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Expression of a cDNA encoding the glucose trimming enzyme glucosidase II in CHO cells and molecular characterization of the enzyme deficiency in a mutant mouse lymphoma cell line

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Glucosidase II is an ER resident glycoprotein involved in the processing of N-linked glycans and probably a component of the ER quality control of glycoproteins. For cloning of glucosidase II cDNA, degenerate oligonucleotides based on amino acid sequences derived from proteolytic fragments of purified pig liver glucosidase II were used. An unamplified cDNA library from pig liver was screened with a 760 bp glucosidase II specific cDNA fragment obtained by RT-PCR. A 3.9 kb glucosidase II cDNA with an open reading frame of about 2.9 kb was obtained. The glucosidase II sequence did not contain known ER retention signals nor hydrophobic regions which could represent a transmembrane domain; however, it contained a single N-glycosylation site close to the amino terminus. All studied pig and rat tissues exhibited an mRNA of approximately 4.4 kb with varying tissue expression levels. The authenticity of the identified cDNA with that coding for glucosidase II was proven by overexpression in CHO cells. Mouse lymphoma PHAR 2.7 cells, deficient in glucosidase II activity, were shown to be devoid of transcripts.

Key words: glucosidase II/glycoprotein processing/N-glycosylation/ER quality control mechanism

Introduction

N-glycosylation of proteins starts in the lumen of the endoplasmic reticulum (ER) by *en bloc* transfer of the lipid-linked oligosaccharide precursor $Glc_3Man_9GlcNAc_2$ to nascent polypeptides. The first steps in its processing to yield the mature oligosaccharide chains of glycoproteins include the removal of all three glucose residues by neutral trimming α-glucosidases (Kornfeld and Kornfeld, 1982; Moremen and Touster, 1988; Roth, 1995). Glucosidase I removes the single terminal α 1,2-linked glucose residue, and the enzyme has been thoroughly characterized (Hettkamp *et al.*, 1984; Schweden *et al.*, 1986; Shailubhai *et al.*, 1987; Bause *et al.*, 1989; Kalz *et al.*, 1995). This is followed by the trimming of the two inner α 1,3-linked glucose residues by glucosidase II, and some of the mannose residues by ER-mannosidases (Moremen and Touster, 1988; Roth, 1995). Glucosidase II has been purified from different sources and studied extensively with regard to its properties (Grinna and Robbins, 1979; Ugalde, 1980; Burns and Touster, 1982; Saunier, 1982; Brada and Dubach, 1984; Martiniuk *et al.*, 1985; Strous *et al.*, 1987; Kaushal *et al.*, 1990). It appears to be composed of 100 kDa subunits carrying an N-linked oligosaccharide chain of high mannose-type (Burns and Touster, 1982; Brada and Dubach, 1984; Strous *et al.*, 1987). By both, immunoelectron microscopy (Lucocq *et al.*, 1986) and biochemical analyses (Burns and Touster, 1982; Brada and Dubach, 1984; Strous *et al.*, 1987), glucosidase II was shown to be a resident ER glycoprotein in hepatocytes. Interestingly, circumstantial biochemical evidence suggests that it is a loosely membrane-associated glycoprotein of the ER (Brada and Dubach, 1984; Strous *et al.*, 1987; Brada *et al.*, 1990). This is in contrast to glucosidase I, which has been shown to represent a transmembrane type II glycoprotein (Kalz *et al.*, 1995).

It has been proposed that glucosidase II plays a role in a recently discovered quality control mechanism for ER to Golgi apparatus transport of glycoproteins in which protein folding and glycosylation are intimately interrelated (Hammond *et al.*, 1994; Helenius, 1994; Hammond and Helenius, 1995). In this model, improperly folded glycoproteins will be retained in the ER prior to further transport by the concerted action of a UDP-glucose:glycoprotein glucosyltransferase, chaperones such as calnexin, calreticulin and BiP, and glucosidase II (Bergeron *et al.*, 1994; Hammond and Helenius, 1994; Hammond *et al.*, 1994; Helenius, 1994). UDP-glucose:glycoprotein glucosyltransferase represents a soluble luminal ER protein (Parodi *et al.*, 1983; Labriola *et al.*, 1995; Sousa and Parodi, 1995). A unique property of this enzyme is to distinguish between native and misfolded glycoproteins present in the ER (Trombetta *et al.*, 1989, 1991; Sousa *et al.*, 1992; Trombetta and Parodi, 1992; Fernandez *et al.*, 1994; Parker *et al.*, 1995). Thus, unfolded, partially folded and misfolded glycoproteins will be bound and reglucosylated by this transferase rendering them a ligand for calnexin (Ware *et al.*, 1995). Calnexin with its lectin-like properties will retain such monoglucosylated glycoproteins in the ER as long as they are not properly folded (Hammond *et al.*, 1994). During this process, the glycoproteins go through cycles of glucose removal by glucosidase II and reglucosylation by UDP-glucose:glycoprotein glucosyltransferase. Once the correct conformation is achieved, the glycoproteins are no more a substrate for the UDP-glucose:glycoprotein glucosyltransferase and calnexin, but solely for glucosidase II. Subsequently, they will be able to exit the ER for the Golgi apparatus. An additional component of this control mechanism, but in a post-ER location, seems to be represented by Golgi apparatus endomannosidase and calreticulin (Spiro *et al.*, 1996).

The importance of both, the UDP-glucose:glycoprotein glucosyltransferase and calnexin in this model has been directly demonstrated (Hammond *et al.*, 1994; Helenius, 1994; Labriola *et al.*, 1995). However, despite some recent indirect evidence (Labriola *et al.*, 1995), a direct proof that glucosidase II by removing an inner glucose residue added on by the glucosyltransferase is involved in the control mechanism is still missing. This is due to the lack of availability of a cDNA coding for glucosidase II.

In this article, we report on the cloning of glucosidase II from pig liver and its expression in bacteria and CHO cells. It appears that glucosidase II is neither a soluble nor a transmembrane ER glycoprotein. The cDNA encoding glucosidase II was found to share significant sequence homology with other known glucose hydrolyzing enzymes. Further, we have analyzed a mutant mouse lymphoma cell line deficient in glucosidase II activity at the molecular level.

