Role of Folic Polyglutamylate Synthetase in the Regulation of Methotrexate Polyglutamate Formation in H35 Hepatoma Cells

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ABSTRACT

The effect of culture conditions on the glutamylation of methotrexate by intact H35 hepatoma cells and folic polyglutamate synthetase (FPGS) activity in the corresponding crude extracts has been examined. The rate of cellular glutamylation of methotrexate observed in quickly dividing confluent cultures of wild-type and folate-depleted H35 cells. However, near-quantitative reductions in cellular glutamylation were caused by media additions of reduced folates and methotrexate to confluent cultures of wild-type and folate-depleted H35 cells. However, these produced relatively modest reductions in FPGS activity in the corresponding crude extracts (approximately 50%). Methionine exclusion resulted in a greater than 50% decrease in FPGS activity in crude extracts of these cells compared to extracts of control cultures. The combination of methionine exclusion and folinic acid addition lowered the FPGS activity to less than 25% of that of control. The data suggest that the changes in the glutamylation rate of methotrexate in whole cells due to culture conditions such as folate restriction, reduced folate addition, methionine exclusion, and growth state are at least in part a consequence of alterations in FPGS activity. This conclusion is consistent with the proposition that the metabolism of slow-acting substrates for FPGS (such as 4-amino antifolates and their corresponding polyglutamates) may be sensitive to changes in enzyme levels or activity (Cook et al., Biochemistry, 26: 530-539, 1987). Analysis of the products formed by FPGS from extracts using methotrexate as the substrate revealed no significant amounts of polyglutamate species higher than 4-NH₂-10-CH₂-PteGlu. In contrast, when using the thymidylate synthase inhibitor N⁴-propargyl-5,8-dideazafolic acid as the starting substrate under identical assay conditions, FPGS from extracts catalyzed the formation of predominantly long chain polyglutamate derivatives (Glu, higher). These results reflect the relative inefficiency of methotrexate and N⁴-propargyl-5,8-dideazafolic acid, as well as their polyglutamate derivatives, as substrates for FPGS.

INTRODUCTION

FPGS (EC 6.3.2.17) catalyzes the conversion of folic and folate analogues into γ-polyglutamate derivatives. The polyglutamylation of folates (1-5) serves several physiological functions. Polyglutamates appear to be the preferred substrates for many of the folate-requiring enzymes of one-carbon metabolism (1, 6), and have been shown to be more efficiently "channeled" than monoglutamates from one active site to another in folate-requiring multienzyme complexes (7). Polyglutamate derivatives of folates and methotrexate are more avidly retained in cells than their monoglutamate counterparts, and thus the FPGS-catalyzed synthesis of methotrexate polyglutamates is a major determinant in the cytotoxicity of this antifolate (1, 8-14). Methotrexate polyglutamates have been shown to be at least as effective as methotrexate against the target enzyme, dihydrofolate reductase (8, 9, 11, 12, 15, 16), and therefore their prolonged cellular retention affords them greater cytotoxic potential than the parent drug.

Mammalian FPGS has been partially purified from rat (17), mouse (18), and beef liver (19), and has only recently been purified to homogeneity from hog liver (20). While studies utilizing these preparations have been extremely useful in elucidating the chemical and physical properties of the enzyme, a limited amount of data are available as to its mode of activity in cells and its role in regulating the balance of cellular polyglutamates of methotrexate. Furthermore, relatively few investigations on FPGS have used tumor cells as an enzyme source. Finally, there appear to be marked differences in the ability of FPGS to glutamylate methotrexate in whole cells versus experiments with isolated enzymes, especially with regard to the distribution of polyglutamate products (13, 14, 21-25).

Previous studies in our laboratory (26, 27) have shown that the ability of H35 hepatoma cells to glutamylate methotrexate can be altered by changes in growth state and culture conditions. In an effort to determine if these alterations are a consequence of changes in FPGS activity, as well as to gain additional insight into the process of glutamylation in intact cells, we have quantitated the FPGS activity in extracts of H35 hepatoma cells grown under those conditions which cause the greatest differences in cellular glutamylation of methotrexate. We have also attempted to understand what factors account for the differences in polyglutamate product distribution with isolated enzyme compared with that seen in cultured tumor cells by evaluating the glutamylation of methotrexate and the thymidine synthase inhibitor PDDF (28-30). It is envisioned that a better understanding of the role of FPGS in the glutamylation of these antifolates will contribute to an enhanced understanding of their activities as chemotherapeutic agents.

