Posttranscriptionally Mediated Decreases in Folylpolyglutamate Synthetase Gene Expression in Some Folate Analogue-resistant Variants of the L1210 Cell

EVIDENCE FOR AN ALTERED COGNATE mRNA IN THE VARIANTS AFFECTING THE RATE OF DE NOVO SYNTHESIS OF THE ENZYME*

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L1210 cell variants resistant to edatrexate (EDX) were isolated by selection in vitro during therapy with this folate analogue. Among the variants selected, seven (L1210/EDX-4 to -7 and L1210/EDX-12 to -14) were found to exhibit 2–23-fold lower levels of folylpolyglutamate synthetase (FPGS) activity compared with parental L1210 cells. Lower levels of FPGS activity in cell-free extract from these variants using EDX as substrate were characterized by the same relative decrease in value for Vmax with no change in apparent Km. The results of an analysis of FPGS activity in mixtures of variant and parental cell extract suggested that no endogenous inhibitors in the variant cells or stimulatory factors in parental cells accounted for the differences observed. Also, FPGS from variant and parental cells showed no difference in thermostability. Decreases in a 60–61-kDa protein as shown by immunoblotting with anti-FPGS peptide antibody were found to occur commensurately with the decrease in FPGS activity in cell extract from the variants compared with parental cells. However, no evidence was obtained for a difference in turnover of FPGS protein during measurement of the decay of FPGS activity in cycloheximide-treated variant and parental cells. In addition, Northern blotting of poly(A)+ RNA did not reveal any difference in the size or level of FPGS mRNA among these various cell types. Studies of in vitro translation of hybridization-selected FPGS mRNA from L1210 cells showed that both mitochondrial and cytosolic forms of FPGS were generated during the reaction. Moreover, FPGS mRNA from the variant cells was significantly less effective in mediating formation of the FPGS peptide product in a manner correlating with FPGS activity and protein found in the cytosol of the various cell types. These results suggest that FPGS gene expression in these variants is posttranscriptionally altered at the level of the cognate mRNA itself and that this alteration constitutively down-regulates the steady-state level of FPGS in these variants.

Biochemical alterations associated with acquired resistance of tumor cells to classical folate analogues are highly diverse (1–5). This phenotypic diversity most likely reflects the common occurrence of multiple genomic alterations among clonal variants selected for resistance to these agents either in vitro or in vivo. As a consequence, acquired resistance to methotrexate and newer folate analogues under development will remain (6–8) a major limitation to their effective clinical utility. One of the determinants of cytotoxicity shared (9–11) by most folate analogues, whether targeted to dihydrofolate reductase or folate-dependent biosynthetic enzymes, is the process of intracellular polyglutamylation mediated by the enzyme folylpolyglutamate synthetase (FPGS).1 This process is not only important to the conservation and efficient utilization by proliferating cells of folate coenzymes (12–18) but is responsible, as well, for converting folate analogues (9–18) to longer chain polyglutamate anabolics that are more retentive and more effective inhibitors of folate-dependent enzymatic targets. Subsequently, examples have been reported from our laboratory (4, 19) and elsewhere (20, 21) of tumor cell variants with acquired resistance to methotrexate and other folate analogues that exhibit lower levels of FPGS activity when compared with parental cells and are cross-resistant to most other classical folate analogues. In one of our own studies with the L1210 leukemia, resistant variants (L1210/EDX) were independently selected (4) during therapy of tumor-bearing mice with edatrexate (EDX). Some of these variants exhibited levels of FPGS activity that were substantially lower than in parental cells. In the current studies, we address the underlying basis for these alterations in FPGS activity in these resistant variants at the level of the FPGS protein elaborated by these cells as well as cognate gene expression. Our results appear to define a novel mechanism underlying these modifications in FPGS activity in these variants that may have broad significance in regard to the regulation of FPGS gene expression and for therapy with classical folate analogues. A preliminary report of some of these findings has already been presented (22) in abstract form.

