cDNA indeed encodes the "classical" RFC, the disparate stoichiometry of carrier protein expression and Mtx transport strongly implies that additional components and/or other undefined mechanisms must regulate RFC function.

MATERIALS AND METHODS

Materials

[3′,5′,7-^{3}H]Mtx (20 Ci/mmol) and [5-^{3}H]2′-deoxyuridine monophosphate (22 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). [α-^{32}P]Deoxycytidine triphosphate was purchased from DuPont/New England Nuclear. Unlabeled Mtx, AMT, and (6*R*,*S*)5-formyltetrahydrofolate (leucovorin) were obtained from the Drug Development Branch, National Cancer Institute, Bethesda, MD. Folic acid was purchased from the Sigma Chemical Co. (St. Louis, MO). ZD1694 [*N*-(5-[*N*-(3,4-dihydro-2-methyl-4-oxyquinazolin-6-ylmethyl)-*N*-methylamino]-2-thenoly)-L-glutamic acid] was obtained from Dr. Ann Jackman (Institute of Cancer Research, Surrey, England) and 1843U89 [(*S*)-2-(5-((1,2-dihydro-3-methyl-1-oxobenzo (F)quinazolin-9-yl)methyl)amino)1-oxo-2-isoindolinyl] glutaric acid from the Glaxo–Wellcome Co. (Research Triangle Park, NC). Both labeled and unlabeled Mtx were purified prior to use by reverse-phase HPLC [19]. The other folates and antifolates were used as provided.

Tissue culture reagents and supplies were purchased from assorted vendors with the exception of fetal bovine serum and iron-supplemented calf serum, which were from the Grand Island Biological Co. (Grand Island, NY) and Hyclone Laboratories, Inc. (Logan, UT), respectively.

Cell Culture

The wild-type K562 erythroleukemia line was obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in RPMI 1640 containing 10% heat-inactivated iron-supplemented calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, in a humidified atmosphere at 37° in the presence of 5% CO_2/95% air. The transport-deficient K500E subline was selected from wild-type K562 cells by cloning in soft agar in the presence of 500 nM Mtx [18]. K500E cells were maintained continuously in the presence of 500 nM Mtx; before assay of [^{3}H]Mtx transport, cells were cultured for 3–5 generations without Mtx. Cell lines were subcultured every 96 hr. Cell numbers were determined by direct microscopic counting with a hemacytometer.

For cytotoxicity determinations, cells were cultured in 24-well culture dishes at 20,000 cells/mL in 2 mL of complete RPMI 1640 containing 10% dialyzed fetal bovine serum. Cells were counted after 120 hr. The IC_{50} values were calculated as the concentrations of Mtx necessary to inhibit growth by 50% compared with control cells grown under identical conditions except that the growth inhibitor was omitted.

Transfection of K500E Cells

The 2.8 kb hRFC cDNA insert (KS43) was directionally ligated into the pcDNA3 expression vector (Invitrogen, San Diego, CA) as described previously [6]. The resulting construct, pC43, was transfected into the transport-deficient K500E cells [18] using lipofectin [20]. Briefly, cells (2×10^6) were washed with opti-MEM I medium (Gibco-BRL) and plated in a volume of 2.5 mL in a 60-mm Petri dish. Cells were then treated with a mixture of pC43 (3 μg)/lipofectin (20 μg) in 200 μL of Opti-MEM I. After 20 hr at 37°, the cells were diluted with 4 mL of complete RPMI 1640 containing 20% supplemented calf serum and antibiotics. Two days later, the cells (500, 1000, 10,000) were plated in 60-mm dishes containing RPMI 1640, 10% supplemented serum, antibiotics, and 0.35% Noble agar, in the presence of 1 mg/mL G418. G418-resistant clones that exhibited increased Mtx sensitivities over the K500E line were selected for further analysis. A mock transfection was also performed in parallel using the pcDNA3 vector without cDNA insert.

Membrane Transport Methodology

Logarithmically growing cells were washed with DPBS [21] and resuspended in HBSS. Transport experiments were performed as previously described [10] using cell densities of $1–2 \times 10^7$ cells/mL. Intracellular accumulations of [^{3}H]Mtx (expressed as pmol/mg protein) were corrected for surface adsorption as described by Sirotnak *et al.* [22]. Kinetic constants (i.e. K_t, V_{max} and K_i values) for assorted transport

substrates were calculated from Lineweaver–Burk and Dixon equations, respectively.

