Alterations in the Expression of Transcription Factors and the Reduced Folate Carrier as a Novel Mechanism of Antifolate Resistance in Human Leukemia Cells*

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The human reduced folate carrier (hRFC) is the dominant transporter mediating the uptake of reduced folate cofactors and antifolate anticancer drugs. Defective antifolate uptake due to inactivating mutations in the hRFC gene is an established mechanism of drug resistance in various tumor cells. However, while antifolate transport is frequently impaired, either no or only a single hRFC allele is inactivated, suggesting that additional mechanism(s) of resistance are operative. Here we studied the relationship between the expression and function of transcription factors and antifolate resistance in transport-defective leukemia cells that poorly express or completely lack RFC mRNA. Stable transfection with a hRFC expression construct resulted in restoration of normal RFC mRNA expression and nearly wild type drug sensitivity in these antifolate-resistant cells. The loss of RFC gene expression prompted us to explore transcription factor binding to the hRFC promoter. The hRFC promoter contains an upstream GC-box and a downstream cAMP-response element (CRE/AP-1-like element. Electrophoretic mobility shift assays and oligonucleotide competition revealed a substantial loss of nuclear factor binding to CRE and GC-box in these drug-resistant cell lines. Consistently, antibody-mediated supershift analysis showed a marked decrease in the binding of CRE-binding protein 1 (CREB-1) and specificity protein 1 (Sp1) to CRE and GC-box, respectively. Western blot analysis revealed undetectable expression of CREB-1, decreased ATF-1 levels, parental Sp1 levels, and increased levels of the short Sp3 isoforms, recently shown to repress hRFC gene expression. Transient transfections into these antifolate-resistant cells demonstrated a marked loss of GC-box-dependent and CRE-driven reporter gene activities and introduction of CREB-1 or Sp1 expression constructs resulted in restoration of hRFC mRNA expression. These results establish a novel mechanism of antifolate resistance that is based on altered expression and function of transcription factors resulting in transcriptional silencing of the hRFC promoter.

Mammalian cells are devoid of reduced folate biosynthesis and therefore meet their folate growth requirement via uptake from exogenous sources (1). Reduced folates are absolutely essential cofactor vitamins involved in a host of one-carbon transfer reactions resulting in the biosynthesis of purines, deoxythymidylate, and methionine (1). Folate antagonists (i.e. antifolates) including methotrexate (MTX) are potent inhibitors of purine and deoxythymidylate biosynthesis and thereby block DNA synthesis. Consequently, MTX is an integral component of chemotherapeutic regimens used in the treatment of various human malignancies (2). Folates and antifolates are hydrophilic divalent anions that cannot cross membranes via diffusion and are therefore taken up into mammalian cells by the reduced folate carrier (RFC) (3). Among the antifolates recognized as transport substrates by the RFC are the dihydrofolate reductase inhibitor, MTX (2), the novel thymidylate synthase inhibitors Tomudex (ZD1694, Raltitrexed) (4) and ZD9331 (5), as well as the glycaminide ribonucleotide transformylase inhibitor AG2034 (6). Various antifolate resistance phenomena pose a major obstacle toward curative cancer chemotherapy. For example, qualitative (i.e. inactivating mutations) (7–11) and quantitative alterations (i.e. decreased or abolished expression) in human RFC (hRFC) gene expression and/or function are documented mechanisms of MTX resistance in acute lymphoblastic leukemia (ALL) (12, 13) and osteogenic sarcoma patients (14). Although decreased expression of hRFC mRNA has been suggested as a mechanism of clinical resistance to MTX, the underlying molecular basis is still unknown (12–14).

