Human Cytosolic and Mitochondrial Folylpolyglutamate Synthetase Are Electrophoretically Distinct

EXPRESSION IN ANTIFOLATE-SENSITIVE AND -RESISTANT HUMAN CELL LINES*

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Folylpolyglutamate synthetase (FPGS) activity in CCRF-CEM human leukemia cells was found in the cytosolic (~67% of total) and mitochondrial (~22%) fractions. A polyclonal antipeptide antibody (430Ab) to human FPGS specifically recognized distinct immunoreactive bands (~60 kDa) present in each subcellular fraction. Human cytosolic FPGS (hcFPGS) migrated more rapidly than mitochondrial FPGS (hmFPGS); their estimated difference in molecular mass was 1 kDa. The human K562 acute nonlymphocytic leukemia and the A253 and FaDu head and neck cancer cell lines also expressed the two FPGS isoforms, and the ratio of hcFPGS to hmFPGS protein in each cell line was similar. Since K562 and A253 cells are intrinsically resistant to pulse methotrexate (MTX) exposure relative to CCRF-CEM and FaDu cells, respectively, because of decreased MTX polyglutamate synthesis (despite having similar levels of total FPGS activity expression), these data suggest that the natural difference in drug sensitivity cannot be explained by compartmentalization of FPGS activity. Higher expression of hmFPGS relative to hcFPGS was observed in some sublines of CCRF-CEM with acquired MTX resistance suggesting that differential expression of the hmFPGS isoform may contribute to MTX resistance caused by decreased FPGS activity.

It has been known for years that mitochondria have their own folate-dependent enzymes and folylpolyglutamate pool, but it has not been established how that pool is acquired or maintained (reviewed in Refs. 1–3). A central enzyme in establishing and maintaining folylpolyglutamate pools in whole cells is folylpolyglutamate synthetase (FPGS)1 (4, 5). Early studies indicated that FPGS activity was present in both cytosol and mitochondria (6, 7), suggesting that folate monoglutamates were transport forms and that the essential (8) folate polyglutamate forms were then synthesized independently in each compartment. Studies with isolated mitochondria have confirmed that folate monoglutamates are transported by a carrier-mediated facilitated-diffusion mechanism (9); if folate polyglutamates pass the mitochondrial membrane, they do so slowly, since the cytosolic and mitochondrial folate pools are not in equilibrium (2, 10).

More detailed studies later showed the presence of FPGS activity in Chinese hamster ovary (CHO) cell cytosol and mitochondria (2). The function of FPGS within the two compartments has recently been studied. Shane and co-workers (2, 11–13) studied CHO AUXB1 cells (which lack expression of both cFPGS and mFPGS) transfected with Escherichia coli or human FPGS that was expressed in either or both compartments. In cells expressing cytosolic FPGS activity, mitochondrial folates were absent, and cells were auxotrophic for glycine and methionine (12). However, cells expressing activity only in mitochondria also contained cytosolic folates, although the levels were low, and as a consequence, folate metabolism was not optimal. The results indicate that both FPGS isoforms must be expressed to establish normal folate metabolism and optimal growth. They also suggest that cytosolic folylpolyglutamates cannot enter mitochondria, but the mitochondrial folylpolyglutamate pool may exit to the cytosol (11), at least slowly and to a limited extent.

The effect of the subcellular localization of FPGS on the mechanism of action of the cancer chemotherapy drug methotrexate (MTX) was also studied (14). Similar to our studies with acquired MTX resistance through decreased MTX polyglutamate (MTXGn) accumulation (15, 16) and with the nonpolyglutamylatable analog γ-FMTX (17, 18), the authors (14) conclude that MTXGn and, thus, FPGS are not required during continuous MTX exposure. Furthermore, the extent of metabolism to MTXGn is dependent on FPGS level; thus, in pulse exposure, where MTXGn allows intracellular retention in the absence of extracellular drug, MTX sensitivity is directly related to FPGS level. In addition, the authors (14) show that MTX does not enter mitochondria and does not affect the pre-existing one-carbon pool in mitochondria, although its continued presence can limit the further accumulation of folates in mitochondria.