MATERIALS AND METHODS

Materials. Swims' medium S-77, folic acid-free Swims' medium, fetal calf serum (dialyzed and undialyzed), and horse serum (dialyzed and undialyzed) were purchased from Grand Island Biological Company. 1-[2,3-3H]Glutamic acid (MET-395, 25 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Cellulose power (CF 11) and DEAE-cellulose (DE 52) were purchased from Whatman. Methotrexate, [3',5',7'-3H]methotrexate, and 4-NH₂-10-CH₂-Pte-G[3H]Glul were purchased from Moravek Biochemicals (La Brea, CA). Methotrexate polyglutamate standards were the kind gift of the National Cancer Institute. PDDF, PDDF polyglutamates, and [14C]PDDF labeled with [14C]glutamate were synthesized by the method of Nair et al. (31). Thymidine, hypoxanthine, and ATP were purchased from Sigma. Folic acid was supplied by ICN Pharmaceuticals, and 5-methyltetrahydrofolic acid was prepared as previously described (32). Methotrexate and all folates were purified by DEAE-cellulose chromatography prior to use (32). All other chemicals were reagent grade.

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2 To whom requests for reprints should be addressed.

The abbreviations and trivial names used are: FPGS, folylpolyglutamate synthase; 4-NH₂-10-CH₂-PteGlu, methotrexate (MTX) or 4-amino-10-methyltetrahydrofolic acid; PDDF, N⁴-propargyl-5,8-dideazafolic acid; N⁴-[2-amino-4-hydroxy-6-quinazolinyl]methyl]prop-2-ynyl amino]benzoyl-1-glutamic acid, also known as CB3717.
Cell Culture. H-11-E-C3 cells derived from the Reuber H35 rat hepatoma (referred to as H35 cells) were grown on 60- or 100-mm Falcon dishes in a 5% CO2 atmosphere and were subcultured weekly. The culture medium was Swims’ medium S-77 supplemented with 5% fetal calf serum, 20% horse serum, and 4 mM glutamine, which was routinely changed at 72 and 120 h after plating. The cells were released from the dishes with 0.05% trypsin, counted with a Model ZBI Coulter counter and diluted to a seeding density of 5 x 10^4 cells/ml. Folate-depleted H35 cells were generated as before (26) and cultured in a similar fashion, but in folate-free Swims’ medium S-77 supplemented with 50 μM thymidine, 50 μM hypoxanthine, 5% dialyzed fetal serum, 20% dialyzed horse serum, and 4 mM glutamine.

Preparation of Crude Extracts. Wild-type or folate-deplete H35 cells were seeded in 100-mm Falcon dishes at a density of 6 x 10^6 cells/dish, and unless otherwise noted, were grown for 96 h prior to changing to the desired culture medium for experiments, which was accomplished by washing each 100-mm plate with 5 ml of the desired medium lacking serum and replenishing with 5 ml of the same medium. After the indicated time, the cultures were cooled on ice, the medium was removed, and each dish was washed with 3-4 ml ice-cold phosphate buffered saline (pH 7.6). The cells were scraped into ice-cold 0.5 M Tris-Cl (pH 8.85), 0.2 M 2-mercaptoethanol (approximately 1 ml buffer/15 mg total cell protein) and lysed by freeze-thawing (33). The debris was removed by centrifugation (4°C) for 1 h at 27,000 x g for 30 min. Following removal of an aliquot for protein determination by the Lowry method (33), the supernatant was stored at -70°C.

The FPGS activity of the crude extracts stored in this manner did not change for 6 weeks. The activity of a particular extract was typically determined on the day following preparation.

Enzyme Assays. Folylpolyglutamate synthetase was assayed according to the method of McGuire et al. (17). Methotrexate was used routinely at saturation (250 μM) as the substrate for FPGS activity determinations (24). The stock solution of folate (50 mM) in dry-ice/ethanol. The debris was removed by centrifugation (4°C) at 27,000 x g for 30 min. Following removal of an aliquot for protein determination by the Lowry method (33), the supernatant was stored at -70°C. The FPGS activity of the crude extracts stored in this manner did not change for 6 weeks. The activity of a particular extract was typically determined on the day following preparation.