It was recently shown that transcription of the hRFC gene is driven by at least two promoter elements: an upstream constitutive element consisting of a GC-box as well as a downstream inducible cAMP-response element (CRE/AP-like element (CRE/AP-1) (15). Reporter gene assays revealed that the binding of Sp1 and the long Sp3 isoforms to the GC-box as well as that of CRE-binding protein (CREB-1) to the CRE/AP-like element result in activation of hRFC transcription (15). Using stepwise antifolate selection, the human CCRF-CEM leukemia cell lines AG2034 (12) and ZD9331 (13) were isolated, which displayed up to 2300-fold resistance to various antifolates due to abolished drug transport (11). This was a result of a marked decrease (or complete loss) of RFC mRNA expression. This major decrease in RFC mRNA expression was associated with alterations in the expression of transcription factors, resulting in a prominently decreased binding to both the constitutive and
Loss of CREB-1, Sp1 Binding, and Activated Antifolate Transport

TABLE I

<table>
<thead>
<tr>
<th>Oligonucleotides used for electrophoretic mobility shift assay</th>
<th>WT, wild type; M, mutant.</th>
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<tr>
<td><strong>Sequence of sense strand (5' → 3')</strong></td>
<td></td>
</tr>
<tr>
<td>CREB WT</td>
<td>TGC ATT GAG TGG CTG GAG AGA G</td>
</tr>
<tr>
<td>CREB M</td>
<td>TGC ATT GAG TGG CTG GAG AGA G</td>
</tr>
<tr>
<td>SP1 WT</td>
<td>AGT CGA TCG GGA CGG GAC</td>
</tr>
<tr>
<td>SP1 M</td>
<td>AGT CGA TCG GGA CGG GAC</td>
</tr>
<tr>
<td>API WT</td>
<td>AGT TAG CAT GAG TAC</td>
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*For double-stranded oligonucleotides used in EMSA, only the sense strand is shown. Binding sites in the API-1, CREB, and SP1 oligonucleotides are underlined with mutations in bold.

MATERIALS AND METHODS

Drugs, Biochemicals, and Radiochemicals—MTX was from Teva Pharmaceuticals Ltd. Novel antifolate drugs were generous gifts from the following sources: ZD9331 from Dr. A. L. Jackman, (Institute of Cancer Research, Sutton, United Kingdom), AG2034 from Dr. T. Bortizki (Agouron Pharmaceuticals, Inc.), and PTZ523 from Dr. W. T. McCulloch (Sparta Pharmaceuticals).

Tissue Culture and Antifolate Drug Selections—CCRF-CEM, a human T-cell leukemia line, and its antifolate-resistant sublines were maintained in RPMI 1640 medium containing 2.5 μM folic acid (Biological Industries, Beth-Haemek, Israel) supplemented with 10% fetal calf serum (Invitrogen), 2 mM glutamine, 100 units/ml penicillin G (Sigma), and 10 μg/ml streptomycin (Schuell). Parental CCRF-CEM cells in gradually increasing concentrations of 5 mM leucovorin as the sole folate source (16).

Parental cells and their antifolate-resistant sublines were harvested by centrifugation and washed with phosphate-buffered saline, cell lysates were prepared, and firefly luciferase activity was assayed using the dual luciferase kit (Promega) and a luminometer. Firefly luciferase activity was normalized with Renilla luciferase, following co-transfection with a Renilla-luciferase plasmid. For transient RFC mRNA expression, after 24 h of growth cells were harvested and total RNA was extracted. Results presented were obtained from at least three independent transfections performed in duplicate cultures and activity determinations.

Stable Transfections with a hRFC Expression Construct—Exponentially growing cells (2 x 10^7) were harvested by centrifugation and washed with phosphate-buffered saline, cell lysates were prepared, and firefly luciferase activity was assayed using the dual luciferase kit (Promega) and a luminometer. Firefly luciferase activity was normalized with Renilla luciferase, following co-transfection with a Renilla-luciferase plasmid. For transient RFC mRNA expression, after 24 h of growth cells were harvested and total RNA was extracted. Results presented were obtained from at least three independent transfections performed in duplicate cultures and activity determinations.

