Molecular Analysis of Murine Leukemia Cell Lines Resistant to 5,10-Dideazatetrahydrofolate Identifies Several Amino Acids Critical to the Function of Folylpolyglutamate Synthetase*

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Four L1210 murine leukemia cell lines resistant to 5,10-dideazatetrahydrofolate (DDATHF) and other folate analogs, but sensitive to continuous exposure to methotrexate, were developed by chemical mutagenesis followed by DDATHF selective pressure. Endogenous folate pools were modestly reduced but polyglutamate derivatives of DDATHF and ALIMTA (LY231514, MTA) were markedly decreased in these mutant cell lines. Membrane transport was not a factor in drug resistance; rather, fopolyglutamate synthetase (FPGS) activity was decreased by >98%. In each cell line, FPGS mRNA expression was unchanged but both alleles of the FPGS gene bore a point mutation in highly conserved domains of the coding region. Four mutations were in the predicted ATP-, folate-, and/or glutamate-binding sites of FPGS, and two others were clustered in a peptide predicted to be β sheet 5, based on the crystal structure of the Lactobacillus casei enzyme. Transfection of cDNAs for three mutant enzymes into FPGS-null Chinese hamster ovary cells restored a reduced level of clonal growth, whereas a T339I mutant supported growth at a level comparable to that of the wild-type enzyme. The two mutations predicted to be in β sheet 5, and one in the loop between NH₂- and COOH-terminal domains did not support cell growth. When sets of mutated cDNAs were co-transfected into FPGS-null cells to mimic the genotype of drug-selected resistant cells, clonal growth was restored. These results demonstrate for the first time that single amino acid substitutions in several critical regions of FPGS can cause marked resistance to tetrahydrofolate antimitabolites, while still allowing cell survival.

Folate cofactors play an essential role in the biosynthesis of purines, thymidylate, glycine, and methionine by providing one-carbon moieties at a variety of oxidation levels. Folates are absorbed through the intestine as monoglutamates and are transported in that form through the circulation and into peripheral cells in mammals. Once in the cell, they are rapidly metabolized to folylpoly-γ-glutamates, the preferred substrates for many of the tetrahydrofolate cofactor-dependent enzymes (1, 2). Folylpoly-γ-glutamate synthetase (FPGS)1 mediates the formation of polyglutamates by FPGS (24). More recently, several classes of antimitabolites have been developed as antitumor agents which are targeted directly against the folate biosynthetic enzymes. These antimitabolites can be subdivided as compounds which are: 1) active against de novo purine synthesis due to inhibition of the third enzyme of this pathway, glycinamide ribonucleotide formyltransferase (GART), 2) active against thymidylate synthase, or 3) active against multiple folate biosynthetic enzymes. Clinically relevant compounds that fall into these three groupings are lomotrexol (DDATHF) and L309887 (25–27), raltitrexed (Tomudex, ZD1694) (28), and ALIMTA (LY231514, MTA) (29, 30), respectively. Most of these agents are strongly activated by polyglutamation, not only because they are better inhibitors of their target enzymes as polyglutamates, but also because they are retained in target cells and accumulate to high levels as these metabolites (26–32).

In this study, we investigate the mechanisms which allow resistance to develop to the prototypical GART inhibitor DDATHF after treatment with the mutagen N-methyl-N-nitrosourea. In several independent cell lines, point mutations in FPGS substantially decreased polyglutamation of DDATHF and several other folate antimitabolites, causing up to a 1700-

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1 The abbreviations used were: FPGS, folylpoly-γ-glutamate synthetase; DDATHF, (6N,N,N-triethyl-5,10-dideazatetrahydrofolate (lometrexol); GART, glycinamide ribonucleotide formyltransferase; CHO, Chinese hamster ovary; 5-CHO-THF, 5-formyltetrahydrofolate; MTX, methotrexate; ALIMTA, pemetrexed disodium, MTA, LY231514; PCR, polymerase chain reaction.
fold decrease in the potency of these drugs. However, the in vivo function of polyglutamation of the naturally occurring folates was sufficient to sustain cell growth and replication.