Our work (15, 16) shows that decreased expression of FPGS activity and protein (19) in whole cells can lead to very high levels of resistance to pulse MTX exposure, a regimen similar to that employed for clinical use of this drug. These findings and the observations that MTX is not accessible to mitochondrial FPGS suggest the hypothesis that selectively decreased expression of hcFPGS relative to hmFPGS could lead to high level resistance to pulse MTX; cytosolic folylpolyglutamate pools essential for growth would be supplied by slow leakage.
from the mitochondria (11). We have explored this hypothesis using activity assays and a recently developed 430Ab polyclonal antipeptide antibody to hFPGS (19). In the course of these studies we discovered that the two FPGS isoforms from whole cells exhibit different electrophoretic mobilities, indicating a physico-chemical difference between them that has not been reported previously.

**EXPERIMENTAL PROCEDURES**

**Materials—**Bovine heart cytochrome c (type Va), NADH, phenylmethylsulfonylfluoride, IGEPAL CA-630 ([octyl]polyethoxysa- nate and Poly Px were from Sigma). Abdomininstained essentially protease inhibitors were from Calbiochem and Roche Molecular Biochemicals, respectively. Other chemicals were reagent grade or higher.

**Cell Culture—**The CCRF-CEM human T-lymphoblastic leukemia cell line (20) and FPGS-deficient, pulse MTX-resistant sublines R2, R3, and R30dm were routinely cultured in RPMI 1640 containing 10% horse serum (16); their generation times were 20–24 h. The MTX-resistant subline R2 (defective MTX transport (21)) was cultured essentially as described (22–24). Generation times of K562, A253, and FaDu were 20–23 h. Using the GenProbe test, all cell lines were negative for *Mycoplasma* contamination.

**Isolation of Human Cytosolic and Mitochondrial Fractions—**Based on results of Lin et al. (2) showing that FPGS activity occurs only in the cytosolic and mitochondrial compartments of CHO cells, a simple separation method was employed to rapidly obtain these two subcellular fractions. Subcellular fractionation of CCRF-CEM cells was performed essentially as described (25); all operations were performed at 0–4 °C. Briefly, logarithmically growing CCRF-CEM cells (3–5 × 10^7/ ml; 4–6 × 10^9 cells total) were harvested by centrifugation (1000 g, 10 min), washed twice with iced 0.9% NaCl, and recovered by centrifugation in a 12-ml graduated conical glass tube. The pellet was loosened by tapping the tube, and five packed cell volumes of iced hypotonic buffer (26) were added. Cells were resuspended by gentle pipetting and allowed to swell for 5 min on ice, then transferred to an iced 7-ml Dounce homogenizer and disrupted with 15 strokes of the tightest-fitting pestle. Microscopic examination at this point indicated that >99% of cells were lysed. The cell lysate was transferred to an iced 12-ml conical glass centrifuge tube and centrifuged for 5 min at 100,000 × g_{max} to pellet nuclei and debris. The postnuclear supernatant (PNS) was removed, and the pellet was discarded. A sample of PNS was removed for activity assays (below), and the remaining PNS was divided and further fractionated by centrifugation for 1 h at 100,000 × g_{max} in a TL-100 tabletop Ultracentrifuge (Beckman Instrument Co.). The supernatant (cytosolic fraction) was removed for assay of FPGS activity (cFPGS; LDH, and cytochrome c oxidase (cyt c oxidase) for Western immunoblot analysis. The pellets contained intact mitochondria as well as other membrane fragments, as confirmed by fixation of one pellet with glutaraldehyde at room temperature and examination by light microscopy (data not shown). One mitochondrial pellet (0.7–0.9 × 10^9 cell equivalents) was resuspended in 1 ml of 250 mM sucrose, 1 mM NaN_3, 0.5 mM EDTA, 0.5 mM Pefabloc, pH 7.8 (2), to maintain mitochondrial integrity until marker enzyme assay. Extraction buffer (100 mM Tris-HCl, pH 8.5, 0.1 mM NaN_3, 0.5 mM EDTA, 0.5 mM Pefabloc, 50 mM 2-mercaptoethanol; 0.6 ml/10^8 cell equivalents) was added to three pellets, and FPGS activity was released into the supernatant by freezing and thawing twice in a dry ice/methanol bath followed by centrifugation at 35,000 × g for 30 min. Protein extraction buffer (50 mM Tris-HCl, pH 7.6, 120 mM NaCl, 0.5% IGEPAL CA-630, 1 mM benzami- dine-HCl, 0.5 mM Pefabloc, 0.75 ml/10^8 cell equivalents (27)) was added to one pellet to extract samples for Western analysis (19).

