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CLONING, SEQUENCING, AND EXPRESSION OF cDNA FOR HUMAN β-GALACTOSIDASE

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Summary: We cloned and sequenced the full-length cDNA for human placental β-galactosidase. The 2379-nucleotide sequence contains 2031 nucleotides which encode a protein of 677 amino acids. The amino acid sequence includes a putative signal sequence of 23 amino acids and 7 potential asparagine-linked glycosylation sites. The cDNA in the expression vector pSVL was used to transfect COS cells. Expression of the cDNA in transfected COS cells produced immunoprecipitable proteins and led to an increase in β-galactosidase activity.

β-Galactosidase (EC 3.2.1.23) catalyzes the hydrolysis of terminal β-galactoside linkage in ganglioside G_{M1} and other carbohydrate-containing compounds. In human fibroblasts, an 84 kDa precursor is processed to an 88 kDa intermediate form, and then to a 64 kDa mature enzyme (1-3). This enzyme protein aggregates with a 32 kDa "protective protein" to form a high molecular weight complex in lysosomes, also involving lysosomal sialidase (4,5). A complete or partial deficiency of β-galactosidase has been observed in diseases of various molecular pathology; G_{M1}-gangliosidosis and Morquio B disease caused by gene defects for the enzyme (6-8), mucolipidosis II and III caused by defect in transport of the precursor enzyme (9,10), and galactosialidosis caused by abnormal degradation of the enzyme molecule due to a defect of the protective protein (11,12). In this communication, we report the nucleotide sequence of a full-length cDNA clone for human placental β-galactosidase and its expression in transfected COS cells.

Materials and Methods

Materials

β-galactosidase was highly purified from human placenta, and two types of specific antisera were raised as described previously (13). Restriction enzymes, T4 DNA ligase, exonuclease III, and mung bean nuclease were purchased from

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Nippon Gene (Toyama, Japan), DNA polymerase I (Klenow fragment) from Boehringer Mannheim Biochemicals (Mannheim, West Germany), the eukaryotic expression vector pSVL, M13 universal primer (17-mer), dextran sulfate, deoxy- and dideoxy-nucleotide triphosphates, and DEAE-dextran from Pharmacia (Uppsala, Sweden), horseradish peroxidase-conjugated second antibody from Bio-Rad Laboratories (Richmond, U.S.A.) and [α-^{32}P]-dATP from Amersham (Buckinghamshire, England). All other reagents used in this study were purchased from Wako Pure Chemicals (Osaka, Japan), unless indicated otherwise.

cDNA library screening

A λ-gt11 cDNA library prepared from human placenta (14) was generously provided by Dr. J. Evan Saddler (Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, U.S.A.). The cDNA library was screened, using two different rabbit antisera (13); one preparation against the β-galactosidase/protective protein complex, and the other against the β-galactosidase monomer. A positive clone GP8 recognized by both antisera was subcloned into pGEM2 (Promega Biotec; Madison, U.S.A.).

cDNA sequencing

cDNA was subcloned into SalI site of the bacteriophage M13mp19 (15) in both directions, and deletions were generated. Each clone was digested first with SacI and XbaI, and then with exonuclease III (16). Aliquots were taken every 30 seconds, and treated with mung bean nuclease. Subsequently, each DNA sample was treated with T4 DNA ligase, and used to transform JM103 to generate a library of human β-galactosidase cDNA deletions. These deletion clones were sequenced by the dideoxynucleotide chain termination method (17). When necessary, 7-deazo-dGTP was used for sequencing through GC-rich regions (18). The entire sequence was determined from the overlapping clones on both strands.

