0026-895X/95/030505-07$3.00/0

MOLECULAR PHARMACOLOGY, 48:505–511 (1995).

Effects of γ-Glutamyl Hydrolase on Folyl and Antifolylpolyglutamates in Cultured H35 Hepatoma Cells

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Received March 15, 1995; Accepted June 12, 1995

SUMMARY

A subline of H35 hepatoma cells (H35D cells) that have been made resistant to 5,10-dideazatetrahydrofolate exhibits an increase in γ-glutamyl hydrolase (GH) activity. GH is a lysosomal enzyme in H35 and H35D cells on the basis of comparison of the distribution of enzyme activity with other known lysosomal enzymes. The hydrolysis rate of methotrexate polyglutamate with isolated, intact lysosomes is 4–5-fold greater in H35D cells than in H35 cells. GH activity in isolated lysosomes is in part dependent on the presence of a reducing agent such as mercaptoethanol. Permeabilization of lysosomal preparations from both cell types by Triton X-100 causes a 10-fold enhancement in GH activity. The result of the enhanced activity of GH in H35D cells is a marked reduction in antifolylpolyglutamate concentration, with the parent antifolate being the predominant intracellular species found under all conditions tested. Unlike antifolates, the total intracellular folate concentration is nearly identical in both cells under standard culture conditions up to 10 μM folic acid. However, the chain length of folylpolyglutamates consists of predominantly triglutamates and tetraglutamates in H35D cells with increased GH, whereas it consists of pentaglutamates and hexaglutamates in the parental cells. At 50 and 100 μM folic acid, the folate accumulation in H35D cells is less than half that of H35 cells, and the predominant polyglutamate species in the H35D cells are the diglutamates through the tetraglutamates. The results demonstrate that the two H35 cell lines having equal folylpolyglutamate synthetase but that one with enhanced lysosomal GH activity exhibits a marked reduction in the amount and γ-glutamyl chain length of folylpolyglutamates and antifolylpolyglutamates.

Folates and classic antifolates are converted into their polyglutamyl forms by the cytosolic enzyme FPGS (EC 6.3.2.17). This enzymatic reaction has been characterized with partially purified enzyme preparations (1–4) demonstrating that folylpolyglutamates are synthesized by the sequential γ addition of glutamate to the terminal glutamyl moiety of folates and antifolates (1, 3–6). Conversion of pteroylmonoglutamate to polyglutamates allows cells to concentrate folates from the extracellular space (7). Moreover, folylpolyglutamates are in general the favored coenzyme forms of the vitamin and display increased affinity or lowered K_m values for most of the enzymes of one carbon metabolism (3, 7). For classic antifolates, the polyglutamates appear to be pharmacologically more effective than the parent compound because of their greater retention and often by their more potent inhibition of folate-dependent enzyme reactions (8–10). Recently, human FPGS was cloned by functional complementation of an *Escherichia coli* folC mutant and also was expressed in a mammalian cell lacking FPGS activity (11).

The process of hydrolysis of folylpolyglutamates and antifolylpolyglutamates is not as well understood. Polyglutamate cleavage is catalyzed by GH (EC 3.4.22.12). The cellular form of the enzyme is in general lysosomal, exhibits an acidic pH optimum, and can require the presence of reduced sulfhydryls for full expression of activity (3, 12–14). Although GH has been partially purified and studied from a number of sources (13–19), its role in the dynamic process of polyglutamate turnover in the cell is not fully established. Recently, Sirotnak *et al.* (20–22) described the facilitative transport and hydrolysis of antifolylpolyglutamate in the lysosomes from S180 cells. The results of those studies establish the lysosomal localization of antifolylpolyglutamate degradation by GH and suggest that intralysosomal turnover of antifolylpolyglutamate is limiting at the level of lysosomal transport and by the intralysosomal availability of fully active GH. Neither a mutant lacking hydrolase activity nor a high affinity, specific inhibitor for the enzyme has been available, which has im-

This investigation was supported in part by Grant CA25933 from the National Cancer Institute.

ABBREVIATIONS: FPGS, folylpolyglutamate synthetase; GH, γ-glutamyl hydrolase; $PteGlu_n$, folylpolyglutamate (*n* indicates the total number of glutamate residues); 10-HCO-H_4PteGlu, 10-formyltetrahydrofolate; DDATHF, 5,10-dideazatetrahydrofolylpolyglutamate; MTX, methotrexate; 4-NH_2-10-$CH_3$$PteGlu_n$, 4-amino-10-methylpteroylglutamate$_n$ (*n* equals the total number of glutamate residues); PDDF, *N*-10-propargyl-5,8-dideazafolate; $PABAGlu_n$, *p*-aminobenzoyl-Glu_n (*n* equals the number of glutamate residues); FBS, fetal bovine serum; MOPS, 3-[*N*-morpholino]-propane sulfonic acid.

peded approaches to understanding the role of enzyme in the intact cell. In addition to its intracellular function, the recently described secretion of GH by numerous cell lines suggests a more complex picture of this enzyme (23). We previously reported a subline of H35 cells resistant to DDATHF that exhibits elevated GH activity and no change in FPGS (24). Using the parental line and this subline, we conducted the present study to understand the role of GH and its characteristics in the parental and resistant cells.

