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Characterization of Human Cellular γ-Glutamyl Hydrolase

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ABSTRACT

A previously identified cDNA encoding a human γ-glutamyl hydrolase was expressed in a baculovirus system. The expressed protein had molecular mass of 37 kDa. Treatment of the protein with PNGase F produced a protein of molecular mass of 30 kDa, indicating that the protein contained asparagine-linked glycosylation. Sequence analysis of the expressed protein indicated that a 24-amino-acid signal peptide had been removed. A polyclonal antibody to the expressed enzyme was used in Western blot analysis of partially purified lysates of HL-60 promyeloid leukemia cells and MCF-7 breast cancer cells. The HL-60 and MCF-7 enzymes appeared as two closely spaced bands with a molecular mass of 37 kDa. Treatment of the HL-60 enzyme with PNGase F produced a protein with a molecular mass of 30 kDa. The activities of the expressed enzyme and the enzyme from HL-60 cells were similar on methotrexate polyglutamates. Methotrexate-γ-Glu is a poor substrate for the human enzyme relative to methotrexate γ-$Glu_{2–5}$. During hydrolysis of methotrexate-γ-Glu_4, all possible pterin-containing cleavage products (methotrexate and methotrexate-γ-$Glu_{1–3}$) appear. The results demonstrated that the human enzyme cleaves both the ultimate and penultimate γ-linkages of methotrexate polyglutamates. Glutamate was released as either glutamic acid or γ-Glu_2. Longer chain species of γ-$Glu_{n>2}$ were not observed. Inhibition by iodoacetic acid suggested that both the expressed enzyme and the HL-60 enzyme may contain a catalytically essential cysteine. These results indicate that the identified cDNA encodes the intracellular γ-glutamyl hydrolase found in a variety of human tumor cells and that the baculovirus-expressed enzyme is a suitable model for further structural and enzymatic studies.

GH is a key enzyme in the metabolism of folic acid and the pharmacology of antifolates such as MTX. Folylmonoglutamates or antifolylmonoglutamates enter cells through specific transport systems. Inside the cell, they are converted to folylpoly-γ-glutamates or antifolylpoly-γ-glutamates by the enzyme FPGS. Glutamate is added sequentially, yielding chain lengths of five to eight poly-γ-glutamates as the predominant intracellular forms. The folylpolyglutamates are retained inside the cell and are better substrates than the monoglutamate for most folate-dependent enzymes. GH catalyzes the removal of γ-linked polyglutamates from these intracellular folylpolyglutamates to yield folylmonoglutamate coenzymes. This allows the folylmonoglutamate to be released from the cell. A similar mechanism is involved in the uptake and metabolism of antifolates such as MTX (Shane, 1995; Priest and Bunni, 1995). MTX polyglutamates are retained inside the cell, while MTX is released. In rat H35 hepatoma cells in culture, increased levels of GH activity have been associated with resistance to antifolates such as MTX and 10-propargyl-5,8-dideazafolate and with a reduction in intracellular polyglutamates of 10-propargyl-5,8-dideazafolate (Rhee *et al*., 1993). Similarly, CCRF-CEM human leukemia cell lines resistant to 5,10-dideaza-5,6,7,8-tetrahydrofolic acid were found to have lower levels of polyglutamylation due to a decrease in FPGS activity, with the most resistant cell lines also having an increased GH level (Pizzorno *et al*., 1995). Decreased MTX polyglutamate formation in the blasts of patients with leukemia has been associated with resistance to MTX treatment, and the GH/FPGS ratio was a predictor of MTX polyglutamylation (Longo *et al*., 1997). The balance between the intracellular activities of FPGS and GH therefore is important both in the cellular maintenance and metabolism of folic acid and in the antitumor efficacy of antifolates. Despite the importance of GH in the pharmacodymanics of antifolates and as a potential therapeutic target, no structural studies have been reported of the intracellular enzyme or detailed kinetic analyses of GH-catalyzed polyglutamate hydrolysis using purified enzyme.

In this laboratory, a cDNA for an hGH (folylpolyglutamate hydrolase) was identified from the expressed sequence tag database and expressed in *Escherichia coli* (Yao *et al*., 1996). The identified clones were from human placenta and brain. The gene for hGH has been located on chromosome 8, region q12.23–13.1 (Yao *et al*., 1997). The full-length protein en-

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ABBREVIATIONS: GH, γ-glutamyl hydrolase; MTX, methotrexate; hGH, human γ-glutamyl hydrolase; FPGS, folylpolyglutamate synthetase; HPLC, high performance liquid chromatography; OPA, *o*-phthalaldehyde; OBG, octyl β-D-glucoside; $PteGlu_n$, folylpolyglutamate; PNGase F, peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

coded by the cDNA has a molecular mass of 36.7 kDa, contains four potential asparagine glycosylation sites, and was predicted to have a 24-amino-acid signal peptide (Yao *et al*., 1996). Isolation and structural characterization of intracellular hGH from cells or tissue have not been carried out. In the current study, the identified cDNA for hGH was expressed as an active enzyme in a baculovirus system, and the expressed protein was used to raise a polyclonal antibody. This antibody was used to characterize the intracellular hGH from HL-60 promyeloid leukemia cells and MCF-7 breast cancer cells. The antibody also was used to purify the enzyme from HL 60 cells by immmunoaffinity chromatography. The catalytic properties of hGH from the enzyme preparations was studied using MTX polyglutamates containing two to six γ-glutamates.

Experimental Procedures

Materials. 4-NH_2-10-CH_3-$PteGlu_{1-6}$ were purchased from Dr. B. Schricks Laboratories (Jona, Switzerland). Mercaptoethanol, Triton X-100, OPA, and sodium acetate were purchased from Sigma Chemical (St. Louis, MO). A protease inhibitor cocktail (Complete) was purchased from Boehringer-Mannheim (Indianapolis, IN). PNGase F was a gift from Dr. Thomas H. Plummer Jr. (Wadsworth Center, Division of Molecular Medicine, New York State Department of Health, Albany, NY). Octyl β-glucoside was purchased from Pierce (Rockford, IL). Iodoacetic acid was purchased from MCB (Cincinnati, OH).