Culture and transfection of COS cells

COS-1 cells (kindly supplied by Dr. Y. Nabeshima, National Institute of Neuroscience, N.C.N.P., Tokyo, Japan) were cultured in Ham's F-10 medium (F-10) supplemented with 10% fetal calf serum (FCS) and antibiotics, and trypsinized frequently. The cDNA was isolated after SalI digestion, and was ligated into the SalI-compatible XhoI site of pSVL. The DEAE-dextran procedure followed by treatment of cells with chloroquine (19) was used to transfect COS cells (20). Subconfluent COS cells, which had been plated 12 h prior to transfection, were washed twice with F-10, and then incubated with transfection buffer (200 μg/ml DEAE-dextran, and 50 mM Tris, pH 7.4, in F-10) containing the plasmid at the concentration of 10 μg/ml for 8 h. At the end of transfection, the cells were washed with F-10 containing 10% FCS, incubated for 2 h with 100 μM chloroquine in this medium, and then incubated in the medium without chloroquine which had been heated at 65^{o}C for 2 h to inactivate serum β-galactosidase. β-Galactosidase activity was assayed with a fluorogenic substrate (4-methylumbelliferyl β-galactoside; Nakarai Chemicals, Kyoto, Japan) as described previously (21) 60 and 80 h after transfection. Protein concentration was determined by the method of Lowry et al (22).

Labeling and immunoprecipitation of human β-galactosidase from transfected COS cells

COS cells were washed 48 h after transfection, incubated for 1 h in leucine-free Eagle's Minimum Essential Medium (MEM), and labeled for 20 h with L-[4,5-^{3}H]-leucine (0.1 mCi per dish, 70 Ci/mmol; Amersham) in leucine-free MEM containing 5% dialyzed FCS (23,24). The cells were harvested, pelleted, and solubilized in 0.4 ml of detergent solution (0.1 M Tris-HCl, pH 7.5, 1% Triton X-100, 0.4 M KCl, 0.6 mM EDTA, and 0.025 mg/ml leupeptin). The samples were precleared by addition of anti-human fibronectin goat serum (5 μl; Cooper, Malvern, U.S.A.), and protein A crude cell suspension (50 μl; Sigma Chemical Co, St. Louis, U.S.A.) was added. The mixture was then incubated at 4^{o}C for 1 h, and centrifugated. Rabbit anti-human β-galactosidase antiserum (5 μl) (13) was added to each supernatant, and the samples were incubated overnight at 4^{o}C. The immunoprecipitates were collected after addition of 50 μl of the crude protein A suspension. The pellets were washed three times with 0.1 M Tris-HCl, pH 7.5, containing 0.15 M NaCl, 0.5% Triton X-100, 0.1% sodium dodecyl sulfate (SDS) and 1 mM EDTA, and then washed once with 10 mM Tris-HCl, pH 7.5.

Finally, the proteins were separated by SDS-polyacrylamide gel electrophoresis (25), and visualized by fluorography.

Results

Isolation of cDNA encoding human β-galactosidase

The anti-human β-galactosidase/protective protein complex antiserum was used to screen a λgt11 library prepared from human placental mRNA (14). Several positive clones were found. One of them, the clone GP8, expressed a fusion protein in Escherichia coli which cross-reacted with the anti-human β-galactosidase monomer antiserum.

Characterization of cDNA

The clone GP8 consisted of a 2379 nucleotide sequence, including a 34-nucleotide 5'-untranslated region, a 2031-nucleotide coding region, and a 314-nucleotide 3'-untranslated region (Fig. 1). The first ATG begins at nucleotide 35. The 3'-untranslated region contains a 3'-cleavage signal, AATAAA, which is followed 16 nucleotide later by a 21-nucleotide stretch of poly(A) sequence. The predicted molecular weight of a protein of 677-amino acid residues is 75 kDa.

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G GGC GCC GAC TGC AGA GCC GGG AGG CTG GTG GTC ATG CCG GGG TTC CTG GTT CGC ATC CTC CCT CTG TTG CTG GTT CTG CTG CTT CTG GGC CCT ACG CGC GGC TTG CGC AAT GCC ACC
                                  Met Pro Gly Phe Leu Val Arg Ile Leu Pro Leu Leu Leu Val Leu Leu Leu Leu Gly Pro Thr Arg Gly Leu Arg Asn Ala Thr