Experimental Procedures

Materials. All culture media and sera were obtained from GIBCO (Grand Island, NY). DDATHF was kindly provided by Dr. S. S. Kerwar (Lederle Laboratories, Pearl River, NY). [3′,5′,7-^{3}H]4-NH_2-10-CH_3PteGlu, [3′,5′,7-^{3}H]4-NH_2-10-CH_3PteGlu$_2$, and [3′,5′,7,9-^{3}H]folic acid were purchased from Moravek Biochemicals, Inc. (Brea, CA) and purified by DEAE column before use (25). [^{14}C]PDDF was kindly provided by Dr. M. G. Nair (University of South Alabama, Mobile, AL). PteGlu$_{2-5}$ and 4-NH_2-10-CH_3PteGlu$_{2-5}$ were purchased from Dr. B. Schirck (Jona, Switzerland). All other chemicals, including Percoll for gradient centrifugation, were purchased from Sigma Chemical Co. (St. Louis, MO). Millipore membrane filters were purchased from Millipore Corp. (Bedford, MA).

Cell culture. H35 and H35D rat hepatoma cells have been maintained in monolayer culture in Swim's medium containing 20% horse serum and 5% FBS as described previously (24, 25). H35D cells have been maintained in the presence of 21 μM DDATHF and subcultured in the absence of drug for 1 week before experimental use.

Lysosomal preparation. Lysosomes from cells grown for 72 hr were isolated according to the method of Barrueco *et al.* (20, 22). Briefly, cells were washed twice in sucrose/EDTA buffer (250 mM sucrose and 1 mM EDTA, pH 7.6), and scraped in 1 mL of this buffer for each 100-mm plate. The cells were homogenized gently by passing the suspension three times through a 200-μl plastic pipette tip fitted to a glass pipette. Centrifugation to generate a crude granular fraction was conducted as described (20, 22). The granular fraction was resuspended in 1 mL of sucrose/EDTA buffer and fractionated in 6 mL of 28% Percoll (250 mM sucrose, 1 mM EDTA, 20 mM MOPS/Tris, pH 7.2) by centrifugation at 37,000 × *g* for 60 min. The top half of the gradient was aspirated and used in some experiments or discarded. The bottom half of the gradient (containing lysosomes) was centrifuged further as described, and the final lysosomal pellet was resuspended in sucrose/MOPS buffer and immediately used for experiments (20, 22).

Extraction and assay of lysosomal marker enzymes. Activity of the lysosomal marker enzyme β-hexosaminidase was measured according to the method of Barrueco *et al.* (20). Latent β-hexosaminidase activity, a measure of lysosomal integrity, was determined for each preparation as the difference in activity measured in the presence and absence of 0.1% Triton X-100. The data are expressed as a function of the latent lysosomal β-hexosaminidase activity that is not less than 70% of the total. One unit of β-hexosaminidase was defined as the amount of enzymatic activity necessary to generate 1 nmol of *p*-nitrophenol/min at 37° in the above assay. The activity of β-hexosaminidase in the lysosomal extracts from wild-type cells was 65 nmol/min/mg lysosomal protein and 79 nmol/min/mg in the resistant cells. The other marker enzymes, β-glucuronidase and acid phosphatase, were assayed with *p*-nitrophenyl β-D-glucuronide and *p*-nitrophenyl phosphate as substrates, respectively (26).

GH assay. GH activity of cell extracts and lysosomal fraction was measured as described previously (23, 27) in the presence or absence of 0.1% Triton X-100. 4-NH_2-10-CH_3PteGlu$_2$ and 4-NH_2-10-CH_3PteGlu$_5$ were used as substrates at the various concentrations in the presence of 100 mM mercaptoethanol in 50 mM sodium acetate buffer, pH 6.

Metabolism of 4-NH_2-10-CH_3PteGlu, 4-NH_2-10-CH_3PteGlu$_2$, and PDDF. Cells were grown as discussed for 72 hr and then placed in folate-free Swim's medium supplemented with insulin (10 mU/mL) for 24 hr and incubated with 10 μM [^{3}H]4-NH_2-10-CH_3PteGlu, [^{3}H]4-NH_2-10-CH_3PteGlu$_2$, or [^{14}C]PDDF. The metabolites were analyzed with high performance liquid chromatography with acetonitrile gradient as previously described (24) and expressed as picomoles/milligram of protein as described by Lowry *et al.* (28).

Cellular folylpolyglutamate chain length. Cells were grown in Swim's medium containing 20% horse serum and 5% FBS with 4 μM or 100 μM [^{3}H]folic acid for 1 week, subcultured in the same medium for 120 hr, and assayed by the method of Shane (29, 30).