**Enzymes and Assays—**CCRF-CEM FPGS was partially purified (28) and assayed (29) as described; one unit of activity incorporated 1 pmol of [3H]glutamato9 into the product. Cyt c oxidase (a mitochondrial-specific marker) and LDH (a cytosolic marker) were assayed essentially as described (30). One unit of cyt c oxidase activity caused one absorbance unit decrease/min at 550 nm (30); one unit of LDH activity caused one absorbance unit/min decrease at 340 nm (30). FPGS activity in PNS, cytosol, and mitochondrial fractions was linear with respect to time and enzyme concentration under the conditions tested. LDH activity was linear with respect to time and enzyme concentration under the conditions tested. Rates for cytochrome c oxidase activity in PNS and mitochondria were nonlinear with respect to time, so the initial velocity was quantitated from the slope of the initial portion of the reaction curve and used to verify enzyme linearity. Thus cyt c oxidase activity in these fractions may be underestimates. LDH activity in mitochondria and cyt c oxidase activity in cytosol were determined in pH 7.6 fractions only, the highest practical pH. Activity was assayed under the conditions of the assay with a range of variable enzyme concentration was tested. Mixing of the cytosolic fraction with the mitochondrial fraction gave 102% and 103% of the expected LDH activity (n = 2) and 106% and 111% of the expected cyt c oxidase activity (n = 2). Mixing of cytosolic fraction with PNS gave 100% and 118% of the expected LDH activity (n = 2). Mixing of the mitochondrial fraction with the PNS gave 91% and 112% of the expected cyt c oxidase activity (n = 2). Nuclear total cFPGS activity, specific activity, and results of mixing studies were obtained in preliminary experiments where only marker activities were measured.

**Western ImmunobLOTS—**Western immunobLOTS of FPGS protein on minigel was performed as described previously (19). High resolution SDS-PAGE (17 cm wide × 16 cm long × 0.75 mm thick) was performed in 7% acrylamide, 0.19% bisacrylamide separating gels with a 2-cm 4% stacking gel in a Bio-Rad Protein Hxi apparatus. In later studies, 7% acrylamide, 0.37% bisacrylamide separating gels were used to increase resolution; under these conditions, different protein loads of the same extract migrated at slightly different rates; thus molecular weight determinations were less accurate. One lane contained Bio-Rad SDS-PAGE [7.5% acrylamide, 0.19% bisacrylamide, high range (16-17 cm)] gel transferred from the gel to a polyvinylidene difluoride membrane (Immobilon P, Millipore, Bedford, MA) in a Hoefer Transfet TE apparatus at 30 V (constant) for 15 h using the same buffer system described for minigel, except that the apparatus temperature was maintained at 4 °C by an external circulator. A second membrane was placed behind the primary membrane during transfer; this membrane was stained with 0.1% Fast Green FCF (20% methanol, 5% acetic acid) for 20 s and destained 3 times with 20% methanol, 5% acetic acid to check for protein blow-through. Western analysis (below) of an unstained secondary membrane showed that no FPGS passed through the primary membrane under the conditions of electrophoret. To detect untransferred material, the residual gel was stained with 0.1% Coomassie Brilliant Blue (40% methanol, 10% acetic acid) for 30 min and destained in 40% methanol, 10% acetic acid.

