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# Cloning and sequence analysis of a cDNA for human glycosylasparaginase

## A single gene encodes the subunits of this lysosomal amidase

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We have isolated a full-length cDNA (HPAsn.6) for human placenta glycosylasparaginase using a 221-bp PCR amplified fragment containing rat liver asparaginase gene sequences. The deduced amino acid sequence from the human clone showed sequence identity to both the α and β subunits of the rat enzyme. The human enzyme is encoded as a 34.6 kDa polypeptide that is post-translationally processed to generate two subunits of approx. 19.5 (α) and 15 (β) kDa. A charge enriched region is present at the predicted site where cleavage occurs. Using polyclonal antibodies against the α and β subunits of rat liver asparaginase, we have shown that the human enzyme is similar in structure to the rat enzyme.

Glycosylasparaginase; Lysosome; cDNA; Aspartylglucosaminuria; Glycoprotein degradation

## 1. INTRODUCTION

Glycosylasparaginase (EC 3.5.1.26) is a lysosomal amidase that cleaves the GlcNAc-Asn bond which joins oligosaccharides to the peptide of asparagine-linked glycoproteins [1]. The enzyme requires a free α-amino and carboxyl group on the Asn [2] and any α-L-fucose at the 6-position of GlcNAc must be removed prior to GlcNAc-Asn hydrolysis [3]. Physiological and biochemical importance of asparaginase in the ordered, bi-directional degradation of asparagine-linked glycoproteins [4] is evidenced by the occurrence of the inborn lysosomal storage disease aspartylglucosaminuria when there is a deficiency of the enzyme [5]. This disorder is almost exclusively localized to Finland where it has been estimated that approx. 1:26 000 are affected and as many as 1:40 in certain areas of the country are heterozygote carriers of the defective gene [6]. The disease is characterized by high intracellular and urinary levels of aspartylglucosamine and related glycopeptide fragments. Although early development of patients with the disorder is normal, they undergo a progressive mental and physical deterioration.

We recently purified glycosylasparaginase to homogeneity from rat liver and found it to have a native molecular mass of 49 kDa and to consist of two subunits of 24 and 20 kDa [7]. Baumann et al. [8] reported human asparaginase to be 60 kDa with three subunits of 24, 18 and 17 kDA, however, we have found the human and rat enzymes to be similar in structure. In the present report we describe the cloning and sequence analysis of a complete cDNA for human glycosylasparaginase. The subunits of the enzyme are encoded by a single gene and therefore are generated post-translationally by proteolytic cleavage*.

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## 2. MATERIALS AND METHODS

### 2.1. *Biochemistry*

Rat liver glycosylasparaginase was purified as previously described [7]. Subunit-specific antisera were obtained by separating the α and β subunits by SDS/PAGE and injecting homogenized gel pieces containing a single subunit into separate rabbits. A partial CNBr digest of gel slices containing the respective rat enzyme subunits was carried out [9]. The CNRr digest was separated by SDS/PAGE and blotted onto a PVDF membrane for sequencing (The Wistar Institute Microchemistry Core Facility, Philadelphia, PA, USA). Human glycosylasparaginase was partially purified from 5 g of liver. The tissue was minced and homogenized in 15 ml 0.05 M phosphate, pH 7.0, containing 0.15 M NaCl and 2% Triton X-100. After centrifugation for 10 min at 10 000 × *g* the supernatant was run through a Concanavalin A and ACA-54 gel filtration step as described previously [7]. For immunodetection the enzyme was subjected to SDS/PAGE Phastgel 8-25% and blotted onto a PVDF-membrane by diffusion at 70°C for 3 h. The antigen was detected with a secondary antibody/alkaline phosphatase conjugate [10]. *N*-glycanase treatment and enzyme activity determination were carried out as done previously [7].

*We have noted a similar α and β subunit structure to exist for glycosylasparaginase from rat, mouse, pig and human liver, but in each case there is some variation in their masses. Therefore, we propose that the heavy and light subunits of this lysosomal amidase from the various species be referred to as α (heavy) and β (light) subunits.



