Identification of a Highly Glycosylated Methotrexate Membrane Carrier in K562 Human Erythroleukemia Cells Up-regulated for Tetrahydrofolate Cofactor and Methotrexate Transport

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ABSTRACT

A K562 human erythroleukemia line (designated K562.4CF) was selected for increased tetrahydrofolate cofactor transport in a growth-limiting concentration (0.4 mM) of (6R,5)-5-formyltetrahydrofolate. K562.4CF cells exhibited elevated methotrexate uptake relative to parental cells, attributable to a 10-fold increased influx \( \nu_{in} \). The rate of methotrexate efflux in K562.4CF cells was somewhat increased (55%) as well. The transport system in K562.4CF cells had similar and high apparent binding affinities for methotrexate and 5-formyltetrahydrofolate and a markedly reduced affinity for folic acid, properties typically associated with the “classical” methotrexate/tetrahydrofolate cofactor carrier in tumor cells. Methotrexate uptake in K562.4CF cells decreased substantially under nonselective conditions; high levels of transport were restored in 0.4 mM 5-formyltetrahydrofolate. Treatment of parental and K562.4CF cells with N-hydroxysuccinimide methotrexate inhibited methotrexate influx. N-Hydroxysuccinimide-(\( ^{3} \)H)methotrexate (700 nmol) radiolabeled a broadly migrating band at \( M_{r}, 76,000-85,000 \). Incorporation from N-hydroxysuccinimide-(\( ^{3} \)H)methotrexate into this band was increased 7-fold in K562.4CF over parental cells and was blocked by unlabeled methotrexate, (6S)-5-formyltetrahydrofolate, or, to a lesser extent, folic acid. Whereas incubation with endoglycosidase F had no effect on the electrophoretic migration of the labeled protein, treatment with endoglycosidase F and glycopeptidase F, or endo-\( \beta \)-galactosidase, reduced the apparent molecular weight to \( M_{r}, \sim 52,000 \) or \( \sim 58,000 \), respectively. These results suggest that the high-affinity transporter in K562.4CF cells is an N-linked glycoprotein containing internal \( \beta \)-galactosidic linkages in, or immediately after, unbranched poly-N-acetyllactosamine sequences. Differences in the level of glycosylation may, in part, account for the disparity in the apparent sizes of the homologous folate transport proteins from human and murine cells.

INTRODUCTION

Membrane transport of chemotherapeutic drugs into tumor cells is a critical event in antitumor activity since most agents achieve their effects at intracellular loci, and drug-target interactions require membrane penetration. In fact, cytotoxic activity is often limited by the inability of sufficient concentrations of drugs to reach intracellular targets.

In certain cases, uptake is mediated by specific membrane receptors. For instance, for methotrexate, a dianion, to enter tumor cells and inhibit dihydrofolate reductase, the antifolate must first bind to the carrier system normally used by 5-methyltetrahydrofolate and other reduced folates to traverse the cell membrane (1, 2). Uptake efficacy by this system correlates with in vivo methotrexate sensitivities for a number of murine models (3) and has been implicated as an important determinant of antifolate activity toward human tumor cells (4, 5). Moreover, incomplete inhibition of dihydrofolate reductase secondary to impaired methotrexate transport is a major mechanism of methotrexate resistance in \textit{in vitro} (6–9) and \textit{in vivo} (10) models and has been suggested to be an important mode of clinical resistance as well (11). A separate membrane folate-binding protein has also been suggested to mediate methotrexate and folate uptake in certain mammalian cells (12, 13). Folate binding proteins typically show extremely high affinities for folic acid and lower affinities for reduced folates and methotrexate.

Considerable interest has focused on the biochemical and molecular determinants for dihydrofolate reductase inhibition by methotrexate in mammalian cells; however, only limited characterization has been reported for the transport system for methotrexate and tetrahydrofolate cofactors. Whereas radioactivity labeling techniques have facilitated the identification of a \( M_{r}, 36,000-48,000 \) methotrexate-binding polypeptide in plasma membranes from murine L1210 cells which has properties expected for the “classical” methotrexate carrier (6, 14–16), this species has not been further characterized. An analogous transport system was described in human tumor cells by standard kinetic approaches (2, 5, 17); however, the carrier protein was not identified.