CAG AGG ATG TTT GAA ATT GAC TAT AGC CGG GAC TCC TTC CTC AAG GAT GGC CAG CCA TTT CGC TAC ATC TCA GGA AGC ATT CAC TAC TCC CGT GTG CCC CGC TTC TAC TGG AAG GAC CGG
Gln Arg Met Phe Glu Ile Asp Tyr Ser Arg Asp Ser Phe Leu Lys Asp Gly Gln Pro Phe Arg Tyr Ile Ser Gly Ser Ile His Tyr Ser Arg Val Pro Arg Phe Tyr Trp Lys Asp Arg

CTG CTG AAG ATG AAG ATG GCT GGG CTG AAC GCC ATC CAG ACG TAT GTG CCC TGG AAC TTT CAT GAG CCC TGG CCA GGA CAG TAC CAG TTT TCT GAG GAC CAT GAT GTG GAA TAT TTT CTT
Leu Leu Lys Met Lys Met Ala Gly Leu Asn Ala Ile Gln Thr Tyr Val Pro Trp Asn Phe His Glu Pro Trp Pro Gly Gln Tyr Gln Phe Ser Glu Asp His Asp Val Glu Tyr Phe Leu

CGG CTG GCT CAT GAG CTG GGA CTG CTG GTT ATC CTG AGG CCC GGG CCC TAC ATC TGT GCA GAG TGG GAA ATG GGA GGA TTA CCT GCT TGG CTG CTA GAG AAA GAG TCT ATT CTT CTC CGC
Arg Leu Ala His Glu Leu Gly Leu Leu Val Ile Leu Arg Pro Gly Pro Tyr Ile Cys Ala Glu Trp Glu Met Gly Gly Leu Pro Ala Trp Leu Leu Glu Lys Glu Ser Ile Leu Leu Arg

TCC TCC GAC CCA GAT TAC CTG GCA GCT GTG GAC AAG TGG TTG GGA GTC CTT CTG CCC AAG ATG AAG CCT CTC CTC TAT CAG AAT GGA GGG CCA GTT ATA ACA GTG CAG GTT GAA AAT GAA
Ser Ser Asp Pro Asp Tyr Leu Ala Ala Val Asp Lys Trp Leu Gly Val Leu Leu Pro Lys Met Lys Pro Leu Leu Tyr Gln Asn Gly Gly Pro Val Ile Thr Val Gln Val Glu Asn Glu

TAT GGC AGC TAC TTT GCC TGT GAT TTT GAC TAC CTC GCG TTC CTG CAG AAG CGC TTT CGC CAC CAT CTG GGG GAT GAT GTG GTT CTG TTT ACC ACT GAT GGA GCA CAT AAA ACA TTC CTG
Tyr Gly Ser Tyr Phe Ala Cys Asp Phe Asp Tyr Leu Ala Phe Leu Gln Lys Arg Phe Arg His His Leu Gly Asp Asp Val Val Leu Phe Thr Thr Asp Gly Ala His Lys Thr Phe Leu

AAA TGT GGG GCC CTG CAG GGC CTC TAC ACC ACG GTG GAC TTT GGA ACA GGC AGC AAC ATC ACA GAT GCT TTC CTA AGC CAG AGG AAG TGT GAG CCC AAA GGA CCC TTG ATC AAT TCT GAA
Lys Cys Gly Ala Leu Gln Gly Leu Tyr Thr Thr Val Asp Phe Gly Thr Gly Ser Asn Ile Thr Asp Ala Phe Leu Ser Gln Arg Lys Cys Glu Pro Lys Gly Pro Leu Ile Asn Ser Glu

TTC TAT ACT GGC TGG CTA GAT CAC TGG GGC CAA CCT CAC TCC ACA ATC AAG ACC GAA GCA GTG GCT TCC TCC CTC TAT GAT ATA CTT GCC CGT GGG GCG AGT GTG AAC TTG TAC ATG TTT
Phe Tyr Thr Gly Trp Leu Asp His Trp Gly Gln Pro His Ser Thr Ile Lys Thr Glu Ala Val Ala Ser Ser Leu Tyr Asp Ile Leu Ala Arg Gly Ala Ser Val Asn Leu Tyr Met Phe