To assess the capacity of (6*R*,*S*)5-formyltetrahydrofolate to trans-stimulate Mtx influx via the RFC [13, 23], cells were loaded with (6*R*,*S*)5-formyltetrahydrofolate (50 μM) for 20 min at 37°, then washed with ice-cold DPBS (3 times), and the cell pellets were stored at 0°. For transport assays, the cell pellets were warmed briefly to 37°, and uptake was initiated by rapid resuspension into 37° HBSS containing 0.5 μM [^{3}H]Mtx.

The inhibitory effects of the activated Mtx ester, NHS-Mtx, on [^{3}H]Mtx influx were assessed. NHS-Mtx was prepared as described previously [10, 24]. Cells were treated for 5 min at 23° (while shaking) with various concentrations of NHS-Mtx (200–2000 nM) in anion-free buffer (20 mM HEPES, 225 mM sucrose, pH 6.8, with MgO); the cells were washed with DPBS (2 times), and resuspended in HBSS for [^{3}H]Mtx transport assay, as described above.

Assay for Dihydrofolate Reductase and Thymidylate Synthase

DHFR enzyme levels were quantitated by measuring the extent of [^{3}H]Mtx binding in the presence of high concentrations of NADPH [18, 19]. Cells (≈1 × 10^7) were sonicated in a buffer containing 50 mM sodium citrate (pH 6), 150 mM KCl, 1 mM EDTA, 50 mM 2-mercaptoethanol and 100 μM NADPH. Following a 10-min incubation at 23° with [^{3}H]Mtx (1 nmol), unbound and DHFR-bound [^{3}H]Mtx were fractionated by chromatography of cell-free extracts on columns of Biogel P6 (200–400 mesh) by rapid centrifugation.

TS levels were determined by measurements of catalytic activity involving the release of ^3H_2O during the conversion of [^{3}H]deoxyuridine monophosphate to thymidine monophosphate [13].

Northern Analysis

Total RNA was isolated from log phase cells using the single-step method of Chomczynski and Sacchi [25]. Northern blots were probed with [^{32}P]dCTP-labeled KS43 hRFC cDNA insert or a 0.8 kb insert of human DHFR cDNA as previously described [6]. The human DHFR cDNA was a gift of Dr. Joseph Bertino (Memorial Sloan-Kettering Cancer Center, New York, NY). Relative expression levels were quantitated on a Molecular Dynamics computing densitometer and analyzed using the Image Quant software (Sunnyvale, CA) and corrected for unequal loading by probing with ^{32}P-labeled β-actin.

Cell Surface Labeling of hRFC Protein

A photoaffinity analogue of Mtx, APA-[^{125}I]ASA-Lys, was synthesized from the unlabeled compound using the procedure of Price and Freisheim [12]. The radiospecific activity of the APA-[^{125}I]ASA-Lys was 2.9 mCi/μmol. Labeling procedures were performed at 0° exactly as described previously [6]; a 0.5 μM concentration of the photoprobe was used in each labeling condition. Equal amounts of labeled proteins were resolved on 7.5% SDS-gels [26]; gels were dried and exposed to film. Relative specific labeling was quantitated using a Bio-Rad PhosphorImager. Deglycosylation of the labeled proteins with *N*-glycanse was performed as previously described [18].

RESULTS

Restoration of Mtx Sensitivity in Human K500E Cells by hRFC cDNA Transfection

To eliminate any ambiguity that may result from the expression of hRFC in the heterologous hamster model [6], the KS43 hRFC cDNA was transfected into an Mtx transport-deficient human K562 subline (K500E). Stable transfectants were selected with G418 (1 mg/mL) and screened for increased Mtx sensitivities. Two transfectants, K43-1 and K43-6, showed increased Mtx sensitivities (IC_{50} values of 64 and 80 nM, respectively) over the Mtx-resistant K500E subline and the mock-transfected KD-1 cells (IC_{50} = 1100 nm and 980 nm, respectively, Fig. 1A). However, the Mtx sensitivities for both transfectants were less than that of the wild-type K562 cells (IC_{50} = 7 nM).