Because of the extremely low levels of the methotrexate carrier in mammalian cells, it is notable that Sirotnak et al. (18) devised a novel strategy for selecting murine L1210 cell variants which “up-regulate” this system. Selection was based on the idea that carrier-mediated uptake is rate limiting to tetrahydrofolate cofactor utilization in rapidly dividing cells and involved growing cultures on progressively decreasing and growth-restricting concentrations of (6R)-5-formyltetrahydrofolate (\textit{i.e.}, leucovorin). Under these conditions, only cells which possessed increased transport capacities for reduced folates were capable of sustained growth.

In this report, a similar approach was used to select a line of K562 human erythroleukemia cells which shows increased activity of a membrane transport system with kinetic properties analogous to the reduced folate/methotrexate transporter described in a number of cultured murine and human cells (1, 2, 5, 9, 17). We report herein the characteristics of this transport up-regulated human line and the identification of a highly glycosylated protein carrier for methotrexate.

MATERIALS AND METHODS

Chemicals. [3,5,7,9-\(^{3}\)H]Methotrexate (20 Ci/mmol), [3,5,7,9-\(^{3}\)H]- (6S)-5-formyltetrahydrofolate (50 Ci/mmol), and [3,5,7,9-\(^{3}\)H]folic acid (40 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA) and purified by reverse-phase high-performance liquid chromatography as previously described (19, 20). [\(^{14}\)C]Inulin was purchased from ICN Biochemicals (Costa Mesa, CA). Unlabeled methotrexate and (6R,5)-5-formyltetrahydrofolate were obtained from the Drug Development Branch, National Cancer Institute (Bethesda, MD). Various chemicals,
including NHS, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, and folic acid, were purchased from Sigma Chemical Company (St. Louis, MO). Unlabeled methotrexate and folic acid were purified by DEAE chromatography (21) and high-performance liquid chromatography (20), respectively, prior to use. Boehringer Mannheim (Indianapolis, IN) was the source of peroxidase-free Triton X-100, endo-β-galactosidase (Bacteroides fragilis), and the endoglycosidase F preparations (Flavobacterium meningosepticum). Bio-Rad (Richmond, CA) was the source of the majority of the electrophoresis reagents. For some experiments, unlabeled and 14C-labeled molecular weight markers for electrophoresis were purchased from Bethesda Research Labs (Gaithersburg, MD). Tissue culture reagents and supplies were purchased from assorted vendors, with the exception of the fetal bovine serum, which was obtained exclusively from Grand Island Biological Company (Grand Island, New York).

Cell Culture. The K562 human erythroleukemia line was obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (0.1 mg/ml). Cells were grown in a humidified atmosphere at 37°C in the presence of 5% CO2/95% air. Cells were routinely subcultured every 72–96 h, and cell numbers were determined by direct microscopic counting with a hemocytometer. K562 cells were initially plated, as previously described (22), into 60-mm culture dishes. After 3–4 weeks, a single colony was chosen and expanded for use as the parental line in the experiments described in this report. For the selection of K562 cells with increased capacity for uptake of 5-formyl-[3H]tetrahydrofolate and [3H]methotrexate, parental K562 cells were previously depleted of endogenous folates by growth in folate-free RPMI 1640 containing adenosine (60 µM) and thymidine (10 µM) for 2 weeks. Soft agar cloning using folate-free RPMI 1640 and diazylated fetal bovine serum was used to select clonal populations capable of growing in 2 mM (6R,5)-5-formyltetrahydrofolate, initially, and, subsequently, 0.75 mM of the reduced folate. The final selection was performed in the presence of 0.4 nM (6R,5)-5-formyltetrahydrofolate. Between steps, lines were expanded from single colonies under the conditions used for selection; these were examined for growth characteristics and transport of [3H]methotrexate and 5-formyl[3H]tetrahydrofolate. Only a single clonal line was used for the next selection step. The final isolate (designated K562.4CF) grew in 0.4 nM (6R,5)-5-formyltetrahydrofolate with a generation time of 25.67 ± 4.60 (n = 2) h in “complete” RPMI 1640 containing 2.3 µM folic acid. Both parental and K562.4CF cells demonstrated low levels of erythroid differentiation as defined by negative benzidine staining (23).

