Identification, Cloning, and Sequencing of a cDNA Coding for Rat γ-Glutamyl Hydrolase*

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Purified γ-glutamyl hydrolase secreted from rat H35 hepatoma cells has been characterized as a diffuse band of 55 kDa on SDS-polyacrylamide gel electrophoresis that is converted to bands of 35 and 33 kDa after enzymatic removal of N-linked carbohydrate. Polyclonal antibodies against 55-kDa γ-glutamyl hydrolase captured the enzyme activity and recognized the glycosylated and both deglycosylated forms of γ-glutamyl hydrolase. A complete cDNA sequence of γ-glutamyl hydrolase was obtained using degenerate oligonucleotides derived from peptide sequences, screening of a rat hepatoma cDNA library, and reverse transcription polymerase chain reaction. Based upon the deduced amino acid sequence the peptide component of γ-glutamyl hydrolase had a molecular weight of 33,400. The results of amino acid analysis of the purified protein agreed with the deduced amino acid sequence in which there are seven potential asparagine-containing glycosylation sites.

γ-Glutamyl hydrolase (EC 3.4.22.12) catalyzes the hydrolysis of the polyglutamate side chain of folyl polyglutamates and anti-folyl polyglutamates (1–16). γ-Glutamyl hydrolase (GH) has been characterized from a number of sources, and it exhibits either endo- or exopeptidase activity, depending upon the tissue of origin (1, 2, 4–11). In many tissues the enzyme is localized to the lysosome, most of the enzyme activity is secreted, a feature that appears to be universal in neoplastic cells (20–22). In order to be able to study this enzyme and its synthesis and regulation in detail, we have cloned a cDNA coding for this enzyme and evaluated some of the properties of the glycoprotein itself. These studies used the H35 rat hepatoma system (16–18, 20–22; this is the first report of a cDNA sequence and corresponding deduced amino acid sequence for GH.

EXPERIMENTAL PROCEDURES

Materials

Cell Culture—H35 hepatoma cells were cultured and transferred to serum-free medium as described previously (16). The pooled medium was stored at -70 °C until used.

Purification of γ-Glutamyl Hydrolase—On the basis of the earlier studies of Lin et al. (23), the individual peaks from TS gel Toyopearl butyl-650S (16) were further purified by chromatography on Matrex gel green A (Amicon Division, W. R. Grace & Co.). Up to 200 μg of protein was applied to a column (1 x 10 cm) of Matrex gel green A that had been equilibrated with 10 mM sodium acetate (pH 6.0) containing 0.1 mM zinc acetate and 50 mM β-mercaptoethanol. The column was washed with 40 ml of equilibration buffer and 40 ml of the same buffer containing 0.15 M sodium chloride. The enzyme was eluted with equilibration buffer containing 0.4 M sodium chloride and 10 mM cysteine. The specific activity of five independent preparations was 107,000 nmol min⁻¹ mg⁻¹ with a S.D. of ±16%. The specific activity of each of the individual TS-gel Toyopearl butyl 650S peaks fell within that range following purification on Matrex gel green A. GH was assayed as described previously (16) using 4-NH₂-10-CH₃PteGlu₂ as substrate.

Methods

Microsequencing of intact GH and Cyanogen Bromide Fragments—Purified enzyme was assayed by SDS, 10% polyacrylamide gel electrophoresis using a Tris-Tricine buffer containing 0.3% thioglycolic acid and 1% glycerol in a Bio-Rad Mini Protein system. The gel was electrophoretically stained with Coomassie Blue and the bands of interest excised. For internal sequencing a single crystal of cyanogen bromide was added to a solution of purified enzyme (30–40 μg) in 70% trifluoroacetic acid and the solution heated at 45 °C for 45 min. The solution was lyophilized and fragments sequenced as above using a SDS, 13% polyacrylamide gel.

Preparation of a Polyclonal Antibody to GH—A single rabbit was immunized with purified GH (an initial immunization of 50 μg followed by three boosts of 50 μg each) using a standard protocol by BioDesign International, Kennebunk, ME. The studies in this report were done with an early test bleed, purified on Protein A-agarose, which had a 1 to 500,000 titer against 50 ng of purified γ-GH in an enzyme-linked immunosorbent assay.

Cloning and Sequencing of GH—The sequence of the open reading frame corresponding to γ-glutamyl hydrolase was determined using a combination of RT-PCR, RACE, and screening of a commercial cDNA library. Total cellular RNA was prepared from rat hepatoma H35 cells using TRI REAGENT (Molecular Research Center, Inc.), according to the manufacturer’s instructions. Poly(A)⁺ mRNA was isolated from total RNA by affinity chromatography using a spin column of microcrystalline oligo(dT)-cellulose (New England BioLabs). Poly(A)⁺ mRNA eluate was precipitated with ethanol and stored at -70 °C for later use.