Results

Intracellular accumulation of antifolylpolyglutamates. H35 and H35D cells were incubated with 10 μM 4-NH_2-10-CH_3PteGlu, 4-NH_2-10-CH_3PteGlu$_2$, or PDDF, and the cellular metabolites were analyzed with high performance liquid chromatography. The results indicated that the total glutamylation in H35D cells was reduced by 96%, 96%, and 65% for 4-NH_2-10-CH_3PteGlu, 4-NH_2-10-CH_3PteGlu$_2$, and PDDF, respectively (Table 1). Because the activity of FPGS is equal in both cell lines, the reduction in polyglutamates is attributed to enhanced GH (24). As indicated in the parentheses, the intracellular antifolates that have not been glutamylated in H35 cells make up only a small component (<20%) of the total. However, in the H35D cells, the nonglutamylated material is by far the predominant species for all three antifolates. In all cases, the polyglutamates represented only a minor proportion of the parent antifolate.

The cellular polyglutamate profile was measured in cultured cells after incubation with 4-NH_2-10-CH_3PteGlu$_2$. The diglutamate is a useful probe in this system because, unlike 4-NH_2-10-CH_3PteGlu and PDDF, it can be acted on by both FPGS and GH once inside the cell. Longer chain length derivatives cannot be readily used for this purpose because they enter the cell too slowly (31–33). Thus, distribution of the products of 4-NH_2-10-CH_3PteGlu$_2$ metabolism are expected to be the result of the balance of activities of FPGS and GH. The results clearly show that the partitioning to the monoglutamate is favored in the GH-enhanced cell line (Fig. 1). In both cell types, large amounts of the diglutamate are seen in the cell, unlike an analogous experiment in which 4-NH_2-10CH_3PteGlu is the substrate (8, 24, 25, 31). In the

TABLE 1

Intracellular accumulation of antifolylpolyglutamates by H35 and H35D cells

Cells were grown for 96 hr and then incubated with 10 μM antifolate for the next 24 hr. Each metabolite was analyzed with high performance liquid chromatography as described in Experimental Procedures. The results are the average of duplicates of one experiment.

Substrate	H35 cells	H35D cells
	Polyglutamates (pmol/mg)	
MTX[a]	83.9 (13)[b]	3.1 (93)
4-NH_2-10-CH_3PteGlu$_2$[c]	75.9 (14)	3.3 (94)
PDDF[a]	19.0 (5.4)	6.7 (78)

[a] Glu_2 derivative and higher.
[b] Numbers in parentheses are the percentages of the total mono- and polyglutamate pool that are nonglutamylated cellular antifolate. With MTX and PDDF, this is only the monoglutamate, whereas with 4-NH_2-10-CH_3PteGlu$_2$, it is the monoglutamate and diglutamate.
[c] Glu_3 derivative and higher.

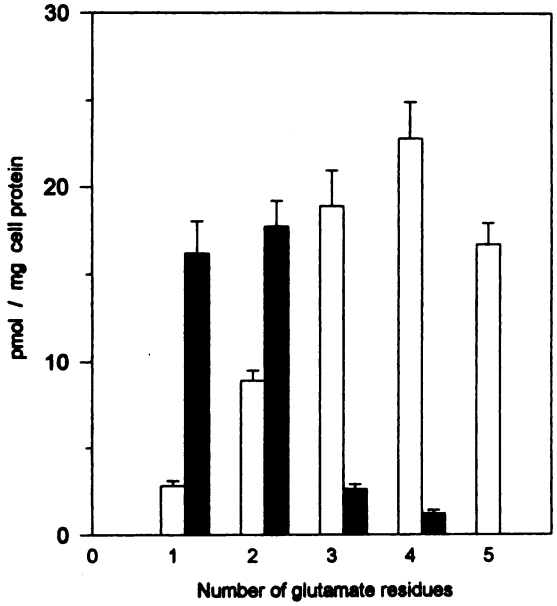

Fig. 1. Metabolism of 4-$NH_2$10-CH_3PteGlu_2 by H35 and H35D cells. Cells were grown for 72 hr as described in Experimental Procedures and then in folate-free Swim's medium for the next 24 hr. At 96 hr, cells received fresh medium and incubated with 10 μM [^{3}H]4-$NH_2$10-CH_3PteGlu_2 for 6 hr. The metabolites in cell extracts of H35 (□) and H35D (■) were analyzed with high performance liquid chromatography, and the abscissa indicates the total number of the glutamate residues in cellular 4-NH_2-10-CH_3PteGlu_n. *Error bars*, mean ± standard deviation (n = 3) from one experiment.

