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Two Isoforms of Trimming Glucosidase II Exist in Mammalian Tissues and Cell Lines but Not in Yeast and Insect Cells

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We previously cloned glucosidase II and provided *in vivo* evidence for its involvement in protein folding quality control. DNA-sequencing of different clones demonstrated the existence of two isoforms of glucosidase II which differed by 66 nucleotides due to alternative splicing. The existence of two enzyme isoforms in various organs of pig and rat as well as human, bovine, rat, and mouse cell lines could be demonstrated by RT-PCR and Western blotting. Furthermore, the two isoforms of glucosidase II could be detected in embryonic and postnatal rat kidney and liver. In yeast, *Saccharomyces cerevisiae*, and in insects, Drosophila S2 cells, only one isoforms of the enzyme was detectable. The ubiquitous occurrence of the two glucosidase II isoforms in mammalian tissues and cell lines might be indicative of a special function of each isoform.

Key Words: **glucosidase II; enzyme isoforms; protein quality control; endoplasmic reticulum; *N*-glycosylation; yeast; insect; mammalian tissue.**

N-glycosylation of proteins is initiated in the endoplasmic reticulum (ER) with the transfer of a lipid-linked precursor, dolichol pyrophosphate $Glc_3Man_9(GlcNAc)_2$, to an asparagine residue of nascent polypeptide chains. Subsequently, ER located trimming glucosidase I and glucosidase II, respectively, remove the terminal α1,2-linked glucose residue and the two α1,3 glucose residues of newly synthesized glycoproteins to obtain the $Man_9(GlcNAc)_2$ structure (1, 2). In addition, ER α-mannosidase I can trim the four α1,2-linked mannose residues to yield the $Man_5(GlcNAc)_2$ structure (2, 3). Trombetta *et al.* (4) reported that glucosidase II from rat liver is composed of a catalytic α-subunit and a tightly bound noncatalytic HDEL-containing β-subunit. Further evidence for such a subunit composition of glucosidase II was obtained in *S. pombe* by the disruption of the gene either encoding glucosidase II α or glucosidase II β (5).

Glucosidase II, in concert with UDP-glucose:glycoprotein glucosyltransferase and chaperones, and ER mannosidase I have been shown to be involved in protein folding control and degradation of misfolded glycoproteins, respectively (for review; 6, 7). In harmony with the involvement of glucosidase II in such a fundamental cellular control mechanism, enzyme activity and immunoreactivity have been detected in yeast and mammalian cells (8–10).

Recently, we isolated the cDNA encoding glucosidase II by screening a pig liver cDNA library (11). DNA-sequencing demonstrated the existence of two isoforms of pig liver glucosidase II. An extra sequence of 22 amino acid residues was inserted at position 188 of pig liver glucosidase II (11) due to alternative splicing. We have separately expressed both isoforms of pig liver glucosidase II in a glucosidase II-deficient yeast strain (12) under the GAL1 promoter in pYES2 in order to test if both isoforms were enzymatically active. Both isoforms were detectable by Western blotting and exhibited enzymatic activity with the artificial substrate 4-methylumbelliferyl-α-D-glucopyranoside (8).

Here, we report the ubiquitous occurrence of the two isoforms of glucosidase II in various adult rat and pig organs as well as embryonic and postnatal rat tissues and cell lines of human, bovine, mouse and rat origin. In insect cells and *Saccharomyces cerevisiae*, however, only one isoform was detectable.

MATERIALS AND METHODS

Materials. Frozen embryonic day 20, postnatal days 2, 7, 14 and adult rat kidneys and livers were purchased from Pel Freez Biologicals (Rogers, AR). Dulbecco's modified Eagle medium (DMEM), Schneider's Drosophila medium, fetal bovine serum (FBS), Taq polymerase from Life Technologies (Basel, Switzerland), protease inhibitor cocktail tablets from Roche Diagnostics (Rotkreuz, Switzerland) and TRI reagent from Lucerna Chem (Luzern, Switzerland). AMV reverse transcriptase, random primers and dNTPs were purchased

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from Promega (Wallisellen, Switzerland), Omniscript reverse transcriptase and QIAquick PCR-purification kit from QIAGEN (Basel, Switzerland). Yeast nitrogen base without amino acids, casamino acids were from Difco Laboratories (Basel, Switzerland). Alkaline phosphatase-conjugated goat anti-rabbit IgG, and goat anti-mouse IgG (all affinity-purified) were purchased from Jackson Immuno-Research Laboratories, Inc. (West Grove, PA).