Membrane Transport Methodology and Measurement of Dihydrofolate Reductase. For membrane transport measurements, logarithmically growing cells were washed with DPBS (24) and suspended in phosphate-buffered Hanks' balanced salt solution (25). Transport experiments were performed in specially designed flasks equipped with motor-driven stirrers under an atmosphere of 95% O2/5% CO2. Cells were incubated at 37°C with [3H]folicates or [3H]methotrexate for various intervals, and transport fluxes were stopped by injecting portions of the cell suspension into 10 volumes of 0°C DPBS. The cell fraction was separated by centrifugation (500 x g for 5 min) and washed twice with ice-cold DPBS. The cell pellets were digested with 0.5 N NaOH (1 ml). Samples of the alkaline digests were solubilized in Ready Value scintillation cocktail (Beckman). Radioactivity was measured with a Tracor Analytic liquid scintillation counter. Counting efficiencies were corrected by internal standardization with [3H]Iolune. Protein determinations were done by the method of Lowry et al. (26). In this fashion, the levels of total intracellular radioactivity were routinely expressed as pmol/mg protein. This provided a direct measure of the level of radioactive methotrexate or folate accumulation since the protein contents (1.93 and 2.2 mg/106 cells) and the intracellular volumes (51.27 ± 6.03 and 63.08 ± 3.02 µl/106 cells; n = 12 and 13, respectively) for parental and K562.4CF cells, respectively, were nearly identical. Intracellular volumes were determined from the difference between the wet and dry weights of a known number of cells minus the [14C]oilulin space (21). The contribution of surface adsorption to the total cell methotrexate was determined by measuring [3H]methotrexate accumulation at 0°C. K, and Kf values for methotrexate and folate inhibitors, respectively, were calculated from Lineweaver-Burk plots.

Dihydrofolate reductase enzyme levels were quantitated by measuring the extent of [3H]methotrexate binding in the presence of high concentrations of NADPH. Cells (1 x 108) were sonicated in a buffer composed of 50 mM sodium citrate (pH 6), 150 mM KC1, 1 mM EDTA, 50 mM 2-mercaptoethanol, and 100 µM NADPH. Following centrifugation (10,000 x g), cell-free extracts were incubated with 3 nmol [3H]methotrexate (10 min, 23°C) and chromatographed by rapid gel filtration using a centrifugal elution technique (19, 27). Both radioactivity and proteins from the column eluates were quantitated.

Quantitation of Total Cellular Folates. Cells were maintained in complete folate-free media in the presence of 60 µM adenosine and 10 µM thymidine for 2 weeks followed by growth for 3–4 generations in folate-free media containing 5-formyl[3H]tetrahydrofolate (without adenosine and thymidine). Total intracellular folates were calculated as pmol/mg protein, as described above.

Radioactivity Labeling of the Reduced Folate/Methotrexate Transporter and Preparation of Cell Membranes. The preparation of unlabeled and radiolabeled NHS-methotrexate was performed as described previously (6, 14). NHS-folic acid was prepared as for the unlabeled methotrexate. The radiospecific activities of the NHS-[3H]methotrexate ranged from 5 to 20 Ci/mmol. Cells (~5 x 107/ml in 20 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES), 225 mM sucrose, pH to 6.8 with MgO) were incubated with affinity reagent (23°C) for 5 min. Cells were washed twice with DPBS, and viabilities were assessed by trypan blue exclusion. The relative levels of [3H]methotrexate incorporation into the membranes were assayed as previously described (14). Briefly, the cell pellets were extracted with 1% peroxidase-free Triton X-100 at room temperature for 30 min. The solubilized membrane proteins were precipitated with an equal volume of acetone at −20°C. The precipitate was solubilized in 0.5 N NaOH for protein determinations and scintillation counting or in Laemmli “sample buffer” for gel electrophoretic analysis (see below). The specificity of radioligand incorporation was determined in parallel incubations containing various concentrations of unlabeled methotrexate, (6S)-5-formyltetrahydrofolate, or folic acid.