Construction of the GH transfer vector. A human EST clone (Genbank H09442) previously shown to encode hGH (Yao *et al*., 1996) was obtained from Genome Systems (St. Louis, MO). A 1.1-kb DNA fragment including the entire open reading frame and some 3′-UTR was amplified from H09442 by polymerase chain reaction and cloned into pCRII (InVitrogen, San Diego, CA). Orientation was determined by restriction analysis. The hGH-cDNA, including the leader sequence, was excised from pCRII using *Not*I (at the 5′-end) and *Kpn*I (at the 3′-end). This cDNA fragment was then ligated into the *Not*I/*Kpn*I sites of the pVL-1392 baculovirus transfer vector (InVitrogen). The integrity of the cloned hGH-cDNA was determined by sequencing analysis. Sequencing of this insert and resequencing of the original cDNA yielded a corrected sequence in which nucleotides 857 and 858 were A rather than C and nucleotide 905 was C rather than G. In the 3′-UTR, nucleotide 1065 was C rather than G. This resulted in reassigning Asn268 to lysine, Pro269 to threonine, and Lys284 to asparagine in the encoded protein. This final baculovirus transfer vector construct was designated pVL-hGH.

Expression of recombinant hGH in insect cells. The transfer vector pVL-hGH (~2 μg) was combined with 0.5 μg of linear baculovirus DNA (BaculoGold; PharMingen, San Diego, CA) and transfected into the Sf9 insect cell line using a calcium phosphate precipitate method modified for insect cells (Guarino and Summers, 1986). After a 4-hr incubation at 27°, the transfection medium was removed and replaced with TNM-FH supplemented with 10% fetal bovine serum. Medium was collected 4 days after transfection. Recombinant virus was amplified, plaque purified and titered according to King and Possee (1992).

Hi-5 cells (InVitrogen) were seeded at a concentration of 3×10^6 cells in 75-cm^2 flasks. After a 2-day incubation period, the cells were infected with the recombinant baculovirus harboring the hGH cDNA at a multiplicity of infection of 5. Medium containing secreted recombinant hGH was collected 4 days after infection.

Purification of the baculovirus-expressed hGH. The pH of the harvested medium was adjusted to 5.3 with acetic acid. A portion (450 ml) of the medium was diluted to 1.35 liters with deionized water to lower the ionic strength. The diluted medium consisted of 50 mM mercaptoethanol, 1 mM octyl β-glucoside, and 1 mM EDTA and applied (10 ml/min) to a column of Protein-Pak CM (2.0 × 10 cm, 40 HR; Waters Division, Millipore, Milford, MA) equilibrated in 25 mM sodium acetate containing 50 mM mercaptoethanol, 1 mM octyl *b*-glucoside, and 1 mM EDTA. The enzyme was eluted at a flow rate of 1.5 ml/min with the same buffer containing 500 mM NaCl. Yields of enzyme were ~15 mg/liter of culture medium.

Preparation of a rabbit polyclonal antibody to the baculovirus-expressed hGH. A female rabbit was immunized with 150 μg of the purified expressed hGH. The rabbit then was administered two additional boosts of 150 μg according to a standard protocol (Wang *et al*., 1993a). The antisera used were from bleeds after the boosts. Before use, the IgG was purified from the antisera by ammonium sulfate precipitation and chromatography on Protein A-coupled Sepharose. The purified antisera could detect 50 ng of the antigen at a 1:100,000 dilution in an enzyme-linked immunoassay.

Isolation of hGH from HL-60 and MCF-7 cell lysates using cation exchange chromatography. HL-60 cells were received from culture and washed three times with RPMI 1640 medium containing no serum. The washed cells (4×10^8 cells/ml) were lysed in 50 mM sodium acetate buffer, pH 5.5, containing 50 mM mercaptoethanol and 1% Triton X-100, pH 5.5. The lysate was dialyzed against 50 mM sodium acetate, 50 mM mercaptoethanol, and 1 mM OBG, pH 5.5, and centrifuged at 10,000 × *g*. The dialysed lysate (10 ml) was applied to a column of CM-52 (1.5 × 18 cm; Whatman; Clifton, NJ) equilibrated in the dialysis buffer. After washing with the equilibration buffer, the γ-glutamyl hydrolase activity was eluted with the same buffer containing 1.0 M NaCl. Fractions containing activity were combined and concentrated using an Amicon (Beverly, MA) stirred cell with a YM-10 membrane. Semipurified MCF-7 cell lysates were similarly prepared. The lysates were analyzed by SDS-12.5% PAGE, blotted onto nitrocellulose, and probed with a 1:50,000 dilution of the anti-hGH rabbit antibody. A goat anti-rabbit IgG horesradish peroxidase conjugate (BioRad, Hercules, CA) was used as secondary antibody, and the blot was developed with an enhanced chemiluminescence system (ECL Western blotting system; Amersham, Arlington Heights, IL).

Isolation of hGH from HL-60 lysate using immunoaffinity chromatography. HL-60 cells (4×10^8 cells/ml) were lysed in phosphate-buffered saline plus 1% Triton X-100 containing a cocktail of protease inhibitors (Complete; Boehringer-Mannheim). A portion of lysate (30 ml) was mixed with 5 ml of a 50% slurry of anti-GH-IgG coupled to cyanogen bromide-activated Sepharose 4B, and the mixture was rotated overnight at 4°. The affinity medium was packed in a column (1.5 × 2.5 cm) and washed with 5 ml of phosphate-buffered saline. The elution buffer was then changed to 0.1 M glycine, pH 2.0. Fractions (0.5 ml) were collected into 50 μl of 1 M sodium acetate/1 M mercaptoethanol, pH 5.5. The fractions were analyzed for activity and by Western blot analysis. Fractions containing hGH were pooled and concentrated to two thirds of the original volume.