EXPERIMENTAL PROCEDURES

Cells and Culture Conditions—Methods for the isolation of variants of the L1210 cell used in these studies were described in detail earlier (4). These variants were independently isolated from ascite fluid in the peritoneal cavity of mice following treatment with EDX. Cloning of each of these variants was by limiting dilution in mice (4), and the cloned variants were maintained in mice under therapy with EDX. Each

1 The abbreviations used are: EDX, edatrexate; fL5CHO-folateH4, natural diastereoisomer of 5-methyltetrahydrofolate; FPGS, folylpolyglutamate synthetase; MPC, magnetic particle concentrator; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.
variant was transplanted into mice not receiving therapy prior to processing cells from ascitic fluid for the various experiments. Cells were usually collected in the form of a pellet by centrifugation, washed once with phosphate-buffered saline (0.14 M NaCl, 0.01 M potassium phosphate), centrifuged again, and frozen at −80 °C for storage.

For Activity for FPGS Activity—After processing of cells (4 and above), the protein concentration of the resulting cell-free extract was determined (23), and the assay for FPGS activity was carried out as described earlier (4) using EDX as substrate. Product formation (edatrexate plus 1 additional glutamate linked by a peptide bond at the γ-carboxyl group) under these conditions was linear for at least 3 h at 37 °C, and the cell-free preparations under the assay conditions used exhibited (24) no detectable folypolyglutamate hydrolase activity. In some experiments, FPGS activity in cell-free extract was determined for cells exposed to 50 μg/ml of cycloheximide for varying periods of time.

Derivation of a Marine FPGS cDNA Probe—A murine FPGS cDNA (ZAP-L1210/RA3−1) 77.3% homologous to a human FPGS cDNA was obtained by hybridization screening (19) of an L1210 cell cDNA library in a gt11 (Stratagene, La Jolla, CA) using the human cDNA, pTZ18U (25), as a probe. This λ gt11 cDNA construct incorporates a 2,247-kilobase insert ligated at the Xho polymer linker site including 3′- and 5′-untranslated region sequences of 460 and 26 base pairs respectively, and an open reading frame of 1761 base pairs that codes for a putative mitochondrial leader peptide as well as the enzyme protein. Sequencing of the insert of the cDNA was accomplished by the method of Sanger et al. (26).

Northern Blot Analysis of FPGS mRNA—A method (27, 28) utilizing rapid isolation of poly(A)+ RNA directly from the cell lysates by means of an oligo(dT)-cellulose column was used. An aliquot (2–10 μg) of the RNA was analyzed (29) by Northern blotting and radioautography using a murine FPGS cDNA, ZAP L1210/RA3−1 (see above), as a probe, and normalized to mRNA content with a human β-Actin probe, PCD- actin (30). Labelling of each probe was by random priming (Random Primers DNA labeling kit; Boehringer Mannheim) using (α-32P)dCTP (3000 Ci/mmol) and 10–20 ng of insert.

Immunoblotting Procedure—Samples of a 0–30% ammonium sulfate fraction (4) of cytosol were solubilized in SDS sample buffer, electrophoresed (31) on a 7.5% polyacrylamide slab gel, and transferred to nitrocellulose using a Bio-Rad Trans-Blot cell (32). Western blotting was performed (32, 33) using anti-human FPGS peptide polyclonal antibody (0.5 μg/ml) prepared in rabbits and purified on a peptide-Sepharose column (33, 34). The human FPGS peptide utilized was deduced from the cDNA nucleotide sequence (23) and linked to keyhole limpet hemocyanin (35). The amino acid sequence of this peptide and its use as an antigen for antibody production has already been described (19). Following blotting, the anti-rabbit horseradish peroxidase conjugate (Sigma) at a 1:3000 dilution was used as the secondary antibody. The blots were used to expose hyperfilm after incubation with enhanced chemiluminescence (ECL) reagents (Amersham Corp.) and quantitated by scanning densitometry (Stratagene).

Hybridization Selection of FPGS mRNA from Parental and Variant L1210 Cells—The isolation of poly(A)+ RNA and the preparation of FPGS cDNA from several different cell types has already been described (4) during therapy with EDX of tumor-bearing mice. Eleven of the 13 variants (L1210/EDX-1 to L1210/EDX-13) showed lower inward transport of folate analogues. The remaining variant (L1210/EDX-4) exhibited the greatest decrease in FPGS activity, showing no alteration in