**MATERIALS AND METHODS**

Chemicals—3',5',7',7'-MHTMX and 3',5',7',7'-HS(6)-5'-CHO-THF were purchased from Amersham Pharmacia Biotech and Moravek Biochemica (Brea, CA), respectively. [3H]ALIMTA, [3H]DDATHF, unlabeled ALIMTA, LY309887, [6R]DDATHF, and DDATHF polyglutamates were kindly provided by Eli Lilly Research Laboratories. ZD1694 and ZD9331 were obtained from IC1 (United Kingdom). Tritiated folates were purified by high performance liquid chromatography prior to use (33, 34). All other reagents were of the highest purity available from various commercial sources.

**Cell Culture**—L1210 murine leukemia cells and its sublines were grown in RPMI 1640 medium containing 2.3 μM folate acid, supplemented with 5% bovine calf serum (HyClone) and either 2 μM [3H]5-CHO-THF. Surviving cells were assayed for cross-resistance to MTX. The clonal cell line was achieved with a multiphase gradient of methanol and tetraethylammonium in the four terminal side chain glutamic acids was prepared for use in [3H]glutamic acid in 200 mM Tris, pH 8.5, containing 36 mM 2-mercaptoethanol.

**Isolation of the DDATHF-resistant Cell Lines**—L1210 cells grown in complete RPMI 1640 medium were treated with 0.4 mM N-methyl-N-nitrosourea for 12 h to achieve about 10% cell survival (35). After cells were washed to remove the mutagen they were placed in 24-well plates at a density of 104 cells/ml and allowed to grow in vitro. Surviving cells were then seeded in fresh complete RPMI 1640 medium containing 400 nM DDATHF and grown for 2 additional weeks. The surviving cells were assayed for cross-resistance to MTX. Cells that were not resistant to MTX were plated in complete RPMI 1640 containing 0.5% soft agar. After an additional 2 weeks, individual clones were picked up and expanded in the absence of DDATHF. The clonal cell lines L7, L15, L44, and L51 were maintained thereafter in DDATHF-free RPMI 1640 medium.

**Growth Inhibition by Antifolates and Growth Requirement for Folates**—Cells in mid-log growth phase were grown in 96-well plates (1 × 105 cells/ml), exposed continuously to the appropriate concentrations of DDATHF, LY309887, MTX, ZD1694, ZD9331, and ALIMTA for 72 h following removal of 90% of the medium. Cells were centrifuged in a Microfuge, and radioactivity in the supernatant was counted. The mixture was centrifuged in a Microfuge, and radioactivity in the supernatant was determined on a liquid scintillation spectrometer.

**FFGS-based DDATHF Resistance**

**Transport Studies**—Influx measurements were performed by methods described previously (36). Briefly, exponentially growing cells were harvested, washed twice, and resuspended in HBS (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl2, 5 mM glucose, pH 7.4) to a density of 1.5 × 106 cells/ml. Cells suspensions were incubated at 37 °C for 25 min following which uptake was initiated by the addition of radiolabeled folate acid. Cells were labeled at the 50% inhibition level, with the addition of 1 ml of the cold folate solution. Cells were centrifuged at 600 × g for 10 min and washed twice with ice-cold HBS and processed for measurement of intracellular radioactivity (36).

**Enzyme Assays**—The pentaglutamate of DDATHF labeled with tritium in the four terminal side chain glutamic acids was prepared for use as a substrate for γ-glutamyl carboxypeptidase assay. [3,4-3H]Glutamylcarboxypeptidase activity was measured by incubating cellular protein with [3H]DDATHF pentaglutamate and separating unreacted substrate from product by adsorption onto activated charcoal. Cells were broken in a hand-held Dounce homogenizer in 50 mM Tris acetate buffer, pH 8.0, containing 50 mM 2-mercaptoethanol, and lysates were centrifuged for 10 min at 4 °C and 14,000 rpm in a Beckman Microfuge. Protein was incubated with 100 μM [3H]DDATHF pentaglutamate in a total volume of 25 μl of 50 mM Tris acetate buffer, pH 6.0, containing 50 mM 2-mercaptoethanol. charcoal slurry (34) was added, the mixture was centrifuged in a Microfuge, and radioactivity in the supernatant was determined on a liquid scintillation spectrometer.

**GART activity** was measured spectrophotometrically as described previously (26, 40). Protein was determined using a dye binding assay (Bradford). 