**Western Blot Analysis**—Nuclear extract proteins (20 μg) were resolved by electrophoresis on 10% polyacrylamide gels containing SDS, electroblotted onto a Protran cellulose nitrate membrane (Schleicher & Schuell), reacted with anti-CREB-1, -ATF-1, -Sp1, and -Sp3 (Santa Cruz Biotechnology Inc.) according to the instructions of the manufacturer. To examine RFC expression in parental and antifolate-resistant cells, microsomes were first isolated (20), and proteins were extracted in a buffer containing 0.5% Triton X-100 as previously described (11). Detergent-soluble proteins (12.5–150 μg) were resolved by electrophoresis, electroblotted onto a nylon membrane and blocked for 1 h at room temperature in TBS-10 (150 mM NaCl, 0.05% Tween 20, 10 mM Tris/Cl at pH 8.0) containing 1% skim milk. The blots were then reacted with a polyclonal antisemur (1:700) prepared in mice against a C-terminal hRFC peptide (11), rinsed in the same buffer for 10 min in room temperature, and reacted with horseradish peroxidase-conjugated goat anti-mouse IgG (1:40,000 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA) at 1 h at room temperature. Following three washes (10 min each) in TBS at room temperature, enhanced chemiluminescence (ECL) detection was performed according to the manufacturer’s instructions (Biological Industries, Beth Haemek, Israel). ECL was recorded on x-ray films using several exposure times, which were evaluated by scanning densitometry.

**RESULTS**

RFC Expression, Drug Transport, and Antifolate Resistance—We studied the molecular basis of antifolate resistance.
in the human leukemia sublines ZD9331R1.5 and AG2034R2. These cell lines displayed up to 2300-fold resistance to various antifolates that use RFC as their primary route of uptake (Fig. 1A). This was associated with a marked decrease (95–97%) in the transport of [3H]MTX (Fig. 1B). While detectable with 25 μg of microsomal proteins from parental CEM cells, RFC was not detectable even with 150 μg of proteins from AG2034R2 and ZD9331R1.5 cells (Fig. 2A). Semiquantitative RT-PCR analysis showed that AG2034R2 cells expressed −1/300th of parental RFC mRNA, whereas ZD9331R1.5 cells had no detectable expression (Fig. 2B). In contrast, AG2034R2 and ZD9331R1.5 cells expressed normal mRNA levels of various housekeeping genes including DHFR, FPGR, MRPI, and GAPDH (Fig. 2C), thereby suggesting that these cells did not suffer from a global transcriptional repression.

Restoration of Antifolate Sensitivity upon Transfection with a hRFC Expression Vector—To examine the impact of restoration of RFC expression on antifolate sensitivity, AG2034R2 and ZD9331R1.5 cells were stably transfected with an expression vector harboring the wild type hRFC cDNA (Fig. 2B, lane 1). This resulted in restoration of normal RFC mRNA expression in these cell lines (Fig. 2B, lane 1). Consequently, AG2034R2 and ZD9331R1.5 cells became up to 170-fold more sensitive to MTX, thereby approaching the sensitivity of wild type cells to this antifolate (Fig. 1C). These results establish that the loss of hRFC gene expression is a major underlying mechanism of antifolate drug resistance in these transport defective cell lines.

Nuclear Factor Binding to [32P]-labeled CRE, AP-1, and GC-box Oligonucleotides—To explore the basis for the loss of RFC mRNA expression, nuclear proteins from parental CEM and antifolate-resistant cells were isolated and examined by an electrophoretic mobility shift assay. This electrophoretic mobility shift assay assessed the binding of nuclear factors to [32P]-labeled CRE, GC-box, and AP-1 double-stranded oligonucleotides (Table I). A 3-band binding pattern to [32P]-labeled CRE (Fig. 3A) and [32P]-labeled GC-box (Fig. 3B) was observed in parental CEM cells, whereas AG2034R2 and ZD9331R1.5 cells had a markedly decreased binding to both oligonucleotides (Fig. 3A and B) but retained normal binding to [32P]-labeled AP-1 (Fig. 3C). To examine the specificity of nuclear factor binding to these consensus binding sites, two independent parameters were used: (a) competition of binding to CRE and GC-box with unlabeled oligonucleotides and (b) binding to mutant CRE and GC-box oligonucleotides (Table I). The binding of nuclear proteins from parental cells to [32P]-labeled CRE, and GC-box was competed in a dose-dependent manner, with a 10–100-fold molar excess of the unlabeled oligonucleotides (data not shown). In contrast, the residual binding of nuclear proteins from AG2034R2 and ZD9331R1.5 cells to CRE and GC-box could not be competed even with a 100-fold molar excess of the unlabeled oligonucleotides. Furthermore, nuclear proteins from both parental and antifolate-resistant sublines failed to bind to the mutant [32P]-labeled CRE and [32P]-labeled GC-box oligonucleotides and displayed a similar nonspecific basal binding (data not shown). These results suggest that the residual binding to CRE and GC-box in these antifolate-resistant sublines was largely nonspecific.