Results

Partial amino acid sequences of pig liver glucosidase II

Glucosidase II purified to homogeneity from pig liver was digested with different proteases as described in *Material and methods.* The proteolytic fragments were separated either by reverse HPLC or by SDS–PAGE. The N-terminal amino acid sequences of the enzyme subunit and the proteolytic peptides were determined by Edman degradation (Figure 1). Degenerate oligonucleotide primers were designed according to amino acid sequences of the N-terminus of the glucosidase II subunit and the peptide 4. Inosine residues were substituted at positions of high degeneracy.

Isolation of cDNA fragments coding for glucosidase II

A 760 bp fragment from a RT-PCR product coding for glucosidase II was labeled with (^{32}P)dCTP and used to screen an unamplified oligo(dT)-primed pig liver cDNA library. Three independent overlapping cDNA clones were obtained and characterized by restriction mapping. Screening of 2 × 10^6 pfu of a random primed pig liver cDNA-library yielded additional upstream sequences. The cDNA clones obtained from the screening of the oligo dT-primed and random primed cDNA library were used to construct a full-length cDNA with an open reading frame of about 2.9 kb. The open reading frame was terminated by a stop codon TAA at position 2833 followed by a 3′ untranslated region of approximately 1 kb ending by a poly(A) tract. The consensus polyadenylation signal AATAAA was found upstream from the poly(A) sequence at position 3781.

N-term	V D R S N F K T L E E S S F X K/L R Q/V
pep 1	T X I R I D E L E
pep 2	G L L N F E H Q R
pep 3	V T E G G X P Y R L Y N L D V
pep 4	V N Q G F D D H N L P X D F
pep 5	X F T X D P X R F P Q
pep 6	D A Q H Y G G X E H R
pep 7	I S I P M X L S L G L V G L S F X G A D
pep 8	A L W V H Y P Q D V T
pep 9	G H F E T P V X I E R V V I I G A G K P
pep 10	G S P E/T S R L S F Q X D-D E/T T
pep 11	K P G V N V A S D X S I H L R

Fig. 1. Partial amino acid sequences of pig liver glucosidase II. The N-terminal amino acid sequence was determined by automated Edman degradation of purified pig liver glucosidase II. The sequences of all other peptides were obtained by Edman degradation after cleavage with proteases.

Analysis of the primary amino acid sequence

Translation of the DNA sequence into a protein sequence (Figure 2A) predicts an acidic (pI 5.5) polypeptide of 106 kb. All peptides obtained by protein sequencing were contained in this sequence indicating its authenticity. The protein sequence starts with a putative signal sequence of 32 amino acids. The signal peptidase cleavage site precedes the N-terminal peptide of glucosidase II subunit obtained by protein sequencing. The hydrophilicity plot (Figure 3) indicates that glucosidase II is a rather hydrophilic protein with no putative transmembrane domains. Further, no double lysine motif (KKXX) was detectable. The primary sequence contains one potential N-glycosylation site, Asn-Met-Th. The sequence at the C-terminus does not contain any known ER retention signal such as KDEL and its variants.

Glucosidase II is evolutionary conserved

A comparative sequence data analysis with the EMBL data bank using the Wisconsin software package revealed homology to a yeast (Z36098) and human (D42041) cDNA of unknown function. The yeast cDNA exhibited 59% similarity and 39% identity and the human cDNA 96% similarity and 92% identity to the pig glucosidase II cDNA.

Glucosidase II exhibits amino acid homology to other glucose-hydrolyzing enzymes but not to glucosidase I

A comparison of peptides 5 and 7 (see Figure 1) with protein sequences deposited in data bases indicated homology with other glucose-hydrolyzing enzymes. The primary amino acid sequence of glucosidase II was of high homology to the sequence of lysosomal α-glucosidase, sucrase-isomaltase, and several yeast glucosidases (yeast family 31 glucosidase, *Candida tsukubaensis* α-glucosidase, *Schwanniomyces occidentalis* glucoamylase). From this analysis it appears that glucosidase II is closer related to the yeast family 31 glucosidase than to sucrase-isomaltase. The homologies occur more frequently in the middle and the C-terminal part of the polypeptide than in N-terminal part (data not shown). Glucosidase II shares apparently also the sequence around the active site (DMNE) of the other glucose-hydrolyzing enzymes (Figure 2B).

Expression of glucosidase II in pig and rat tissues

Northern blot analysis of total and mRNA from pig liver revealed a message size of about 4.4 kb (Figure 4). A transcript of the same size was found in other pig (Figure 5) and rat liver as well as kidney, small and large intestine, heart, adrenal gland, brain, submaxillary and parotid gland, thymus, lung, ovary, and testis (data not shown). However, the levels of glucosidase II expression varied between the tissues. Glucosidase II mRNA was more abundant in pig liver than in brain and heart (Figure 5). This was positively correlated with the amount of enzyme protein in these tissues as detected by Western blotting (Figure 6).

Mutant mouse lymphoma PHAR 2.7 cells are transcriptionally deficient in glucosidase II

The mutant mouse lymphoma cell line PHAR 2.7 has been previously shown to be deficient in glucosidase II activity

A

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1     ATG GCG GCG GTA GCG GCA GTG GCG GCG CGT AGG AGG CGG TCT TGG ACG GGT TTG GTA CTG GCT TGT TTA GGG GTC TGC CTG GGA CTT ACC CTT GCT GTG GAT AGA AGC AAC TTT AAG ACC
      Met ala ala val ala ala val ala ala arg arg arg arg ser trp thr gly leu val leu ala cys leu gly val cys leu gly leu thr leu ala val asp arg ser asn phe lys thr   40

121   TGT GAA GAG AGT TCC TTC TGC AAG AGG CAg CGA AGC ATA CGG CCA GGC CAG TCT CCA TAC CGA GCC TTG CTG GAC TCT CTG CAG CTT GGT CCT GAT ACC CTC ACA ATC CAT CTA ATC AAC
      cys glu gln ser ser phe cys lys arg gln arg ser ile arg pro gly gln ser pro tyr arg ala leu leu asp ser leu gln leu gly pro asp thr leu thr ile his leu ile asn   80