H35 cells, the longer chain length derivatives clearly predominate. In contrast, relatively rapid hydrolysis of 4-NH_2-10-CH_3PteGlu_2 is apparent in the H35D cells as the monoglutamate is a predominant product with little polyglutamate accumulation. At the end of the 6-hr incubation (Fig. 1), 20–26% of the radiolabel in the medium is converted to 4-NH_2-10-CH_3PteGlu. We cannot know whether this is due to extracellular cleavage or cell entry, cleavage, and efflux. However, experiments in which cultures are incubated with 4-NH_2-10-CH_3PteGlu_2 containing 25% 4NH_2-10-CH_3PteGlu throughout the incubation with [^{3}H] in either or both species reveal that the contribution from the monoglutamate is 1) equilibrated throughout the polyglutamate pool and 2) makes up no more than 10% of any of the polyglutamate species. In addition, a shorter incubation (4 hr) results in only 15% conversion of diglutamate to monoglutamate in the medium and results in a profile identical to that in Fig. 1 but with lower amounts of 4-NH_2-10-CH_3PteGlu_{3-5}. Thus, the profile in Fig. 1 shows primarily the contribution from the diglutamate, with minor amounts coming from the monoglutamate that arises in the medium or the cell.

Hydrolysis of polyglutamates in subcellular fractions. An earlier study established that H35D cell extracts have enhanced levels of GH (24). The study shown in Fig. 1 demonstrates that the enhanced GH activity is reflected in the greater rate of cleavage of 4-NH_2-10-CH_3PteGlu_2 by intact H35D cells than wild-type cells. The results in Table 2 extend that observation to subcellular fractions of the two different cell lines. Because GH is a lysosomal enzyme in many cell and tissue systems (3, 20–22), it became important to determine the intracellular location of the enzyme activity in the H35 parent and resistant cell lines. H35D cell extracts

TABLE 2

GH activity in cell extracts, intact lysosomes, and lysosomal extracts of H35 and H35D cells

GH activity was measured using 100 μM 4-NH_2-10-CH_3PteGlu_2 as substrate as described in Experimental Procedures.

	H35 cells	H35D cells
	GH activity (nmol/hr/mg)	
Cell extracts[a]	1.0 ± 0.23[b]	6.8 ± 0.7
Cell extracts with Triton X-100[c]	5.8 ± 0.7	26.2 ± 2.0
Intact lysosomes[d]	1.2 ± 0.2	5.3 ± 0.9
Disrupted lysosomes[d]	14.7 ± 0.8	75.1 ± 2.5
Lysosome-depleted cell extracts[e]	0.5 ± 0.13	2.1 ± 0.2

[a] Prepared by treating cells in 0.2 M Tris/acetate (pH 6) with a Dounce homogenizer.
[b] Mean ± standard deviation (3 experiments).
[c] Prepared by treating the cells in 0.2 M Tris/acetate (pH 6) containing 0.1% Triton X-100, which resulted in disruption of the lysosomes.
[d] Prepared as described in Experimental Procedures.
[e] The lysosomes are removed from cell extracts in sucrose/MOPS buffer (pH 7.2) by centrifugation at 37,000 × g for 60 min.

made in the absence of Triton X-100 showed a 7-fold higher hydrolase activity than wild-type cells. Inclusion of Triton X-100 in extract preparation, which causes fragmentation of the lysosomes (21), caused the activity in extracts of both cell types to be increased by severalfold. The difference in activity between H35 and H35D cells is maintained when the hydrolysis of 4-NH_2-10-CH_3PteGlu_2 is measured with the use of isolated intact lysosomes. Isolation of the lysosomes followed by Triton X-100 treatment increases the specific activity of both cell types due to elimination of the lysosomal membrane, which impedes substrate GH access (21). The postlysosomal fraction also showed a 4-fold enrichment in GH activity in H35D cells compared with H35 cells. However, the specific activity of the postlysosomal fraction was only 3% of that of the disrupted lysosomes, documenting the enrichment of activity in the lysosomes.

The present study was extended with the use of the substrate 4-NH_2-10-CH_3PteGlu_5 because the longer chain derivatives are frequently predominant in H35 cells treated with MTX (8, 23–25). Approximately 3-fold greater activity was observed with intact and disrupted lysosomes from H35D cells (Fig. 2A). Treatment of the lysosomes with Triton X-100 caused a 10-fold increase in the rate of cleavage of 4-NH_2-10-CH_3PteGlu_5 (Fig. 2B). The differences in the rate of substrate cleavage by lysosomes from H35 and H35D cells appear to be less in Fig. 2 than in Table 2. This is because the results in Table 2 are based on protein, whereas the results in Fig. 2 are based on units of hexosaminidase, which is 25% higher in H35D cells than in H35 cells (Table 3). When measured with the same units, the relative rates of hydrolysis of 4-NH_2-10-CH_3PteGlu_2 and Glu_5 at 50 μM by lysosomes are approximately equal.[1] This result is the same with lysosomes from H35 and H35D cells, although as noted the rate of hydrolysis of both substrates by H35D cell lysosomes is severalfold more rapid than that by H35 cell lysosomes. Furthermore, detailed kinetic analyses are under way to compare the interaction of different polyglutamate substrates with lysosomes isolated from both cell types.