Treatment of expressed hGH and hGH from HL-60 cells with PNGase F. The pH of an aliquot (200 μl) of the pool of purified expressed hGH was adjusted to 7.4 with 10 μl of 0.1 N NaOH and 20 μl of 250 mM EDTA added. An aliquot of 10% SDS solution (10 μl, 0.42% final) was added, and the solution was placed in a boiling water bath for 3 min. An aliquot of 10% Nonidet P-40 solution (13 μl, 0.51% final; Pierce) was added, and the solution was divided into two portions of 50 and 150 μl. An aliquot (5 μl) of PNGase F solution (0.69 μg/μl) was added to the 150-μl portion, and both solutions were incubated at room temperature for 48 hr. The solutions were frozen at −20° until they were analyzed. An aliquot (200 μl) of immunoaffinity-purified hGH from HL-60 cells was treated similarly. The samples were analyzed by SDS-12.5% PAGE followed by Western blotting onto nitrocellulose. Prestained protein molecular mass standards (BioRad) were used to determine approximate molecular masses on the blot. A 1:10,000 dilution of the anti-hGH antibody and a goat anti-rabbit IgG alkaline phosphatase conjugate (BioRad) as secondary antibody were used. The blot was developed with an

alkaline phosphatase conjugate substrate kit (BioRad) according to the manufacturer's directions.

Enzyme activity assays. The enzyme activity was measured using 100 μM MTX polyglutamates (4-NH_2-10-CH_3PteGlu$_n$) as substrate in pH 6.0 buffer and incubation at 37° for varying times. The pterin-containing products were separated by HPLC (Rhee *et al.*, 1995) or capillary electrophoresis (Takemura *et al.*, 1996). For kinetic studies, the rate was measured as the disappearance of the substrate. With 4-NH_2-10-CH_3PteGlu$_6$ and 4-NH_2-10-CH_3PteGlu$_5$ as substrates, there was the potential for generating products that would be competing substrates. For this reason, V_{max} rates were measured when no more than 15% of the substrate was used. Under these conditions, 90% of the hydrolysis products are due to the initial cleavage. The released glutamic acid and γ-glu$_n$ were derivatized with orthophthaldehyde and analyzed by HPLC using fluorescence detection (Wang *et al.*, 1993).

Amino-terminal analysis of hGH samples. Amino-terminal amino acid sequence analysis of expressed proteins was carried out by blotting onto Immobilon (BioRad), excising the bands of interest, and sequencing as described previously (Yao *et al.*, 1996a).

Inhibition of expressed and HL-60 hGH by iodoacetic acid. Solutions (15 μl) of recombinant hGH in either 50 mM NaAc, 50 mM mercaptoethanol, pH 5.5, or 50 mM MES, and 2 mM dithiothreitol, pH 5.5, were incubated at 37° with various concentrations of iodoacetic acid (0–0.5 mM final concentration). At time intervals of 0–120 min, aliquots were removed and diluted 1:49 with 50 mM MES, and 2 mM dithiothreitol, pH 3.65, containing 100 μM 4-NH_2-10-CH_3PteGlu$_5$. The assay mixtures were incubated for 1 hr at 37°, boiled for 3 min, and analyzed by capillary electrophoresis to determine MTX product distribution. The inhibition of recombinant hGH by 500 μM *p*-hydroxymercuribenzoate (Sigma Chemical) was measured in a similar way. The inhibition of cation exchange-purified hGH from HL-60 cell lysates was carried out in an identical way, except the dilution of incubation mixture with assay mixture was 1:5 because of lower amounts of enzyme.

Results

The cDNA was readily expressed in a baculovirus system, and the expressed protein was partially purified by ion exchange chromatography. After this purification step, yields of protein were ~15 mg/liter [specific activity, 6.4 nmol of product/μg of protein/min], and the enzyme appeared on analysis by SDS-PAGE as a broad band with a molecular mass of 37 kDa and a faint band due to a baculovirus protein with a molecular mass of 65 kDa (Fig. 1). Amino-terminal analysis of the expressed enzyme before ion exchange chromatography yielded Arg^{25}-Pro-His-Gly. This sequence begins with Arg25 of the encoded amino-acid sequence, confirming a previous suggestion (Yao *et al.*, 1996) that the first 24 amino acids constitute a signal peptide that is removed during processing of the protein. After ion exchange chromatography, ~80% of the protein had Gly28 as the amino terminus, presumably due to the action of a contaminating protease. This proteolysis could be prevented and the amino terminus maintained as Arg25 by carrying out the purification in the presence of 1 mM EDTA (data not shown).

The baculovirus-expressed enzyme purified by ion exchange chromatography was used for antibody production. The rabbit polyclonal antibody readily detected the baculovirus-expressed enzyme in a Western blot assay (Fig. 2, *lane 1*). The predicted sequence for the enzyme contains four potential glycosylation sites (Yao *et al.*, 1996). After treatment with the enzyme PNGase F to remove asparagine-linked carbohydrate, the expressed enzyme migrated as a band with a molecular mass of 30 kDa (Fig. 2, *lane 2*), consistent with the calculated molecular mass of the encoded mature enzyme (33.6 kDa). Lysates of HL-60 promyeloid leukemia cells and MCF-7 breast cancer cells after ion exchange chromatography then were examined by Western blot analysis to determine whether the intracellular enzyme corresponded to the protein encoded by the identified cDNA and the baculovirus-expressed enzyme. When analyzed by Western blot analysis, both lysates contained an immunoreactive protein with a molecular mass of 37 kDa. When lysates of MCF7 and HL-60 cells purified by ion exchange chromatography were analyzed using Western blotting and a chemiluminescence detection system, the HL-60 cell and MCF-7 cell hGH proteins could be resolved into two closely spaced bands rather than a single band (Fig. 3). These two bands were not resolved when samples were analyzed by Western blotting

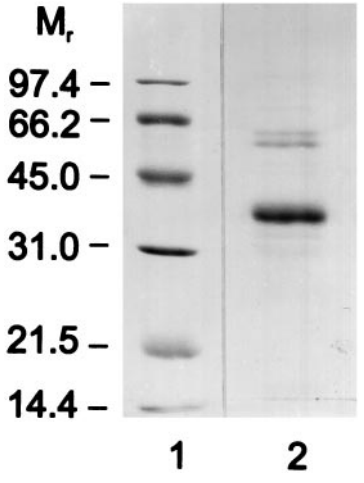

Fig. 1. SDS-12.5% PAGE analysis of purified expressed hGH. The enzyme was purified as described in Experimental Procedures. The sample of purified enzyme (4 μg, *lane 2*) was electrophoresed at 200 V for 45 min. The gel was stained with 0.25% Coomassie Brilliant Blue. *Lane 1*, standard marker proteins (BioRad).