Immunoblotting was performed as described previously (19). Most studies used immunoaffinity-purified rabbit polyclonal 430Ab (19) elicited by a multiple antigen peptide to residues 275–290 of the human FPGS sequence (31); 430Ab was obtained during a pH 2.5 elution of the immunoaffinity column. Some experiments, however, used 430Ab immunoaffinity purified by an alternate means. Briefly, after pH 2.5 elution (19), the immunoaffinity column was rewarshed with 10 mM Tris-HCl, pH 8.8, until equilibrated, and then the column was eluted with 100 mM triethylamine-HCl, pH 11.5 (32). The resulting basic eluate (10 ml) was neutralized to pH 8.5 by the addition of 2 ml of 1 M Tris-HCl, pH 8.0, and concentrated by adsorption and elution from protein A-Sepharose (19). After purification and concentration, ~60% of the total activity was recovered in the low pH elution of the immunoaffinity column (19). Only ~12% of the antibody was recovered from the basic elution, but this antibody reacted only with FPGS (data not shown) and not with the nonspecific bands noted with the acid-eluted antibody (19). Since this base-eluted antibody was obtained in smaller amounts, it was used only in selected experiments despite its greater specificity.

**RESULTS AND DISCUSSION**

**Detection of hcFPGS and hmFPGS Activity and Protein—**FPGS activity has been previously localized to both the cyto- solic and mitochondrial fractions of sheep liver, rat liver, and CHO cells (2, 6, 7). As part of our effort to explore the role of FPGS in antifolate activity and resistance in human leukemia, we examined hcFPGS and hmFPGS activity in CCRF-CEM human leukemia cells. Separation by differential centrifugation of the cytosolic and mitochondrial compartments from CCRF-CEM cells was achieved by differential centrifugation (Table I). The separation of the cytosolic (LDH) and mitochondrial (cyt c oxidase) marker activities; based on these markers, fractions were also obtained in good overall yield (30). FPGS activity was detected in the cytosolic and mitochondrial fractions of the CCRF-CEM cell line (Table I). About 67% (range 63–71% in two studies) of the total FPGS activity of the PNS was found in the
Latency is 0%. Mixing experiments (“Experimental Procedures”) indicated that inhibitors or activators of each activity were not present. The entire experiment was repeated with similar results. Sp. Act., specific activity.

| Fraction | Units/10⁸ cells | Yield % | Sp. Act. | Relative Sp. Act. |
|----------|----------------|---------|----------|-------------------|
| PNS      | 101            | 100     | 11.5     | 1                 |
| Cytosol  | 111            | 110     | 27       | 2.4               |
| Mito     | 0.001          | 0.001   | 0.0002   | 0.00002           |

**TABLE I**

Mixing experiments (“Experimental Procedures”) indicated that inhibitors or activators of each activity were not present. The entire experiment was repeated with similar results. Sp. Act., specific activity.

Cytosol, and about 22% (range 19–24% in 2 studies) was found in the mitochondria. The specific activity was approximately equal in the two compartments, although it should be noted that the mitochondrial fraction was not pure (see “Experimental Procedures”). Mixing of PNS + cytosol or PNS + mitochondria gave 80% (range 79–81%; n = 2) and 89% (range 70–107%; n = 2), respectively, of the expected FPGS activity, indicating the validity of the activity measurements. Because of the low levels of FPGS activity detected in assays of mitochondria, the latency of this activity was not measured; FPGS from CHO cell mitochondria was latent, however (2).

The proportion of activity in the two compartments of CCRF-CEM cells differs slightly from those in rat liver (7), where about 75% was in the cytosol and 13% in the mitochondria, and from CHO cells (2), where about 50% was in each compartment. This may represent species or tissue differences, but given the low measured activity in the mitochondrial fraction, these differences may represent inaccuracy in the measurements.

Western immunoblot analysis on minigels showed that polyclonal 430Ab, developed to a deduced peptide of human FPGS (19), detected an immunoreactive band at the appropriate (60 kDa) molecular mass in each fraction (data not shown). Reaction with both isoforms was expected, since the two isoforms are translated from mRNA species derived from alternate transcription start sites within a single gene (33–35), and the internal peptide used to elicit the antibody is common to the isoforms (19).