In some experiments, purified plasma membranes were isolated from NHS-[3H]methotrexate-labeled cells. In this case, cells were suspended in 10 mM Tris, pH 7, containing proteolytic inhibitors (hereafter referred to as “proteolytic inhibitor cocktail”) including: 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin, 10 µg/ml antipain, 10 µg/ml bestatin, 3 µg/ml aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride. The cells were disrupted with a Parr nitrogen cavillator at 500 psi for 20 min. The homogenate was spun at 600 x g to remove cell debris and nuclei; the supernatant was centrifuged at 200,000 x g in a Beckman 50Ti rotor for 90 min. The membrane pellet was suspended in 600 µl of 10 mM Tris-HCl, pH 7, containing the proteolytic inhibitor cocktail layered on a discontinuous sucrose gradient (2.0 ml 60% and 2.5 ml 20% sucrose in a SW50.1 rotor). Centrifugation was for 1 h at 58,000 x g. The 20%–60% interface containing plasma membranes was removed, diluted approximately 10-fold with 10 mM Tris-HCl (pH 7.0, containing proteolytic inhibitors), and again centrifuged at 200,000 x g for 60 min to pellet the membranes.

The purified membranes were solubilized in an appropriate detergent-containing buffer in the presence of the proteolytic inhibitor cocktail for the glycosidase digestions or the extraction of membrane proteins for Laemmli SDS gel electrophoresis.

Enzyme Deglycosylation. Plasma membranes were prepared as described above. Membrane proteins were treated with endo-β-galactosidase (0.75 unit/ml in 50 mM sodium phosphate (pH 6.0), 2 mM dithiothreitol, 2 mM EDTA, 0.5% 3-[3-cholamidopropyl]dimethyl-3421

3 The abbreviations used are: DPBS, Dulbecco's phosphate-buffered saline; NHS, N-hydroxysuccinimide; SDS, sodium dodecyl sulfate.
Characterization of 5-Formyltetrahydrofolate and Methotrexate Transport in K562 Cells with Up-regulated Influx Capacity. A clonal variant of the K562 human erythroleukemia (designated K562.4CF) was selected in soft agar in the presence of 0.4 nM (6R,5S)-5-formyltetrahydrofolate. By comparison, 10 nM (6R,5S)-5-formyltetrahydrofolate is the minimum concentration capable of supporting maximal growth of parental K562 cells.

K562.4CF cells and parental cells were initially assayed for uptake of 5-formyl[3H]tetrahydrofolate (0.1 μM) and [3H]methotrexate (0.5 μM) (Fig. 1). For both compounds, transport at 37°C was markedly enhanced in K562.4CF cells. This was reflected in both the initial influx rates (increases of 4.4- and 6.8-fold, respectively) and the apparent steady-state accumulation (elevated 3.1- and 4.2-fold, respectively). Negligible accumulation of [3H]methotrexate by both parental and K562.4CF cells was observed at 0°C (less than 0.05 pmol/mg after 210 s), direct evidence that the drug was being "internalized" via a specific temperature-dependent uptake process. First-order drug efflux at 37°C in K562.4CF cells was slightly increased over that of parental cells (55%; Fig. 2). Conversely, there was no loss of intracellular drug at 0°C, in either the presence or the absence of 200 μM unlabeled methotrexate. In contrast to the results with 5-formyltetrahydrofolate and methotrexate, folic acid (4.5 μM) uptake was increased only 56% in K562.4CF over parental cells (data not shown).

Kinetic analyses of the influx rates for [3H]methotrexate in parental and K562.4CF cells were performed. The kinetic constants are summarized in Table 1. These values were calculated by Lineweaver-Burk analyses of initial uptake velocities at 37°C, measured during the first 25-80 s of exposure to various concentrations of methotrexate (0.5-5 μM), prior to the appearance of appreciable unbound intracellular drug. Influx was constant with time (data not shown) at all of the concentrations used. The calculated Kᵢ values between K562.4CF and parental K562 cells showed only minor differences. However, the influx Vₘₐₓ in K562.4CF cells was elevated approximately 10-fold. Table 1 also summarizes data that show dihydrofolate reductase levels between the two cell lines were essentially unchanged.