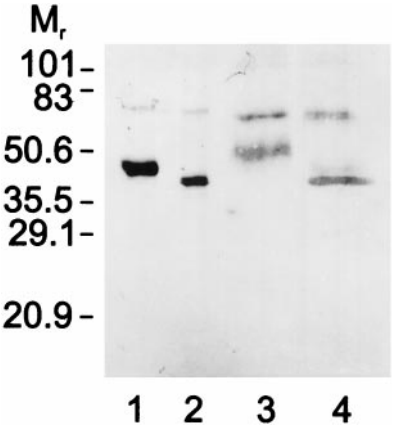

Fig. 2. Western blot analysis of expressed hGH and immunoaffinity-purified hGH from HL-60 cells with and without treatment with PNGase F to remove asparagine-linked carbohydrate. Samples were separated by SDS-12.5% PAGE, transferred to nitrocellulose, and probed with the polyclonal antibody to expressed hGH (1:10,000 dilution) as described in Methods. Detection was by enzyme-catalyzed color reaction. Prestained protein molecular mass standards were used to generate approximate molecular masses. *Lane 1*, expressed hGH (0.05 μg). *Lane 2*, expressed hGH treated with PNGase F (0.09 μg). *Lane 3*, immunoaffinity-purified hGH from HL-60 cells (1.9 μg). *Lane 4*, immunoaffinity-purified hGH from HL-60 cells after treatment with PNGase F (1.9 μg).

using an enzyme-catalyzed color reaction detection system. This may be related to the higher concentration (1:10,000 dilution) of primary antibody required in this type of assay. An immunoaffinity chromatography matrix was prepared by coupling the polyclonal antibody against baculovirus-expressed hGH to Sepharose 4B. This matrix was used to purify hGH from HL-60 cell lysates. The enzyme activity was retained on the column and could be eluted by using a low pH buffer (data not shown). The pooled fractions had the previously identified 37-kDa band when analyzed by Western blotting (Fig. 2, *lane 3*). The HL-60 cell enzyme when treated with PNGase yielded a protein of molecular mass of 30 kDa, similar to the baculovirus-expressed enzyme (Fig. 2, *lane 4*). The protein with molecular mass of ~53 kDa in Fig. 2 (*lanes 3 and 4*) seemed to be a contaminant that was not observed in the ion exchange-purified material (Fig. 3).

The expressed enzyme was evaluated for its mode of hydrolysis of 4-NH_2-10-CH_3PteGlu$_{2-6}$. Initially, we examined the dependence of the reaction rate on the concentration of MTX polyglutamates of different glutamate chain lengths (two to six) (Fig. 4). As shown in Fig. 4, 4-NH_2-10-CH_3PteGlu$_2$ was a poor substrate for expressed hGH in regard to both V_{max} and enzyme saturation, with the latter achieved at 400 μM (data not shown). The lack of activity on 4-NH_2-10-CH_3PteGlu$_2$ corroborates the results of an earlier study that we conducted on a panel of hGH enzymes secreted by several human tumor cell lines (Rhee *et al*., 1995). The V_{max} values for the other substrates decreased in the order 4-NH_2-10-CH_3PteGlu$_6$ >4-NH_2-10-CH_3PteGlu$_4$ > 4-NH_2-10-CH_3PteGlu$_5$ > 4-NH_2-10-CH_3PteGlu$_3$.

In the next experiment, we sought to determine the properties of appearance of the MTX products during the cleavage of 4-NH_2-10-CH_3PteGlu$_{3-6}$. Previous studies (Yao *et al*., 1996) had shown that *E. coli*-expressed hGH catalyzed the cleavage of 4-NH_2-10-CH_3PteGlu$_5$, yielding all possible pteroyl-containing products (4-NH_2-10-CH_3PteGlu$_4$, 4-NH_2-10-CH_3PteGlu$_3$, 4-NH_2-10-CH_3PteGlu$_2$, and 4-NH_2-10-CH_3PteGlu$_1$) of MTX polyglutamates. The data shown in Fig. 5 are representative of numerous studies that followed the expressed enzyme catalyzed reaction through ~50% hydrolysis of the particular substrate. In all cases, each intermediate product appeared. For example, the cleavage of 4-NH_2-10-CH_3PteGlu$_6$ resulted in the appearance of 4-NH_2-10-CH_3PteGlu$_{5-1}$. An interesting feature of the expressed human enzyme-catalyzed reaction was the more rapid appearance of the product containing two fewer glutamates than the product lacking a single glutamate. This preferential cleavage occurred in all substrates containing three to six glutamate residues. A similar result had been observed with unpurified hGH secreted from human tumor cell lines, but further studies were not conducted at that time to elucidate the cause (Rhee *et al*., 1995).

An analysis of the γ-Glu products in a separate experiment revealed the reason for the results in Fig. 5. All substrates, except 4-NH_2-10-CH_3PteGlu$_2$, were hydrolyzed to release both γ-Glu$_2$ and glutamic acid (Table 1). In some cases, γ-Glu$_2$ exceeded glutamic acid, which is consistent with the release of greater amounts of the product containing MTX polyglutamates with two glutamates removed. These results clearly demonstrate that the human enzyme expressed in the baculovirus system catalyzes the hydrolysis of both the ultimate and penultimate γ-Glu linkages. In no case was a γ-Glu$_n$ product found that contains more than two glutamic acid residues. The partially purified enzymes from MCF-7 and HL-60 cells had the same mechanism of hydrolysis with the release of γ-Glu$_2$ and glutamic acid exclusively (data not shown).