Cell lines. The mouse lymphoma cell lines BW5147 and PHAR2.7 were kindly provided by Dr. I. Trowbridge (Salk Institute, San Diego, CA) and Drosophila S2 cells were obtained from Dr. W. G. Gehring (Biozentrum, University of Basel, Switzerland). Human HepG2, rat liver clone 9 and BRL 3A and bovine MDBK cells were obtained from American Type Culture Collection (Manassas, VA).

Strains and medium. The following yeast strains were used: *S. cerevisiae* SS328 which is wild type for glucosidase II: MATα ade2-101 ura3-52 his 3Δ200 lys2-801. YG427 which is a glucosidase II deficient strain: MATα ade2-101 ura3-52 his 3Δ200 lys2-801 Δ gls2::KanMX (12). *S. cerevisae* were grown at 30°C in YPD medium (2% Bacto-Peptone, 1% yeast extract, 2% glucose). Drosophila cells were grown in Schneider's Drosophila medium at 25°C.

RT-PCR. Total RNA from frozen embryonic, postnatal and adult rat organs as well as from the various mammalian cell lines, insect cells and yeast were isolated with the TRI reagent (13). Total RNA was reverse transcribed by AMV reverse transcriptase using random oligonucleotide primers. The 20 μl reaction mixture contained 1 μg of RNA, 20 U of RNAsin, 0.5 μg of random primer, 1 mM of each dNTP, 5 mM $MgCl_2$, and 20 U of AMV reverse transcriptase in the supplied reverse transcription buffer. Alternatively, cDNA was synthesized using total RNA, omniscript reverse transcriptase and random oligonucleotide primers following the protocol of the manufacturer. A 5 μl aliquot of the reaction mixture was used as template in the subsequent PCR-amplification with the following species specific glucosidase II oligonucleotides (A–C, mammalian tissues and cell lines; D–F, *S. cerevisiae;* G–I, Drosophila): oligonucleotide A, 5′-CAAGAGGCAGCGAAGCATAC; oligonucleotide B, 5′-TACCTC-CACGCTGTTGTCAT; oligonucleotide C, 5′-CTCTGCCTTCCCCTG-GATCTCCTCT; oligonucleotide D, 5′-TAACAAGGAAAACCAT-CACC; oligonucleotide E, 5′-TTCACCGAGACCACCAGCAC; oligonucleotide F, 5′-GAAAAGTCCAGAGCCACAGC; oligonucleotide G, 5′-AGAGAATGCCAACCAACAGC; oligonucleotide H, 5′-TGTCTGCAGTGGAGTTTGAA; oligonucleotide I, 5′- AGAACCCAT-GAAAGAGAAAT. The PCR was performed in a reaction volume of 100 μl containing 1.5 mM $MgCl_2$, 0.5 μM of each primer, 200 μM of each dNTP and 2.5 U of Taq polymerase. The cycling parameters were set as follows: 60 s at 94°C (1 cycle), then 60 s at 94°C, 60 s at the appropriate annealing temperature and 60 s at 72°C (30 cycles) followed by a final extension at 72°C. PCR products derived from pig organs were separated using a 5% polyacrylamide TBE gel and silver stained. All other PCR products were resolved in 2% agarose gel containing ethidium bromide. For DNA-sequencing PCR-products were separated in 2% agarose gels and the bands were excised and purified using the QIAquick PCR-purification.

SDS-PAGE and immunoblotting. Tissues were homogenized at 4°C in PBS (pH 7.4) containing protease inhibitors and proteins extracted with 1% Triton X-100 for 1 h at 4°C. Cell debris was removed by centrifugation at 10,000*g* for 10 min at 4°C. The extracts were mixed with Laemmli buffer and boiled for 5 min at 100°C (14). Proteins (150 μg/lane) were resolved by SDS–PAGE using 7.5% polyacrylamide gels. Protein concentrations were determined by the Bradford procedure (15). The gels were transferred to nitrocellulose membranes using a semi-dry blotting apparatus. For immunoblotting, the membranes were conditioned with PBS containing 1% defatted milk powder and 0.05% Tween 20 for 1 h at room temperature, incubated with rabbit anti-pig liver glucosidase II antibodies (10) for 18 h at 4°C, followed by alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies (0.12 μg/ml). Color reaction was performed using nitroblue tetrazolium/BCIP-phosphate as substrates.