Subcellular distribution of lysosomal marker enzymes and GH. To determine whether H35 cell GH enrichment in lysosomes was consistent with other lysosomal enzymes, it was compared with a battery of "marker" enzymes in H35 and H35D cells (Table 3). In all cases, 3–6% of the enzymes were found in the top, nonlysosomal proportion of the Percoll gradient. Thus,

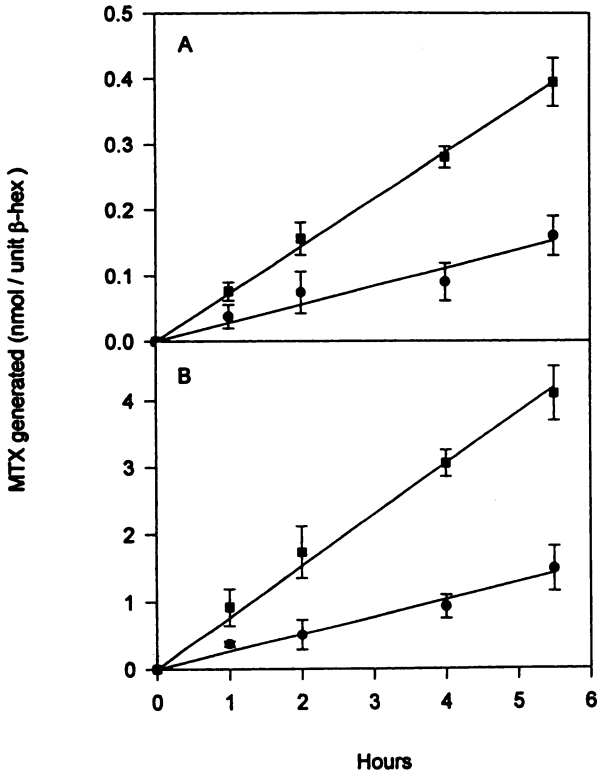

Fig. 2. The hydrolysis of 4-NH_2-10-$CH_3PteGlu_5$ by intact and permeabilized lysosomes of H35 and H35D cells. Lysosomes prepared as described in Experimental Procedures from H35 (●) or H35D (■) cells were incubated at 37° in buffer containing 50 μM 4-NH_2-10-$CH_3PteGlu_5$ in the absence (A) or presence (B) of 0.1% Triton X-100 for the indicated times. The amount of 4-NH_2-10-CH_3PteGlu formed was analyzed with high performance liquid chromatography and expressed as nanomoles/unit of β-hexosaminidase. *Error bars*, mean ± standard deviation (3 independent experiments).

TABLE 3

Fractional location of lysosomal enzymes compared with GH

Cells were cultured and extracted for lysosomal preparation as described in Experimental Procedures. The isolated top and bottom (lysosomal) fractions were treated with 0.1% Triton X-100 and assayed for the individual enzyme as described.

		H35	H35D
		nmol/min/mg	
β-Hexosaminidase	Top fraction	2.8 ± 0.24	3.0 ± 0.23
	Bottom fraction	64.2 ± 9.48	78.9 ± 12.6
β-Glucuronidase	Top	1.2 ± 0.10	1.9 ± 0.21
	Bottom	24.0 ± 3.12	47.2 ± 7.17
Acid phosphatase	Top	4.1 ± 0.38	14.9 ± 1.43
	Bottom	136.8 ± 22.6	249.6 ± 36.2
GH	Top	0.009 ± 0.002	0.035 ± 0.003
	Bottom	0.245 ± 0.013	1.25 ± 0.042

by these criteria, GH is a lysosomal enzyme in wild-type and H35D cells. Several other results are apparent from this comparison. Enhancement of the other lysosomal enzymes in H35D cells varies between a modest increase of 25% (β-hexosaminidase) to an approximate doubling (β-glucuronidase and acid phosphatase) compared with 5-fold for GH. The activity of the marker enzymes is much greater than that of GH. However, the total production of GH activity by the cells is not unlike that of the other enzymes. The main difference is the cellular trafficking of the enzymes because <10% of the markers are secreted (23), whereas 99% of GH is secreted. Thus, although GH is primarily a secreted enzyme in H35 cells, its major intracellular location is lysosomal. The amount of GH activity secreted by H35D cells exceeds that of H35 cells by approximately 3-fold.[1]

Effect of mercaptoethanol on GH activity in intact lysosomes. The presence of exogenous sulfhydryls has been shown to be critical for the activity of the secreted enzyme from H35 cells (23). In addition, intact lysosomes from S180 cells exhibit a near-absolute requirement for added sulfhydryls to hydrolyze 4-NH_2-10-$CH_3PteGlu_2$ (21). Assessment of the sulfhydryl requirement of intact lysosomes from H35 cells for GH activity demonstrated a partial response (Fig. 3). In both H35 and H35D cells, the rate of cleavage was increased by 1.7-fold by the presence of mercaptoethanol. Thus, the isolated lysosomes from this source had the ability to maintain relatively high levels of GH activity compared with S180 cells in the absence of added sulfhydryl. The relatively high apparent reducing activity in the lysosomes may be a result of the H35 cells being of hepatic origin.