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‡The nucleotide sequences reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U 38379.
The poly(A)–mRNA from H35 cells was reverse transcribed into cDNA using Moloney murine leukemia virus-reverse transcriptase and an oligo(dT) primer (Stratagene). The cDNA was subjected to PCR amplification using degenerate primers derived from the amino acid sequence of the N terminus and of CNBr-generated fragments. The primers used for RT-PCR were modified by carrying deoxyinosine residues at positions corresponding to ambiguous nucleotides (24). The RT-PCR generated a 605-base fragment denoted pGH-1.

The cDNA prepared from H35 mRNA was synthesized by Moloney murine leukemia virus-reverse transcriptase and ligated to the Marathon cDNA adapter (Clontech, Palo Alto, CA). RACE-ready cDNA was amplified with nested γ-GH-specific primers in combination with Marathon adaptor primers, AP1 and AP2, respectively. For sequencing, PCR products generated with Taq polymerase were cloned into a PCR II vector in the TA cloning system, as the 3′ A overhangs are not removed (Invitrogen, San Diego, CA). The amount of PCR product needed to ligate with 50 ng of PCR II vector was estimated according to the manufacturer’s directions.

A rat hepatoma cDNA library was obtained from Stratagene (Palo Alto, CA). A total of 10⁶ λZAP II recombinant phage plaques on E. coli strain XL1-blue cells were screened. Phage plaques were lifted twice onto nitrocellulose membranes, denatured in 0.5 N NaOH/1.5 M NaCl, and neutralized in 1.5 M NaCl/1.0 M Tris HCl, pH 7.5. The membranes were baked for 2 h at 80 °C in a vacuum oven, and then prehybridized 16 h at 37 °C in prehybridization solution containing 5X SSC, 5X Denhardt’s, 0.1% SDS, 50% formamide, and 0.2 mg/ml salmon sperm DNA. This was followed by hybridization for 20 h in the above buffer with [32P]-randomly-labeled pGH-1. Three partial cDNA’s were obtained (pGH-3, pGH-4, and pGH-5).

cDNA inserts were excised from the λZAP II cDNA library and subcloned into pBluescript according to the in vivo excision procedure described by Stratagene. Plasmid DNA was prepared with a Wizard miniprep DNA purification system and used as a sequencing template. Initial sequencing for pGH-1 and pGH-2 was done using M13 forward and M13 reverse primers on the PCR II vector (Invitrogen). For the clones of pGH3, -4 and -5, T3 and T7 primers on pBluescript were used for determining the sequences at both ends of the insert. The dioxyanucleotide clad termination method of Sanger et al. (25) was used with Sequenase (U. S. Biochemical Corp.). Primers were synthesized by the Molecular Genetic Core at the Wadsworth Center.

RESULTS AND DISCUSSION

Purification of GH—Rat GH was previously shown to elute in three peaks of activity from TSK-gel Toyopearl butyl-650S (16). These three peaks (peaks 1, 2, and 3) have now been individually purified to homogeneity by chromatography on Matrex gel green A. Each peak when purified appeared as a individually purified to homogeneity by chromatography on Matrex gel green A. Each peak when purified appeared as a

| M1 | 97.4 | 66.2 |
|----|------|------|
| 45 | 31   | 29   |
| 15 | 12   | 10   |
| 5  | 3    | 2    |

| M2 | 97.4 | 66.2 |
|----|------|------|
| 45 | 31   | 29   |
| 15 | 12   | 10   |
| 5  | 3    | 2    |

| M3 | 97.4 | 66.2 |
|----|------|------|
| 45 | 31   | 29   |
| 15 | 12   | 10   |
| 5  | 3    | 2    |

| PRODUCT FORMED (%) |
|--------------------|
| 0  | 1  | 2  | 3  | 4  |
|     |   |    |    |    |

FIG. 1. A, SDS, 12.5% polyacrylamide gel electrophoresis of purified GH. GH from peaks 1 (lane 1), peak 2 (lane 2), and peak 3 (lane 3) from TSK-gel Toyopearl butyl-650S were purified to homogeneity on Matrex gel green A as described under “Experimental Procedures.” A portion of the purified peak (0.5 μg) was applied to each lane. B, analysis of native and deglycosylated GH. Purified pooled peaks 1 and 2 (1 μg of protein) were utilized without further treatment in lanes 1 and 3. Samples (lane 2, 0.5 μg; lane 4, 0.125 μg) were incubated with PNGase F (10 and 5 μg, respectively) in 0.1% SDS and 0.1% Triton X-100 for 20 h at 30 °C. Following electrophoresis, the gel was stained with 0.05% Coomassie Brilliant Blue (lanes 1 and 2) or evaluated by Western blot using a 1:50,000 dilution of rabbit anti-GH (lanes 3 and 4). Although PNGase F also migrates at approximately 33 kDa, it did not stain because of the low amount of protein used in the incubation. 2C capture assay of GH. Plates were incubated with goat anti-rabbit IgG (1:1000, 100 μl, Tago-immunologics) overnight at 4 °C, washed with phosphate-buffered saline (7 mM sodium phosphate buffer, pH 7.0, containing 140 mM sodium chloride, 3 × 200 μl), and incubated with 1% bovine serum albumin for 2 h at 23 °C. The plates were then incubated with 100 μl of rabbit anti-GH IgG (1:5000) for 5 h at 23 °C, followed by washing with phosphate-buffered saline (3 × 200 μl). GH (200 ng in 100 μl) was added and incubated overnight at 4 °C. Following washing as above, the reaction was initiated by the addition of 10 μM 4-NH2-10-CH2-PteGlu and the enzyme activity assay (2 h) conducted as described under "Experimental Procedures." A, assay mixture lacking anti-GH antibody; 2, complete reaction; 3, lacking GH. The results are presented as the percent of the total substrate converted to 4-NH2-10-CH2-PteGlu (methyloctrate). Replacement of anti-GH with protein A-purified prebleed rabbit serum gave the same results as in lane 1.