**Identification of Mutations in FPGS—**Total RNA was isolated using the TRIzol reagent (Life Technologies). RNA (20 μg) was resolved by electrophoresis on 1% agarose gels containing formaldehyde, and sequenced (39) in which cytosolic protein, prepared by a 110,000 × g centrifugation with 90% Percoll, was allowed to incin 5 days in HEPES-buffered saline (4, 42). Dual transfection plates were treated with 5 μg of each plasmid and cloned fragments were sequenced using the two primers described above for the PCR reaction and two additional FPGS-based primers: 5'-CCCTTCTATTCTCGGTTCTCTC-3' and 5'-CACCTGTGTTCCGCCCATCC-3' (41). The PCR amplifications were performed for 35 cycles of 45 s at 95 °C, 45 s at 60 °C, and 4 min at 72 °C. The 1683-base pair long predicted PCR product was purified on an agarose gel (Qiagen) and cloned into a pCR-Blunt vector (Invitrogen). Both the whole cDNA population and cloned fragments were sequenced using the two primers described above for the PCR reaction and two additional FPGS-based primers: 5'-CCCTTCTATTCTCGGTTCTCTC-3' and 5'-CACCTGTGTTCCGCCCATCC-3' (41). The PCR amplification product was resolved by agarose gel (42). Dual transfection plates were treated with 5 μg of each plasmid and 15 μg of carrier DNA. After 30 min, the microprojectiles were added to cells and transfected cells were osmotically shocked with 10% Me2SO in complete medium the next morning. After 2 days inoculation in vitro function of polyglutamation of the naturally occurring folates was sufficient to sustain cell growth and replication.
ditation at 37 °C in non-selective conditions, the medium was changed to either minimal essential medium α containing 10% fetal calf serum and 1 mg/ml G418 as a transfection efficiency control, or minimal essential medium α formulated without nucleosides containing 10% dialyzed fetal calf serum and G418 to test for the ability of a construct to confer FPGS activity to the cells. Plates were fixed and stained after 2 weeks of selection, and macroscopic colonies were counted.

RESULTS

Selection of DDATHF-resistant Cells That Are Not Cross-resistant to Continuous Exposure to MTX—L1210 cells were exposed to a mutagenic concentration of N-methyl-N-nitrosourea, followed by selective pressure with 0.4 μM (6R)-DDATHF. This selection strategy minimized the likelihood of acquired resistance due to changes in the level of expression of genes critical to drug action. A total of 53 DDATHF-resistant clones were initially identified; the majority were cross-resistant to MTX and were not studied further. Six clones which were not cross-resistant to continuous exposure to MTX exhibited stable resistance to DDATHF after passage in drug-free medium for 6 months. These clones maintained a doubling time similar to that of the parent L1210 cells. Four of these cell lines had very similar biochemical and pharmacological phenotypes and are the subject of this report.

Cross-resistance Patterns to Antifolates and Growth Requirements for Folic Acid and 5-CHO-THF—The levels of DDATHF required to inhibit these four cell lines were 13–40-fold higher than those inhibitory to wild-type L1210 cells (Table I). In contrast, the IC50 for MTX was unchanged or slightly decreased compared with parental cells. All four cell lines were cross-resistant to the second generation GART inhibitor LY309887. Interestingly, the resistance of the L15 cell line to LY309887 was only 4-fold greater than that of L1210 cells, whereas these cells were 40-fold more resistant to DDATHF than the parental L1210 cells. A much higher level of cross-resistance to the TS-inhibitor D1694 was observed in several of these cell lines; for instance, the L7 cells were nearly 2000-fold less sensitive to ZD1694 than were L1210 cells, but only 17-fold resistant to DDATHF, the agent used in the original selection procedure. However, none of these cell lines were resistant to a non-polyglutamatable TS inhibitor, ZD9331 (43); this suggested alterations in polyglutamation as the mechanism of resistance. All four resistant cell lines displayed moderate (15–60-fold) resistance to ALIMTA, similar in each case to that for DDATHF.

The growth requirement of these mutant cell lines for folic acid and for 5-CHO-THF was examined. All mutant cells required higher levels of either folate source for half-maximal growth (Table I); the increased requirement of each of the cell lines for folic acid was nearly identical to that for growth on 5-CHO-THF. The increased growth requirement for exogenous folates of these mutant cell lines was much lower than the degree of resistance to DDATHF or ALIMTA, and differed from the degree of resistance to ZD1694 by factors of as much as 250.