241   GAA GTC ACC AAG GTG TTG CTG GTG CTG GAG CTC CAG GGG CTT CAA AAG AAC ATG ACT CGG ATC CGG ATT GAT GAA CTA GAG CCC CGA CGG CCC CGA TAC CGT GTG CCA GAC GTG TTG GTG
      glu val thr lys val leu leu val leu glu leu gln gly leu gln lys asn met thr arg ile arg ile asp glu leu glu pro arg arg pro arg tyr arg val pro asp val leu val   120

361   GCT GAG CCC CCC ACC GCT CGG CTT TCT GTC TCT GGC CAG GAT GAC AAC AGC GTG GAG GTA ACC GTG GCT GAG GGA CCC TAT AAA ATC ATC TTG ACC GCG CGG CCA TTC CGG CTG GAC CTG
      ala glu pro pro thr ala arg leu ser val ser gly gln asp asp asn ser val glu val thr val ala glu gly pro tyr lys ile ile leu thr ala arg pro phe arg leu asp leu   160

481   CTG GAG GAC CGC AGC CTT CTG CTC AGT GTC AAT GCC CGA GGA CTC TTA AAT TTT GAG CAC CAG AGG GCC CCC AGG GTC TCG CAA GGA TCA AAA GAC CCA GCT GAG GGC GAT GGG GCC CAG
      leu glu asp arg ser leu leu leu ser val asn ala arg gly leu leu asn phe glu his gln arg ala pro arg val ser gln gly ser lys asp pro ala glu gly asp gly ala gln   200

601   CCC GAG GAA GCA CCT GGG GAT GGA GAC AAG CCA GAG GAG ATC CAG GGG AAG GCA GAG AAA GAT GAG CCA GGA GCC TGG GAA GAG ACA TTC AAA ACT CAC TCT GAC AGC AAG CCC TAT GGC
      pro glu glu ala pro gly asp gly asp lys pro glu glu ile gln gly lys ala glu lys asp glu pro gly ala trp glu glu thr phe lys thr his ser asp ser lys pro tyr gly   240

721   CCC ACG TCT GTG GGT TTG GAT TTC TCT CTG CCA GGC ATG GAA CAT GTG TAT GGG ATC CCC GAG CAT GCA GAC AGC CTG AGG CTA AAA GTC ACT GAG GGT GGG GAT CCA TAT CGC CTC TAC
      pro thr ser val gly leu asp phe ser leu pro gly met glu his val tyr gly ile pro glu his ala asp ser leu arg leu lys val thr glu gly gly asp pro tyr arg leu tyr   280

841   AAT TTG GAC GTG TTC CAG TAT GAG CTG TAC AAC CCC ATG GCC CTG TAC GGC TCC GTG CCT GTG CTC CTG GCA CAC AGC CCT CAC AGG GAC CTG GGC ATC TTC TGG CTC AAC GCT GCA GAG
      asn leu asp val phe gln tyr glu leu tyr asn pro met ala leu tyr gly ser val pro val leu leu ala his ser pro his arg asp leu gly ile phe trp leu asn ala ala glu   320

961   ACC TGG GTT GAC ATA TCC TCC AAC ACT GCA GGG AAG ACC CTG TTT GGG AAG ATG CTG GAC TAC CTA CAG GGC TCT GGG GAG ACC CCA CAG ACA GAT GTT CGC TGG ATG TCG GAG AGC GGC
      thr trp val asp ile ser ser asn thr ala gly lys thr leu phe gly lys met leu asp tyr leu gln gly ser gly glu thr pro gln thr asp val arg trp met ser glu ser gly   360

1081  ATC ATC GAT GTC TTC CTG CTC CTT GGG CCC TCC GTC TTC GAT GTC TTC CGG CAG TAC GCT AGT CTC ACT GGG ACC CAG GCA TTG CCC CCG CTC TTC TCC CTC GGC TAC CAC CAG AGC CGC
      ile ile asp val phe leu leu leu gly pro ser val phe asp val phe arg gln tyr ala ser leu thr gly thr gln ala leu pro pro leu phe ser leu gly tyr his gln ser arg   400

1201  TGG AAC TAT CGA GAT GAG GCC GAT GTC CTG GAA GTT AAT CAG GGC TTT GAT GAT CAC AAC CTG CCC TGT GAT TTC ATC TGG CTG GAC ATC GAG CAT GCT GAT GGC AAG CGG TAC TTC ACC
      trp asn tyr arg asp glu ala asp val leu glu val asn gln gly phe asp asp his asn leu pro cys asp phe ile trp leu asp ile glu his ala asp gly lys arg tyr phe thr   440

1321  TGG GAC CCC AGC CGC TTC CCC CAG CCC CGC ACC ATG CTT GAG CAC TTG GCC TCT AAG AGG CGC AAG CTG GTA GCC ATT GTG GAC CCT CAT ATC AAG GTG GAC TCC AGC TAC CGC GTA CAT
      trp asp pro ser arg phe pro gln pro arg thr met leu glu his leu ala ser lys arg arg lys leu val ala ile val asp pro his ile lys val asp ser ser tyr arg val his   480

1441  GAA GAG TTG CAG AAC CTG GGT CTG TAT GTT AAA ACC CGG GAT GGC TCT GAC TAT GAG GGC TGG TGC TGG CCA GGT GCA GCT AGT TAC CCT GAT TTT ACC AAT CCC AAG ATG AGA GCC TGG
      glu glu leu gln asn leu gly leu tyr val lys thr arg asp gly ser asp tyr glu gly trp cys trp pro gly ala ala ser tyr pro asp phe thr asn pro lys met arg ala trp   520

1561  TGG GCT GAC ATG TTT CGC TTT GAG AAT TAC GAG GGC TCA TCT TCC AAC CTC TAT GTC TGG AAT GAC ATG AAC GAA CCG TCC GTG TTC AAT GGC CCT GAG GTC ACC ATG CTC AAG GAT GCC
      trp ala asp met phe arg phe glu asn tyr glu gly ser ser ser asn leu tyr val trp asn asp met asn glu pro ser val phe asn gly pro glu val thr met leu lys asp ala   560