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Ammonium Sulfate Fractionation. Solid ammonium sulfate was added to the crude supernatant over 20 min at 0°C with stirring until 35% saturation was reached. The solution was stirred at the same temperature for 1 h and centrifuged (4°C) for 1 h at 27,000 x g. The supernatant was discarded, and the pellet was suspended in a minimum volume of 20 mM potassium phosphate buffer (pH 7.5), 50 mM 2-mercaptoethanol, 20% glycerol. Following protein determination (33) the sample was divided into 1.5-ml portions and stored at -70°C. FPGS activity was determined on the day following preparation.

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Texture of PDDF. Solid ammonium sulfate was added to the crude supernatant over 20 min at 0°C with stirring until 35% saturation was reached. The solution was stirred at the same temperature for 1 h and centrifuged (4°C) for 1 h at 27,000 x g. The supernatant was discarded, and the pellet was suspended in a minimum volume of 20 mM potassium phosphate buffer (pH 7.5), 50 mM 2-mercaptoethanol, 20% glycerol. Following protein determination (33) the sample was divided into 1.5-ml portions and stored at -70°C. FPGS activity was determined on the day following preparation.

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The linearity of the assay suggested that γ-glutamyl hydrolase (conjugase) was not interfering with the measurement of methotrexate polyglutamate formation in assays of crude extracts. To determine the relative activity of γ-glutamyl hydrolase under the conditions of the FPGS assay, the hydrolysis of 200 pmol of 4-NH2-10-CH3-Pte-[G-3H]Glu2 (a typical amount formed in an assay) was measured by incubation with 400 μg of extract for 1 h or 200 μg of extract for 2 h at pH 8.4. High-performance liquid chromatography analysis (34) showed that in each case less than 0.3% of the substrate had been converted to the monoglutamate. Thus γ-glutamyl hydrolase, which has a relatively low activity in these cells (36) did not interfere with the measurement of FPGS under these conditions.

Methotrexate was compared to tetrahydrouracil as a substrate for FPGS from crude extracts under the standard assay conditions of the FPGS assay (10 μM/ml) * 4 mM glutamine for 24 h (total culture time, 120 h).

The medium was changed to folate-free Swims’ medium S-77 with insulin (10 μM/ml) + 4 mM glutamine for 24 h (total culture time, 120 h).

### Table 1 Product formation from [3H]glutamate and methotrexate with FPGS from extracts of wild-type H35 hepatoma cells with respect to time and protein concentration

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>Incubation time (min)</th>
<th>Protein added (μg)</th>
<th>[3H]Glutamate incorporated (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confluent</td>
<td>60</td>
<td>400</td>
<td>260</td>
</tr>
<tr>
<td>Confluent</td>
<td>120</td>
<td>200</td>
<td>251</td>
</tr>
<tr>
<td>- Serum</td>
<td>60</td>
<td>400</td>
<td>285</td>
</tr>
<tr>
<td>- Serum</td>
<td>120</td>
<td>200</td>
<td>320</td>
</tr>
<tr>
<td>Log phase</td>
<td>60</td>
<td>400</td>
<td>520</td>
</tr>
<tr>
<td>Log phase</td>
<td>120</td>
<td>200</td>
<td>621</td>
</tr>
</tbody>
</table>

The results are the average of identical duplicate experiments. Confluent and log phase cells were harvested after 96 and 48 h in culture medium, respectively. Extracts of cells lacking serum in culture were harvested after exposure of cells grown as described in "Materials and Methods" to Swims’ S-77 medium + insulin (10 μM/ml) + 4 mM glutamine for 24 h (total culture time, 120 h).
REGULATION BY POLYGLUTAMATE SYNTHETASE

conditions. At saturating concentrations (250 and 35 μM for methotrexate and tetrahydrofolate, respectively) methotrexate typically incorporated approximately 1.8-fold as much glutamate as did tetrahydrofolate (data not shown).