The Intracellular Accumulation of Folates, ALIMTA, and DDATHF in DDATHF-resistant Cells—The accumulation of folates and antifolates was studied in these mutant cell lines. Cells were grown in folate-free medium supplemented with either 2 μM [³H]folic acid or 25 μM [³H]5-CHO-THF. After 1 week of exponential growth, cells were harvested and the total intracellular folate content determined. Total cellular folates were lower in the mutant cell lines than in parental L1210 cells after growth on either folic acid or 5-CHO-THF (Fig. 1A). Typically, the intracellular folate pools were decreased by 45–55% in L7, L44, and L51 cells, and by 60–75% in L15 cells grown on either folic acid or 5-CHO-THF.

The accumulation of DDATHF and ALIMTA was studied over a period of 3 days in medium supplemented with nucleo-
cells, but the time-dependent synthesis of FPGS products in all four mutant cell lines was still at the limits of sensitivity of the assay (data not shown). Previous studies have shown that mammalian cells can survive, and indeed can maintain adequate folate pools, on levels of FPGS that are only a few percent of those in leukemic cells (3, 45). Northern analysis of transcripts from the FPGS gene (Fig. 4) indicated negligible differences in steady-state levels of message in all mutants and L1210 cells. These data suggested that the sequence of the message may have been changed to alter the catalytic activity or stability of the expressed FPGS protein.

Identification of Mutations in the Structural Gene for FPGS—
cDNAs encompassing the entire coding region of FPGS in the mutant and wild-type L1210 cells were amplified by reverse transcription-PCR, cloned, and sequenced. Single nucleotide mutations were found in the expressed coding region of the FPGS gene for cDNA from each mutant cell line (Table III).

| Table I |
|---|
| Growth inhibition by continuous exposure to antifolates (IC\textsubscript{50}) and growth requirements (EC\textsubscript{50}) in DDATHF-resistant L1210 cell lines |
| L1210 and mutant cells grown in RPMI 1640 medium were exposed continuously to the appropriate concentrations of drugs for 72 h following which cell numbers were determined by hemocytometer count. For measurement of total folate pools, L1210 and mutant cell lines were grown exponentially in folate-free RPMI 1640 medium supplemented with either 2 µM [\textsuperscript{3}H]folic acid or 25 nM [\textsuperscript{3}H]5-CHO-THF for 1 week. For determination of ALIMTA or DDATHF polyglutamate derivatives, the cells were grown with 50 nM [\textsuperscript{3}H]ALIMTA or [\textsuperscript{3}H]DDATHF for 3 days in RPMI 1640 medium supplemented with GAT to circumvent the cytotoxicity of the drugs. Intracellular radioactivity was assessed as described under “Materials and Methods.” Data are the mean ± S.E. of three separate experiments. |
| Cell line | IC\textsubscript{50} | EC\textsubscript{50} |
| L1210 | 27.9 | 1.6 (1) |
| L7 | 12 | 1.0 (1) |
| L5 | 9.5 | 1.0 (0.6) |
| L44 | 5.3 | 1.0 (0.4) |
| L51 | 5.3 | 0.4 (0.4) |
| L7 | 25.9 | 1.6 (1) |
| L15 | 10.0 | 0.6 (0.6) |
| L4 | 5.3 | 0.4 (0.4) |
| L51 | 5.3 | 0.4 (0.4) |
| L1210 | 27.9 | 1.6 (1) |
| L7 | 12 | 1.0 (1) |
| L5 | 9.5 | 1.0 (0.6) |
| L44 | 5.3 | 1.0 (0.4) |
| L51 | 5.3 | 0.4 (0.4) |
| ALIMTA | 62.6 | 220 (700) |
| 5-CHO-THF | 28.0 | 11 (1.1) |
| DDATHF | 22.0 | 3 (23) |

Fig. 1. Intracellular accumulation of natural folate metabolites (A), ALIMTA, and DDATHF (B) in L1210 cell mutants. For measurement of total folate pools, L1210 and mutant cell lines were grown exponentially in folate-free RPMI 1640 medium supplemented with either 2 µM [\textsuperscript{3}H]folic acid or 25 nM [\textsuperscript{3}H]5-CHO-THF for 1 week. For determination of ALIMTA or DDATHF polyglutamate derivatives, the cells were grown with 50 nM [\textsuperscript{3}H]ALIMTA or [\textsuperscript{3}H]DDATHF for 3 days in RPMI 1640 medium supplemented with GAT to circumvent the cytotoxicity of the drugs. Intracellular radioactivity was assessed as described under “Materials and Methods.” Data are the mean ± S.E. of three separate experiments.