**High Resolution Western Immunoelectrophoretic Detection of hcFPGS and hmFPGS**—High resolution SDS-PAGE analysis (Fig. 1) showed that the immunoreactive FPGS in CCRF-CEM PNS actually appeared as a doublet and that the separated cytosolic and mitochondrial fractions contained single immunoreactive bands that corresponded to the lower and upper bands, respectively. A mixture of cytosolic and mitochondrial fractions reproduced the doublet of the PNS. Although the individual molecular weights of the hcFPGS and hmFPGS as determined from 11 separate analyses were nearly identical (60 kDa), within each experiment, the two always differed by 1 kDa. Neither band was detected if 430Ab was preincubated with its cognate multiple antigen peptide (19) or if an irrelevant IgG replaced 430Ab (data not shown). These data support a specific antibody interaction with FPGS isoforms. Direct solubilization of intact CCRF-CEM cells and subsequent Western analysis showed that both bands were again detected (below), suggesting that the difference in mobility is not a proteolysis artifact. Partially purified (23) CCRF-CEM FPGS was also resolved into two species at high resolution (data not shown). The biochemical basis of the difference in electrophoretic mobility is currently under study.

**hcFPGS and hmFPGS in Other Human Cell Lines**—Whole cell extracts of other human cell lines were analyzed (Fig. 2) to determine whether the different mobilities of FPGS isoforms were specific to CCRF-CEM. K562 acute nonlymphocytic leukemia cells and two HNSCC cell lines, A253 and FaDu HNSCC cells. Whole cell extracts (100 µg of protein) were resolved by SDS-PAGE on a 16-cm separating gel, electrotransferred to polyvinylidene difluoride, and visualized with anti-FPGS peptide antibody 430Ab and chemiluminescent detection as described under “Experimental Procedures.” The two bands appear at ~60 kDa. Note that differences in signal intensity between cell lines do not indicate quantitative differences in protein expression because of variability in transfer efficiency and detection. Exposures shown are representative of data from at least two extracts. Panel A, expression of FPGS isoforms in CCRF-CEM and K562 leukemia cells and in A253 and FaDu HNSCC cells. Whole cell extracts (100 µg of protein) were resolved by SDS-PAGE on a 16-cm separating gel, electrotransferred to polyvinylidene difluoride, and visualized with anti-FPGS peptide antibody 430Ab and chemiluminescent detection as described under “Experimental Procedures.” The two bands appear at ~60 kDa. Note that differences in signal intensity between cell lines do not indicate quantitative differences in protein expression because of variability in transfer efficiency and detection. Exposures shown are representative of data from at least two extracts. Panel A, expression of FPGS isoforms in CCRF-CEM and K562 leukemia cells and in A253 and FaDu HNSCC cells. Panel B, expression of FPGS isoforms in A253 and FaDu HNSCC cells. Note that the apparent difference in migration in these samples is a result of a small difference in the protein loaded in each lane (see “Experimental Procedures”).

**FIG. 1.** Western immunoblot analysis of human FPGS from postnuclear supernatant, purified cytosol, and mitochondria from CCRF-CEM cells after high resolution SDS-PAGE. The PNS (75 µg), cytosolic (C; 75 µg), mitochondrial (M; 75 µg), and cytosolic + mitochondrial (75 µg each) fractions were resolved by high resolution SDS-PAGE. Analysis of these samples was repeated with similar results; the entire experiment was also repeated with similar results. The PNS actually appeared as a doublet and that the separated cytosolic and mitochondrial fractions contained single immunoreactive bands that corresponded to the lower and upper bands, respectively. A mixture of cytosolic and mitochondrial fractions reproduced the doublet of the PNS. Although the individual molecular weights of the hcFPGS and hmFPGS as determined from 11 separate analyses were nearly identical (~60 kDa), within each experiment, the two always differed by 1 kDa. Neither band was detected if 430Ab was preincubated with its cognate multiple antigen peptide (19) or if an irrelevant IgG replaced 430Ab (data not shown). These data support a specific antibody interaction with FPGS isoforms. Direct solubilization of intact CCRF-CEM cells and subsequent Western analysis showed that both bands were again detected (below), suggesting that the difference in mobility is not a proteolysis artifact. Partially purified (23) CCRF-CEM FPGS was also resolved into two species at high resolution (data not shown). The biochemical basis of the difference in electrophoretic mobility is currently under study.