ATA GGT GGG ACC AAT TTT GCC TAT TGG AAT GGG GCC AAC TCA CCC TAT GCA GCA CAG CCC ACC AGC TAC GAC TAT GAT GCC CCA CTG AGT GAG GCT GGG GAC CTC ACT GAG AAG TAT TTT
Ile Gly Gly Thr Asn Phe Ala Tyr Trp Asn Gly Ala Asn Ser Pro Tyr Ala Ala Gln Pro Thr Ser Tyr Asp Tyr Asp Ala Pro Leu Ser Glu Ala Gly Asp Leu Thr Glu Lys Tyr Phe

GCT CTG CGA AAC ATC ATC CAG AAG TTT GAA AAA GTA CCA GAA GGT CCT ATC CCT CCA TCT ACA CCA AAG TTT GCA TAT GGA AAG GTC ACT TTG GAA AAG TTA AAG ACA GTG GGA GCA GCT
Ala Leu Arg Asn Ile Ile Gln Lys Phe Glu Lys Val Pro Glu Gly Pro Ile Pro Pro Ser Thr Pro Lys Phe Ala Tyr Gly Lys Val Thr Leu Glu Lys Leu Lys Thr Val Gly Ala Ala

CTG GAC ATT CTG TGT CCC TCT GGG CCC ATC AAA AGC CTT TAT CCC TTG ACA TTT ATC CAG GTG AAA CAG CAT TAT GGG TTT GTG CTG TAC CGG ACA ACA CTT CCT CAA GAT TGC AGC AAC
Leu Asp Ile Leu Cys Pro Ser Gly Pro Ile Lys Ser Leu Tyr Pro Leu Thr Phe Ile Gln Val Lys Gln His Tyr Gly Phe Val Leu Tyr Arg Thr Thr Leu Pro Gln Asp Cys Se rAsn

CCA GCA CCT CTC TCT TCA CCC CTC AAT GGA GTC CAC GAT CGA GCA TAT GTT GCT GTG GAT GGG ATC CCC CAG GGA GTC CTT GAG CGA AAC AAT GTG ATC ACT CTG AAC ATA ACA GGG AAA
Pro Ala Pro Leu Ser Ser Pro Leu Asn Gly Val His Asp Arg Ala Tyr Val Ala Val Asp Gly Ile Pro Gln Gly Val Leu Glu Arg Asn Asn Val Ile Thr Leu Asn Ile Thr Gly Lys

GCT GGA GCC ACT CTG GAC CTT CTG GTA GAG AAC ATG GGA CGT GTG AAC TAT GGT GCA TAT ATC AAC GAT TTT AAG GGT TTG GTT TCT AAC CTG ACT CTC AGT TCC AAT ATC CTC ACG GAC
Ala Gly Ala Thr Leu Asp Leu Leu Val Glu Asn Met Gly Arg Val Asn Tyr Gly Ala Tyr Ile Asn Asp Phe Lys Gly Leu Val Ser Asn Leu Thr Leu Ser Ser Asn Ile Leu Thr Asp

TGG ACG ATC TTT CCA CTG GAC ACT GAG GAT GCA GTG CGC AGC CAC CTG GGG GGC TGG GGA CAC CGT GAC AGT GGC CAC CAT GAT GAA GCC TGG GCC CAC AAC TCA TCC AAC TAC ACG CTC
Trp Thr Ile Phe Pro Leu Asp Thr Glu Asp Ala Val Arg Ser His Leu Gly Gly Trp Gly His Arg Asp Ser Gly His His Asp Glu Ala Trp Ala His Asn Ser Ser Asn Tyr Thr Leu

CCG GCC TTT TAT ATG GGG AAC TTC TCC ATT CCC AGT GGG ATC CCA GAC TTG CCC CAG GAC ACC TTT ATC CAG TTT CCT GGA TGG ACC AAG GGC CAG GTC TGG ATT AAT GGC TTT AAC CTT
Pro Ala Phe Tyr Met Gly Asn Phe Ser Ile Pro Ser Gly Ile Pro Asp Leu Pro Gln Asp Thr Phe Ile Gln Phe Pro Gly Trp Thr Lys Gly Gln Val Trp Ile Asn Gly Phe Asn Leu