1681  CAG CAT TAT GGG GGC TGG GAG CAC CGA GAC CTG CAC AAC ATC TAT GGC TTC TAC GTG CAC ATG GCA ACT GCT GAC GGG CTG GTG CTG CGC TCT GGG GGT GTA GAA CGG CCC TTT GTC CTG
      gln his tyr gly gly trp glu his arg asp leu his asn ile tyr gly phe tyr val his met ala thr ala asp gly leu val leu arg ser gly gly val glu arg pro phe val leu   600

1801  AGC AGG GCT TTC TTC GCT GGC TCC CAG CGC TTT GGA GCC GTG TGG ACT GGG GAC AAC ACT GCT GAA TGG GAC CAT TTG AAG ATC TCT ATC CCT ATG TGT CTC AGC TTG GGG CTG GTG GGA
      ser arg ala phe phe ala gly ser gln arg phe gly ala val trp thr gly asp asn thr ala glu trp asp his leu lys ile ser ile pro met cys leu ser leu gly leu val gly   640

1921  GTT TCC TTC TGT GGA GCG GAT GTG GGT GGC TTC TTC AAA AAT CCA GAG CCA GAG CTG CTT GTG CGC TGG TAC CAG ATG GGT GCT TAC CAG CCA TTC TTC CGG GCA CAT GCC CAT TTG GAC
      val ser phe cys gly ala asp val gly gly phe phe lys asn pro glu pro glu leu leu val arg trp tyr gln met gly ala tyr gln pro phe phe arg ala his ala his leu asp   680

2041  ACT GGT CGG CGA GAG CCG TGG CTG TTA CCG ACT CAG TAC CAG GAC ATG ATC CGA GAT GCC CTG GGC CAG AGA TAC TCC TTA TTG CCC TTC TGG TAC ACT CTC TTC TAT CAG GCC CAT CGC
      thr gly arg arg glu pro trp leu leu pro thr gln tyr gln asp met ile arg asp ala leu gly gln arg tyr ser leu leu pro phe trp tyr thr leu phe tyr gln ala his arg   720

2161  GAA GGC GTT CCT GTC ATG AGG GCC CTG TGG GTG CAT TAT CCT CAG GAC GTG ACG ACC TTC AGT ATA GAT GAC GAG TTC CTG CTT GGG GAT GCA CTG CTG GTT CAC CCT GTA ACG GAC TCT
      glu gly val pro val met arg ala leu trp val his tyr pro gln asp val thr thr phe ser ile asp asp glu phe leu leu gly asp ala leu leu val his pro val thr asp ser   760

2281  GAG GCA CAT GGC GTG CAG GTC TAT CTG CCG GGC CAA GGG GAG GTG TGG TAC GAT GTT CAC AGC TAC CAG AAG TAT CAT GGT CCG CAG ACC CTG TAC CTG CCT GTA ACT CTA AGC AGC ATC
      glu ala his gly val gln val tyr leu pro gly gln gly glu val trp tyr asp val his ser tyr gln lys tyr his gly pro gln thr leu tyr leu pro val thr leu ser ser ile   800

2401  CCT GTG TTC CAG CGC GGA GGG ACC ATT GTG CCC CGA TGG ATG CGA GTG CGG CGT TCC TCA GAC TGC ATG AAG GAC GAC CCC ATC ACT CTC TTC GTT GCA CTC AGT CCC CAG GGT ACA GCC
      pro val phe gln arg gly gly thr ile val pro arg trp met arg val arg arg ser ser asp cys met lys asp asp pro ile thr leu phe val ala leu ser pro gln gly thr ala   840

2521  CAA GGA GAG CTC TTT CTC GAC GAT GGG CAC ACA TTC AAC TAT CAG ACT GGC CAT GAG TTC CTG CTG CGT CGA TTC TCA TTC TCT GGC AAC ACC CTT GTC TCC AGC TCA GCA GAC TCC AAA
      gln gly glu leu phe leu asp asp gly his thr phe asn tyr gln thr gly his glu phe leu leu arg arg phe ser phe ser gly asn thr leu val ser ser ser ala asp ser lys   880

2641  GGC CAC TTT GAG ACA CCT GTC TGG ATT GAG CGG GTG GTG ATA ATA GGG GCT GGA AAG CCA GCA ACT GTG GTA CTC CAG ACA AAA GGA TCT CCT GAA AGC CGC CTG TCC TTC CAG CAT GAC
      gly his phe glu thr pro val trp ile glu arg val val ile ile gly ala gly lys pro ala thr val val leu gln thr lys gly ser pro glu ser arg leu ser phe gln his asp   920

2761  CCT GAG ACC TCT GTG TTG ATC CTG CGC AAG CCT GGC GTC AAT GTG GCA TCC GAC TGG AGC ATT CAC CTG CGA TAA CCC ATG GGA TGT TGG GAT GGG GTG CGG TGG TGA TTG AGA GTT ACA
      pro glu thr ser val leu ile leu arg lys pro gly val asn val ala ser asp trp ser ile his leu arg ***