**hcFPGS and hmFPGS in Other Human Cell Lines**—Whole cell extracts of other human cell lines were analyzed (Fig. 2) to determine whether the different mobilities of FPGS isoforms were specific to CCRF-CEM. K562 acute nonlymphocytic leukemia cells and two HNSCC cell lines, A253 and FaDu, displayed the same two isoforms. Thus the presence of the distinct isoforms appears to be a general feature of FPGS in human cells. Since eukaryotic FPGS display significant homology (31, 33, 35, 36), this phenomenon may be universal in eukaryotes. This could not be tested, however, because the 430Ab was elicited to a peptide that is not conserved across species.
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Western blots of whole cell extracts of cell lines, including the blots of Fig. 2, were quantitated by densitometry, and the ratios of the intensity of cFPGS and mFPGS were calculated. Values are averages ± S.D. of the number of individual exposures listed in parentheses and are from at least two extracts prepared at different times, each of which was analyzed in separate experiments.

Table II

| Cell line     | FPGS activity (pmol/hr/mg) | cFPGS/mFPGS (relative protein expression) |
|---------------|-----------------------------|---------------------------------------------|
| CCRF-CEM      | 1575 ± 150                  | 0.6 ± 0.1 (48)                              |
| K562          | 1475 ± 15                  | 0.9 ± 0.1 (35)                              |
| A253          | 2110 ± 540                  | 0.9 ± 0.2 (61)                              |
| FaDu          | 3270 ± 590                  | 0.9 ± 0.2 (37)                              |

a Data are from Ref. 39; MTX was used as folate substrate.

hcFPGS and hmFPGS in Drug Sensitivity and Resistance—

Decreased antifolate polyglutamate accumulation is one mechanism by which tumor cells can display natural resistance or acquire resistance to pulse exposure to antifolates that require polyglutamylmation for retention (such as MTX) and/or resistance to antifolates that require polyglutamylation for potent inhibition of their target enzymes (reviewed in Ref. 37). This decreased polyglutamate accumulation is often a result of decreased expression of FPGS activity. In some cases, however, the difference in total cellular FPGS activity is small relative to the decrease in polyglutamylation of the antifolate (e.g. Ref. 38). In this regard, it has been shown that MTX does not enter mitochondria (9, 14), but that mitochondrial folate polyglutamate forms may exit to the cytosol at a slow rate and allow for cell viability in the absence of cFPGS (11). This suggests that reduced hcFPGS activity with preservation of hmFPGS activity would lead to resistance of cells to pulse exposure to drugs like MTX; MTXGn would not accumulate in the cytosol, but the essential cytosolic folylpolyglutamate pools would be supplied from the mitochondria. Since most studies to date have examined only total cellular FPGS, if hcFPGS were selectively decreased, the effect on total activity would be minimized, perhaps leading to the discordance between MTXGn synthesis in intact cells and the FPGS activity measurements. Because of the compartmentalization of (anti)folate metabolism in the cell (1, 3), measurement of FPGS in both compartments is, thus, essential. Since the two isoforms can be measured in high resolution Western blots (above), this method was used to explore this hypothesis without physically separating the mitochondrial and cytosolic compartments.

Relative expression of hcFPGS and hmFPGS was determined in pairs of human cell lines that show widely different activity to pulse MTX exposure of these cell pairs cannot be explained by differential expression of one FPGS isoform and, hence, not by compartmentalized MTX metabolism.

Relative expression of FPGS isoforms was also assessed in sublines of CCRF-CEM with acquired MTX resistance (Fig. 3; Table III). The R2 subline (defective MTX uptake (21)), which shows elevated FPGS activity (~2-fold (40)), displayed a slightly greater proportion of the activity in the cytosol. R3 (moderate decrease of whole cell FPGS activity (16)) had no change in relative expression of the isoforms despite the decrease in total FPGS activity in these cells. In contrast, R30 and its clonal progeny, R30dm, both of which express very low total FPGS activity, have a greater preservation of the mitochondrial isoform. These latter data support the hypothesis that decreased relative expression of cFPGS may contribute to resistance to pulse MTX exposure. The basis for this difference in isoform expression is now under study.

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