**RESULTS**

**Characterization of 5-Formyltetrahydrofolate and Methotrexate Transport in K562 Cells with Up-regulated Influx Capacity.** A clonal variant of the K562 human erythroleukemia (designated K562.4CF) was selected in soft agar in the presence of 0.4 nM (6R,5S)-5-formyltetrahydrofolate. By comparison, 10 nM (6R,5S)-5-formyltetrahydrofolate is the minimum concentration capable of supporting maximal growth of parental K562 cells.

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**Table 1** Characteristics of parental K562 and K562.4CF cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MTX transport parameters</th>
<th>DHFR levels*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Influx Kᵢ (μM)</td>
<td>Influx Vₘₐₓ (pmol/mg/s)</td>
</tr>
<tr>
<td>Parent</td>
<td>4.64 ± 2.11</td>
<td>0.081 ± 0.021</td>
</tr>
<tr>
<td>K562.4CF</td>
<td>7.48 ± 1.31</td>
<td>0.807 ± 0.162</td>
</tr>
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* Kinetic constants for methotrexate (MTX) transport were calculated by Lineweaver-Burk analysis of initial rate data over a range of concentrations (0.5-5 μM).

Fig. 1. Uptake of 5-formyl[3H]tetrahydrofolate (5-CHO-FH₄; 0.1 μM) and [3H]methotrexate (MTX; 0.5 μM) by parental and K562.4CF cells. Cells (1 x 10⁷/ml) were incubated at 37°C in Hanks’ balanced salt solution with the radioactive compounds. Uptake was determined as described in “Materials and Methods.” The dihydrofolate reductase binding capacity for both parental and K562.4CF cells in this analysis was 2.3 pmol/mg protein.

Fig. 2. Rates of methotrexate efflux in parental and K562.4CF cells. Parental and K562.4CF cells were incubated for 20 min with [3H]methotrexate (10 μM and 2 μM, respectively). Cells were washed with DPBS (0°C) and resuspended in drug-free Hanks’ balanced salt solution at 37°C. Aliquots of the cell suspension were removed over time and processed as described in “Materials and Methods.” Data are plotted as the percentage of free methotrexate (in excess of the dihydrofolate reductase level) on a logarithmic ordinate for the calculation of the first-order efflux rate constants. The rate constants for the parental and K562.4CF lines were 0.148 and 0.228 min⁻¹, respectively. MTX, methotrexate.
was measured for the radiolabeled compounds from 0 to 180 s. Data are shown percentage of methotrexate uptake rate relative to an untreated control.

Fig. 3. Inhibition of [$^3$H]methotrexate (MTX; 1 μM) and 5-formyl[$^3$H]tetrahydrofolate (CHO-FH2, 2 μM) influx in K562.4CF cells by assorted folates. Influx was measured for the radiolabeled compounds from 0 to 180 s. Data are shown for the effects of varying folate acid (open symbols), (6S)-5-formyltetrahydrofolate (•), and methotrexate (A) on these uptake processes and are expressed as the percentage of methotrexate uptake rate relative to an untreated control.

Table 2 Correlation between cellular folate levels and [$^3$H]methotrexate uptake capacity*

<table>
<thead>
<tr>
<th>Line</th>
<th>Cellular folate</th>
<th>MTX uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(pmol/mg peptide)</td>
<td>(pmol/mg/s)</td>
</tr>
<tr>
<td>Parent</td>
<td>10</td>
<td>0.0052</td>
</tr>
<tr>
<td>K562.4CF</td>
<td>10</td>
<td>0.0111</td>
</tr>
<tr>
<td>K562.4CF</td>
<td>0.4</td>
<td>0.0316</td>
</tr>
<tr>
<td>K562.4CF</td>
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<td>0.0144</td>
</tr>
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</table>

* Cells were depleted of endogenous folates in folate-free media containing 60 μM adenosine and 10 μM thymidine for 2 weeks, followed by growth in (6S)-5-formyltetrahydrofolate (5-CHO-H4PteGlu; 10 or 0.4 nM) for 3-4 generations.