Fig. 2. Influx of MTX, 5-CHO-THF, and DDATHF in wild-type and mutant L1210 cells. After 25 min incubation in HBS, L1210 and mutant cells were exposed to 1 µM [\textsuperscript{3}H]MTX, [\textsuperscript{3}H]5-CHO-THF, or [\textsuperscript{3}H]DDATHF and portions of the suspension were then taken at various times for measurement of intracellular drugs. Influx was calculated from the slope of the initial linear time course of uptake. Data are expressed as the mean ± S.E.
Multiple individual cloned cDNAs were sequenced in the region of these mutations; a minimum of 10 randomly picked clones were analyzed for each mutant cell line. The mutations involved either C → T (5/8) or G → A (3/8) substitutions, consistent with the known action of the mutagen N-methyl-N-nitrosourea (35). In all four resistant cell lines studied, both copies of the FPGS gene were mutated. Thus, in L7 cells, approximately half of the cDNAs sequenced had a serine to phenylalanine mutation at codon 67; those which were not mutated at codon 67 had a glycine to glutamic acid substitution at codon 178. L44 cells had two closely spaced mutations: half of the clones (Gly → Asp) and a premature stop introduced at codon 445 in the other half. L51 cells had a stop mutation at codon 180 (Ser → Phe) in half of the sequenced cDNAs and the same mutation (67Ser → Phe) as was seen in L7 cells in the other half of the cDNAs. For all of the cell lines analyzed, each cDNA sequence harbored only one mutation. The results suggested that both alleles of the FPGS gene were independently mutated, yet both alleles were still expressed in the mutant cells. This is in contrast to previous studies on the L1210/D3 (46) and

Table II

| Cell line | FPGS activitya | GART activityb | γ-Glutamylcarboxypeptidase activityc |
|-----------|----------------|----------------|-------------------------------------|
| L1210     | 0.49 ± 0.12    | 3.2 ± 0.51     | 0.09 ± 0.05                         |
| L7        | 0.006 ± 0.005  | 3.0 ± 0.48     | 0.08 ± 0.04                         |
| L15       | 0.006 ± 0.003  | 3.4 ± 0.26     | 0.06 ± 0.05                         |
| L44       | 0.004 ± 0.004  | 3.6 ± 0.30     | 0.09 ± 0.05                         |
| L51       | 0.010 ± 0.002  | 2.4 ± 0.53     | 0.03 ± 0.04                         |

a Measured with (65)-tetrahydrofolate, ATP, and glutamic acid as substrates, using a microassay procedure (39), modified as described in the text. Each value represents the slope of time curves with eight points per curve ± S.D. (n = 3).

b Measured spectrophotometrically (26, 40) using 10-formyl-5,8-dideoxofolic acid and γ-glutamylamine ribonucleotide as substrates. Values are means ± S.D. of three experiments. The values listed are not significantly different from each other nor from previous measurements of GART in L1210 cells (34).

c Measured by the release of [3H]glutamic acid from 5,10-dideazatetrahydrofolate, ATP, and glutamic acid as substrates, using a microassay procedure (39), modified as described in the text. Each value represents the slope of time curves with eight points per curve ± S.D. (n = 3).

MTX'A cell lines (16) in which mutation of one allele, and silencing of the other wild-type allele, of the reduced folate carrier occurred when resistant cells were produced by continuous selection pressure. Care was taken in these experiments to avoid PCR-generated sequence artifacts, and several lines of evidence verified that these multiple mutations in the FPGS gene were not due to reverse transcriptase-PCR errors. Thus, one mutation or the other was found in each of the 10–12 clones sequenced or partially sequenced from each cell line; wild-type FPGS sequence was not found in any clone sequenced from any of these mutant cell lines. As a confirmation, PCR-generated cDNA was sequenced directly in a repeat experiment without intermediate cloning of PCR products. In these experiments, at the nucleotide identified as being mutated in the initial sequencing results, two nucleotides were found by direct sequencing, and the abundance of the two nucleotides at each position was compatible with a 1:1 ratio. In addition, the restriction enzyme NarI was used to verify the A337V mutation in L44 cells, since the C → T substitution eliminates a unique restriction site in the FPGS cDNA (data not shown). Finally, the expression of a wild-type allele of FPGS in these mutants is incompatible with the results of the FPGS activity assays described in Table II.