Folate accumulation in H35 and H35D cells. The effect of elevated GH activity on cellular antifolylpolyglutamates is evident from these and previous results (24). However, those results may not be predictive for the status of cellular folylpolyglutamates. To assess the effects of elevated GH activity on cellular folylpolyglutamates, both H35 and H35D cultures were depleted of folates and then incubated with [^{3}H]folate for 12 days to reach steady state. The amount of total [^{3}H]folate was unaffected by elevated GH in the H35D cells at the normal culture concentration of folate (4 μM) (Fig. 4). When the medium concentration was elevated to 10 μM, both cell lines had experimentally identical amounts of intracellular folates, but at 50 and 100 μM, the intracellular folate in the culture medium with enhanced GH retained only 40% that found in the parent cells. Thus, at excessive levels of folate, the total accumulation of folylpolyglutamates appears to be limited by the additional GH activity.

Distribution of glutamate chain length of folate in H35 and H35D cells. The altered activity of GH in H35D cells was not reflected in total folate accumulation in medium containing the normal amounts of folate. However, an effect on the glutamate chain length remained possible. The pattern of folylpolyglutamates in H35 and H35D cells is shown in Fig. 5. At 4 μM folic acid, the distribution of polyglutamate derivatives was altered in H35D cells in which triglutamates and tetraglutamates were predominant, whereas parental H35 cells showed pentaglutamates and hexaglutamates as the major species. At this folate concentration, the relative amounts of 5,10-$CH_2H_4PteGlu_n$, $H_4PteGlu_n$, and 10-$HCOH_4PteGlu_n$, the major cellular species, were identical in H35 and H35D cells when measured by the linked thymidylate synthase [^{3}H]fluorodeoxyuridylate assay (34).[1] At 100 μM folate, a similar but more exaggerated effect was observed. In the control cells, 100 μM extracellular folate resulted in a downward shift in chain length compared with the same cells with 4 μM folate. This is consistent with earlier results showing an inverse relationship between extracellular MTX concentration and intracellular polyglutamate chain length (36), suggesting similar control mechanisms for polyglutamate formation and degradation of folates and antifolates. With 100 μM folate, folylpolyglutamate chain length

[1] J. Galivan and M. S. Rhee, unpublished observation.

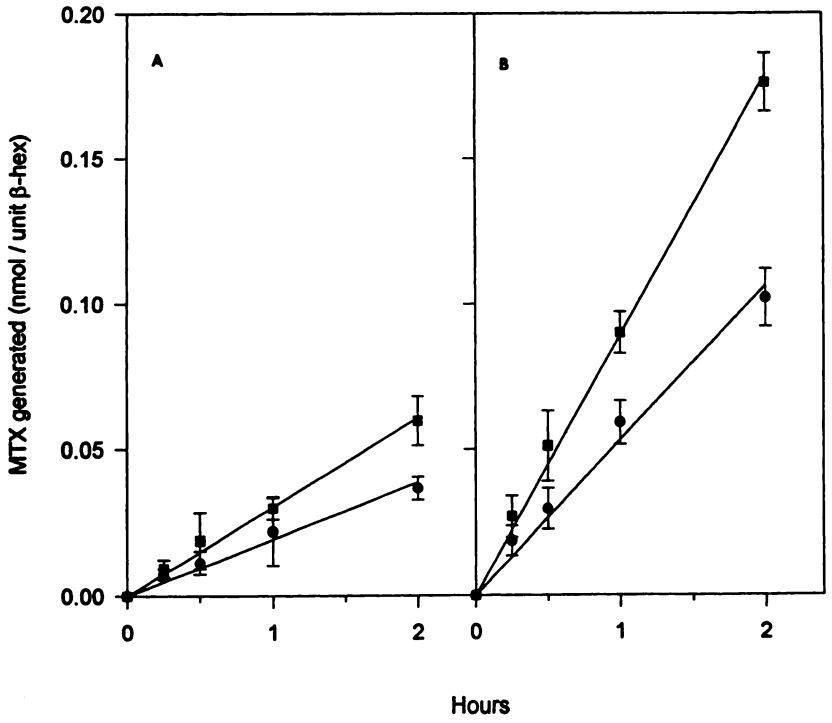

Fig. 3. The effect of exogenous thiols on GH in isolated lysosomes. Lysosomes from H35 (A) and H35D (B) cells were isolated as described in Experimental Procedures and incubated with 50 μM 4-NH_2-10-CH_3PteGlu_2 in the absence (●) or presence (■) of 50 mM mercaptoethanol. GH activity is expressed as in Fig. 2. *Error bars*, mean ± standard deviation (3 independent experiments).

in H35D cells is reduced to diglutamate through tetraglutamate species. Regardless of the specific molecule used to test glutamylation, in all cases there is not only a reduction in polyglutamates but also an increase in the monoglutamate and diglutamate in the H35D cells relative to wild-type.