The incomplete recovery of Mtx sensitivity in the K500E transfectants compared with the wild-type cells likely reflects the 7.7-fold increase in DHFR levels (16.55 ± 2.70 vs 2.15 ± 0.18 pmol/mg for wild-type K562 cells) and a 49% decrease in TS activity (54.63 ± 10.92 vs 105.62 ± 21.84 pmol/mg/min for wild-type K562) in K500E cells. Consistent with this concept, DHFR enzyme (20.88 ± 1.36 pmol/ml) and mRNA levels (not shown), and TS activity (51.74 ± 5.65 pmol/mg/min) in K43-6 cells were nearly identical to those in K500E cells. While K500E cells and the KD-1 mock-transfected cells were 7-fold resistant to the TS inhibitor ZD1694 (IC_{50} values of 34 and 32 nM, respectively, vs 4.6 nM for wild-type K562 cells), K43-6 cells (IC_{50} = 5 nM) were as sensitive to ZD1694 as the wild-type cells (Fig. 1B). This suggests that the resistance to ZD1694 in K500E cells was due mainly to a transport deficiency.

Reconstituted RFC Transport Activities of K43-1 and K43-6 Cells

Initial rates of [^{3}H]Mtx influx were assayed over 180 sec in wild-type K562, K43-1, K43-6, K500E, and the mock-transfected KD-1 cells (Fig. 2). At 0.5 μM Mtx, only low levels of transport were detected in the K500E and mock-transfected KD-1 lines over 180 sec (approximately 11% of wild-type cells). The K43-6 and K43-1 transfected cell lines were able to accumulate [^{3}H]Mtx at rates 2.8- and 2.6-fold greater than wild-type K562 cells, respectively.

Since the transport activities for both transfectants were essentially identical, the K43-6 subline was selected for the more detailed transport characterization. Kinetic analyses of influx rates over a range of [^{3}H]Mtx concentrations were

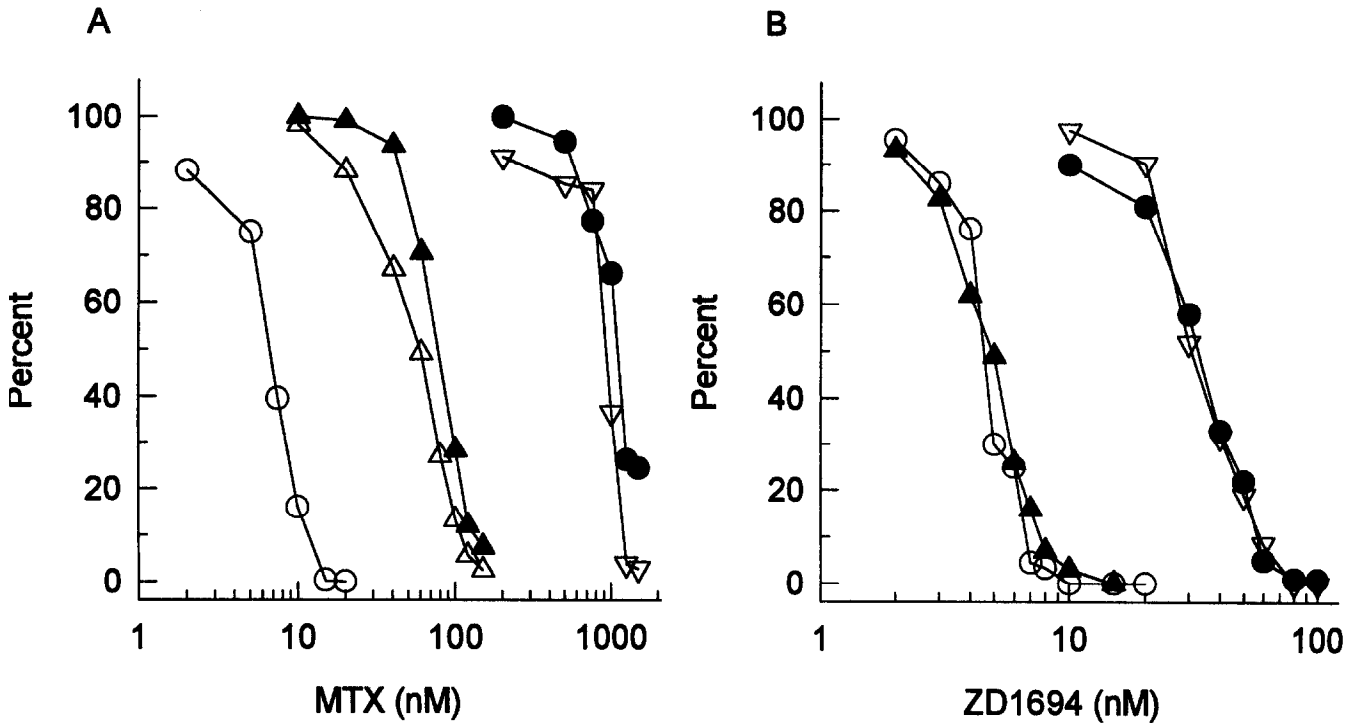

FIG. 1. Cytotoxicity of Mtx and ZD1694. Cytotoxicity curves of Mtx (A) and ZD1694 (B) for wild-type K562 (○), transport-deficient K500E (●), mock-transfected KD-1 (▽), and the K43-6 (▲) and K43-1 (△) transfected cells. Results are expressed as percent of survival at each drug concentration relative to control cells.