Transfection of the Cloned FPGS Mutant cDNAs into FPGS-null Cells—Classical studies on the selection of CHO cells that were auxotrophic for the products of folate metabolism (47) resulted in the establishment of the AUXB1 cell line, which was subsequently found not to express measurable FPGS ac-

FIG. 3. Metabolism of DDATHF to polyglutamate derivatives in L1210 cell mutants. Cell lines were exposed to 0.2 μM [3H]-DDATHF for 8 h. Folate compounds were then extracted at 100 °C, the extracts were mixed with authentic DDATHF markers and analyzed on a 10-cm 3-μM C18 HPLC column eluted with a gradient of methanol and tetrabutylammonium phosphate as described under “Materials and Methods.” Results shown are from a representative experiment.

FIG. 4. Northern blot analysis of total RNA from mutant and wild type L1210 cells. Total RNA (20 μg) was resolved on a 1% agarose gel containing formaldehyde. After transferring and fixing to the membrane, the blot was probed successively with the full-length murine FPGS, GART, and β-actin cDNA. The blot is a representative autoradiogram of two separate experiments. The mouse GART probe hybridized with two transcripts, one for the trifunctional GART and the other for a monofunctional glycaminide ribonucleotide synthetase (GARS) protein, as expected (65).

Table III

| Cell lines | Nucleotide change | Amino acid change | Mutation frequency |
|------------|-------------------|-------------------|-------------------|
| L7         | 203TCC → TTC      | 67Ser → Phe       | 6/10              |
|            | 533GGA → GAA      | 173Gly → Glu      | 4/10              |
| L15        | 955GCC → GAC      | 326Gly → Asp      | 5/10              |
|            | 1335TGG → TGA     | 446Trp → stop     | 5/10              |
| L44        | 1013CCC → GTC     | 337Ala → Val      | 7/12              |
|            | 1014ACC → ATC     | 337Thr → Ile      | 5/12              |
| L51        | 203TCC → TTC      | 67Ser → Phe       | 5/10              |
|            | 539TCC → TTC      | 180Ser → Phe      | 5/10              |
FIG. 5. Functional complementation of AUXB1 cells by cDNAs for L1210 FPGS and its mutants. cDNAs were mixed with carrier DNA and transfected into AUXB1 cells as calcium phosphate co-precipitates. Colony growth requiring the action of FPGS was obtained by addition of medium containing G418 but lacking nucleosides. Colonies were counted after 14 days growth under these selective conditions. The results shown are the mean ± S.D. of three plates for each condition from each of two experiments.

activity (48). cDNAs for each mutant FPGS species found in the DDATHF-resistant L1210 variant lines were cloned into a mammalian expression vector, pcDNA3, and the cDNAs were transfected into AUXB1 cells. Transfectants were screened for the ability to grow on medium which required folate metabolism as an in vitro test of function of the mutant FPGS species. Under conditions in which several hundred colonies were formed from AUXB1 cells transfected with wild-type mouse leukemic cell FPGS, three of the mutant FPGS cDNAs (G178E, S180E, and G320D) did not allow any colony survival (Fig. 5).

Two cDNA constructs (S67F and A337V) allowed the formation of a decreased number of colonies, relative to the control dishes transfected with a cDNA for the wild-type L1210 FPGS, but the size of the colonies formed were quite small. The FPGS cDNA bearing the T339I mutation allowed large numbers of colonies to survive selection and, notably, the numbers of large colonies approximated those found with the wild-type construct. Of particular interest, the cDNA construct harboring a premature stop codon at position 445 supported the growth of some colonies, which did not give rise to any colonies when the wild-type cDNA was transfected into AUXB1 cells. This was observed in each of three experiments using cDNA in which the sequence of the construct was confirmed. No colonies were observed on plates of AUXB1 cells transfected with the pcDNA3 plasmid alone.

It should be noted that three of the four L1210 cell variants selected in this study were growing at rates not appreciably different from that of L1210 cells, and one (L7) was growing only marginally slower. In order to determine whether simultaneous expression of sets of two mutant FPGS species in AUXB1 cells would mimic the behavior of the selected L1210 cell mutants, pairs of cDNAs corresponding to the mutated alleles found in L7 (S67F and G178E), L15 (G320D and W445*), L44 (A337V and T337I), and L51 (S67F and S180F) were co-transfected into AUXB1 cells. Significant numbers of viable colonies were seen with each combination of cDNAs corresponding to the mutant species of FPGS found in vivo (Fig. 5). Hence, the occurrence of colonies with each of these co-transfections recapitulated the growth of the L1210 mutants during the selection procedure.