In an additional study, we evaluated the hydrolysis process for the substrate 4-NH_2-10-CH_3PteGlu$_3$. This product anal-

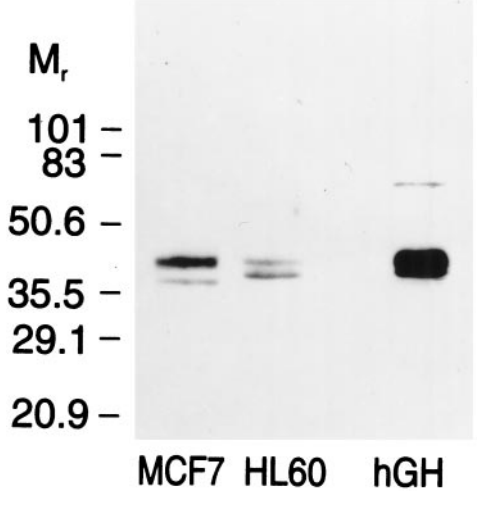

Fig. 3. Western blot analysis of expressed GH, cation exchange chromatography-purified HL-60 cell lysate, and cation exchange chromatography-purified MCF-7 cell lysate using chemiluminescent detection. Samples were separated by SDS-12.5% PAGE, transferred to nitrocellulose, and probed with the polyclonal antibody to expressed hGH at a 1:50,000 dilution. Prestained protein molecular weight standards were used to generate approximate molecular masses. *MCF-7*, MCF-7 cell lysate (4 μg of protein). *HL60*, HL-60 cell lysate (10 μg of protein). *hGH*, expressed hGH (0.34 μg). Detection was by chemiluminescence substrate as described in Methods.

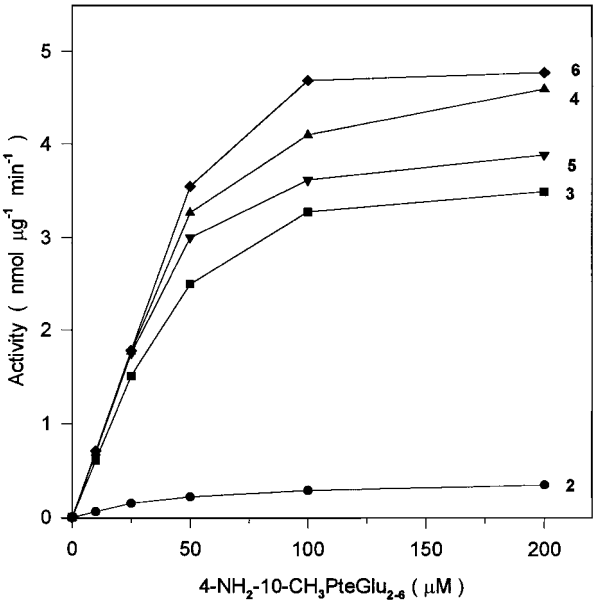

Fig. 4. Concentration dependence of the rates of hydrolysis of MTX polyglutamates as substrates for recombinant hGH. The purified expressed GH was incubated with various concentrations of substrates 4-NH_2-10-CH_3PteGlu$_{2-6}$ in 100 μl of reaction buffer at 37° for 5–30 min. The substrates and the reaction products were analyzed by HPLC, and hGH activity was calculated by the disappearance of the substrate. The maximum reaction rate of each substrate concentration, expressed as nmol of product/μg of hGH/min, was plotted against the concentrations of substrates. *Each point*, average of two experiments.

ysis is simplified because it yields only two MTX-containing products (4-NH_2-10-CH_3PteGlu$_2$ and 4-NH_2-10-CH_3PteGlu$_1$) and two γ-Glu-containing products (γ-Glu$_2$ or glutamic acid). This substrate also has a relatively high V_{max} value (Fig. 4). The results clearly show that the primary pterin-containing cleavage product is 4-NH_2-10-CH_3PteGlu, with lesser amounts of 4-NH_2-10-CH_3PteGlu$_2$ (Fig. 6). Consistent with this, γ-Glu$_2$ production exceeds that of glutamic acid, although quantitatively it is not as great as the predominance of 4-NH_2-10-CH_3PteGlu$_1$ over 4-NH_2-10-CH_3PteGlu$_2$. This may be due to the concomitant hydrolysis of γ-Glu$_2$, because γ-Glu$_2$ is also a substrate for hGH. Replication of the experiment described in Fig. 6 with hGH from HL-60 and MCF-7 cells and that expressed in *E. coli* (Yao *et al.*, 1996) gave similar results.

The baculovirus-expressed hGH was inhibited by iodoacetic acid in a time- and concentration-dependent manner (Fig. 7). The recombinant enzyme also was inhibited by *p*-hydoxymercuribenzoate (500 μM), suggesting that like the rat enzyme (Yao *et al.*, 1996a), the human enzyme may contain a catalytically essential cysteine. The partially purified hGH from HL-60 lysates also was readily inhibited by 500 μM iodoacetic acid (Fig. 7, *inset*).