performed; kinetic constants were calculated from Lineweaver–Burk plots and are summarized in Table 1. The K_t values for wild-type K562 and K43-6 cells were nearly identical; however, the V_{max} for K43-6 was increased 1.7-fold. The transport capacity (V_{max}/K_t) for K43-6 was increased 2-fold over wild-type cells and approximately 20-fold over the K500E subline.

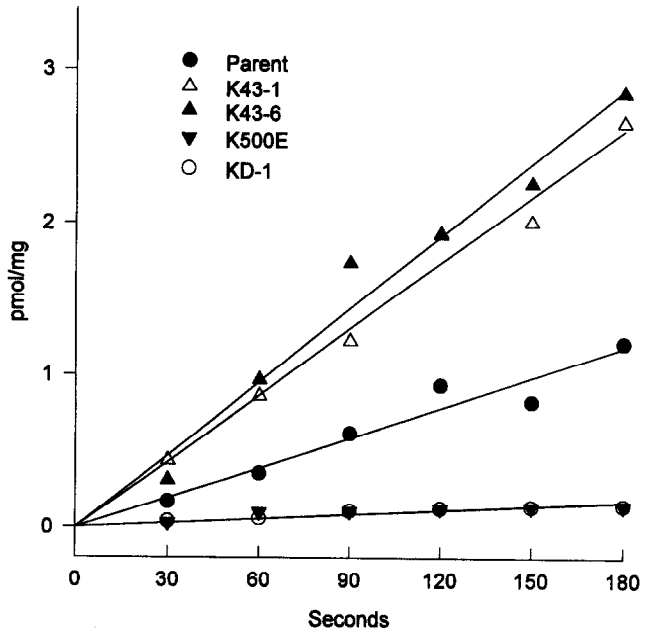

FIG. 2. Initial uptake of [^{3}H]Mtx in wild-type K562 and transfected sublines. Uptake of [^{3}H]Mtx (0.5 μM) was measured at 37° over 180 sec. Results were corrected for cell surface binding. Data are from a single representative experiment performed 3 separate times with similar results.

Expression of RFC Protein

Using the KS43 hRFC cDNA as a probe in northern analysis, the levels of hRFC transcript were quantitated in the transfected lines for comparison with wild-type K562 and K500E cells (Fig. 3). Consistent with our earlier report [6], hRFC transcript level in K500E subline was decreased 2- to 3-fold; a similarly decreased transcript level was detected in the KD-1 mock-transfected line. Both K43-6 and K43-1 transfectants showed marked increases in hRFC transcript levels. Compared with wild-type cells, hRFC transcript levels were increased 11- and 19-fold, respectively.

To investigate the level of RFC protein expression in the transfected lines, cells were labeled with APA-[^{125}I]ASA-Lys, a photoaffinity inhibitor of RFC [6, 12, 27]. For all

TABLE 1. Kinetic constants for Mtx influx*

Cell line	K_t (μM)	V_{max} (pmol/mg/min)	V_{max}/K_t
K562	2.80 ± 1.05	4.89 ± 2.43	1.75
K43-6	2.32 ± 1.27	8.43 ± 2.82	3.63
K500E	4.60 ± 1.65	0.84 ± 0.31	0.18

* Kinetic constants for Mtx transport were calculated by Lineweaver–Burk analysis of initial rate data expressed as means ± SEM from 4 experiments.