Restoration of RFC mRNA Expression upon Transient Transfection with CREB-1 and Sp1 Expression Constructs—The putative loss of function of transcription factors that are involved

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<tr>
<th>Gene</th>
<th>Sense primer</th>
<th>Antisense primer</th>
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<tr>
<td>RFC</td>
<td>5′-CGGGCTTCCACCTCCCTCC-3′</td>
<td>5′-CCGAGTTGGAACCCACCCAGA-3′</td>
</tr>
<tr>
<td>DHFR</td>
<td>5′-CTGACAAATAGGAGGAGAGG-3′</td>
<td>5′-TATGCTTTCTCCTCCTCCTG-3′</td>
</tr>
<tr>
<td>FPGR</td>
<td>5′-CCGCTAGAAGACTCACTC-3′</td>
<td>5′-CTTTGAGCCATGGAGATGCTTCT-3′</td>
</tr>
<tr>
<td>MRPI</td>
<td>5′-CGAGGAGGAGTCAGCAGAGA-3′</td>
<td>5′-TGATGCTGCTACAGCCGAGGAGG-3′</td>
</tr>
<tr>
<td>Sp1</td>
<td>5′-AAGAGGGATCCTCACCCTC-3′</td>
<td>5′-TACATGCTGGGGTGTGTTGA-3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′-AGGGGGGAGCCGAAAAAGG-3′</td>
<td>5′-GAGGAGGTTGGTGTCGCTGTTG-3′</td>
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</table>

| FIG. 1. Antifolate resistance and [3H]MTX transport in parental CEM cells and their antifolate-resistant sublines. Levels of antifolate resistance in AG2034R2 and ZD9331R1.5 cells prior to (A) or after stable transfection with a hRFC cDNA (C). Initial rates of [3H]MTX transport (B) were determined as described under "Materials and Methods." The influx of [3H]MTX in parental CEM cells was 4.0 ± 0.6 pmol [3H]MTX/min × 10⁸ cells. Results shown are the means of four independent experiments ± S.D.
in the binding to CRE and GC-box prompted us to explore the impact of the introduction of CREB-1 and Sp1 on RFC mRNA expression (Fig. 2D). Transfection of AG2034R2 and ZD9331R1.5 cells with expression constructs harboring CREB-1 (pRSV-CREB1) or Sp1 (pPacSp1) resulted in restoration of normal RFC mRNA expression (Fig. 2D).

Loss of CRE- and GC-box-driven Reporter Gene Activity—To assess CRE- and GC-box-mediated transcription, parental cells and their antifolate-resistant sublines were transiently transfected with CRE-luciferase and GC-box-luciferase (Fig. 4A), GC-box-β-actin luciferase (Fig. 4B), and GC-box-CAT (Fig. 4C), and reporter gene activity was determined. The activities of firefly luciferase and CAT were determined. The activities of firefly luciferase and CAT were determined. The activities of firefly luciferase and CAT were determined.

Antibody-mediated Supershift Analysis—It was recently shown that CREB-1 and Sp1 bind to CRE and GC-box in the hRFC promoter, respectively (15). To identify alterations in the binding of specific transcription factors to CRE (Fig. 5A) and GC-box (Fig. 5B), antibody-mediated supershift analysis was used. Preincubation of nuclear proteins either with antibodies to CREB-1 and ATF-1 or a combination of the two followed by [32P]-labeled CRE protein-antibody complex formation revealed supershifts in parental CEM cells (Fig. 5A, complexes A–C in lanes 2–4, respectively; note the elimination of bands 1 and/or 2). Likewise, [32P]-labeled GC-box supershifts in parental CEM cells were obtained with anti-Sp1 (Fig. 5B, complex A in lane 2; note the elimination of band 2) and anti-Sp3 antibodies (Fig. 5B; note the elimination of bands 1 and 3 in lane 3). Furthermore, the combination of anti-Sp1 and -Sp3 antibodies eliminated all three [32P]-labeled GC-box bands and formed a high molecular weight complex (Fig. 5B, complex B in lane 4).