Radioaffinity Labeling of the Methotrexate Transport System with NHS-[H]Methotrexate. As in L1210 cells, NHS-methotrexate is an inhibitor of [H]methotrexate influx into K562.4CF (Fig. 4) and parental K562 cells (not shown). Conversely, NHS-olic acid was notably less inhibitory (Fig. 4). On this basis, NHS-[H]methotrexate was used as a specific radioaffinity ligand for the methotrexate transport system in these lines. Following incubations with various concentrations of this agent (140–700 nM), cells were washed with DPBS, and the relative levels of radiolabeled membrane proteins were assayed by the method of Henderson et al. (14). The levels of “nonspecific” incorporation in this assay were measured in parallel incubations, containing 0.5 mM unlabeled methotrexate in addition to the radioaffinity reagent. Total and nonspecific [H]methotrexate incorporations from NHS-[H]methotrexate are illustrated in Fig. 5. The levels of specific labeling were calculated as the difference between total and nonspecific components. At 700 nM, specific labeling for K562.4CF cells was 6.7-fold greater than for the parental line.

A distinguishing feature among the different folate transporter systems in mammalian cells relates to their affinities for methotrexate and reduced folates, relative to folic acid. In Fig. 3, data are shown for the concentration-dependent inhibitions of [H]methotrexate uptake by folic acid and 5-formyltetrahydrofolate and of 5-formyl[H]tetrahydrofolate uptake by folic acid and methotrexate. In both cases, folic acid was significantly less inhibitory than the other compounds. Although the experiments depicted in Fig. 3 were performed with the K562.4CF subclone, essentially identical results were obtained in parental K562 cells (not shown).

K, values for 5-formyltetrahydrofolate and folic acid were calculated from Lineweaver-Burk double-reciprocal plots. For K562.4CF cells, the K, values for (6R,S)-5-formyltetrahydrofolate and folic acid were 7 and 110 μM, respectively. For parental K562 cells, the calculated values were 7.2 and 164 μM, respectively. These results confirm that the transport system in K562 cells has a significantly lower binding affinity for folic acid, compared to methotrexate or 5-formyltetrahydrofolate. Moreover, they strongly suggest that the marked enhancement of methotrexate and 5-formyltetrahydrofolate uptake in K562.4CF cells derives from increased activity of the “classical” reduced folate and methotrexate transport system (1, 2).

Stability of Up-regulated Transport in K562.4CF Cells. The increased methotrexate uptake capacity in K562.4CF cells decreased approximately 65% following growth for 3-4 generations in 10 nM 5-formyltetrahydrofolate (Table 2). This effect correlated with the repletion of the cellular cofactor pools to an extent exceeding that in parental cells under these conditions. However, transport was also suppressed in K562.4CF cells grown without any folates (i.e., in folate-free media containing 60 μM adenosine and 10 μM thymidine; Table 2). Maximal influx rates (95% of maximum) could be reinduced in both folate-replete and -deplete K562.4CF cells in the presence of 0.4 nM 5-formyltetrahydrofolate for 3-4 generations (data not shown).

Radioaffinity Labeling of the Methotrexate Transport System with NHS-[H]Methotrexate. As in L1210 cells, NHS-methotrexate is an inhibitor of [H]methotrexate influx into K562.4CF (Fig. 4) and parental K562 cells (not shown). Conversely, NHS-olic acid was notably less inhibitory (Fig. 4). On this basis, NHS-[H]methotrexate was used as a specific radioaffinity ligand for the methotrexate transport system in these lines. Following incubations with various concentrations of this agent (140–700 nM), cells were washed with DPBS, and the relative levels of radiolabeled membrane proteins were assayed by the method of Henderson et al. (14). The levels of “nonspecific” incorporation in this assay were measured in parallel incubations, containing 0.5 mM unlabeled methotrexate in addition to the radioaffinity reagent. Total and nonspecific [H]methotrexate incorporations from NHS-[H]methotrexate are illustrated in Fig. 5. The levels of specific labeling were calculated as the difference between total and nonspecific components. At 700 nM, specific labeling for K562.4CF cells was 6.7-fold greater than for the parental line.

To associate the specific incorporation of radioactivity from NHS-[H]methotrexate with the reduced folate/methotrexate transporter, experiments were performed which examined the relative effects of folic acid and 5-formyltetrahydrofolate on this process (Fig. 6). In these experiments, folic acid was significantly less effective than 5-formyltetrahydrofolate over a 25-fold concentration range in inhibiting covalent incorporation of radioaffinity label. These effects are similar to those on [H]methotrexate influx (Fig. 3).