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2.2. *PCR amplification and identification of a partial rat asparaginase clone*

Mixed oligonucleotide primers were synthesized (Penn State Biotechnology Institute) using amino acid sequence data from amino terminal analysis and a CNBr peptide of the β subunit of rat liver asparaginase. Deoxyinosine was substituted at selected wobble (degenerate) positions to reduce the number of primer sequence mixtures [11]. PCR reactions were performed using the GeneAmp DNA Amplification Reagent Kit (Perkin-Elmer/Cetus) according to the manufacturer's instructions with the 5′ (sense) and 3′ (antisense) primers at 0.5 μM each. The PCR template was 5 μg of first strand cDNA generated from random hexanucleotide primed rat liver poly(A)$^+$ mRNA. Thermal cycling was done in an automated heating/cooling block (DNA Thermal Cycler:Perkin-Elmer/Cetus) programmed for a temperature-step cycle of 92°C (1 min), 48°C (1 min), and 72°C (2 min). A total of 40 cycles were performed with the 72°C step extended to 15 min in the final cycle. Amplified fragments were resolved on a 10% polyacrylamide gel and either visualized with ethidium bromide or Southern blotted onto a nylon membrane (Amersham). Southern blots were hybridized with a mixed-sequence 3′-end labeled oligonucleotide probe synthesized from the amino acid sequence of a CNBr peptide predicted to reside between the sequences used for the 5′ and 3′ PCR primers. Fragments of interest were excised from the gel, electroeluted and subcloned into M13mp18/19 and pUC18 for sequence analysis and preparative growth [10].

2.3. *Northern blotting and isolation of a human glycosylasparaginase cDNA*

The PCR amplified fragment PCR/RLAsn. 1 was random primer labeled with [α-$^{32}$P]dCTP and used to probe a Northern blot of rat liver and human placenta poly(A)$^+$ mRNA. PCR/RLAsn.1 was also random primer labeled with biotin-11-dUTP and used to screen a λgt11 human placenta cDNA library (Clonetech) [10]. Phage DNA was purified from a single putative positive clone and the cDNA insert subcloned into M13mp18/19 and pUC18 for DNA sequencing and preparative growth. Nucleotide and protein sequences were analysed with the computer software DNA Strider [12].

## 3. RESULTS AND DISCUSSION

### 3.1. *Immunological and biochemical similarity of rat and human glycosylasparaginases*

Antisera against rat α and β subunits crossreacted with corresponding subunits of the human enzyme (Fig. 1). Structural similarity between human and rat asparaginases was further verified by comparing loss of enzyme activity in relation to temperature- and pH-induced dissociation of human α and β subunits in the presence of SDS as was done previously with purified rat liver enzyme [7]. The biochemical and physical behaviors of human asparaginase mimicked those of the rat in these experiments (data not shown). Two heterogeneous forms of human β subunits having masses of 17 and 18 kDa were specifically detected on a Western blot with antibody against the rat β subunit (Fig. 1, lanes 1 and 2). These appear to be the same two light subunits reported in purified human asparaginase isolated by Baumann et al. [8]. After *N*-glycanase treatment only a single band of 15.5 kDa (lane 3) was detectable. A single polypeptide having an *N*-linked oligosaccharide of variable sugar composition therefore would account for the heterogeneity in human β subunits. The human α subunit was specifically detected by antibody against rat α subunit and had a mass of 23 kDa (lanes 1 and 4) which decreased to 20.5 kDa after *N*-glycanase treatment (lane 5). Using similar biochemical and immunological methods we have so far noted a basic α and β subunit structure to be common to lysosomal glycosylasparaginase from rat, human, mouse and pig (data not shown).

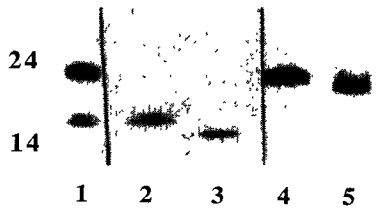


Fig. 1. Immunoblot of human glycosylasparaginase using antisera against rat enzyme subunits. A total of 0.06 U/ml of partially purified human enzyme (0.003 U/mg) was subjected to SDS/PAGE and immunoblotted as described in section 2. Lane 1: antisera against both subunits were used; 2 and 3: antiserum against β subunit; 4 and 5: antiserum against α subunit. In lanes 3 and 5 the enzyme was pretreated with *N*-glycanase.

### 3.2. *PCR amplification of a partial rat asparaginase cDNA*

The strategy that was employed to obtain a DNA fragment encoding rat asparaginase gene sequences is outlined in Fig. 2. The correct mapping of the two CNBr sequences relative to the amino terminus was accomplished by performing partial CNBr digests of the purified β (20 kDa) subunit of rat asparaginase. Based on the size difference between the undigested β subunit and CNBr-2, it was predicted that the amplified fragment should be approx. 150–200 bp in length. Although attempts to selectively amplify the sequence between the two PCR primers appeared inconclusive from ethidium bromide stained gels (i.e. numerous distinct fragments were generated), the internal probe synthesized from CNBr-1 amino acid sequence hybridized to only a single band of approx. 200 bp on Southern blot hybridization of the reaction products. This fragment, designated PCR/RLAsn.1, had an open reading frame 221 bp in length (Fig. 3). The deduced amino acid sequence from PCR/RLAsn.1 was found to correspond to the collective rat asparaginase protein sequence that aligned between the two PCR primers.