Alterations in Cellular MTX Glutamylation and Extract FPGS Activity as a Function of Growth State and Culture Condition. A comparison of the rate of glutamylation of methotrexate by intact H35 hepatoma cells and FPGS activity in crude extracts of the same cultures is shown in Table 2. The greater rate of cellular glutamylation of methotrexate observed in rapidly dividing cultures (>4-fold higher than confluent cultures) was accompanied by an increase in extract FPGS activity (2.2-fold). The depletion of cellular folates produced comparable increases in both cellular methotrexate glutamylation and extract FPGS activity (approximately 1.8-fold). The addition of exogenous reduced folates or methotrexate to confluent cultures of wild-type and folate-depleted H35 cells, which in some cases caused near-quantitative reductions in cellular methotrexate glutamylation, produced relatively modest reductions in FPGS activity in the corresponding crude extracts (approximately 50%). To determine if the reduction in FPGS activity in the cytosol of the folate-treated cells represented substrate competition between the added folate in the cell extracts and the substrate, methotrexate, an extract of folate-deplete cells which had been exposed to 20 μM folinic acid for 24 h was treated with 2.5% dextran-treated charcoal and reassayed for FPGS activity. No significant change compared to a charcoal-untreated extract was observed, indicating that an increase in cellular folates following exposure to folinic acid was not altering the apparent FPGS activity in extracts.

The relationship between extract FPGS activity and growth state of wild-type H35 cells was further investigated by measuring the enzyme activity in extracts of cells harvested at various points in the normal growth cycle. The results of this study are depicted graphically in Fig. 1. Extract FPGS activity was shown to gradually increase for the first 48 h and then peak sometime between 48 and 54 h before returning to the initial level in extracts of confluent cells (500–600 pmol/h/mg).

Alterations in FPGS Activity in Extracts of Folate-Deplete H35 Hepatoma Cells Deprived of Methionine. Earlier studies from this laboratory have shown that the exclusion of methionine from the culture media caused nearly a 70% reduction in the glutamylation of methotrexate by intact folate-deplete H35 hepatoma cells (14). As can be seen in Table 3, methionine exclusion resulted in a greater than 50% decrease in FPGS activity in crude extracts of these cells compared to extracts of control cultures. The combination of methionine exclusion and folinic acid addition lowered the FPGS activity to less than 25% that of control. The combination of methionine exclusion and 5-methyltetrahydrofolate addition reduced the activity to approximately half that seen with extracts of control cultures.

Analysis of Products Formed from Methotrexate and PDDF in Intact H35 Hepatoma Cells and by FPGS from Crude Extracts or Resuspended 0–35% Ammonium Sulfate Pellets. Previous investigations with liver FPGS indicated a limited ability of the enzyme to form longer chain length methotrexate polyglutamates (Glu, and higher) (21, 22, 24, 25), as was the case with cultured hepatocytes during short term incubations (14). Since the rat hepatoma cells readily formed longer chain derivatives under similar conditions (13, 26), an evaluation of the products formed with hepatoma cell extracts was conducted to determine if the enzyme from this source could more readily produce 4-NH2-10-CH3-Pte-Glu and Glu. Methotrexate at an extracel-

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Table 2. Alterations in the glutamylation of methotrexate by intact H35 hepatoma cells and FPGS from crude extracts by growth state and culture conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>MTX Glutamylation (nmol/4 h/mg)</th>
<th>Extract FPGS Activity (pmol/h/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cell</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type H35 cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Log phase</td>
<td>211</td>
<td>1426</td>
</tr>
<tr>
<td>Control (confluent)</td>
<td>47</td>
<td>638</td>
</tr>
<tr>
<td>+ Folinic acid</td>
<td>0.2</td>
<td>346</td>
</tr>
<tr>
<td>+ Methotrexate</td>
<td>12</td>
<td>362</td>
</tr>
<tr>
<td>Folate-deplete H35 cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (confluent)</td>
<td>83</td>
<td>1198</td>
</tr>
<tr>
<td>+ Folinic acid</td>
<td>7</td>
<td>775</td>
</tr>
<tr>
<td>+ 5-Methyltetrahydrofolate</td>
<td>2</td>
<td>616</td>
</tr>
<tr>
<td>+ Methotrexate</td>
<td>15</td>
<td>675</td>
</tr>
</tbody>
</table>

* Data from Ref. 26.

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Table 3. Effect of methionine exclusion and folate addition on FPGS activity in extracts of folate-deplete H35 hepatoma cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>FPGS Activity (pmol/h/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1103 ± 93</td>
</tr>
<tr>
<td>Methionine</td>
<td>476 ± 96</td>
</tr>
<tr>
<td>Methionine + folinic acid</td>
<td>245 ± 60</td>
</tr>
<tr>
<td>Methionine + 5-methyltetrahydrofolate</td>
<td>515*</td>
</tr>
</tbody>
</table>

* Two determinations.