| Cell line | Specific activity | V<sub>max</sub> | V<sub>max</sub>/K<sub>m</sub> |
|----------|-----------------|-------------|----------------|
| L1210    | 1.52 ± 0.3     | 29.8 ± 4    | 1.74 ± 0.7    |
| L1210/EDX-4 | 0.39 ± 0.07 | 34.6 ± 5    | 0.44 ± 0.07   |
| L1210/EDX-5 | 0.23 ± 0.04 | 28.2 ± 5    | 0.25 ± 0.4    |
| L1210/EDX-6 | 0.066 ± 0.02 |              |              |
| L1210/EDX-7 | 0.48 ± 0.06 | 31.0 ± 5    | 0.56 ± 0.8    |
| L1210/EDX-12 | 0.78 ± 0.13 | 26.3 ± 4    | 0.85 ± 0.1    |
| L1210/EDX-13 | 0.74 ± 0.08 | 32.9 ± 5    | 0.91 ± 0.1    |
| L1210/EDX-14 | 0.56 ± 0.08 | 28.6 ± 4    | 0.84 ± 0.07   |

<sup>a</sup> Activity was inadequate for a kinetic analysis.

TABLE I Kinetic properties and level of FPGS activity in EDX-resistant, L1210 cell variants

Methodological details are provided under “Experimental Procedures.” The values shown were derived with edatrexate as the substrate.

Some Phenotypic Characteristics of the L1210/EDX Variants—A total of 14 L1210/EDX-resistant variants were isolated (4) during therapy with EDX of tumor-bearing mice. Eleven of these variants when compared with parental L1210 cells exhibited increases in dihydrofolate reductase activity; nine exhibited decreases in transport inward of folate analogues, and seven showed a decrease in FPGS activity. Among these latter seven variants, FPGS activity was determined in crude cell-free extracts of L1210/EDX variants (Table 1). From 2- to 23-fold lower than the level seen in parental L1210 cells. In addition to this enzymic alteration, four of these variants (L1210/EDX-4, -5, -13, and -14) exhibited (4) elevated levels of dihydrofolate reductase, and two variants (L1210/EDX-7 and -12) showed lower inward transport of folate analogues. The remaining variant (L1210/EDX-6), exhibiting the greatest decrease in FPGS activity, showed no alteration in posttranscriptional effects on FPGS synthesis.
either one of these other properties. In every case, where adequate kinetic measurements could be made, the decrease in FPGS activity observed in these variants compared with parental L1210 cells using EDX as substrate was associated (Table I) with a commensurate decrease in value for $V_{\text{max}}$ for catalytic activity and no change in value for apparent $K_m$. Otherwise, FPGS activity from variant and parental L1210 cells exhibited (4) similar requirements and preferences among various folate compounds as substrate. Preferences were in the order of $L5\text{CHO-folateH}_4 \gg$ aminopterin $> EDX >$ methotrexate.

**FPGS Activity Obtained during Mixing Experiments with Cell-free Extract from Variant and Parental L1210 Cells**—In order to rule out the possible role of endogenous stimulatory or inhibitory factors other than FPGS, itself, in determining the relative level of FPGS activity among variant and parental cell-free extracts, a series of mixing experiments were carried out. In these experiments, equal amounts of crude cell-free extract from parental and variant (L1210/EDX-4 to -7 and L1210/EDX-12 to -14 cells) were added to the reaction mixture, and FPGS activity was compared with that obtained with the same amount of each extract added alone to each reaction mixture. In the case of all of the mixtures prepared with parental and variant-derived cell-free extract, the results (data not shown) showed that the level of FPGS activity obtained with these mixtures was very similar to that activity expected from the calculated sum of the activity found in each extract when assayed alone. Thus, the difference in FPGS activity seen with variant and parental cell-free extract appeared to be related to the amount or properties of FPGS itself. In support of this conclusion, it was also found that the same relative level (variant/parental cell) of FPGS activity was obtained (data not shown) in preparations of partially purified FPGS from variant and parental cells following ammonium sulfate fractionation of cell-free extract.