DISCUSSION

In this study, cell lines were selected for resistance to the prototypical GART inhibitor DDATHF that retained sensitivity to continuous exposure to MTX, thus probing for mechanisms of resistance that were particularly crucial to the newer generation folate analogs. Four out of six clones selected with this phenotype bore mutations in each allele of the FPGS gene. The DDATHF-resistant cell lines were cross-resistant to the second generation GART inhibitor LY309887 and the multiply targeted antifolate ALIMTA, and were even more strikingly resistant to the thymidylate synthase inhibitor ZD1694 (ralitrexed). This pattern of cross-resistance strongly reinforces the centrality of polyglutamation to the mechanism of action of all these compounds and the causality of polyglutamation defects as the mechanism of antifolate resistance in these cells. The more marked resistance of ZD1694 in these cell lines likely reflects the fact that the polyglutamates of ZD1694 bind to thymidylate synthase about 100 times tighter than the ZD1694 monoglутamate. On the other hand, DDATHF and LY309887 are tight binding inhibitors of GART prior to metabolism (31), and become better enzyme inhibitors with polyglutamation (31), but to a lesser degree than does ZD1694 (28).

The individual mutations in FPGS seem to have had a selective impact on the polyglutamation of folates and of the various antifolates studied. Thus, there were only small effects of the FPGS mutations selected in these experiments on cellular accumulation of the natural folates as compared with DDATHF and ALIMTA. This selective suppression of the polyglutamation of the antifolates compared with the naturally occurring folates was an obvious outcome, in retrospect; that is, cells with mutations in both alleles of FPGS which did not allow polyglutamation of the natural folates would be lethal and hence, would never be selected. Nevertheless, the mutant enzymes created during the selection process appear to distinguish between ALIMTA and DDATHF and the natural cellular folates, favoring the latter, despite the fact that ALIMTA and DDATHF are among the most efficient substrates for the pure wild-type L1210 enzyme.2 We are currently studying the active site of these mutant enzymes in more detail.

The sequences of the FPGS gene from seven species have been published, and the sequence of an open reading frame corresponding to FPGS in yeast, Caenorhabditis elegans, blue-green algae, and a few bacterial strains are available from large scale sequencing studies (49–58). A comparison of the homology of primary sequence of FPGS across species reveals that the mammalian enzymes are remarkably similar over their entire length, but the homology between the eukaryotic and prokaryotic enzymes is much more limited. Each of the FPGS mutations selected in this study occur in patches of very high homology across species from bacteria to man (Fig. 6). In addition to homology considerations, previous site-directed mutagenesis studies on the L. casei (59) and human enzymes (38), and the x-ray crystal structure of the L. casei FPGS (60) give direct insight into the functions of the peptides surrounding these mutations, particularly residues 61–67 and residues 334–341. Serine 67 is conserved in all species except Neurospora crassa and is part of a highly conserved motif commonly found in nucleotide-binding proteins, such as the synthetases. The peptide GTKGKGS was previously identified as a Walker B half-site (38, 59, 61), which is usually responsible for alignment of ATP, and mutagenesis (59) of the central lysine in this

2 R. Zhao, S. Titus, F. Gao, R. G. Moran, and I. D. Goldman, unpublished results.
peptide has been shown to dramatically decrease the binding of ATP to the L. casei FPGS. The crystal structure of the L. casei enzyme (60) shows that serine 52 of L. casei FPGS, equivalent to serine 67 in the mouse protein, is part of the p-loop surface involved in ATP binding; substitution at this position with the bulky and hydrophobic phenylalanine would be expected to disrupt nucleotide binding and cause substantial changes in the kinetics of the polyglutamation process. In this light, it is interesting that the S67F mutant cDNA still supports the growth of small colonies in the AUXB1 complementation experiments (Fig. 5).