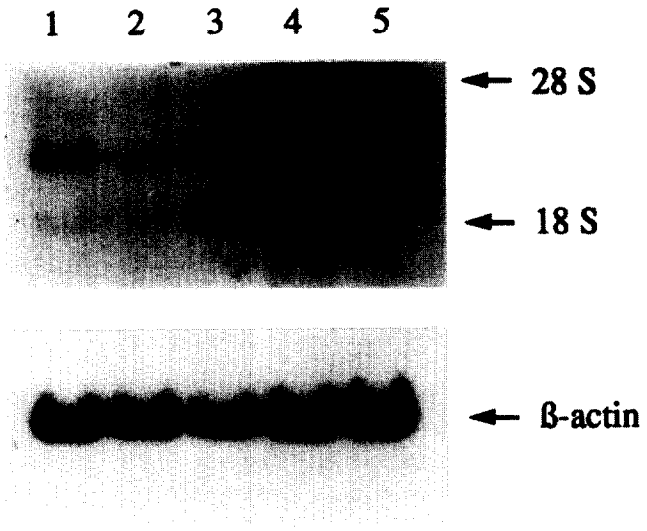

FIG. 3. Northern blot analyses of RNAs prepared from K562 and various sublines. Upper panel: total RNAs (20 μg/lane) were size-fractionated and hybridized to [^{32}P]dCTP-labeled hRFC cDNA insert (KS43). Lane 1, wild-type K562; lane 2, K500E; lane 3, KD-1; lane 4, K43-1; and lane 5, K43-6. Lower panel: the same membrane was stripped and rehybridized with a human β-actin cDNA probe.

lines, photoaffinity-labeled RFC proteins migrated as broad bands (≈80–94 kDa, Fig. 4). Radiolabel incorporation was abolished in the presence of 100 μM AMT, establishing its specificity. When compared with the wild-type cells, an approximate 90% reduction in photoaffinity labeling was detected in K500E cells, which is identical to the decrease

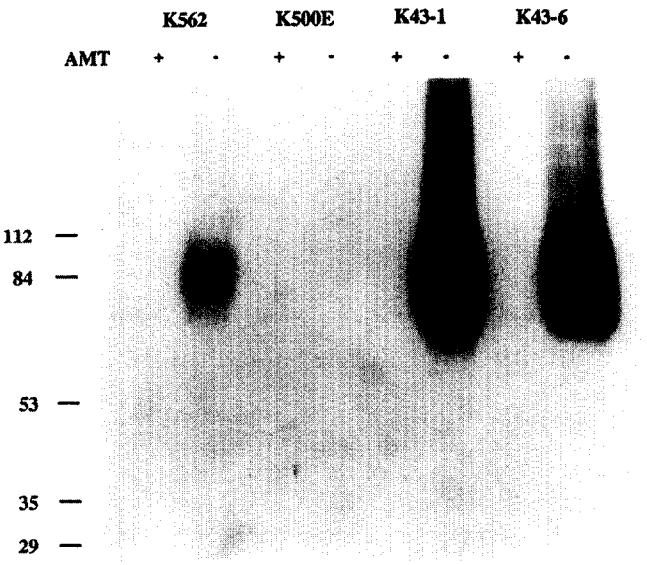

FIG. 4. Photoaffinity labeling of RFC proteins with APA-[^{125}I]ASA-Lys. For each cell line, 1 × 10^8 cells were treated with 0.5 μM APA-[^{125}I]ASA-Lys in the presence (+) or absence (-) of 100 μM AMT. Equal amounts of membrane proteins (250 μg/lane) were electrophoresed on a 7.5% gel in the presence of SDS. The figure shows the autoradiograph of the dried gels. The molecular weights (in kDa) of standard proteins are indicated.

in Mtx transport (Table 1). However, for the K43-1 and K43-6 sublines, the 2- to 3-fold increased Mtx uptake over wild-type cells was accompanied by 15- and 19-fold increased levels of radiolabeled RFC, respectively. Hence, RFC protein expression in the K43-1 and K43-6 transfectants approximates relative levels of hRFC transcripts (Fig. 3).

Characteristics of Expressed RFC in Transfected Human Cells

To assess the structural specificities of the RFC in the K43-6 transfectant, K_i values for a number of transport substrate inhibitors [1, 28] were calculated from Dixon equations and the K_t and V_{max} values in Table 1. For all compounds tested (1843U89, bromosulfophthalein, folic acid, leucovorin, and ZD1694), there were only minor differences in the calculated K_i values between wild-type K562 cells and the K43-6 transfected line (Table 2).

In addition, K43-6 cells were examined for transport characteristics commonly associated with RFC such as the capacity to be trans-stimulated by a high intracellular concentration of leucovorin [13, 23] and sensitivity to inhibition by NHS-Mtx, an affinity inhibitor of RFC [24]. Following preloading with 50 μM leucovorin for 20 min, increases of 91 and 136% in initial rates of Mtx uptake were observed for K43-6 and wild-type cells, respectively (Fig. 5). Further, sensitivities to inhibition by NHS-Mtx were identical for wild-type K562 and K43-6 cells (Fig. 6).