2881  CTT CCT TCT GCC TTG GAG TTT GAC CTT CCC CAG ACT TCA CCT TTC TTA TGC CGA CGT CTG GGC AGG GTG AGA GGG CTG TCC CTG GGC TTG AAT TCC TTT TGT GAC CTG ATC TCT CCC ACC
3001  CCA CTG ACA CCA GAT CTC CCT TCA TCT CCC AGT ACT CTG TTG CTC GAA CTG GAG CAC AAT CAC CTG TGA AGA CCT GGG GAC CAT AGG GCC CTT GCT TTC CCT CCT CTT TTC TTT CTT TTG
3121  GGG GCC CTG AAT CTC CTC CAG ACC CTC TCC ATT CAT GTC TCT TGT GTG TTG ATG CCA TTT CTT GGA AGA AGA TAA GGG CAG TGA GCT TTA GGG CTG CTT TTC TCC TTC CCC TTC CCT CCC
3241  CAC CGA ACT GCT CTC CCT CCT TTT ATT TCT TCC TCT GTC AGC CCT TCC CTT TTA ATG CCC CAT CGA TAC ACT GGG ACC ACC CCT TAC CTG ATG AGG GAT GAA TGG ATC ACA GGA GTG AGG
3361  TTG CTG GAA AAC GTC CTC TTC CCT GGC TCC CAA CTT TTC CTC TCC CCG CTT CTT TCT AGA GCT GCT GCA GTT CTG ACA GGG GCA GTT CTA CCT CCC CTG TCC TTT GGG GGA AGG AAG TTT
3481  CCA CTC CCT GAG AGG GGA TAA ACA AAA CTT CTC TTG CCT CCT AAA ATT TTG TCC CGT TGA GGG GCA TTC AAG ATG GAG AAA TCA GTT GTG GTT TCA TCG AAT CAC GGT CAT CTG TAT TTA
3601  TTG CTG GGA GAA GGC TGA GGG TGG GGG AGA GAT GAT CAT GTG CGC CCA GGG GTG GCC CAA AGC CCT GGG TAG GGG GTG GGG TGG GCA AGG ACT AAC TGG GGG CGA GGG GGA ATA TTT GTG
3721  GGA ATT TTT TTT ACT TCC TCT TGG CCT CCA GCC GTG ACA GGT TTT GAT AAA AGG AGA AAC AAT AAA AGA GAT AAA CCA TAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA A
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Fig. 2. (A) Nucleotide sequence of pig liver glucosidase II and the deduced amino acid sequence. Numbers on the right site show the amino acid residues from 1 to 944 in the ORF, numbers on the left the nucleotide sequence. All sequenced peptides are underlined (dotted line). The possible N-glycosylation site (bold letters) and the polyadenylation signal AATAAA (double underlined) are marked. The GenBank accession number for the pig liver glucosidase II is U71273.

B

pig liver glucosidase II	- - - N Y E G S S S N L Y V W N	D M N E	P S V	F	- - - - - - - N G P E V T M L K	558
human cDNA(alpha-glucosidase-related)	- - - N Y E G S A P N L F V W N	D M N E	P S V	F	- - - - - - - N G P E V T M L K	575
H. sapiens lysosomal glucosidase	- V A E F H D Q V P F D G M W I	D M N E	P S N	F	T R G S E D - - G C P N N E L E	539
S. cerevisiae glucosidase	- - - D L P A D L T N L F I W N	D M N E	P S I	F	- - - - - - - D G P E T T A P K	553
S. pombe glucosidase	G S N Y S Y D L - P F S G L C L	D M N E	P T S	F	C I G S C G - - - - - - - - - -	514
S. tsukunbaensis glucosidase	- - - - - - E I V D F S G I W L	D M N E	P S S	F	V I G N A A - - - - - - - - - -	539
S. occidentalis glucoamylase	- - K D W Y E L T P F D G I W A	D M N E	V S S	F	C V G S C G - - - - - - - - - -	483
H. sapiens sucrase-isomaltase	- C S I F H Q E V Q Y D G L W I	D M N E	V S S	F	I Q G S T - - K G C N V N K L N	526
O. cuninculus sucrase-isomaltase	- C N I F H Q E V N Y D G L W I	D M N E	V S S	F	V Q G S N - - K G C N D N T L N	526
R. norvegicus sucrase-isomaltase	- C N L F H Q Q V E Y D G L W I	D M N E	V S S	F	I Q G S L N L K G V L L I V L N	537

Fig. 2. (B) Comparison of the amino acid sequences of glucosidase II with other glucose-hydrolyzing enzymes. Aligned pig liver glucosidase II, human glucosidase II (accession number D42041) *H.sapiens* lysosomal glucosidase (Y00839), *S.cerevisiae* glucosidase (Z36098), *S.pombe* glucosidase (Z67961, Z69728), *S.tsukunbaensis* glucosidase (X56024), *S.occidentalis* glycoamylase (M60207), *H.sapiens* sucrase–isomaltase (X63597, M22616), *O.cuninculus* sucrase–isomaltase (M14046), *R.norvegicus* sucrase–isomaltase (L25926, M62889) around the active site. Boxes are placed around the amino acids identical in all 10 sequences.

(Reitman, 1982). Western blot analysis of electrophoretically resolved protein extracts from the parental BW5147 line and the mutant PHAR 2.7 cells showed absence of an immunoreactive band in the mutant cells (Figure 7A). Moreover, by Northern blot analysis of total RNA, mRNA encoding glucosidase II could not be detected in the mutant cells (Figure 7B).

Expression of glucosidase II in bacteria, CHO cells and mutant mouse lymphoma PHAR2.7 cells

Extracts of transformed bacteria and of stably transfected CHO cells were assayed for glucosidase II activity. In bacteria, no enzymatic activity could be measured although glucosidase II immunoreactivity was detectable by Western blotting (not shown). In CHO cells transfected with pcDNA3-glu II, the enzymatic activity was increased up to threefold compared to untransfected and mock-transfected cells (Table I) and this was associated with enzyme protein amounts (Figure 8). Transfection of mutant PHAR2.7 cells with a vector containing the cDNA for glucosidase II failed.

Discussion

Glucosidase II plays a key role in the processing of N-linked oligosaccharide chains of glycoproteins and seems to be involved in the ER quality control mechanism of glycoproteins. Using degenerated oligonucleotides based on the amino acid sequences from purified pig liver glucosidase II, a 1.2 kb cDNA fragment could be amplified by RT-PCR, which was used for the screening of a pig liver cDNA library to obtain a full-length clone. Six different clones were identified, and two of them were used for the construction of a full-length cDNA consisting of a single open reading frame of about 2.9 kb which coded for the entire glucosidase II enzyme. Several lines of evidence indicated the authenticity of the cDNA clone. All peptides obtained by protein sequencing were encoded in the open reading frame. Further, expression of the cDNA in CHO cells resulted in threefold overexpression of an active enzyme. Western blot analysis showed an immunoreactive band at about 100 kDa, which is in agreement with our earlier biochemical data (Brada and Dubach, 1984). Notably, expression in bacteria resulted in the synthesis of enzymatically inactive, glucosidase II immunoreactive protein.