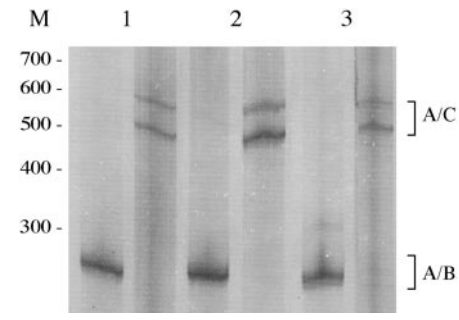

FIG. 1. Existence of two isoforms of glucosidase II in various adult pig organs. RNA from kidney (lane 1), liver (lane 2), and brain (lane 3) was isolated and random primed cDNA prepared. PCR amplification using primer pair A/C, flanking the variably expressed fragment, demonstrates the existence of two glucosidase II isoforms. Primer pair A/B was used as internal control. PCR-products were visualized by silver staining.

RESULTS

Cloning the cDNA encoding pig liver glucosidase II (11) proved the existence of two isoforms of the enzyme due to alternative splicing. The sequence of the two enzyme isoforms were identical with the exception of an extrapeptide sequence, FSDKVSLTLGSIWDKIKN-LFSR, inserted between position 188 and 209. Western blot analysis of protein extracts from pig liver, heart, and kidney showed two immunoreactive bands for glucosidase II (11). The existence of two isoforms of glucosidase II in pig brain and kidney, in addition to pig liver, was demonstrated by RT-PCR, using flanking primers A/C. As an internal standard for the PCR-amplification, we amplified a product upstream of the extra sequence using the primers A/B which resulted in a PCR-product of 279 bp (Fig. 1).

Analysis of protein extracts from rat pancreas, spleen, kidney, liver, thymus, brain, placenta, lung, and testis revealed the presence of two immunoreactive bands for glucosidase II with an apparent molecular mass of 107 kDa and 112 kDa (Fig. 2A). In order to exclude that the lower immunoreactive band seen in Western blots was due to degradation, RNA from the various rat organs was prepared and reverse transcribed using AMV reverse transcriptase. PCR-amplification using the primers A/C, flanking the variably expressed fragment always generated two DNA-fragments of 517 bp and 583 bp, respectively, demonstrating the presence of two mRNA for glucosidase II. Two PCR products could be detected in all studied tissues when omniscript reverse transcriptase was used for cDNA synthesis followed by PCR amplification (data not shown), indicating that the PCR products were not dependent on reverse transcriptase reaction. Furthermore, we analyzed embryonic, postnatal and adult rat kidney and liver to establish whether the expression of the two isoforms was devel-

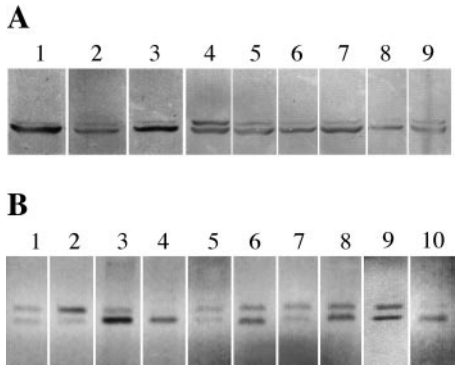

FIG. 2. Two isoforms of glucosidase II exist in various rat organs. (A) Western blot analysis of protein extracts (150 μg/lane) from pancreas (lane 1), spleen (lane 2), kidney (lane 3), liver (lane 4), thymus (lane 5), brain (lane 6), placenta (lane 7), lung (lane 8), and testis (lane 9) revealed two immunoreactive bands. (B) RT-PCR amplification was performed using the primer pair A/C and reverse-transcribed RNA isolated from testis (lane 1), adrenal gland (lane 2), thymus (lane 3), spleen (lane 4), pancreas (lane 5), placenta (lane 6), stomach (lane 7), heart (lane 8), small intestine (lane 9), and ovary (lane 10). PCR-Products were separated in a 2% agarose gel and visualized by ethidium bromide staining.

opmentally regulated. Embryonic day 20 and postnatal day 7 kidneys showed a high amount of the long glucosidase II isoform, whereas in the adult kidney the short isoform was prominently present (Fig. 3A). Both isoforms of glucosidase II could be detected in embryonic, postnatal and adult rat liver (Fig. 3B). The short and the long PCR products obtained from embryonic day 20, postnatal day 7 and adult rat kidney were subjected to DNA sequencing. The amino acid sequence of the extrapeptide, FSDKVSLTLGSIWDKIKNLFSR, as deduced from the DNA sequence of the PCR product was identical in all samples. In various cell lines such as human HepG2, rat liver clone 9 and

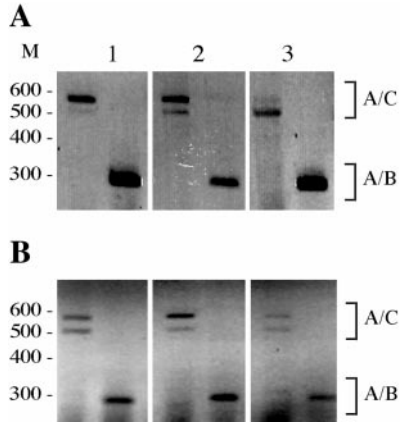

FIG. 3. Existence of two isoforms of glucosidase II in embryonic, postnatal, and adult rat kidney and liver. Random primed cDNA prepared from RNA from kidney (A) or liver (B) was amplified using primers A/C: embryonic day 20 (lane 1), postnatal day 7 (lane 2), and adult (lane 3). Primer pair A/B was used as internal control.