Discussion

A cDNA encoding a human GH was identified previously and expressed in *E. coli* (Yao *et al.*, 1996). This cDNA has now been expressed in a baculovirus system to yield an active glycosylated enzyme. Analogous to the rat enzyme (Yao *et al.*, 1996a), a 24-amino-acid signal peptide was removed during protein processing to yield the mature protein. A polyclonal antibody raised against the baculovirus-expressed enzyme cross-reacted with the intracellular hGH from HL-60 cells and MCF-7 cells, indicating that the three proteins were antigenically similar, of similar molecular mass, and glycosylated on asparagine. The resolution of the HL-60 cell enzyme and the MCF-7 enzyme into two closely spaced bands when the proteins were analyzed by Western blotting using chemiluminescent detection is presumably due to post-translational modification of the proteins. Further studies will be necessary to determine the nature of this modification. A

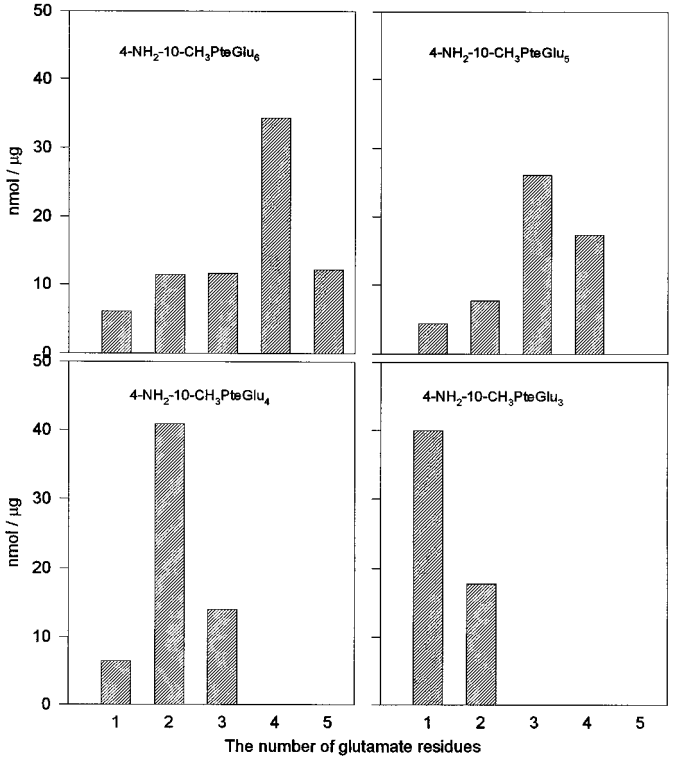

Fig. 5. Distribution of the hydrolysis products of 4-NH_2-10-CH_3PteGlu$_{3-6}$ catalyzed by recombinant hGH. Substrates (100 μM) were incubated with purified expressed hGH at 37° for 10 min. Pterin-containing hydrolyzed products were analyzed by HPLC, and each product was expressed as nmol of product/μg of hGH.

TABLE 1

Formation of γ-Glu products from the hydrolysis of 4-NH_2-10-CH_3PteGlu$_n$ catalyzed by recombinant hGH

Substrates 4-NH_2-10-CH_3PteGlu$_{2-6}$ (50 μM), were incubated with recombinant hGH at 37° for 15 min. The γ-Glu$_n$ species were analyzed as described in the text. Under no conditions could γ-Glu$_3$, γ-Glu$_4$, or γ-Glu$_5$ be detected.

Substrate	γ-Glu$_2$	Glu
	nmol of product / μg of hGH	
4-NH_2-10-CH_3PteGlu$_2$		3.3
4-NH_2-10-CH_3PteGlu$_3$	17.0	16.3
4-NH_2-10-CH_3PteGlu$_4$	23.6	16.4
4-NH_2-10-CH_3PteGlu$_5$	16.8	20.1
4-NH_2-10-CH_3PteGlu$_6$	20.5	31.1

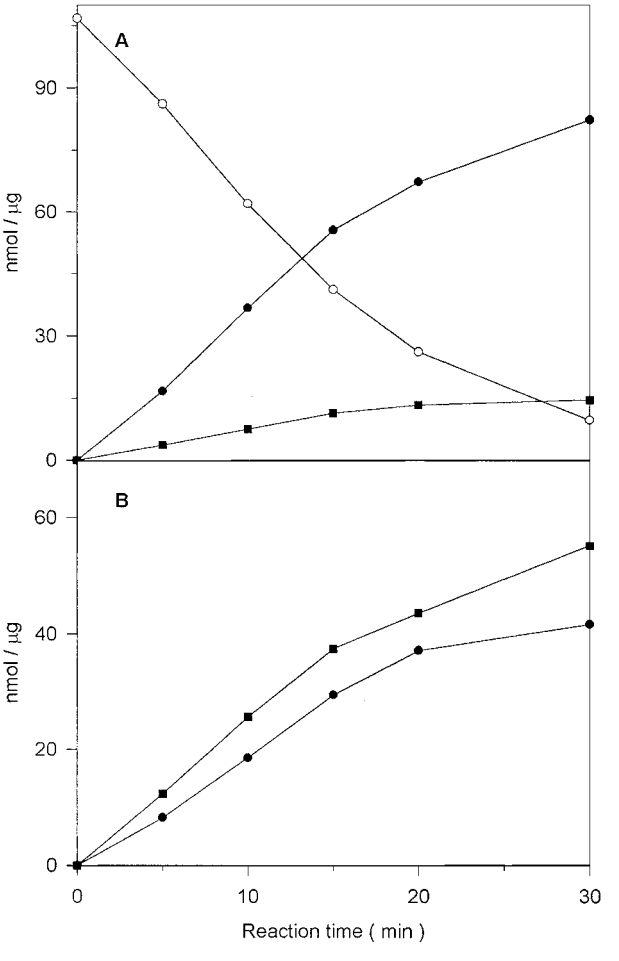

Fig. 6. Time dependence of the appearance of the hydrolysis products of 4-NH_2-10-CH_3PteGlu$_3$ catalyzed by recombinant hGH. 4-NH_2-10-CH_3PteGlu$_3$ (100 μM) was incubated with purified expressed hGH at 37° for the indicated time. Reactants were analyzed for (A) pterin-containing products (*top*) by HPLC/UV detection and (B) released γ-Glu$_n$ species (*bottom*) by HPLC/fluorescence detection. A: ○, Substrate; ●, 4-NH_2-10-CH_3PteGlu$_1$; ■, 4-NH_2-10-CH_3PteGlu$_2$. B: ●, Glutamic acid; ■, γ-Glu$_2$.

similar observation of two protein forms was made with the rat enzyme (Yao *et al*., 1996a). Both the baculovirus-expressed enzyme and the intracellular enzyme from HL-60 cells were inhibited by iodoacetic acid, suggesting that they both contained a catalytically essential cysteine.