### 3.3. *Isolation and characterization of a human placenta cDNA for glycosylasparaginase*

Northern blot analysis of rat liver and human placenta poly(A)$^+$ mRNA with the amplified fragment PCR/RLAsn.1 identified an approx. 2.0 kb message in both species (Fig. 4). A second band of 1.7 kb was also observed in the rat sample that appears to be the predominant message based on signal intensities. Upon screening approx. $10^6$ pfu's of the human placenta cDNA library with the biotin-labeled PCR/RLAsn.1 fragment, only a single putative positive clone

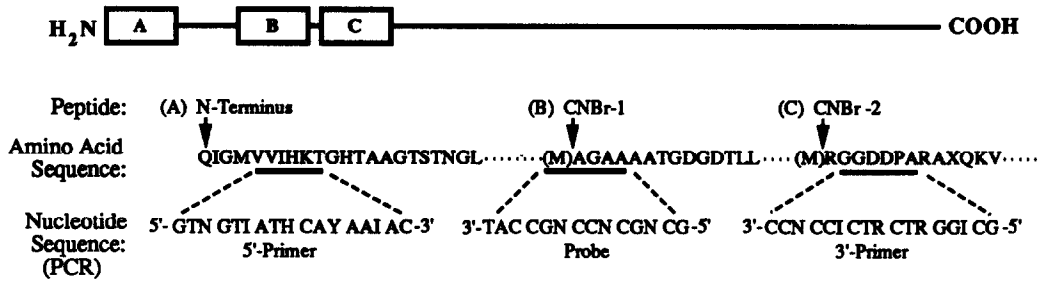


Fig. 2. Amino acid sequences from the $\beta$ subunit of rat liver glycosylasparaginase that were used for PCR amplification of a rat asparaginase cDNA. The bold underlined amino acid sequences from the N-terminus of the $\beta$ subunit of purified rat liver glycosylasparaginase (A) and one of its cyanogen bromide peptides [(C), CNBr-2] were selected to design the indicated 5'- and 3'-primers for PCR amplification of a rat cDNA fragment. The indicated DNA probe designed from a second cyanogen bromide peptide [(B), CNBr-1] was used to detect the rat cDNA fragment amplified by PCR between regions (A) and (C).

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                V  V  I  H  K  T  G  H  T  A  A  G  T  S  T  N  G  L  K  F
PCR/RLAsn.1     GTT GTG ATT CAC AAG ACG GGA CAT ACT GCT GCT GGC ACA TCT ACA AAT GGT TTA AAA TTC  60
5'-PCR Primer (5')GTN GTI ATH CAY AAI AC(3')
                V  V  I  H  K  T  G  H  T  A  A  G  T  S  T  N  G  L

                K  I  P  G  R  V  G  D  S  P  I  P  G  A  G  A  Y  A  D  D
                AAA ATA CCT GGT CGT GTA GGA GAC TCA CCA ATC CCT GGA GCT GGA GCC TAT GCT GAT GAC  120

                M  A  G  A  A  A  A  T  G  D  G  D  T  L  L  R  F  L  P  S
                ATG GCT GGA GCA GCT GCG GCC ACT GGA GAT GGT GAC ACA CTC CTG AGA TTT CTG CCA AGC  180
Internal Probe (3')TAC CGN CCN CGN CGN CG(5')
                M  A  G  A  A  A  A  T  G  D  G  D  T  L

                Y  Q  A  V  E  Y  M  R  G  G  D  D  P
                TAC CAA GCT GTA GAA TAT ATG AGA GGC GGC GAC GAC CCC GC                        221
                                        (3')CCN CCI CTR CTR GGI CG(5')  3'-PCR Primer
                                     M  R  G  G  D  D  P  A
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Fig. 3. Sequence of the PCR amplification product PCR/RLAsn.1. The PCR primer and internal probe sequences that were used are given below the sequence of PCR/RLAsn.1. The relevant protein sequence from the $\beta$ subunit of rat asparaginase that was used to construct the PCR primers and internal probe, and to establish the identity of the amplified fragment are also given.