Discussion

In a previous study, we demonstrated that a subline of H35 hepatoma cells resistant to DDATHF has elevated GH activity. There was a ~7-fold elevation in GH activity in cell extracts that was accompanied by a reduced ability to glutamylate antifolates and reduced drug sensitivity. The results are consistent with other studies on acquired DDATHF resistance, which is usually accompanied by an impaired capacity to glutamylate this folate analogue (37). Earlier results were related to altered transport or defective FPGS (38), although a more recent study has shown that GH is also elevated in human CCRF-CEM cells (37). The H35D cell line is the only one in which a major cause of reduced glutamylation is an increase in GH activity without a change in FPGS, offering the unique opportunity to evaluate the role of changes in GH activity in altering cellular antifolylpolyglutamates and folylpolyglutmates.

The effect of enhanced GH activity on the glutamylation of three antifolates is profound. For MTX and PDDF, the cell line with enhanced GH had a reduction in polyglutamates of 96% and 65%, respectively. A useful substrate with which to illustrate the effect of enhanced GH is 4-NH_2-10-CH_3PteGlu_2, which can be acted on by both FPGS and GH once inside the cell. Clearly, the latter enzyme predominates in H35D cells because the monoglutamates and diglutamates are nearly exclusively present.

Intracellular GH has been established as a lysosomal enzyme in other cell systems, but it has also been reported to have other subcellular locations (3). It is clear that the enzyme from H35 and H35D cells has a distribution that is consistent with other "marker" enzymes used to demonstrate a lysosomal location (Tables 2 and 3, Figs. 2 and 3). The main difference between GH and the marker enzymes used in the present study (Table 3) is that most of the GH activity

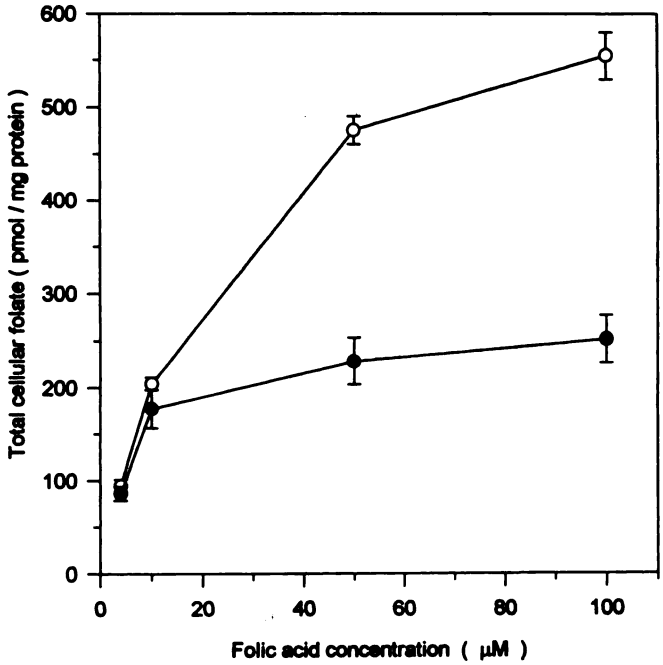

Fig. 4. Effect of folate concentration on total folate accumulation by H35 and H35D cells. Cells were grown in folate-free Swim's medium containing 10% FBS for 1 week to deplete the cells of folate and then subcultured in the same medium supplemented with the various concentrations of [^{3}H]folic acid for 120 hr. Cells were washed three times with PBS and extracted with 1 N NaOH to determine dpm and cell protein. Total cellular folate in H35 (○) or H35D (●) cell extracts was expressed as picomoles per milligram of cell protein. *Error bars*, mean ± standard deviation (4 experiments).