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Fig. 1. The relationship between extract FPGS activity (H) and growth state (O) of wild-type H35 cells. The cells were seeded at a density of 5 x 10⁵ cells/ml (2 x 10⁵ cells/60-mm dish), cultured for the indicated time in Swims' S-77 medium, 5% fetal calf serum, 20% horse serum, and 4 mM glutamine. Cell numbers were determined by visual counting using a Zeiss model E invertoscope equipped with a reticle. Preparation of extracts of these cultures and measurement of FPGS activity were accomplished as described in "Materials and Methods." The results are the average of three experiments except for the 24-h assay for FPGS which is the mean of two observations.
lular concentration of 10 \( \mu M \) was extensively glutamylated by intact wild-type H35 hepatoma cells in 6 h (Table 4), with the predominating products (73%) containing four or more glutamate residues. In contrast, FPGS from crude extracts synthesized polyglutamates from methotrexate only up to the triglutamate time (Table 5). Partial purification of the extracts by ammonium sulfate fractionation did not result in a preparation which could catalyze the formation of significant amounts of the tetraglutamate (Table 6). This partially purified extract was capable of converting 4-NH\(_2\)-10-CH\(_3\)-PteGlu\(_3\) to 4-NH\(_2\)-10-CH\(_3\)-Pte-Glu\(_4\), and the amount formed was highly dependent on the amount of substrate (Table 6). The same reaction occurred in crude extracts, but at a 50–70% reduction amount (data not shown).

The monoglutamate of the thymidylate synthase inhibitor PDDF (10 \( \mu M \)) was also glutamylated by intact H35 hepatoma cells (Table 4), with the glutamylated products consisting nearly exclusively of the tetra- or higher polyglutamate derivatives. A radiolabeled peak near the expected retention time for the pentaglutamate derivative of PDDF was detected during the high-performance liquid chromatography product analysis, which increased when the incubation time was extended from 6 to 24 h. Lack of a pentaglutamate standard prevented positive identification. In contrast to methotrexate, PDDF was readily able to form substantial amounts of the tetra- and higher polyglutamate derivatives when using crude extracts of folate-deplete H35 hepatoma cells as an enzyme source (Table 5). Using a resuspended 0–35% ammonium sulfate pellet from crude extracts as a source for FPGS (Table 6), the product distribution formed after 6 h from PDDF at initial concentrations lower than 1 \( \mu M \) was virtually identical to that seen in the experiments with intact cells (i.e., near-exclusive formation of the tetra- and higher polyglutamate derivatives).

### DISCUSSION

Several detailed investigations using FPGS from normal mammalian tissues have appeared in the recent literature (17–20), but relatively few studies have used rapidly dividing or transformed cells as an enzyme source. Taylor and Hanna showed that the FPGS activity of suspension-cultured Chinese hamster ovary cells was not altered by the growth state of the cells or the addition of several metabolites (glycine, adenosine, thymidine, folates, and methionine) to the media (37), suggesting that changes in growth state and culture conditions did not result in altered activities or amounts of the enzyme. However, the finding that the ability of H35 hepatoma cells to glutamylate methotrexate could be markedly altered by changes in growth state, cellular folate levels, and the exclusion of methionine from the media (14, 26) suggested the possibility that changes in FPGS activity in cells could be involved in regulating the glutamylation of methotrexate. This possibility has been tested in the present study by measuring FPGS activity in extracts of H35 hepatoma cells grown under those culture conditions which caused the greatest alterations in whole cell glutamylation.

Using crude extracts of H35 cells as a source for FPGS, linear incorporation of \(^{1}H\)glutamate into 250 \( \mu M \) methotrexate took place for up to 1 h with 400 \( \mu g \) of added protein, and up to 2 h with 200 \( \mu g \) of added protein (Table 1). These data, along with the finding that \( \gamma \)-glutamyl hydrolase (optimum pH 7.2 (34)) was virtually inactive under these conditions (pH 8.4), indicated that a reliable comparison of FPGS activity could be made by assaying the extracts in duplicate for 1 h at 400 \( \mu g \) of added protein and for 2 h at 200 \( \mu g \) of added protein. At saturating substrate concentrations, the 1.8-fold higher glutamate incorporation for methotrexate versus tetrahydrofolate compared favorably with the results of similar experiments by McGuire et al. using partially purified rat liver FPGS, which showed an approximate 1.6-fold greater glutamate incorporation with methotrexate (21).