**Thermotability of FPGS Activity in Cell-free Extract from Variant and Parental Cells**—A possible basis for differences seen between FPGS activity derived from variant and parental cells may relate to the stability of the enzyme in each case. Consequently, the relative stability with time of FPGS was determined by the standard assay after incubation of cell-free extract at 50°C in the reaction buffer without ATP, glutamate, MgCl$_2$, 2-mercaptoethanol, or folate. The results in Fig. 2 show that a product of the translation reaction occurred only with parental L1210 FPGS mRNA, which occurred in the form of a doublet, was constant with time for a period of at least 50 min. By comparison, product formation utilizing FPGS mRNA from L1210/EDX-4 cells also occurred as a doublet but was appreciably less after 50 min. In subsequent experiments comparing the rate of translation mediated by FPGS mRNA from variant and parental L1210 cells, a reaction interval of 50 min was selected to ensure that the comparison of translation effi-

**Decline in FPGS Activity in Cell-free Extract from Variant and Parental L1210 Cells following Cycloheximide Treatment**—Variant and parental L1210 cells were harvested from mice and resuspended at $1 \times 10^7$ cells in RPMI medium in the presence of 50 μg/ml of cycloheximide. Following various periods of incubation at 37°C, aliquots of cell suspension were removed, and the cells were processed for protein determination and FPGS activity. The results are given in Fig. 2, where the data show that loss of FPGS activity with time occurred with a half-time of 259 ± 30 min. Measurements of FPGS activity in cycloheximide-treated variant (L1210/EDX-4, -5, -7, -12, and -13) cells yielded similar values ($t_{1/2} = 236–268$ min) for half-time for the loss of activity with time.

**Relative Level of FPGS mRNA among Variant and Parental L1210 Cells**—Northern blotting of poly(A)$^+$ mRNA from variant and parental L1210 cells with a murine FPGS cDNA probe (19) revealed (Fig. 3) no differences in FPGS mRNA level among these cell types. In each case, a single 2.3-kilobase pair mRNA was detected at the same relative intensity when compared with a control blot of γ-actin mRNA. These blots were repeated with different amounts of mRNA from each cell type and different hybridization conditions with the same result (data not shown).

**Relative Rate of in Vitro Translation of FPGS mRNA from Variant and Parental Cells**—Following hybridization selection of FPGS mRNA, the relative ability of this mRNA derived from both variant and parental L1210 cells to mediate translation in an in vitro assay system was determined. In the initial experiments, the time course for accumulation of $[^{35}]$methionine-labeled product of the translation reaction was determined by SDS-PAGE for parental cell mRNA. The results given in Fig. 4 show that a product of the translation reaction occurred only when FPGS mRNA or another mRNA, in this case encoding luciferase, was added to the reaction mixture. The results in Fig. 4 also show that the product of the FPGS mRNA-mediated reaction occurred as a doublet (~61 kDa). These products could be blotted (data not shown) by anti-FPGS polyclonal antibody. Data in Fig. 5 show that the rate of product formation obtained with parental L1210 FPGS mRNA, which occurred in the form of a doublet, was constant with time for a period of at least 50 min. By comparison, product formation utilizing FPGS mRNA from L1210/EDX-4 cells also occurred as a doublet but was appreciably less after 50 min. In subsequent experiments comparing the rate of translation mediated by FPGS mRNA from variant and parental L1210 cells, a reaction interval of 50 min was selected to ensure that the comparison of translation effi-

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**Fig. 1. Immunoblotting of FPGS from variant and parental L1210 cells.** Aliquots of 40 μg of partially purified FPGS from cell-free extract were solubilized in SDS-PAGE sample buffer and electrophoresed. Following transblotting, the protein in the blot was probed with purified anti-FPGS peptide antibody. The data shown are for one representative blot of several carried out. Additional details are given under “Experimental Procedures.”
Fig. 2. Half-time for loss of FPGS activity in variant and parental L1210 cells during cycloheximide treatment. Cells were obtained as ascite suspensions in mice and incubated at 37 °C at a density of 2-3 \times 10^7 \text{cells/ml} following resuspension in RPMI medium with 50 \mu g/ml of cycloheximide. At the time periods indicated, the cell suspension was cooled to 0 °C, pelleted by centrifugation, and frozen at -85 °C. Additional experimental details are given under "Experimental Procedures." The data shown represent the average of two experiments that differed by no more than 15% at each time point.

Fig. 3. Northern blot analysis of FPGS poly(A)^+ mRNA from variant and parental L1210 cells. Preparation of poly(A)^+ mRNA from the various cells are given under "Experimental Procedures." Aliquots of 5 \mu g of mRNA from each cell type were added to the gel prior to blotting with ZAP-L1210/R83–1. Additional experimental details are as given under "Experimental Procedures." The data shown are for one representative blot of several different blots that were done.