The open reading frame encodes a polypeptide of 944 amino acids with a pI of 5.5 and a deduced molecular mass of 106 kDa. Further, the potential N-glycosylation site is apparently used *in vivo* since glucosidase II was shown to carry a single N-linked oligosaccharide of the high mannose-type (Strous *et al.*, 1987). The N-terminus contains an arginine-rich, cleavable signal sequence of 32 amino acid residues. This signal sequence is unusual in its length but contains the recognition site for the signal peptidase. Kyte and Doolittle hydropathy plot analysis of the sequence did not reveal any hydrophobic region which could serve as transmembrane domain. Further, the absence of a double lysine motif at the C-terminus (Jackson *et al.*, 1990) and of a double arginine motif at the N-terminus (Schulze *et al.*, 1994) characteristic of ER transmembrane proteins, strongly indicates that the purified glucosidase II protein is not a proteolytic fragment (Brada and Dubach, 1984). On the other hand, the C-terminal part of the deduced amino acid sequence did not contain the KDEL ER retention signal or

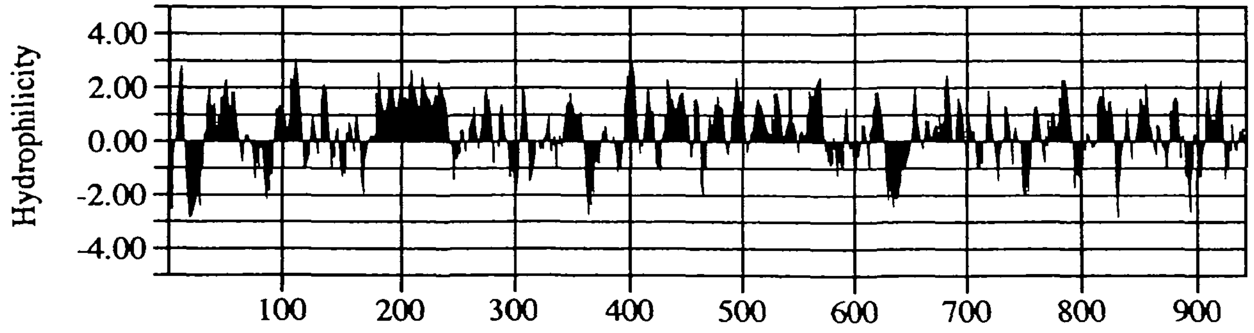

Fig. 3. Hydrophilicity plot of the amino acid sequence of glucosidase II. The hydrophobicity profile was calculated by the Kyte–Doolittle method with a window size of seven amino acids (Kyte and Doolittle, 1982).

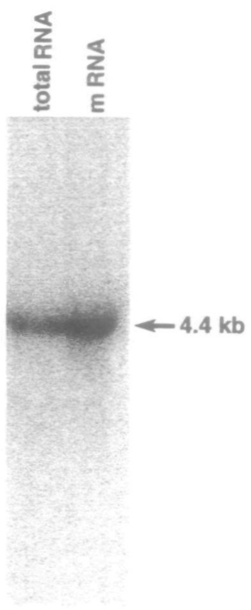

Fig. 4. Northern blot analysis of pig liver. Northern blots containing 50 μg of total and 1 μg mRNA from pig liver were hybridized with a radiolabeled glucosidase II cDNA fragment revealing a message size of approximately 4.4 kb.

variants thereof characteristic of soluble ER glycoproteins (Munro and Pelham, 1987; Pelham, 1995). This is in good accordance with our earlier biochemical results indicating that glucosidase II is a loosely membrane-associated, luminally oriented glycoprotein (Brada and Dubach, 1984). The recent successful cloning of glucosidase I clearly demonstrated this first acting trimming enzyme to be a transmembrane type II glycoprotein (Kalz *et al.*, 1995). In contrast, glucosidase II, the second acting trimming enzyme, seemingly belongs to another class of ER resident proteins and may be retained by a different mechanism. Another example for such a type of glycoprotein is lysyl hydroxylase (Kellokumpu *et al.*, 1994) an enzyme involved in collagen processing, which neither contains a C-terminal KDEL sequence nor a double lysine motif (Hautala *et al.*, 1992). The mechanism by which glucosidase II and lysyl hydroxylase are retained in the ER is unknown. It is tempting to speculate that this is achieved upon interaction with another ER protein and experiments will be performed to clarify this important aspect. When our manuscript was under review, we became aware of the work of Trombetta *et al.* (1996) on glucosidase II purified from rat liver. These authors reported presence of two tightly bound proteins in the glucosidase II activity containing fractions obtained from MonoQ column with apparent molecular weights of 110 kDa and 80 kDa, respectively. They proposed that glucosidase II is an heterooligomer with the larger protein being catalytically active glucosidase II. The smaller, noncatalytic protein contained the HDEL sequence and was proposed to be responsible for glucosidase II ER retention. Under our experimental conditions, we never observed copurification of such an 80 kDa protein from pig kidney and liver (Brada and Dubach, 1984; M.Ziak, unpublished observations). All our evidence points to glucosidase II purified from these tissues as being composed of identical subunits of 100 kDa. It remains to be demonstrated if the 80 kDa HDEL containing rat liver protein represents truly a glucosidase II subunit (Trombetta *et al.*, 1996) or rather a protein generally

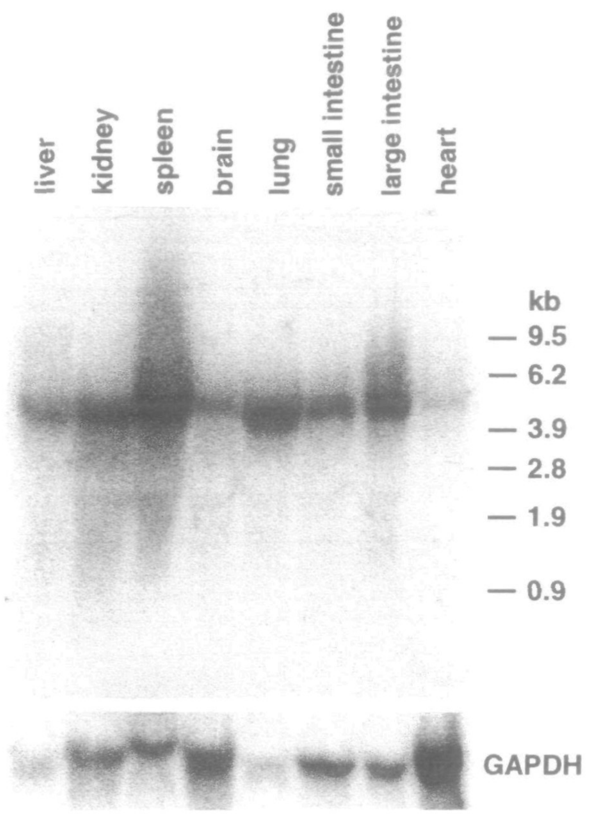

Fig. 5. Northern blot analysis of pig tissues. Northern blots containing 50 μg of total RNA from various pig tissues were hybridized with a radiolabeled glucosidase II cDNA fragment and showed a transcript of the same size as in liver. The expression levels varied in the different tissues.