The human enzyme has been shown to degrade MTX polyglutamates by removing either the outermost glutamate individually or the two outermost glutamates together. It has been suggested that because of the appearance of all possible MTX polyglutamates during the course of degradation of a long chain MTX polyglutamates, the enzyme could have been an exopeptidase (Yao *et al*., 1996; Waltham *et al*., 1997). Exopeptidase refers to the cleavage of only the outermost γ-linkage. It now seems clear that the situation is more complex because either of the two outermost γ-linkages can be cleaved. However, we could not detect any evidence for removal of the more interior linkages because γ-Glu_n with n > z were never found.

In many cases, the MTX polyglutamate product with two fewer glutamates exceeds the product with one fewer glutamate, and γ-Glu_2 exceeds glutamic acid. It is somewhat difficult to quantify this because all products except MTX and glutamate are substrates. However, the predominance suggests that the penultimate γ-linkage may be favored. If this is so, it may be the reason 4-NH_2-10-CH_3PteGlu_2 is such a poor substrate. It is clear, however, that more detailed studies on the reaction will be required because the resulting product profile also contains substrates and presents a very complex problem.

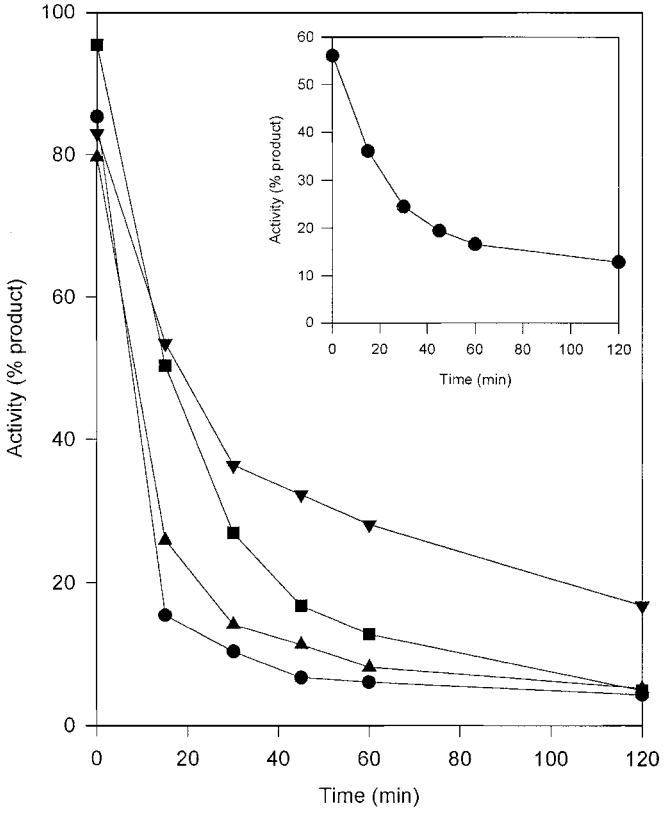

Fig. 7. Inhibition of recombinant hGH and hGH from HL-60 cells by iodoacetic acid. Solutions of recombinant hGH were incubated with varying concentrations of iodoacetic acid (●, 0.5 mM; ▲, 0.25 mM; ■, 0.1 mM; ▼, 0.04 mM), and aliquots were removed at the indicated times. The aliquots were assayed for activity on 4-NH_2-10-CH_3PteGlu_5 as described in Methods. *Inset*, inhibition of immunoaffinity-purified hGH from HL-60 cell lysates using 0.5 mM iodoacetic acid.

The possibility of ultimate and penultimate γ-cleavage was established by Wang *et al*. (1986) with the cellular enzyme of human jejunal mucosa. They determined that incubation of hGH preparations with PteGlu_2[^{14}C]Glu liberated [^{14}C]Glu and [^{14}C]γ-Glu_2. However, they did not examine longer chain polyglutamates or evaluate the cellular enzymes from other sources. Thus, the current work supports their initial observation and generalizes to longer chain polyglutamates and to the GH from other human sources.

The triglutamate derivative makes an interesting model substrate. It has a high V_{max} value, but unlike the longer chain polyglutamates, it produces only two pterin-containing products, the monoglutamate and diglutamate. It could be used to examine tissues for the pattern of cleavage of MTX or folate polyglutamates. If the appearance of the pteroyl monoglutamate early in the reaction exceeds that of the diglutamate, it is suggestive of both γ-linkages being cleaved. It is very unlikely that the degradation of 4-NH_2-10-CH_3PteGlu_3 proceeds slowly and the product 4-NH_2-10-CH_3PteGlu_2 is cleaved very rapidly to 4-NH_2-10-CH_3PteGlu, because 4-NH_2-10-CH_3PteGlu_2 is such a poor substrate for hGH (Fig. 4). In these experiments in which we examined the hydrolysis of 4-NH_2-10-CH_3PteGlu_3, cleavage of the penultimate γ-linkage clearly seems to be favored.

It is possible that hGH from human tissues and cells that have not yet been examined have different hydrolysis patterns from those we describe here. Two others that will be particularly interesting are the membrane-associated hGHs: one from the jejunal brush border (Chandler *et al*., 1986) and one from prostate membrane (Pinto *et al*., 1996). These are distinct from cellular hGH with regard to sequence and structure. Chandler *et al*. (1986) presented evidence that the jejunal brush border enzyme is an exopeptidase with PteGlu_2[^{14}C]Glu used as the substrate. The prostate membrane enzyme superficially appears like an exopeptidase (Pinto *et al*., 1996), but analysis of the γ-Glu_n products has not been done, nor has pteroyl polyglutamate with labeled glutamate been used as a substrate. The human enzymes described in this report have similar physical properties (molecular mass, pH optimum, and essential sulfhydryl group) to the intracellular human enzyme identified in the soluble fraction of jejunal mucosa (Reisenauer *et al*., 1977; Wang *et al*., 1986) and to the enzyme from human sarcoma HT-1080 cells (Waltham *et al*., 1997).