For the K562.4CF cells, SDS gel electrophoresis of membrane proteins on 3–17% gradient gels showed that radioactivity from NHS-[H]methotrexate was associated with a single
Membrane proteins were solubilized with 1% Triton X-100 and precipitated with acetone, and the incorporation of radioactivity was determined as previously described (14). The "nonspecific" and "specific" fractions were calculated as described in "Materials and Methods." Data are shown for total (——) and nonspecific (—–—–) incorporations into parental and K562.4CF cells. Nonspecific incorporation was determined in the presence of 0.5 mM unlabeled methotrexate. "Specific" labeling was calculated as the difference between total and nonspecific levels.

**Glycosylation as the Basis for the Microheterogeneity of the NHS-[3H]Methotrexate-labeled Transporter.** It was of particular interest that the apparent molecular weight of the radioaffinity labeled protein from K562.4CF cells was somewhat dependent on the gel system used for analysis. Hence, on 7.5% gels, the band shifted to $M_r$ 85,000–99,000 (median $M_r$, 92,220 ± 3,450; $n = 10$; Fig. 8). This property, coupled with the broad electrophoretic profile for NHS-[3H]methotrexate-labeled carrier, is characteristic of highly glycosylated proteins (30). To evaluate the possibility that the methotrexate transporter protein is a glycoprotein, K562.4CF cells were labeled with NHS-[3H]methotrexate, membranes were prepared, and detergent-solubilized proteins were digested with commercial glycosidases. As illustrated in Fig. 8, endo-ß-galactosidase treatment resulted in a sharpening of the broad radiolabeled band and a shift to a lower molecular weight ($M_r$, 57,800 ± 7,730; $n = 5$), establishing its glycosylation. In additional experiments, endoglycosidase F alone had no effect on the electrophoretic migration of the NHS-[3H]methotrexate-labeled carrier; however, endoglycosidase F:glycopeptidase F (1:1) converted the carrier to a $M_r$ 52,390 (±5,110; $n = 2$) polypeptide (not shown).
DISCUSSION

The strategy used for the selection of a human erythroleukemia line with increased transport capacity for tetrahydrofolates was based on a concept originally elaborated by Sirotnak et al. (18) for studying the regulation of this membrane process in murine leukemia cells. Selection involved growing cells on progressively decreasing concentrations of (6R,5′)-5-formyltetrahydrofolate, conditions under which only variants with enhanced cofactor transport were capable of sustained growth. As with these up-regulated L1210 lines (18), uptake of methotrexate in K562.4CF cells was augmented as well. Methotrexate efflux was increased to a much reduced extent, further direct evidence for the separateness of influx and exit processes in tumor cells (31).

Increased methotrexate uptake in K562.4CF over parental cells was associated with greater incorporation from NHS-[3H] methotrexate into an anomalously electrophoresing membrane protein, identified as the methotrexate transporter. Incorporation was selectively blocked by folic acid or 5-formyltetrahydrofolate, to extents paralleling the inhibition of [3H]methotrexate transport by these compounds. The increased amount of labeling in K562.4CF over parental cells implies that their differential uptake capacities derive from corresponding changes in the levels of the carrier system for methotrexate. However, unrecognized factors (i.e., endogenous folate pools; Ref. 32) may influence carrier accessibility to this reagent.

The high apparent binding affinities of the K562 membrane carrier for methotrexate and 5-formyltetrahydrofolate relative to folic acid are typically associated with the "classical" folate transport system, described in numerous cultured murine and human lines (1, 2, 5, 9, 17). This system is clearly distinct from the homologous folic acid-binding proteins described in KB cells (12) and human placenta (13) since the latter show extremely high affinities for folic acid (nm) compared to reduced folates and methotrexate and appear to function by endocytosis. Whereas no immunological cross-reactivity can be demonstrated between the folate-binding protein from KB cell membranes and the K562.4CF methotrexate transporter, when assayed on Western blots with anti-KB folate binder antisera,6 there may be at least a partial conservation of epitopes for antibody binding between the human placental high-affinity folate binder and the murine L1210 tetrahydrofolate cofactor carrier (33).