Alterations in the Expression and Function of Transcription Factors—As alterations in the function of transcription factors could also result from changes in their expression, we determined CREB-1, ATF-1 (Fig. 6A), Sp1 (Fig. 6B), and Sp3 levels (Fig. 6C) by Western blot analysis. Parental CEM cells expressed both the long and short isoforms of Sp3 (Fig. 6C, lane 1), whereas AG2034R2 and ZD9331R1.5 cells (lanes 9–12) neither showed [32P]-labeled CRE supershifts with anti-CREB-1 or ATF-1 antibodies (Fig. 5A) or [32P]-labeled GC-box supershifts with antibodies to Sp1 (Fig. 5B). However, anti-Sp3 antibodies eliminated band 3 of [32P]-labeled GC-box in both AG2034R2 and ZD9331R1.5 cells (Fig. 5B, lanes 7 and 11, respectively).
These results suggest that an inhibitory component was present in the nuclear extract from ZD9331R1.5 cells that appeared to disrupt GC-box binding.

**DISCUSSION**

The aim of the current study was to identify the molecular basis underlying the loss of RFC mRNA expression in antifolate-resistant human leukemia cell lines displaying impaired MTX transport. We find substantial alterations in the expression of CREB-1, ATF-1, and the short isoforms of Sp3. These alterations were associated with a prominent decrease in the binding of transcription factors to the inducible (CRE) and constitutive (GC-box) elements in the hRFC promoter, presumably leading to a marked decrease in RFC mRNA expression. Consistently, introduction of CREB-1 and Sp1 into these antifolate-resistant cells resulted in restoration of RFC mRNA expression. Importantly, stable transfection of a hRFC cDNA driven by a potent cytomegalovirus promoter, resulted in restoration of normal RFC mRNA expression and nearly wild type sensitivity to MTX. These results provide the first demonstration of a novel mechanism of antifolate drug resistance that is based upon altered expression and function of transcription factors resulting in transcriptional silencing of the hRFC promoter.

It was recently found that the hRFC promoter is driven by an inducible CRE/AP-1-like element and a constitutive GC-box element (15). This study showed that CREB-1 and c-Jun in human fibrosarcoma HT1080 cells, as well as CREB-1 and ATF-1 in human hepatocellular carcinoma HepG2 cells, were the cell-specific transcription factors involved in CRE binding (the inducible element) in the hRFC promoter. We consistently find here that CREB-1 and ATF-1 were expressed at substantial levels in wild type human leukemia CEM cells and displayed a functional binding to CRE. The marked decrease or loss of RFC mRNA expression in AG2034R2 and ZD9331R1.5 cells, respectively, was associated with a decreased expression (or lack) of CREB-1 and ATF-1. These results strongly suggest that CREB-1 and ATF-1 are essential components for the transcriptional activation of the inducible element in the hRFC promoter in leukemia cells. However, antifolate-resistant cancer cell lines and malignant tumors of different cell lineages may potentially express alternative, cell-specific transacting factors of the basic region-leucine zipper (bZip) family, which may also contribute to hRFC transcription.

Transient transfections of either Sp1 or the long Sp3 isoform resulted in a potent activation of the constitutive GC-box element in the hRFC promoter (15). In contrast, introduction of the short Sp3 isoform resulted in a potent repression of the Sp1-dependent activation of the hRFC promoter. Consistently, ZD9331R1.5 and AG2034R2 cells showed elevated levels of the short Sp3 isoforms; the latter is likely to act as a repressor of the Sp1-mediated activation of the constitutive GC-box element in the hRFC promoter. This suggestion could gain support from the following results and considerations. First, the
1:1 mixing of nuclear proteins from parental and ZD9331 R1.5 cells markedly blocked GC-box binding but did not interfere with CRE binding. Second, it is likely that transfection of ZD9331R1.5 and AG2034 R2 cells with an Sp1 expression construct should result in Sp1 levels that exceed those of the short Sp3 isoforms, resulting in restoration of RFC mRNA expression. Thus, the short Sp3 isoforms that are expressed at elevated levels in these antifolate-resistant cells appear to competitively block Sp1 binding to the GC-box, thereby repressing transcriptional activation of the hRFC gene.