Fig. 4. Northern blot analysis of rat and human mRNAs with PCR/RLAsn.1. mRNAs from rat liver (1.5 and 3.0 $\mu$g, lanes 1 and 2) and human placenta (1.5 $\mu$g, lane 3) were electrophoresed and probed with $^{32}$P-labeled PCR/RLAsn.1.

(λHPAsn.6) hybridized to the PCR probe in the primary and subsequent screening rounds.

The 1.2 kb cDNA insert (HPAsn.6) that was retrieved from λHPAsn.6 contained an open reading frame of 1065 bp (Fig. 5). The first in-frame methionine (ATG) encoded by HPAsn.6 was preceded by the sequence CAGGG that conforms to a concensus sequence for eukaryotic mRNAs by containing a purine in the −3 position relative to the initiation codon [13]. A stretch of amino acid (Ser-24 to Trp-44) downstream from Met-1 was colinear with the amino terminal sequence from the $\alpha$ subunit of the rat enzyme with only a single amino acid difference between the two species. The 23 residue sequence encompassing Met-1 through Cys-23 is characteristic of a eukaryotic signal sequence suggesting the site of signal peptidase cleavage to be Cys-23/Ser-24. This is supported by the weighted-matrices method of von Heijne [14].

The deduced amino acid sequence from HPAsn.6 revealed additional regions with sequence identity to rat asparaginase including the amino terminus of the $\beta$ subunit, CNBr peptide sequences from the $\alpha$ and $\beta$ subunits, and the PCR amplified fragment PCR/RLAsn.1 from the $\beta$ subunit. Of the 131 amino acids that overlap between rat asparaginase protein/cDNA sequence and the human asparaginase clone HPAsn.6,