Finally, experiments were performed to assess whether the disparity between the relative molecular mass of the photoaffinity-labeled RFC determined by gel mobility (≈80–94 kDa, Fig. 4) and the molecular mass of 65 kDa predicted from amino acid sequence [6] was the result from post-translational modifications, particularly N-glycosylation. When digested with *N*-glycanase, the apparent molecular mass of the radiolabeled protein in both wild-type and K43-6 cells shifted to ≈65–70 kDa, a size consistent with the predicted value (Fig. 7). This confirms that the single consensus N-glycosylation site at asparagine 58 is glycosylated.

DISCUSSION

We previously demonstrated that transfection of transport-defective CHO cells with hRFC cDNAs results in the expression of a transport system with kinetic properties resembling both the human and hamster carriers [6]. It seemed possible that the basis for this peculiar transport phenotype may be that additional regulatory/modulatory factors unique to human cells are required for the expression of a human carrier system and/or involves the host cells used in the transfection experiments. In the present study, expression of hRFC in transport-deficient human K500E cells was, likewise, sufficient to fully restore [^{3}H]Mtx uptake. The restored transport exhibited a wide range of properties characteristic of the "classical" RFC in human

TABLE 2. K_i values for assorted transport substrates*

Cell line	K_i (μM)				
	1843U89	BSP	Folic acid	Leucovorin	ZD1694
K562	1.07 ± 0.27	32.06 ± 11.86	263.56 ± 32.66	9.33 ± 1.06	2.61 ± 0.91
K43-6	1.45 ± 0.28	34.78 ± 6.60	360.00 ± 67.52	16.19 ± 2.47	5.54 ± 0.94

* K_i values are expressed as means ± SEM for the inhibition of [^{3}H]Mtx (1 μM) from 3–4 separate experiments. Undefined abbreviation: BSP, bromosulfophthalein.

cells. These include kinetic parameters, competitive inhibition by various transport substrates and inhibitors [1, 28], and sensitivity to the RFC affinity inhibitor NHS-Mtx [24]. Other features commonly associated with the human RFC proteins such as its glycosylation [10] and trans-stimulation by leucovorin [13, 23] were also found in the transfected cells. Similar transport characteristics for the murine RFC1-encoded gene product were reported recently by Brigle *et al.* [11]. While the data in the present study suggest that the hamster/human hybrid transport properties previously observed in the CHO transfectants were, at least in part, a function of the host cells that were used, the possibility of additional regulatory factors or components for the RFC system still remains.

The demonstration of corresponding high levels of hRFC mRNA and photoaffinity labeling of RFC proteins implies an efficient translation of the cDNA-encoded transcripts in transfected human cells, in contrast to the hamster model [6]. The labeled RFC migrated as a broad, high molecular weight band (80–94 kDa) on SDS gels; the basis for the difference in size when compared with the 56 kDa RFC protein identified in ZR-75-1 breast cancer cells by western analysis [9] is unclear, but may reflect differences in N-glycosylation. In spite of the apparent ability of surface RFC proteins to bind transport substrate, the disproportionate increase in affinity labeling relative to Mtx uptake strongly suggests that only a small portion of the expressed RFCs are actively involved in facilitating Mtx membrane translocation. A possible explanation for this observation is that attainment of maximal rates of carrier translocation in the transfected lines is, in some fashion, restricted by the availability of a limiting transport component(s). For example, in K500E cells, RFC protein is initially limiting to Mtx uptake; however, upon high level hRFC expression in K43-6 cells, the non-RFC component(s) could then become limiting. Consistent with this argument, nearly identical transport activities were observed for both the K43-1 and K43-6 transfectants despite apparent differences in RFC protein levels. An analogous regulatory mechanism was suggested for the SGLT1 [29, 30]. In this case, a puta-