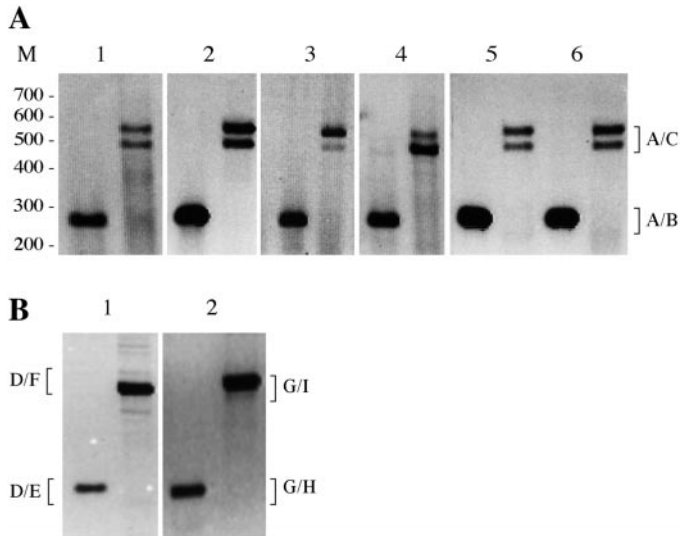

FIG. 4. (A) Two isoforms of glucosidase II are detectable in human, bovine, mouse, and rat cell lines. RNA was extracted and reverse transcribed using random primers. PCR amplification was performed using the primers A/C and A/B as a control. Human HepG2 (lane 1), rat clone 9 (lane 2), rat BRL 3A (lane 3), bovine MDBK (lane 4), mouse BW5147 (lane 5), and mouse PHAR2.7 cells (lane 6). (B) One isoform of glucosidase II is detectable in yeast and insect cells. Reverse transcribed cDNA, prepared from RNA isolated from *S. cerevisiae* SS328, was amplified using the primers D/F and D/E as control (lane 1). RNA from Drosophila S2 cells was isolated and random primed cDNA prepared. PCR amplification using primer pair G/I demonstrates the existence of one glucosidase II isoform. Primer pair G/H was used as internal control (lane 2).

BRL 3A, bovine MDBK and mouse BW5147 cells the existence of the two isoforms of glucosidase II could be established as well. Like the parental BW5147 cells, the glucosidase II-deficient mutant cell line PHAR2.7 also showed the presence of two PCR products (Fig. 4A). This indicates that at least the 5′ end of the glucosidase II gene is present in this mutant cell line. Furthermore, the RT-PCR assay was used to investigate whether the two glucosidase II isoforms are also present in *S. cerevisiae* and Drosophila S2 cells (Fig. 4B). Although both contain glucosidase II activity, the gene coding for gls2 has been only identified in *S. cerevisiae* (GenEMBL Accession No. Z36098). In *S. cerevisiae* SS328, one isoform of glucosidase II could be detected when the primers D/F were used. As expected, in the glucosidase II deficient yeast strain, YG427, no PCR-product could be detected, using the primers mentioned above (data not shown). Using the amino acid sequence derived from the pig liver glucosidase II (8) we searched the Drosophila gene data bank and identified an open reading frame of Drosophila with a sequence identity of 46% (GenEMBL Accession No. AE003122). Based on this DNA sequence, we designed the Drosophila specific oligonucleotides G, H and I, which were used for PCR amplification. In Drosophila S2 cells one DNA-fragment of 486 bp was detected

using the primers G/I. These data demonstrate, that in *S. cerevisiae* and Drosophila S2 cells only one isoform of glucosidase II is present.