Peptide LDGAHT (mouse residues 334–340; Fig. 6) constitutes one of the most highly conserved motifs in the known FPGS sequences. Recent studies identified this peptide as being solvent exposed on the basis of very rapid labeling of cysteine 346 with iodoacetamide which was prevented by ATP and folate (38). Subsequent alanine scanning mutagenesis of the ionic residues of this peptide produced two very informative mutants, D335A and H338A (38). Kinetic analysis of these mutants revealed that His338 is central to the binding of glutamic acid within the active site and that the D335A mutant had lower binding of all three substrates, folate, ATP, and glutamic acid. Hence, the A337V and T339I mutations are located on a peptide which clearly constitutes the binding site for glutamic acid and probably also approximates the point of intersection of the binding surfaces for all three substrates. In the L. casei FPGS structure, the amino acids equivalent to both mouse FPGS alanine 337 and threonine 339 connect the β-sheet B12 and α-helix A10 of the carboxyl-terminal domain; both of these structural elements appear to be involved in the folate binding pocket (38, 60). It is interesting to note that the T339I mutation was the most selective of all those uncovered in this study: the in vitro enzyme assays indicated quite low FPGS activity (<2% of wild-type) in cells expressing both T339I and A337V (Table II), yet transfection of AUXB1 cells with the T339I cDNA allowed the formation of large colonies of cells similar to those seen with wild-type mouse L1210 FPGS (Fig. 5). This result also highlights the power of the genetic selection approach used in this study: substitution of isoleucine in place of threonine at this location would be a very unlikely choice in any planned site-directed mutagenesis experiment, as would be the A337V substitution.

The structure of the L. casei FPGS is organized in two large domains: an amino-terminal domain which has clear homology with other ATP-binding proteins and a carboxyl-terminal domain which appears to contain the folate-binding site and at least some of the residues involved in catalysis (60). We have previously concluded that human FPGS residue Arg377 was directly involved in catalytic processes on the basis of the loss of 95% of the $k_{cat}$ of the wild-type protein for a R377A mutant enzyme (38). It would appear that catalysis is occurring in FPGS in a region of the active site at the interface between the COOH-terminal and NH$_2$-terminal domains. One of the mutants detected in this study was located on the linker peptide between these two large domains. It is not immediately apparent whether the loss of enzyme activity of the G320D mutant FPGS observed in these experiments (Table II) reflects an intrinsic role of this peptide in the binding of substrates or catalysis or whether the structure of this linker is required for alignment of the substrate binding surfaces located at the interface of the domains. However, we note that both Gly320 and Trp318 are completely conserved among FPGS from divergent species (Fig. 6).

Two mutations selected in this studies, G178E and S180F, are on a peptide equivalent to the β-strand B5 in the L. casei structure (60). This β-strand appears in the crystal structure to be in close contact to the p loop and would likely alter the flexibility, and perhaps the function of this region of the protein. Both enzyme assays (Table II) and complementation analysis in AUXB1 cells (Fig. 5) indicated a complete lack of FPGS activity for these mutants. It is of interest that both of these mutations occurred in cell lines in which the other allele of the FPGS gene was mutated at codon 67 (S67F), although this might conceivably be just happenstance in a small collection of mutant cell lines.

Finally, the most surprising mutation selected in this study was the premature stop introduced at codon 445. Although no detectable FPGS activity was found in cell line L15, which harbored both the W445* and G320E mutations, a number of AUXB1 colonies survived selection for cells which could use folic acid to form cofactor pools in the absence of nucleosides in the medium. This was observed in each of several experiments and the mutation was repetitively confirmed in the transfected cDNA. Several of the colonies formed in these analyses were quite large, an occurrence which seemed to make the interpretation that the W445* cDNA had residual enzyme activity in vivo, despite the fact that the most carboxyl-terminal sequence of FPGS (residues 536–546 of the L1210 cytosolic FPGS: VTGSLHLVGVG) is one of the most highly conserved peptides in this protein.

In summary, selection for point mutations in genes essential for the action of the tetrahydrofolate class of antimetabolites repetitively and frequently resulted in isolation of cell lines in which both alleles of the FPGS gene were independently mutated. Each mutation was informative and further biochemical analysis of the proteins encoded is being pursued. Yet, the low residual FPGS activity of these several mutants still allowed functional cofactor pools to be made and the survival and growth of drug-resistant tumor cells. While resistance to DDATHF has been related to a decrease in transport (62), decrease in FPGS activity (63) or increase in y-glutamyl hydro-lase activity (64) this paper represents the first report that single amino acid substitutions in several critical regions of
FPGS-based DDATHF Resistance

FPGS can cause marked resistance to tetrahydrofolate antimetabolites.

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