The results of these experiments suggest that the alterations in the cellular glutamylation of methotrexate are in part a consequence of changes in the activity of FPGS (Table 2). The reduction of cellular folates (i.e., comparing control cultures of wild-type versus folate-deplete H35 cells), caused comparable increases in both whole cell glutamylation and extract FPGS activity (approximately 1.8-fold), indicating that alterations in the enzyme itself may be largely responsible for this effect. However, media additions of folinic acid and 5-methyltetrahydrofolate to wild-type and folate-deplete cultures for 24 h, which caused a near-quantitative reduction in cellular methotrexate glutamylation [Table 2, (26)], resulted in only a 50% decrease in extract FPGS activity, demonstrating that these changes in enzyme activity are probably not large enough to totally account for alterations in glutamylation in intact cells. Thus, other factors which could alter cellular glutamylation of the drug, such as the competition between reduced folates and metho-
trexate for transport (38) and FPGS (21, 22, 39), can also import an additional effect.

The increased FPGS activity in extracts of H35 cells harvested in log phase (Table 2 and Fig. 1) indicates that changes in enzyme activity are also involved in the increased ability of dividing cells to glutamylate methotrexate. These data are the first of their kind and are in contrast to the results of similar experiments by Taylor and Hanna (37), who reported that FPGS activity of suspension-cultured Chinese hamster ovary cells was not altered by growth state. A report of increased FPGS activity in dividing LS178Y lymphoma cells has recently appeared in abbreviated form (40).

Methionine has been shown to alter both folypolyglutamate product distribution in Chinese hamster ovary cells (41) and the rate of polyglutamylation in hepatic cells. In the latter case, glutamylation was reduced by the presence of methionine in normal hepatocytes and increased by the presence of methionine in folate-restricted hepatoma cells (14). FPGS activity in extracts of H35 cells deprived of methionine was decreased 57% compared to extracts of control cultures (Table 3), while the corresponding decrease in whole cell glutamylation was 70% (14). The combination of folinic acid addition and methionine exclusion caused the largest reduction in extract FPGS activity measured during this study (>75%). The mechanism by which methionine alters FPGS activity is not understood, but the effect is particularly interesting because of its magnitude and divergent nature in hepatocytes and hepatoma cells. Experiments in which FPGS activity was measured in extracts of hepatocytes indicated that the reduction in glutamylation is not associated with changes in enzyme activity.

One possible additional mechanism for alterations in glutamylation is an alteration in the steady state intracellular levels of methotrexate as a result of different conditions. Several positive effectors of the rate of MTX glutamylation which include insulin, methionine, and folate-lacking medium (26) caused no discernible alteration in the steady state levels of methotrexate during the period of polyglutamate formation in intact cells. Similarly the other substrates for FPGS (ATP and glutamate) are not altered in amount by the changes in growth conditions. However dividing hepatoma cells accumulated more intracellular methotrexate than confluent cultures when incubated in the presence of a concentration of the drug (10 μM) that is saturating for transport and glutamylation. Under these conditions confluent cells had 8.8 ± 3.7 nmol/g and dividing cells had 14.2 ± 6.7 nmol/g methotrexate (N = 6). Thus, this 1.6-fold increase in cellular MTX may also contribute to the enhanced rate of glutamylation observed in dividing cells.

Shane and his coworkers have conducted a series of elegant studies in which purified FPGS from hog liver was used to model folate and folate analog metabolism, and concluded that changes in cellular folypolyglutamate synthetic rates and distributions can be explained by substrate specificities and affinities for the enzyme (25). Furthermore, Shane has pointed out that an exception to autoregulatory control by substrate specificity would exist in the case of substrates such as methotrexate (and its polyglutamates), whose metabolism is relatively slow. Under these conditions, modest changes in the level or activity of FPGS would play an important role in the rate of conversion to polyglutamates (25). Thus, the present finding that extracts of H35 hepatoma cells with differential capacities for glutamylation have correspondingly altered FPGS activities suggests that the cellular levels or activity of this enzyme may play an important role in the regulation of methotrexate metabolism in transformed mammalian tissue. The activity of γ-glutamyl hydrolase, the enzyme which catalyzes the hydrolysis of folyl and antifolyl polyglutamates, does not appear to be a major factor in altering glutamylation in intact H35 cells as a result of growth state, folate, and methionine content, as its activity in extracts of cells grown under these conditions is not significantly changed.