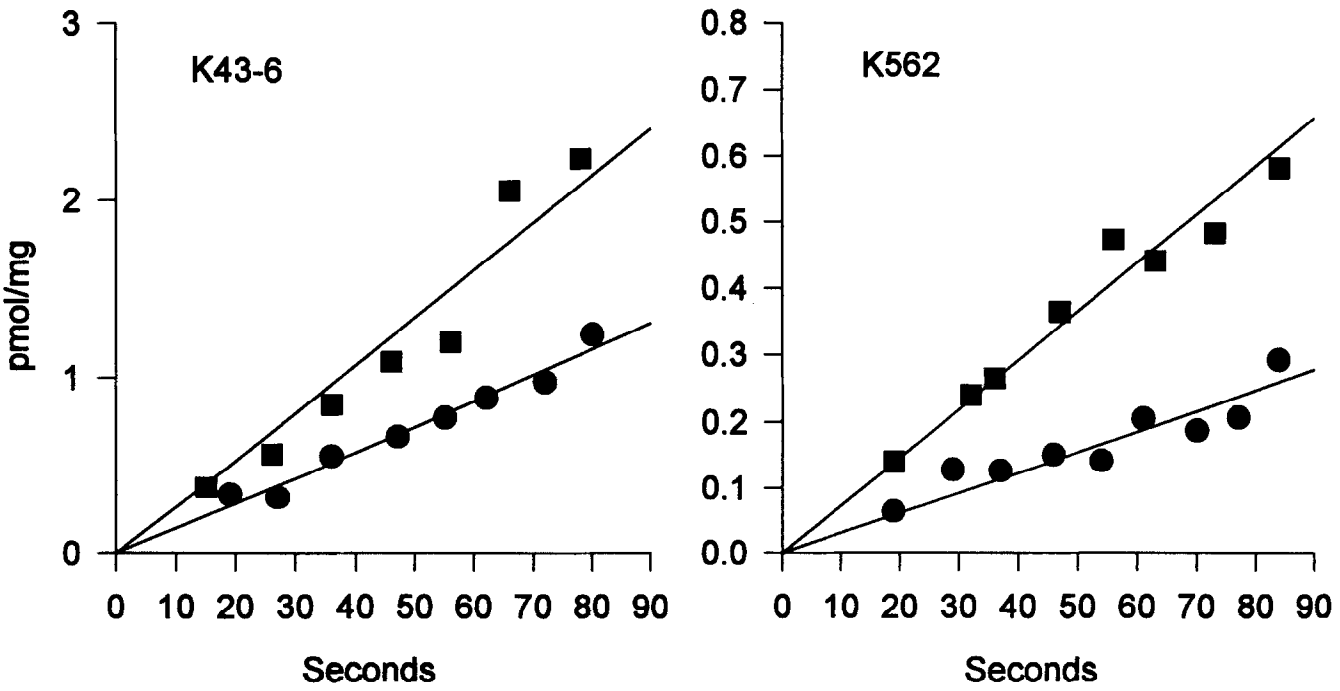

FIG. 5. Trans-stimulation of [^{3}H]Mtx influx by leucovorin. K43-6 (left panel) and K562 (right panel) cells were incubated with 50 μM leucovorin for 20 min at 37°, then were washed and resuspended in HBSS for [^{3}H]Mtx transport measurements. Influx of [^{3}H]Mtx is shown for cells with (■) and without (●) leucovorin pretreatment. Data are from a single representative experiment performed 3 separate times with similar results.

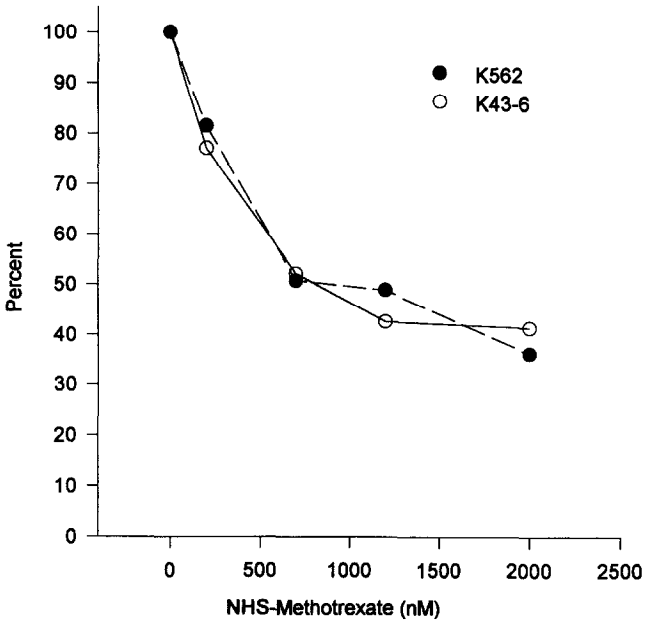

FIG. 6. Inhibitory effect of NHS-MTx on [^{3}H]Mtx uptake by wild-type K562 and K43-6 cells. Cells were treated with various concentrations of NHS-Mtx (200–2000 nM) for 5 min at 23°, washed, and resuspended in HBSS for [^{3}H]Mtx transport assays. Results are expressed as percent uptake of [^{3}H]Mtx (0.5 μM) over 180 sec relative to cells not treated with NHS-Mtx. Data are the means of 3 separate experiments.