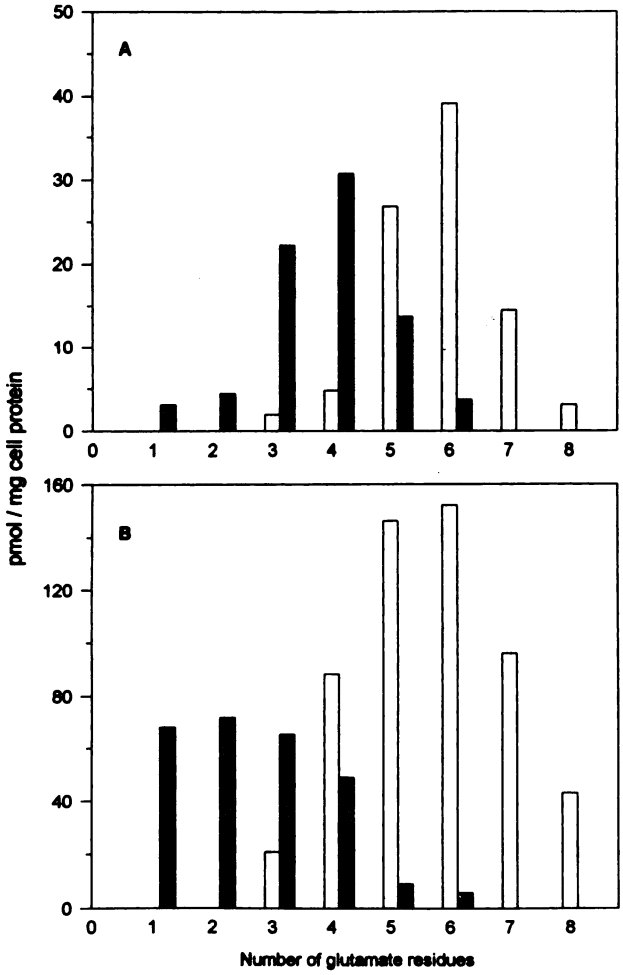

Fig. 5. Distribution of glutamate chain length of cellular folate in H35 and H35D cells. Cells were grown as described in Experimental Procedures and extracted with 0.1 N HCl. Glutamate chain length was analyzed with high performance liquid chromatography as described in Experimental Procedures. Symbols are for H35 (□) cells and H35D cells (▨). *Bars*, A, Mean of three independent observations (4 μM folic acid, average standard deviation of 14%); B, average of two independent observations (100 μM folic acid).

(>98%) is secreted from the cell (23, 35). Because these are all glycoproteins, it is likely that the high level of secretion found with GH is dictated by a specific carbohydrate structure that serves to target these molecules to their ultimate destination (39). It is not unknown for lysosomal enzymes to also be targeted for secretion because the cathepsins are also found in both of these locations (40).

The intact lysosomes have the capacity to cleave 4-NH_2-10-$CH_3$$PteGlu_5$ and do so by hydrolyzing the innermost γ linkage. Samples of enzyme from all subcellular fractions of H35 and H35D cells in Table 2 and the secreted enzyme (35) demonstrate an identical mechanism.[1] Thus, there appears to be no reason to assume that the enzyme found at any of the cellular locations has unique catalytic properties. On the basis of the distribution of enzymes shown in Table 3, it is possible that the activity found in the cytosol is present as a contaminant from the isolation procedure used for lysosomes. Therefore, no evidence for a unique cytosolic form of the enzyme exists in the H35 cell system.

The results indicate that the main difference between the H35 and H35D cells in polyglutamate metabolism is greater ability of the lysosomes from the latter cells to hydrolyze polyglutamates and that this is related to greater amounts of enzyme activity. Initial rates of uptake of 4-NH_2-10-$CH_3$$PteGlu_n$ (5–100 μM) by lysosomes failed to reveal significant differences between H35 and H35D cells.[1] Unfortunately, at the present we cannot tell whether the enhanced GH activity is due to enzyme amplification or to a more active form of GH in the H35D cells. Studies are under way to answer that important and intriguing question.

The response of the cellular folylpolyglutamates to enhanced activity of GH is less extensive than that of the antifolates. At 4 and 10 μM folic acid, the total cellular folate concentration is the same in both cell types, but the glutamyl chain length profile is reduced by approximately two glutamate residues. In comparison, the total antifolate concentration at similar extracellular concentrations is reduced by 70–90% with a corresponding result in chain length (Table 1, Fig. 1). More notably, the monoglutamate is the principal intracellular species except when the diglutamate is the molecule being tested. The reason for this is likely to be complex and is certainly unanswered. Several parameters may be involved. The predominant folate in H35 cells is 10-HCO-$H_4$$PteGlu_n$ (34), and it is known to be a relatively good substrate for FPGS (41), whereas MTX and PDDF are relatively poor substrates (42, 43). Thus, the higher activity of GH in the H35 cells on poor FPGS substrates can result in most of the cellular material being driven to the monoglutamate. The reduced folates have the advantage of being better FPGS substrates in many cases and can be extensively protein bound within the cell (44). We have already established that protein-bound polyglutamates are remarkably insensitive to GH from H35 cells (27).

It is possible that different substrate affinities of GH for folates and antifolates could contribute to these effects. We have little information on this point, but the brief analysis comparing 4-NH_2-10-$CH_3$$PteGlu_5$ and $PteGlu_5$ showed no obvious differences between them (35). However, more data are needed on this point. It is also possible that GH from H35 and H35D cells differ in their properties. Initial studies have been conducted showing that its chromatographic behavior is similar to that of wild-type cells.[1] It also has the same mechanism of cleavage, showing endopeptidase activity, and the same affinity for the substrate 4-NH_2-10-$CH_3$$PteGlu_2$.[1] Further definitive studies on these points will be conducted after purification of the required amounts of the enzyme.

The role of GH in effecting polyglutamate formation and turnover is just being understood. Shane *et al.* have shown that a 10-fold increase in FPGS activity can result in a ~10-fold increase in MTX polyglutamates (45). The data from the present study show a qualitatively similar, inverse response of antifolylpolyglutamates to changes in GH activity and, to a lesser extent, of folylpolyglutamates.

Acknowledgments

We thank Dr. M. G. Nair of the University of South Alabama for kindly providing [^{14}C]PDDF.

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