Long chain methotrexate polyglutamates (those with four or more glutamate residues) can be formed in hepatoma cells as early as 2 h after exposure to methotrexate and can be the major species within 4 h (13, 26), while virtually no methotrexate species above the level of the triglutamate are formed in extended (>36 h), enzyme-supplemented incubations with partially purified FPGS from rat and mouse liver (22, 24). The latter is consistent with the reduced capacity of cultured hepatocytes to form longer chain length polyglutamates during short-term incubations (<6 h). In this investigation, FPGS from crude extracts synthesized polyglutamates from methotrexate only up to the triglutamate level (Table 5), in contrast to observations with intact cells (13, 26). The absence of higher polyglutamate products was not a consequence of γ-glutamyl hydrolase activity, since ammonium sulfate fractionation [which greatly reduced the hydrolase activity in the extracts (23)] failed to yield a preparation which could form significant amounts of 4-NH2-10-CH3-Pte-Glu4 from methotrexate (Table 6). Additionally, the ammonium sulfate fraction was able to form 4-NH2-10-CH3-Pte-Glu4 from 4-NH2-10-CH3-Pte-Glu3, indicating that this conversion could take place provided the triglutamate was present at effective concentrations. Thus in spite of the fact that the hepatoma cells have a greater capacity to form long chain polyglutamates of methotrexate relative to hepatocytes, the FPGS extracted from both sources has a limited capacity to convert the drug to its high polyglutamates.

In contrast to the result with methotrexate, both intact hepatoma cells and FPGS from extracts metabolized the monoglutamate of the thymidylate synthase inhibitor PDDF to long chain polyglutamates (Tables 4 and 5). Furthermore, a similar product distribution for PDDF as was seen in whole cell experiments was noted when using FPGS from crude extracts or resuspended 0–35% ammonium sulfate pellets (Table 6). These results suggest that the inability of methotrexate to form long chain polyglutamates outside of the cellular environment is due to the triglutamate being formed in quantities insufficient to overcome its poor kinetic properties as a substrate for further glutamylation. Shane et al. have shown that the diglutamate was the highest chain length formed for several 4-amino antifolates tested with the purified hog liver enzyme (25). Decreased substrate activity of methotrexate polyglutamates as the glutamate chain length is increased has been reported by Schoo et al., who showed that 4-NH2-10-CH3-Pte-Glu3, had about 3–5% of the substrate activity of methotrexate for the beef liver enzyme (22), and by Clarke and Waxman, who found that the relative Vmax/Km ratios of 4-NH2-10-CH3-Pte-Glu4, for the human liver enzyme were each less than 20% that of methotrexate (42). It does not appear that the catalytic ability of FPGS is severely compromised outside of the cell, since the use of other substrates such as reduced folates (4, 17, 19, 23, 25) and PDDF (Tables 5 and 6) can result in the formation of long chain polyglutamates. Other possible factors contributing to the limited glutamylation of methotrexate in experiments with isolated enzyme may be the inability to achieve the effective cellular concentration of FPGS, and the absence of cellular proteins which may alter glutamylation.

* T. B. Johnson and J. Galivan, unpublished results.
The results of this study show that the activity of FPGS may be an important parameter in the regulation of methotrexate polyglutamate formation in H35 hepatoma cells, and that the decreased ability to form long chain methotrexate polyglutamates in assays with isolated enzyme may be a consequence of the decreased substrate activity of the polyglutamates of this antifolate, rather than solely an impairment of the enzyme catalytic ability. The interaction of methotrexate and PDDF with intact cells and FPGS may take on added importance since it has recently been shown that this combination and others like it can exhibit synergistic cytotoxicity against hepatoma cells in vitro (43).

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REFERENCES

Role of Folylpolyglutamate Synthetase in the Regulation of Methotrexate Polyglutamate Formation in H35 Hepatoma Cells

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