GGC CGC TAT TGG CCA GCC CGG GGC CCT CAG TTG ACC TTG TTT GTG CCC CAG CAC ATC CTG ATG ACC TCG GCC CCA AAC ACC ATC ACC GTG CTG GAA CTG GAG TGG GCA CCC TGC AGC AGT
Gly Arg Tyr Trp Pro Ala Arg Gly Pro Gln Leu Thr Leu Phe Val Pro Gln His Ile Leu Met Thr Ser Ala Pro Asn Thr Ile Thr Val Leu Glu Leu Glu Trp Ala Pro Cys Ser Ser

GAT GAT CCA GAA CTA TGT GCT GTG ACG TTC GTG GAC AGG CCA GTT ATT GGC TCA TCT GTG ACC TAC GAT CAT CCC TCC AAA CCT GTT GAA AAA AGA CTC ATG CCC CCA CCC CCG CAA AAA
Asp Asp Pro Glu Leu Cys Ala Val Thr Phe Val Asp Arg Pro Val Ile Gly Ser Ser Val Thr Tyr Asp His Pro Ser Lys Pro Val Glu Lys Arg Leu Met Pro Pro Pro Pro Gln Lys

AAC AAA GAT TCA TGG CTG GAC CAT GTA TGA TGATGAAAGCCTGTGTCTTTGAGGGATTCTACCCTGAACATACCTCACAGATCCTCCCTGTCATGCCACATTTCACTGATTGGAATGTGGAAATGGAAAAGGAATTTAGGATGTGCATT
Asn Lys Asp Ser Trp Leu Asp His Val *

TTCACCTGAGGTTTCCCTGCATCCCTGCAGTGCCAAAGCCCCACCTTCAGGGACCACCTGGAATGTGTGAGGGGCTGACAGCACAGTAACGTGCATACATATCTGCAGGGCTGGAATGGAAGCTTTAAAGGTGGTAGTGATTTTTATTTTGGAAGAATC

ATGTTACCTTTTTGTTAAATAAAATTTGTACTC
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Fig. 1 Nucleotide and deduced amino acid sequences of human β-galactosidase. The methionine encoded by the first ATG is designated amino acid 1. The putative signal sequence cleavage site is indicated by an arrow. The potential asparagine-linked glycosylation sites are boxed. The amino acid sequence that is homologous to monkey testis β-galactosidase and the AATAAA sequence in the 3'-untranslated region are underlined.

Table 1 Expression of human β-galactosidase in transfected COS cells

Plasmid	Cell extract		Medium	
	60 h	80 h	60 h	80 h
	unit/mg protein		total unit/dish	
pSVL	92	100	0.5	0.7
pSVL(GP8)	234	298	1.7	4.9

COS cells were transfected with pSVL or pSVL(GP8) as described in the text. β-Galactosidase activity in the cells and the culture medium was assayed 60 h and 80 h after transfection. One unit of the enzyme activity was defined as the activity releasing 1 nmol of 4-methylumbelliferone per h.

The amino acid sequence contains a 23-amino acid putative signal sequence at the NH_2 terminus. This signal sequence includes a long hydrophobic stretch of amino acids, helix-breaking residues (glycine and proline) at positions -5 and -4 from a probable cleavage site marked by an arrow in Fig. 1, a small neutral residue (threonine) at position -3, a large polar residue (arginine) at position -2, and glycine at position -1. This sequence agrees with the consensus sequence for a signal sequence cleavage site (26,27). Seven potential N-linked glycosylation sites are located at positions 26, 247, 464, 498, 542, 545, and 555. The sequence of this protein was compared with the sequences in the protein database of the National Biochemical Research Foundation (release 15, 1988). No significant protein homology was detected.