The physiological importance of neither the high-affinity folate binder, typified by that in KB cells, nor the methotrexate/tetrahydrofolate transporter, described herein, is firmly established. However, it appears from earlier reports (34, 35) that the latter system can suffice as the sole uptake route for reduced cofactors under physiologically relevant folate concentrations. A pharmacological role for the reduced folate transporter is equally probable since it mediates methotrexate uptake over a wide range of clinically relevant concentrations (1, 2, 36).

While elevated methotrexate uptake in K562.4CF cells was sustained as long as cells were maintained in 0.4 nm 5-formyltetrahydrofolate, transport capacity markedly decreased under "nonselective" growth conditions (i.e., in the presence of 10 nm 5-formyltetrahydrofolate or in the absence of folates altogether). A similar effect of preincubation with "physiological" concentrations of reduced folates on methotrexate uptake was reported in transport-up-regulated CCRF-CEM (CEM-7A) cells (37). For both K562.4CF and CEM-7A cells, high levels of transport could be restored upon resuspension into "selective" folate-free medium containing 0.4 and 0.25 nm 5-formyltetrahydrofolate, respectively. The reversible nature of the up-regulated transport in these experiments directly implicates reduced folates or folate-dependent products (i.e., nucleotides) as regulators of cofactor uptake in these cells. In such a case, the increased transport capacity of K562.4CF versus parental K562 cells may derive from an enhanced sensitivity of these control mechanisms to regulatory effectors rather than a protein overexpression/gene amplification phenomenon. A gene amplification-related cytogenetic abnormality (i.e., homogeneously staining region) was associated with up-regulated folate transport in an L1210 line (38). In our karyotype analyses, neither homogeneously staining regions nor double minute chromosomes were detectable in the K562.4CF subclone.7

Although the apparent molecular weight for the reduced folate/methotrexate transporter in K562.4CF cells varied slightly with the gel system used, in both cases it was notably different from its presumed counterpart in L1210 cells (reported as M, 36,000–48,000 for the NHS-[3H]methotrexate-labeled protein; Refs. 6, 14–16). A number of higher-molecular-weight (M, 56,000, 63,000, and 67,000) methotrexate-binding membrane proteins were also identified in murine cells (39); however, their relationship, if any, to the methotrexate carrier was not established. In the present study, difficulties with protein aggregation were not uncommon, particularly during electrophoretic analyses of "crude" membrane homogenates (i.e., Fig. 8); however, this does not seem to account for the disparity in the apparent molecular weights between the homologous folate transport proteins from the human and murine systems.

The anomalous electrophoretic profile of the transporter protein in K562.4CF cells was suggestive of a microheterogeneity typical of glycoproteins. Carrier glycosylation was confirmed by treatment with endo-β-galactosidase, which reduced the estimated molecular weight of the broad polypeptide to M, ~58,000. Whereas endoglycosidase F had no effect on the carrier, a mixture of endoglycosidase F and glycopeptidase F decreased the molecular weight by approximately 52,000. These results indicate that the transporter is an N-linked glycoprotein containing internal β-galactosidic linkages in, or immediately after, unbranched poly-N-acetyllactosamine sequences (40). The absence of an effect by endoglycosidase F, alone, implies that the glycoprotein may contain bisected hybrid, triantennary complex, or tetraantennary complex structures which are readily cleaved by glycopeptidase F (40). In any case, differences in the extent of glycosylation between the human and murine methotrexate carriers may account for the disparity in their apparent sizes.

In summary, in this report we have identified the high-affinity membrane carrier for methotrexate and reduced folates in a transport up-regulated human erythroleukemia line. Our demonstration that human transporter is extensively glycosylated raises the possibility that lectin-agarose affinity chromatography may be useful for purifying this species. Indeed, our initial studies confirm this supposition since the NHS-[3H]methotrexate-labeled glycoprotein from K562.4CF cells can be isolated in nearly homogeneous form by Ricinus communis agglutinin I-agarose chromatography (41). These results will be described in a more complete form elsewhere.

* L. H. Matherly and C. A. Czajkowski, unpublished observation.

7 A. Mohamed and L. H. Matherly, unpublished data.
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Identification of a Highly Glycosylated Methotrexate Membrane Carrier in K562 Human Erythroleukemia Cells Up-regulated for Tetrahydrofolate Cofactor and Methotrexate Transport

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