Fig. 4. Product formation obtained during in vitro translation of hybridization-selected FPGS mRNA from parental L1210 cells. The data show the level of [35S]methionine-labeled product obtained during in vitro translation with either FPGS (200 ng) or luciferase (1 \mu g) mRNA after SDS-PAGE of 20 \mu l of reaction mixture and radioautography. The incubation time was 30 min at 30 °C. Additional details are given under "Experimental Procedures." The data shown are from one of several representative experiments.

Fig. 5. Product formation obtained during in vitro translation of hybridization-selected FPGS mRNA from variant and parental L1210 cells. The data show the level of [35S]methionine-labeled product obtained after various reaction times from 5 to 50 min at 30 °C with 200 ng of FPGS RNA in and from a 50-min reaction with L1210/EDX-4 mRNA. The data shown are from one of several representative experiments. See "Experimental Procedures" for additional details.

cell-free extract derived from these different cell types.

The lower levels of FPGS activity and protein that were characteristic of the L1210/EDX variant cell lines, in light of evidence for no alteration in cognate mRNA level, could have had as its basis more rapid turnover of this protein. The results presented here strongly suggest that this was not the case and that the lower levels of FPGS synthesis observed in cell-free extract from these variants were determined at least in part by alterations in their FPGS mRNA. These alterations could possibly affect ribosomal binding to FPGS mRNA, translation initiation or elongation, and/or the interaction of various stimulatory or inhibitory factors (36) with the mRNA. It was of interest to note that the magnitude in the difference between variant and parental cell FPGS mRNA in the ability to mediate product formation in the in vitro translation assay, while appreciable, was less than that expected in view of the differences in FPGS activity and protein among these different cell types. However, this may reflect the fact that the in vitro translation in this assay occurred out of context with respect to the normal environment of the cytosol and that differences seen within the cell were not fully expressed under these conditions. Alternatively, changes involving molecular factors (see above) in addition to the FPGS mRNA, itself, may play a role in mediating these apparent posttranscriptional modifications of FPGS gene expression in these variants. In addition, it should also be noted that we have not directly excluded the possibility of a separate mutational alteration in some of these variants that affected the catalytic turnover of FPGS. Such an effect could account in part for the lower level of FPGS activity observed in these variants given the limitation on the precision with which FPGS levels could be quantitated by Western blotting. Further
work will be required to address these issues and to distinguish between the various possibilities.

It was also of interest to observe from the data given in Figs. 5–7 that multiple peptide products were generated by \textit{in vitro} translation of FPGS mRNA from variant and parental cells. In view of their molecular mass relative to each other, these products would appear to represent (25, 37–39) mitochondrial and cytosolic forms of FPGS. If so, the data also suggest that the predominant product of the \textit{in vitro} translation reaction is the mitochondrial form rather than the cytosolic form of FPGS. This is an unexpected result, since some of our other findings (37) show that the most common splice variant (variant II) of FPGS mRNA found in L1210 cells does not encode a mitochondrial leader peptide. Consequently, it is possible that within the somewhat artificial environment of the \textit{in vitro} translation assay, their was preferential initiation of FPGS synthesis at the upstream ATG start codon, which is present only in variants I and III, which are less prevalent than variant II.

Whether this is actually the case remains to be seen and will require further work. Of greatest interest were our findings showing that formation of both products of the translation reaction were decreased in the case of the variant FPGS mRNA. This would suggest that an alteration of the mRNA modulating translation occurred either in the nucleotide sequence encoded by exon 1b (37) or further downstream.

Including those variants described here, three different classes of antifolate-resistant variants of the L1210 cell have been isolated that exhibit altered FPGS activity. A recent publication (19) from this laboratory reported on the selection of a variant resistant to methotrexate with a decrease in the rate of FPGS mRNA transcript formation. Thus, resistance to classical folate analogues in this murine tumor resulting in lower FPGS activity can occur from both transcriptional and post-transcriptional alterations of FPGS gene expression. In the same report (19), an even more novel group of variants were described that were selected for resistance to the lipophilic inhibitor of dihydrofolate reductase, metoprine. These variants exhibited elevated levels of FPGS activity as a result of the increase in rate of FPGS mRNA transcript formation. The availability of these three classes of variants with altered levels of FPGS synthesis should greatly facilitate studies of the regulation of expression of the corresponding gene at both transcriptional and posttranscriptional levels and perhaps the role of sequence-dependent topology of mRNA and other cytosolic factors in determining translation efficiency.

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