We have recently shown that a predominant mechanism of resistance to MTX and various novel hydrophilic antifolates is the loss of RFC-mediated drug transport in multiple human leukemia sublines (8, 10, 11). These transport-defective sublines harbored various inactivating mutations that primarily clustered in the first transmembrane domain of the hRFC (8, 10, 11). In contrast, AG2034R2 cells were devoid of RFC mutations (11), had an extremely poor expression of RFC mRNA, and had no detectable levels of RFC protein. These cells consequently displayed an abolished antifolate transport. Thus, the loss of RFC expression in AG2034R2 cells constitutes the first mechanism of antifolate-resistance that appears to rely solely on the loss of transcriptional activation of the hRFC promoter. In contrast, ZD9331R1.5 cells harbored a heterogeneous premature translation termination mutation (Glu257Stop) but retained a wild type hRFC allele (11). Although this wild type allele could have been substantially transcribed, ZD9331R1.5 cells were devoid of RFC mRNA expression. These results suggest that although mutations are frequently observed in the human (7–11) and the murine (21) RFC genes, the mutational inactivation of a single allele is insufficient and may be accompanied by transcriptional silencing of both alleles via alterations in the expression and function of certain transcription factors. A strong support to this suggestion derives from the fact that various antifolate-resistant human (7–11) and mouse (21) leukemia sublines harbored both RFC-inactivating mutations and prominently decreased RFC mRNA levels.

The present findings have potentially important implications for the development of clinical resistance to MTX and novel antifolates, including ZD1694 (Raltitrexed, Tomudex) (4) and multitargeted antifolate (Pemetrexed) (22), which use RFC as their primary route of entry. Several studies with specimens derived from ALL (12, 13) and osteosarcoma (14) have shown that defective drug transport via the RFC may be a mechanism of resistance to MTX. Consistently, decreased RFC mRNA expression has also been documented in specimens from ALL (12, 13) and osteosarcoma patients (14). Surprisingly however, while inactivating mutations in the RFC gene were a frequent mechanism of antifolate resistance in human (7–11) and animal tumor cell lines (21), the frequency of RFC mutations in

| FIG. 5. Antibody-mediated supershift analysis with [32P]-labeled CRE and [32P]-labeled GC-box oligonucleotides. Nuclear proteins (6 μg) from parental CEM and antifolate-resistant cell lines were first incubated with antibodies (2–4 μg) to CREB-1, ATF-1, Sp1, and Sp3 for 1 h at 4°C. Then, the binding to [32P]-labeled CRE (A) or [32P]-labeled GC-box (B) oligonucleotides was examined by phosphorimaging. The antibody-nuclear protein(s)-oligonucleotide complexes are denoted on the left by A, B, and C. |
| FIG. 6. Western blot analysis of transcription factor expression in parental and antifolate-resistant cell lines. Nuclear proteins (20 μg) from parental CEM and antifolate-resistant sublines were resolved by polyacrylamide gels containing SDS, transferred to a cellulose nitrate Protran membrane, and reacted with antibodies to human CREB-1/ATF-1 (A) as detailed under “Materials and Methods.” The blots were then reprobed with antibodies to Sp1 (B) and Sp3 (C). |
ALL specimens was extremely low. These results suggest that transcriptional silencing of the hRFC gene either by altered expression and function of transcription factors or alternatively by hRFC promoter methylation (23) may prove as factors that contribute to antifolate resistance. Importantly, the collateral sensitivity of various transport-defective phenotypes to lipophilic antifolates including trimetrexate (11) may be exploited to eradicate antifolate-resistant tumors (24).

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REFERENCES