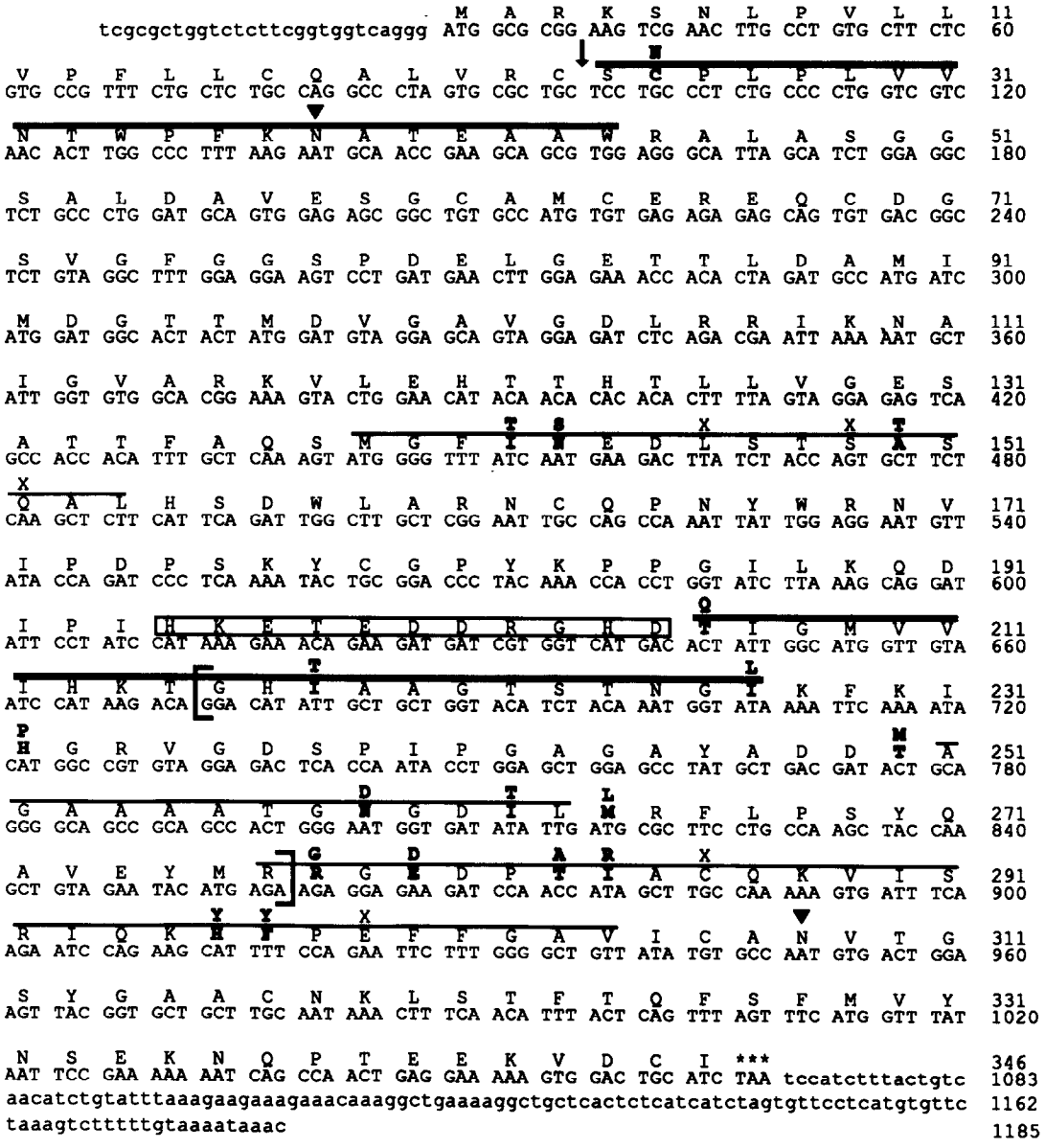

Fig. 5. Nucleotide and deduced amino acid sequence of human placenta glycosylasparaginase clone HPAsn.6. The protein coding region is given in capital letters and untranslated sequences are in lowercase letters. The deduced amino acid sequences from Ser-24 to Trp-44 and from Thr-206 to Ile-227 that are overscored with a double-line are homologous to the amino terminal sequence obtained from the $\alpha$ and $\beta$ subunits of rat liver glycosylasparaginase, respectively. The deduced amino acid sequence from Met-139 to Leu-154 that is overscored with a single line is homologous to a CNBr peptide sequence from the $\alpha$ subunit of rat glycosylasparaginase. The deduced amino acid sequences from Ala-251 to Leu-263 and from Arg-277 to Val-304 are homologous to CNBr peptide sequences obtained from the $\beta$ subunit of rat glycosylasparaginase. The sequence between the brackets is homologous to the DNA fragment PCR/RLAsn.1 that was amplified from rat liver mRNA. Residues in bold print indicate differences between the deduced amino acid sequence of HPAsn.6 and the partial rat asparaginase sequence compiled from amino terminal analysis, CNBr peptides and PCR/RLAsn.1. Amino acid residues with an X above were inconclusive during protein sequencing of the rat enzyme. Potential asparagine-linked glycosylation sites are indicated by a solid triangle. The vertical arrow shows the predicted site of cleavage by signal peptidase. The boxed sequence shows a hydrophilic region proposed to be susceptible to proteolytic cleavage.

86% of the residues are identical. The presence of HPAsn.6 sequence homologous to both the $\alpha$ and $\beta$ subunits of rat asparaginase suggests human asparaginase is encoded as a 34.6 kDa polypeptide that is subsequently processed to generate two fragments of approx. 19.5 ($\alpha$ subunit) and 15 kDa ($\beta$ subunit). The predicted site of post-translational cleavage is a stretch of 11 hydrophilic residues immediately upstream of the sequence that corresponds to the amino terminus of the $\beta$ subunit of rat asparaginase (Fig. 6). Hydrophilic regions such as this have been proposed to be susceptible to proteolytic hydrolysis in the lysosomes [15]. Two Asn-linked glycosylation sites ($Asn_{38}$ and $Asn_{308}$) encoded by HPAsn.6 are distributed such that each of the

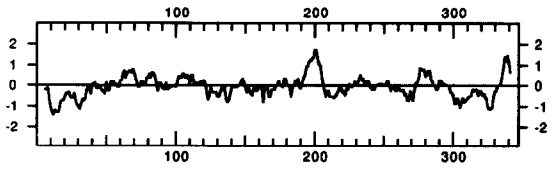


Fig. 6. Hydrophilicity plot of human glycosylasparaginase [16]. Within the hydrophilic region between amino acid residues 195–205 is the site proposed to be cleaved by cathepsins to form mature $\alpha$ and $\beta$ subunits in the lysosomes.

two subunits has the potential to be glycosylated. $Asn_{308}$ in the sequence corresponding to the 15 kDa fragment may display heterogeneity in the structure of the attached oligosaccharide thereby resulting in the two immunologically related fragments shown in Fig. 1. This information on the biochemistry and molecular biology of human glycosylasparaginase can now be used to characterize the mutation(s) responsible for aspartylglucosaminuria.

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