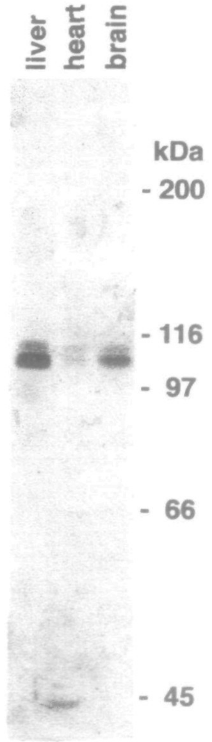

Fig. 6. Western blot analysis of pig tissues. Western blot analysis of electrophoretically resolved extracts (100 μg) from liver, heart, and brain revealed differences in glucosidase II protein amounts.

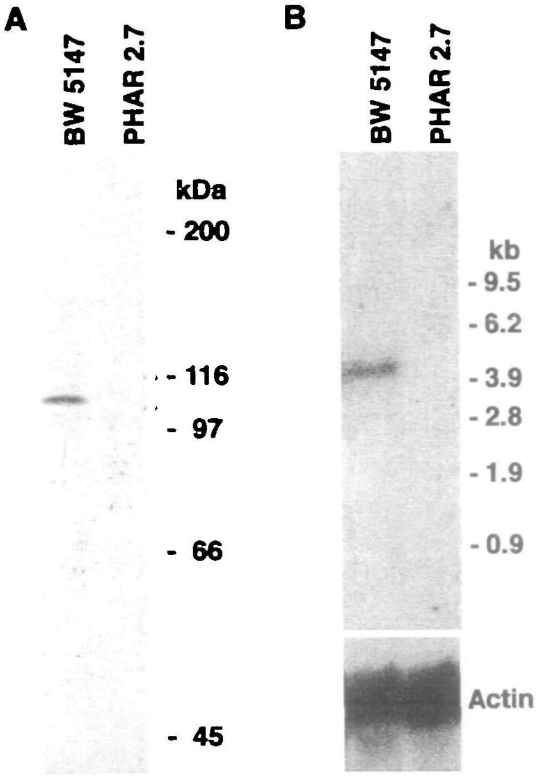

Fig. 7. Mutant lymphoma cell line PHAR 2.7 is deficient in glucosidase II protein and message. The mutant mouse lymphoma cell line PHAR 2.7 has been previously shown to be deficient in glucosidase II activity. (**A**) Western blot analysis of electrophoretically resolved extracts from the parental BW 5147 cells shows a glucosidase II immunoreactive band that is undetectable in the mutant PHAR 2.7 cells. (**B**) Northern blot analysis of total RNA reveals the presence of mRNA encoding glucosidase II in the parental BW 5147 cells which is absent in the mutant PHAR 2.7 cells.

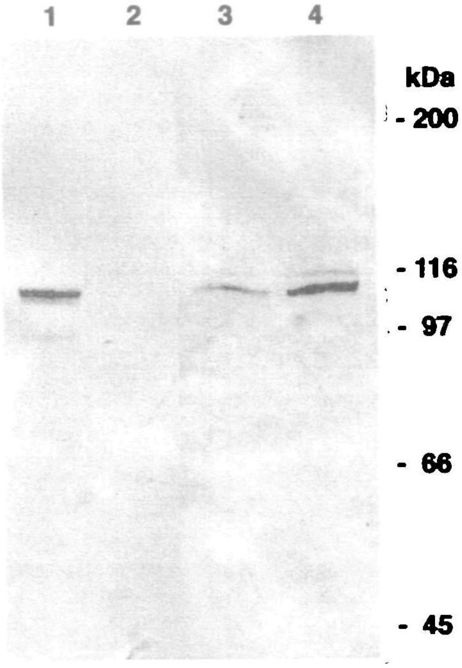

Fig. 8. Overexpression of glucosidase II in CHO cells. Extracts of CHO cells transfected with pcDNA3-gluII (lane 1, 10 μg protein) or pcDNA3 (lane 2, 10 μg protein) and extracts from pig liver (lane 3, 10 μg protein; lane 4, 50 μg protein) were analyzed by Western blotting.

functioning in the ER retention of glucosidase II, lysyl hydroxylase, and related yet unknown ER proteins.

Computer assisted comparison of neutral trimming glucosidase II amino acid sequence with other deposited sequences revealed that this enzymes is highly conserved from yeast to mammals. Furthermore, it revealed a striking homology to lysosomal acidic α-glucosidase, sucrase-isomaltase, and several yeast glucosidases. The sequence similarity between lysosomal α-glucosidase and both subunits of the intestinal sucrase-isomaltase enzyme complex was demonstrated previously (Hoefsloot *et al.*, 1988). Apparently, the enzymes comprise a group whose members contain conserved single amino acids or clusters throughout the sequence. Homologous amino acids are present most frequently in the middle and C-terminal parts of the sequences. The homology is low in the N-terminal part apparently reflecting the fact that the enzymes are located in different cellular organelles. Comparison of the deduced amino acid sequence of glucosidase I (Kalz *et al.*, 1995) with that of glucosidase II failed to reveal sequence similarities. Likewise, the mammalian α-mannosidases exhibited no sequence similarities (Bischoff *et al.*, 1990; Moremen and Robbins, 1991; Bause *et al.*, 1993). Thus, the two trimming glucosidases, which differ in their substrate specificity, are apparently coded for by evolutionary unrelated genes. Currently, nothing is known about the sequence of the active site of glucosidase II. The active sites of the lysosomal α-glucosidase and the sucrase-isomaltase have been shown to consist of the sequence DMNE. Glucosidase II contains this amino acid sequence, and it is very likely that it represents part of the active site. This can now be tested by *in vitro* site-directed mutagenesis.