tive RS1 component was identified which in co-transfection experiments alters the kinetics of SGLT1 transport [29, 30]. Interestingly, RFC cDNA transfection studies in murine [11] or hamster cells [5, 6] resulted in apparently similar levels of RFC protein expression and Mtx transport (2- to 5-fold increase over wild-type cells). This difference, presumably, reflects the cell model employed for transfection and/or the exceedingly high RFC expression (15- to 19-fold over wild-type cells) in the transfected human K500E sublines. The finding that several transport "up-regulated" cell lines had transport capacities comparable to or exceeding RFC proteins (10, 22, 31), suggests that the putative non-RFC component may also be increased in response to the folate-limiting conditions used to select these lines.

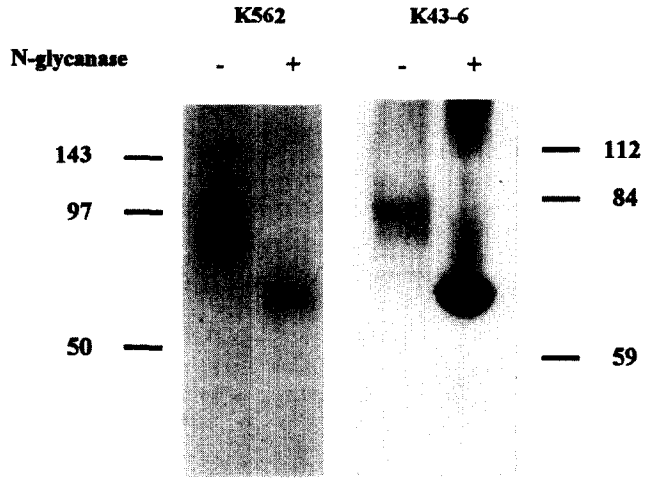

FIG. 7. Deglycosylation of RFC protein. APA-[^{125}I]ASA-Lys-labeled membrane proteins from K562 and K43-6 cells were digested with (+) or without (-) *N*-glycanase, then electrophoresed, and the dried gels autoradiographed. Molecular weights (in kDa) of standard proteins are indicated. The K562 and K43-6 cells for this experiment were labeled with different lots of photoprobe and were analyzed on separate gels.

Early evidence for non-complementary RFC components was presented by Underhill and Flintoff [32] for two transport-defective hamster lines. More recently, a 46 kDa protein designated RFT was purified from up-regulated L1210/R83 cells [15] and found to be immunologically distinct from the 58 kDa murine RFC protein [16, 17]. In human cells (HL-60, CCRF-CEM), RFT antibody detected an 83 kDa protein [15, 17]. A similar 92 kDa NHS-Mtx binding glycoprotein (GP-Mtx) separate from RFC was identified previously in this laboratory from transport up-regulated K562 cells [18]. Finally, an unidentified 38 kDa cytosolic or peripheral membrane protein was implicated in RFC function in CCRF-CEM and L1210 cells affinity labeled with APA-[^{125}I]ASA-Lys [12, 27]. Collectively, these results suggest the functional complexity of reduced folate transport including the possibility that RFC may exist as a multi-component system.

Other explanations for the discrepancy between transport activity and RFC protein in the K562 transfectants include effects on protein folding, membrane insertion/orientation, or post-translational modifications of RFC (e.g. protein phosphorylation). An additional, perhaps related possibility is that RFC transport is subject to regulatory controls responsive to folate cofactors or other cellular metabolites. This may occur directly at the level of the RFC or be mediated by a secondary membrane or non-membrane component(s). It was demonstrated previously that enhanced RFC-mediated transport may be induced by culturing cells in low folate medium [10, 31]. Moreover, such up-regulated transport was decreased upon pretreatment with elevated folates or adenosine [10, 31]. By a similar reasoning, K43-6 cells were cultured (greater than six generations) in folate-free RPMI 1640 containing 0.4, 2, or 10 nM leucovorin as the sole source of folates in an attempt to recruit a seemingly latent RFC fraction to participate in Mtx transport. While the absence of an effect in these experiments (data not shown) might seem to exclude an analogous regulatory mechanism in the K43-6 transfectant, these results may further reflect the role of a limiting regulatory component separate from the RFC in folate transport, as suggested above.

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