The role of hGH in determining the therapeutic activity of MTX and other antifolates has not been determined. Enhancement of hGH activity in host tissues or inhibition of hGH in target tissues could potentially enhance the therapeutic index of these drugs. Using *in vitro* models of rat and human tumor cells in culture, it has been demonstrated that elevated GH is associated with reduced antifolate activity (Rhee *et al*., 1993; Pizzorno *et al*., 1995). In addition, resistance of human leukemia to MTX has been associated with high levels of hGH relative to FPGS (Longo *et al*., 1997).

GH-dependent antifolate resistance presents an interesting and complex case. Antifolate resistance could result from an increase in the amount of GH enzyme causing greater GH activity, as seems to be the case with rat H35 hepatoma cell lines (Rhee *et al*., 1993). Studies from this laboratory have

shown that the cDNAs for rat and human GH encode proteins containing leader sequences and consensus glycosylation sites that result in the enzyme being targeted to the lysosomes and for secretion (Rhee *et al*., 1995; Yao *et al*., 1995). Mutations in any of these sites could result in altered trafficking of the enzyme. If a mutation caused significant amounts of the enzyme to be rerouted to the cytosol, a dramatic reduction in MTX polyglutamate formation and cytotoxic activity would result. Hence, a complete understanding of the structure and activity of GH is required to assess the contribution of GH to the cytotoxicity of MTX and other antifolates. The baculovirus-expressed human GH seems to be the same hGH as that found intracellularly in a variety of normal and abnormal human cell lines and may serve as a model for structural and kinetic studies aimed at the design of therapeutic inhibitors.

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References

Chandler CJ, Wang TTY, and Halsted CH (1986) Pteroylpolyglutamate hydrolase from human jejunal brush borders. *J Biol Chem* **261:**928–933.

Guarino LA and Summers M (1986) Functional mapping of a transactivating gene required for expression of a baculovirus delayed-early gene. *J Virol* **57:**563–571.

King LA and Possee RD (1992) *The Baculovirus Expression System: A Laboratory Guide*, Chapman and Hall, London.

Longo GS, Gorlick R, Tong WP, Lin S, Steinhertz P, and Bertino JR (1997) *gamma*-Glutamyl hydrolase and folylpolyglutamate synthetase activities predict polyglutamylation of methotrexate in acute leukemias. *Oncol Res* **9:**259–263.

Pinto JT, Suffoletto BP, Berzin TM, Qiao CH, Lin S, Tong WP, May F, Jukherjee B, and Heston WDW (1996) Prostate-specific membrane antigen: a novel folate hydrolase in human prostatic carcinoma cells. *Clin Cancer Res* **2:**1445–1451.

Pizzorno G, Moroson BA, Cashmore AR, Russello O, Mayer JR, Galivan J, Bunni MA, Priest DG, and Beardsley GP (1995) Multifactorial resistance to 5,10-dideazatetrahydrofolic acid in cell lines derived from human lymphoblastic leukemia CCRF-CEM. *Cancer Res* **55:**566–573.

Priest DG and Bunni MA (1995) Folate and folate antagonists in cancer chemotherapy, in *Folates in Health and Disease* (Bailey L, ed) pp 379–404, Marcel Dekker, New York.

Reisenauer AM, Krumdieck CL, and Halsted CH (1977) Folate conjugase: two separate activities in human jejunum. *Science (Washington D C)* **198:**196–197.

Rhee MS, Ryan TJ, and Galivan, JH (1995) γ-Glutamyl hydrolase secreted from human tumour cell lines. *Cell Pharmacol* **2:**289–292.

Rhee MS, Wang Y, Nair GM, and Galivan J (1993) Acquisition of resistance to antifolates caused by enhanced γ-glutamyl hydrolase activity. *Cancer Res* **53:** 2227–2230.

Shane B (1995) Folate chemistry and metabolism, in *Folates in Health and Disease* (Bailey L, ed) pp 1–22, Marcel Dekker, New York.

Takemura Y, Kobayashi H, and Sekiguchi S (1996) Separation of methotrexate polyglutamates by capillary electrophoresis and its application to the measurement of *gamma*-glutamyl hydrolase activity in human leukemia cells in culture. *Rinsho Byori (Jpn J Clin Pathol)* **44:**51–56.

Waltham MC, Li WW, Gritsman H, Tong WP, and Bertino J R (1997) γ-Glutamyl hydrolase from human sarcoma HT-1080 cells: characterization and inhibition by glutamine antagonists. *Mol Pharmacol* **51:**825–832.

Wang TTY, Chandler CJ, and Halsted CH (1986) Intracellular pteroylpolyglutamate hydrolase from human jejunal mucosa. *J Biol Chem* **261:**13551–13555.

Wang Y, Dias JA, Nemic Z, Rotundo R, O'Connor BM, Freisheim J, and Galivan J (1993a) The properties and function of γ-glutamyl hydrolase and poly-γ-glutamate. *Adv Enzyme Regul* **33:**207–218.

Wang Y, Nimec Z, Ryan TJ, Dias JA, and Galivan J (1993) The properties of secreted γ-glutamyl hydrolase from H35 cells. *Biochim Biophys Acta* **1164:**227–235.

Yao R (1997) Studies of γ-glutamyl hydrolase: human tissue distribution and chromosome location, in *Chemistry and Biology of Pteridines and Folates* (Pfleiderer W and Rokos H, eds) pp 475–478, Blackwell, Berlin.

Yao R, Nimec Z, Ryan TJ, and Galivan J (1996a) Identification, cloning and sequencing of a cDNA coding for rat γ-glutamyl hydrolase. *J Biol Chem* **271:**8525–8528.

Yao R, Rhee MS, and Galivan J (1995) The effects of γ-glutamyl hydrolase on folyl and antifolyl polyglutamates in cultured H35 cells. *Mol Phamacol* **48:**505–511.

Yao R, Schneider E, Ryan TJ, and Galivan J (1996) Human γ-glutamyl hydrolase: cloning and characterization of the enzyme expressed in vitro. *Proc Natl Acad Sci USA* **93:**10134–10138.

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