Table I. Glucosidase II activity in cell lysate

		GluII-activity mU/mg protein
CHO-K1 wt[a]		21.3
CHO-pcDNA4[a]	Clone 23	24.1
	Clone 24	20.1
	Clone 25	22.0
	Clone 26	21.2
	Clone 27	17.5
CHO-pcDNA3-GluII[a]	Clone 2	24.6
	Clone 4	31.9
	Clone 6	38.6
	Clone 8	36.9
	Clone 10	32.7
	Clone 12	50.4
	Clone 14	28.3
	Clone 16	31.0
	Clone 18	35.9
	Clone 20	34.1

[a] CHO-K1 wt represents a polyclonal cell population and the transfected cell lines are clonal.

Glucosidase II has been implicated in the ER quality control for newly synthesized glycoproteins but thus far the evidence is indirect. The availability of the full length cDNA will permit investigations to directly prove that the removal of a single glucose residue by glucosidase II is an essential element of the ER quality control system for glycoproteins.

Nonetheless, the availability of a glucosidase II cDNA has already allowed to define the molecular basis of the enzyme deficiency in the mutant mouse lymphoma PHAR2.7 cells. It is expected that it will assist in the clarification of the cell-type-specific variation in glucosidase II subcellular distribution (Brada *et al.*, 1987). In contrast to liver hepatocytes (Lucocq *et al.*, 1986), various kidney tubular epithelia exhibited immunolabeling for glucosidase II additionally in the Golgi apparatus, the plasma membrane and a system of vesicular structures involved in exo- and endocytosis (Brada *et al.*, 1987). Enzymatically active and sialylated glucosidase II was detected in plasma membrane (brush border) fractions, and evidence could be obtained for a ligand for glucosidase II present in this location.

Materials and methods

Preparation of protein sequence data

Glucosidase II from pig liver was purified to homogeneity as described by (Brada and Dubach, 1984) with modifications and using an FPLC system (Pharmacia, Uppsala, Sweden). The purified enzyme was digested by trypsin according to the manufacturer's instructions (Boehringer, Mannheim, Germany) and the tryptic fragments separated by reverse-phase HPLC. In addition, partially purified glucosidase II was resolved by SDS–PAGE (4–15% gradient gels), transferred to PVDF-membrane and digested with endoprotease glu C (Boehringer, Mannheim, Germany). The purified peptides were subjected to automated Edman degradation.

Cloning of the cDNA

Standard techniques were performed as described (Sambrook, 1989). Protein sequences of the N-terminus of glucosidase II subunit and of peptide 4 were chosen for preparation of the sense and antisense degenerate oligonucleotides 5′-GTIGAT/CC/AGIA/TG/CIAAT/CTTT/CAAA/GACIC/TTIGAA/GGA-3′ and 5′-GGIAA/GA/GTTA/GTGA/GTCA/GTCA/GAAICCT/CTGA/GTT-3′, respectively). The degenerate primers were used in PCR amplification with a first-strand cDNA template obtained from pig liver poly(A) mRNA using Superscript II reverse transcriptase (GibcoBRL). A 1.2 kb PCR product was subcloned into pBluescript KS (Stratagene, La Jolla, CA) and sequenced using an automated sequencer from ABI. A (^{32}P) labeled PstI-PstI fragment of the PCR-product (760 bp) was then used to screen an unamplified pig liver cDNA library. The library prepared in Uni-ZAP XR vector (Zap-cDNA Synthesis Kit, Stratagene, La Jolla, CA) was oligo(dT) primed; 10^6 pfu were screened and four independent clones of different sizes isolated. The clones were characterized by restriction mapping. Overlapping cDNA fragments were sequenced on both strands. Additional 5′ end sequences upstream from the already received cDNA sequence were obtained by screening 2×10^6 pfu of a random primed Uni-ZAP library. The sequence data were analyzed and compared with the EMBL and the Swissprot data banks using the Wisconsin software package (Genetics Computer Group, Madison, WI).

Northern blot analyses

Total RNA was isolated by the single step method (Chomczynski and Sacchi, 1987) or with TRI Reagent (Chomczynski, 1993). Isolation of mRNA, preparation of formaldehyde gels, labeling of cDNA fragments, and hybridizations in 50% formamide were carried out according to standard protocols (Sambrook, 1989). "Oligolabeling" was performed according to Feinberg and Vogelstein (1983).

Western blot analysis and glucosidase II activity measurement

SDS–PAGE, Western blotting and enzyme activity measurements were performed as described previously (Brada and Dubach, 1984). For Western blotting, rabbit polyclonal antibodies raised against the denatured enzyme subunit were used (Brada and Dubach, 1984; Lucocq *et al.*, 1986).

Expression of the cDNA coding for glucosidase II in E.coli

The full-length cDNA was subcloned into EcoRI site of the expression vector pGEX-4T-1 (Pharmacia, Uppsala, Sweden). Expression of the fusion protein, purification and digestion with thrombin were performed according to the manufacturer's instructions.

Cell culture and transfection of CHO and PHAR2.7 cells

The cell lines BW5147 and PHAR2.7 were kindly provided by Dr. I. Trowbridge (Salk Institute, San Diego, CA) and were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C and 10% CO_2. CHO-K1 cells were obtained from ATCC.

The full-length cDNA coding for glucosidase II was constructed as follows. The cDNA fragment starting at the single PvuII site and containing the whole 3′ end region inclusive poly(A) tail was ligated together with a cDNA fragment lying upstream of the PvuII site into the expression vector pcDNA3 (pcDNA3-gluII). The cDNA fragment containing this PvuII site and the upstream 5′ end region was generated by RT-PCR. Both the mutant PHAR2.7 cells and CHO-K1 cells were transfected with pcDNA3-glu II using the lipofectamine method (Hawley-Nelson, 1993).

CHO cells were transfected with 5μg of either pcDNA3 or pcDNA3-gluII using 50 μl of lipofectamine according to standard protocol. Selection on G418 (1.5 μg/μl) was started 3 days after transfection. Clones were isolated using cloning rings (Sigma)

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