DISCUSSION

The results of the present study provide strong evidence for the widespread occurrence of two isoforms of glucosidase II. Due to alternative splicing, an additional peptide of 22 amino acid residues is inserted into position 188 of pig liver glucosidase II (8, 11, and present study). The additional peptide did not show any significant homology to known protein sequences. Not unexpected, the amino acid sequence of the peptide identified in pig liver was identical to the amino acid sequence found in embryonic, postnatal and adult rat kidneys. Recently, Arendt *et al.* (16) reported the existence of splice variants of glucosidase II in mouse T lymphoma cells. Interestingly, the additional peptide detected in pig liver as well as in embryonic, postnatal and adult rat kidney showed 90% identity to a variably expressed segment, box A1, in the mouse T lymphoma cells (16). During the preparation of our manuscript, Pelletier *et al.* (17) reported the existence of two isoforms of the α subunit of human glucosidase II differing in 22 amino acid residues. The extrapeptide found in human glucosidase II (17) showed 95% identity to the extrapeptide of the pig and rat enzyme (8, 11, and present study).

We designed species specific oligonucleotides, which were used to amplify a possible extrapeptide sequence of glucosidase II in *S. cerevisiae* and Drosophila. However, in *S. cerevisiae* and in Drosophila S2 cells only one isoform of glucosidase II was detectable, indicating that the two isoforms of the enzyme must have arisen later in evolution.

Glucosidase II is ubiquitously expressed in mammalian tissues (4, 11, 18). However, the expression level may vary as demonstrated for various pig tissues by Northern and Western blot analysis (11). Two immunoreactive bands migrating at about 107 and 112 kDa were detectable when extracts of pig tissues were analyzed by Western blotting. The intensity of the 107 kDa band was always stronger than the 112 kDa band (11). In agreement with our observations, immunoblot analysis of crude microsomes prepared from pig brain, rat liver, calf liver, and monkey kidney cells using an anti-pig liver glucosidase II antibody showed two reactive bands with a molecular mass of 107 and 112 kDa (18). These data, together with the results obtained by RT-PCR, might indicate that the two immunoreactive bands represent the two isoforms of glucosidase II.

Glucosidase II sequentially cleaves the two inner α1,3-linked glucose residues. However, it is noteworthy that these glucose residues are either α1,3-linked to glucose, or α1,3-linked to mannose. Furthermore, pulse chase studies demonstrated that the removal of the outermost of the two glucose residues occurs faster than the removal of the inner one (19). Three major questions arise. First, are both isoforms enzymatically active; second, do they have different substrate specificity; and third, are there differences in their kinetics? During the purification of glucosidase II from rat liver (20) or pig liver (11) it was not possible to separate the glucosidase II isoforms by their physical properties. Therefore, the measured enzymatic activity could not be attributed to one of the isoforms. In order to answer the first question we have expressed the cDNA encoding either the long or short isoform of pig liver glucosidase II in a glucosidase II-deficient *S. cerevisiae* strain. Both isoforms showed enzymatic activity, using the artificial substrate 4-methylumbelliferyl α-D-glucoside (8). Expression of $\alpha1/\beta$ and $\alpha2/\beta$ isoforms of human glucosidase II in Sf9 insect cells and subsequent enzyme purification resulted in a heteroenzyme exhibiting *p*-nitrophenyl α-D-glucopyranoside activity (17). Both isoforms, α1 and α2, showed the same specificity for the $Glc_2Man_9GlcNAc_1$ and $Glc_1Man_9GlcNAc_1$ substrates (17). Although Pelletier *et al.* (17) have purified the recombinant human glucosidase II by Ni^{2+}-NTA and anion exchange chromatography, obviously the possibility that the recombinant human α subunit can form a complex with the endogenous glucosidase II of Sf9 cells cannot be excluded. It has been shown that in pig kidney (10) and pig liver (18) glucosidase II exists as a tetrameric complex under native conditions. Therefore, the measured glucosidase II activities and isoform specificity of human glucosidase II expressed in Sf9 cells (17) might not be unequivocally attributed to the recombinant enzyme.

The two glucosidase II isoforms exist in embryonic, postnatal and adult mammalian tissues. Furthermore, the sequence of the additional 22 amino acid residues is highly conserved in rat, pig and human. Thus, it is tempting to speculate that this high level of conservation might be indicative of different functions of each isoform. It has been speculated that the additional peptide might modulate protein-protein interactions such as the association between the α- and the β-subunit of glucosidase II or an interaction of the extrapeptide with other ER proteins or substrates (16). Recently, Arendt *et al.* (21) demonstrated a strong interaction between glucosidase II and CD45 in mouse T cells. Subsequently, the same authors demonstrated in transfection experiments that the glucosidase isoform containing the alternatively spliced sequence Box A1 is capable of binding to CD45 (22).

Monoglucosylated oligosaccharides play an important role with regard to folding of proteins present in the ER (6, 7). Experiments are now in progress to reveal the possible functional role of the two isoforms of glucosidase II in this quality control mechanism and their subcellular distributions.

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