Expression and immunoprecipitation

The insert of GP8 was subcloned into the SV40 expression vector pSVL and designated pSVL(GP8). The results of experiments after transfection of COS cells with PSVL(GP8) or pSVL are summarized in Table 1. Transfection with pSVL(GP8) led to a 3-fold increase in β-galactosidase activity in the cells and the medium 60 h after transfection as compared with the enzyme activity in the cells transfected with the vector alone. β-Galactosidase activity was increased 7-fold in the medium after 80 h. Also a large increase of the immunoprecipitable protein was found in the cells transfected with pSVL(GP8) (Fig 2). Protein bands of 88, 84 and 60 kDa were detected by immunoprecipitation from the extract of the cells transfected with pSVL(GP8).

Discussion

We reported here the results of isolation and sequencing of the full-length cDNA for human placental β-galactosidase. This cDNA was expressed in COS cells with an increase of the enzyme activity and synthesis of immunoprecipitable proteins. The deduced amino acid sequence revealed some characteristic features of a lysosomal enzyme, β-galactosidase. There are a signal sequence

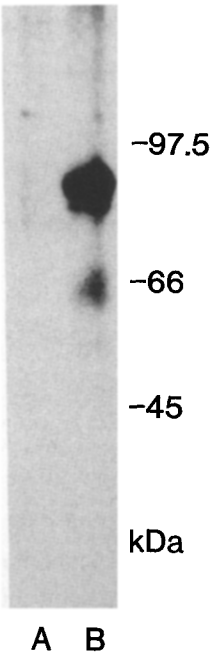

Fig. 2 Immunoprecipitation of human β-galactosidase from transfected COS cells. COS cells were transfected with pSVL (lane A) or with pSVL(GP8) (lane B), and metabolically labeled with L-[4,5-^{3}H]-leucine as described in the text. The proteins precipitated from the cell extracts with rabbit anti-β-galactosidase antiserum were separated by SDS-polyacrylamide gel electrophoresis and visualized by fluorography.

and seven potential N-linked glycosylation sites. The carbohydrate content of β-galactosidase precursor was estimated at 21-28% on the basis of the molecular mass of this molecule (28). This estimation agrees with the number of potential N-linked glycosylation sites, and accounts for an apparent discrepancy between the molecular weight of the immunoprecipitable protein (88 kDa) and that of the protein calculated from the amino acid sequence after cleavage of the signal sequence (73.5 kDa). The deduced amino acid sequence at positions 235-254 is 75% homologous with an amino acid sequence of monkey testis β-galactosidase (Try-Glu-Pro-Arg-Gly-Pro-Leu-Ile-Asn-Ser-Glu-Phe-Tyr-Tyr-Gly-Trp-Leu-Asp-Phe-Tyr) determined by Edman degradation (29).

Expression of the cloned cDNA for human β-galactosidase, GP8, led to biosynthesis of a large amount of 88 kDa and 84 kDa and a relatively small amount of 60 kDa immunoprecipitable proteins. The former two were visualized as sharp and narrow bands, but the latter always appeared as a broad band. Mature human β-galactosidase may not have been processed properly, or may be unstable and partially degraded in COS cells which are derived from monkey kidney.

Studies on patients with a human inherited metabolic disease, galactosialidosis, demonstrated the presence of a specific protein ("protective protein") for stabilization of β-galactosidase (12,13). The expression product (newly synthesized

β-galactosidase) may therefore have been degraded excessively in COS cells, due to oversaturation of the stabilizing system involving the protective protein, as the enzyme protein is expected to increase at least 100-fold in the transfected cells according to the calculation based on the theoretical efficiency of transfection. Galactosialidosis is a disease with defect in protective protein resulting in accelerated degradation of β-galactosidase molecule (30). Further experiments are necessary to elucidate this problem, including those on expression of the cloned cDNA in human cell lines.

The full-length cDNA reported here will be useful for the study of transport, processing, and stabilization of β-galactosidase, and will make it possible to clarify the pathogenesis of the disorders with β-galactosidase deficiency.

Acknowledgments

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