bands upon deglycosylation is not presently understood. It may be due to some residual PNGase F-resistant carbohydrate or O-linked carbohydrate. It is also possible that differences occur in other posttranslational modifications or that some variation
A full-length cDNA for the GH coding region was prepared by PCR amplification using poly(A) + mRNA from H35 cells and primers that flanked the coding region (Fig. 2). The insert consisted of 1,204 nucleotides. The first 9 nucleotides of the 5' end of the sequence correspond to part of a consensus sequence for the initiation of translation in vertebrates (27).

Characteristics of the Encoded GH—The cDNA coded for a protein of 317 amino acids. Based on the N-terminal sequence of the purified enzyme, there is a leader sequence (residues 1–24) with Gly-25 as the N terminus of the mature enzyme. The calculated molecular weight of the mature protein was 33.4 kDa, which is within the range of the deglycosylated proteins (Fig. 1). The calculated amino acid analysis of the protein was consistent with the determined amino acid composition of the purified enzyme (Table I). The deduced amino acid sequence (Fig. 2) contained seven potential N-linked glycosylation sites (28), supporting the observation that the protein is highly glycosylated. There are three Cys residues in the sequence (Fig. 2). The finding that the enzyme activity of GH is enhanced by the presence of sulfhydryl-containing compounds (16) and the observed inhibition of GH by iodoacetic acid3 suggest that the enzyme may contain an active site or structurally critical cysteine.

The availability of the cDNA and amino acid sequence for GH and a polyclonal antibody to the protein offers the possibility of investigating a number of questions concerning this enzyme. The role of GH in the cellular metabolism of folylpolyglutamate coenzymes and in the cytotoxic activity of antifolates can be evaluated in detail. GH activity is known to be altered by a number of factors including insulin (18), estrogen (19), and selection for resistance with 5,10-didiazatetrahydrofolate in rat (17, 21) and human (29) cell lines. With the availability of the molecular and immunological probes described in this report, the mechanism of alterations in GH activity can be investigated. The cellular trafficking of the glycoprotein can be approached with an emphasis on the mechanism and significance of secretion. Studies are under way to

TABLE I

| Amino acid | Theoreticala | Peak 1 | Peak 2 | Peak 3 |
|------------|--------------|-------|-------|-------|
| Asp + Asn  | 30           | 30.99 | 30.47 | 28.41 |
| Thr        | 17           | 17.16 | 15.92 | 16.1  |
| Ser        | 26           | 21.8  | 21.29 | 26.6  |
| Glu + Gln  | 29           | 32.6  | 32.5  | 29.4  |
| Pro        | 11           | 5.33  | 4.93  | 4.76  |
| Gly        | 18           | 14.57 | 21.55 | 26.17 |
| Ala        | 17           | 17.47 | 19.63 | 19.86 |
| Cys        | 3            | NDb   | ND    | ND    |
| Val        | 14           | 14.83 | 14.69 | 14.35 |
| Met        | 5            | 4.82  | 5.91  | 6.54  |
| Ile        | 16           | 15.64 | 16.33 | 16.1  |
| Leu,       | 30           | 40.06 | 36.10 | 36.22 |
| Tyr        | 14           | 11.59 | 8.78  | 7.81  |
| Phe        | 22           | 22.1  | 23.21 | 19.86 |
| Lys        | 20           | 20.20 | 18.83 | 18.37 |
| His        | 6            | 6.91  | 6.31  | 6.28  |
| Trp        | 4            | NDb   | ND    | ND    |
| Arg        | 11           | 10.76 | 10.44 | 10.07 |

a Calculated amino acid composition of the translated cDNA sequence corresponding to γ-glutamyl hydrolase expressed as number of amino acid residues/mol of enzyme.
b ND, not determined.

Glucosamine coelutes with leucine on this amino acid analyzer resulting in potential falsely elevated leucine values.

Further studies are under way to resolve this question and to determine whether the deglycosylated enzyme retains catalytic activity.
determine if the sequences of the rat and human cDNAs are homologous and the cross-reactivity of the anti-rat GH antibody. If feasible, these probes will be used to evaluate the abundant secretion of GH by human breast cancer cell lines in culture (22), which is potentially related to the high levels of GH in serum of metastatic breast cancer patients (30). In addition, relatively large amounts of enzyme should become available for the first time when an appropriate expression system is established, and this will allow detailed analysis of the structure and mechanism of GH.

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