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# The heterodimeric structure of glucosidase II is required for its activity, solubility, and localization *in vivo*

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**Glucosidase II is an ER heterodimeric enzyme that cleaves sequentially the two innermost α–1,3-linked glucose residues from *N*-linked oligosaccharides on nascent glycoproteins. This processing allows the binding and release of monoglucosylated ($Glc_1Man_9GlcNAc_2$) glycoproteins with calnexin and calreticulin, the lectin-like chaperones of the endoplasmic reticulum. We have isolated two cDNA isoforms of the human α subunit (α1 and α2) differing by a 66 bp stretch, and a cDNA for the corresponding β subunit. The α1 and α2 forms have distinct mobilities on SDS–PAGE and are expressed in most of the cell lines we have tested, but were absent from the glucosidase II-deficient cell line PHA$^R$ 2.7. Using COS7 cells, the coexpression of the β subunit with the catalytic α subunit was found to be essential for enzymatic activity, solubilization, and/or stability, and ER retention of the α/β complex. Transfected cell extracts expressing either α1 or α2 forms with the β subunit showed similar activities, while mutating the nucleophile (D542N) predicted from the glycoside hydrolase Family 31 active site consensus sequence abolished enzymatic activity. In order to compare the kinetic parameters of both α1/β and α2/β forms of human glucosidase II the protein was expressed with the baculovirus expression system. Expression of the human α or β subunit alone led to the formation of active human/insect heteroenzymes, demonstrating functional complementation by the endogenous insect glucosidase II subunits. The activity of both forms of recombinant human glucosidase II was examined with a *p*-nitrophenyl α-D-glucopyranoside substrate, and a two binding site kinetic model for this substrate was shown. The $K_{M1\text{-}2}$ values and apparent $K_{i1\text{-}2}$ for deoxynojirimycin and castanospermine were determined and found to be identical for both isoforms suggesting they have similar catalysis and inhibition characteristics. The substrate specificities of both isoforms using the physiological oligosaccharides were assessed and found to be similar.**

*Key words:* calnexin cycle/ER quality control apparatus/glycan processing/N-linked glycosylation

## Introduction

Nascent glycoproteins entering into the ER are substrates for oligosaccharyltransferase which transfers from a dolichol-phosphate precursor a $Glc_3Man_9GlcNAc_2$ glycan to a Asn-X-Ser/Thr sequon. The α-glucosidases of the endoplasmic reticulum (ER) then sequentially hydrolyze the three terminal glucoses on the *N*-linked oligosaccharide. Glucosidase I cleaves the first α-1,2-linked glucose of the glycan structure, then glucosidase II sequentially cleaves the two inner α-1,3-linked glucoses to yield $Man_9GlcNAc_2$ (Grinna and Robbins, 1979, 1980; Burns and Touster, 1982). The intermediate of this reaction ($Glc_1Man_9GlcNAc_2$) is specifically bound by the ER lectin-like chaperones calnexin and calreticulin (Ou *et al.*, 1992; Bergeron *et al.*, 1994; Hammond and Helenius, 1994; Rodan *et al.*, 1996; Zapun *et al.*, 1997; Cannon and Helenius, 1999). Then ERp57, a protein disulfide isomerase which is specific for monoglucosylated glycoproteins, associates with calnexin and calreticulin, and catalyses disulfide bond exchange on the lectin bound glycoproteins (Zapun *et al.*, 1998). The interaction between the glycoprotein and the lectin is abrogated upon cleavage of the last glucose residue by glucosidase II. If the protein has not attained its native conformation, it is then recognized and reglucosylated by the ER enzyme UDP-glucose glycoprotein:glucosyl transferase (UGGT) (Sousa *et al.*, 1992; Sousa and Parodi, 1995; Tessier *et al.*, 2000), which allows rebinding to calnexin and calreticulin. However, if correct folding is achieved, the nascent glycoprotein escapes recognition by UGGT and leaves the ER to continue its maturation along the secretory pathway. This process has been termed “The Calnexin Cycle” (Hammond and Helenius, 1995; Zapun *et al.*, 1999).

Inhibitors of the ER α-glucosidases have been useful in elucidating the effects of glycan processing on the maturation of secretory and membrane glycoproteins (Ou *et al.*, 1992; Kearse *et al.*, 1994; Balow *et al.*, 1995; Hebert *et al.*, 1995; Ora and Helenius, 1995; Flura *et al.*, 1997). While not all glycoproteins have been shown to interact with calnexin/calreticulin, impaired association with these lectins can lead to minor

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structural changes and loss of function, or ultimately to retro-translocation through the translocon and degradation in the cytosol by the ubiquitin-proteasome pathway (Brodsky and McCracker, 1997; Kopito, 1997; Sommer and Wolf, 1997). In earlier studies with cultured cells (Karpas *et al.*, 1988; Taylor *et al.*, 1988; Block *et al.*, 1994; Fischer *et al.*, 1995; Zitzmann *et al.*, 1999), and more recently with animal model systems (Block *et al.*, 1998; Mehta *et al.*, 1998), α-glucosidase inhibitors have shown also to inhibit viral propagation by blocking viral envelope glycoprotein-ER lectin associations (Elbein, 1991; Mehta *et al.*, 1998).

Glucosidase II has been described both biochemically and genetically as a heterodimer with the α subunit displaying sequence homology with the glycoside hydrolase Family 31 (Trombetta *et al.*, 1996; Arendt and Ostergaard, 1997; Hentges and Bause, 1997; D'Alessio *et al.*, 1999). The α subunit contains the catalytic domain, which is characterized by the consensus sequence (G/F)-(L/I/V/M)-W-X-D-M-N-E (Henrissat and Bairoch, 1993), while the β subunit is presumably involved in ER localization through its C-terminal HDEL sequence. Although mostly localized to the ER, Brada *et al.* (1990) found glucosidase II in endocytic structures beneath the plasma membrane as well as associated with vacuoles in kidney brush border cells. These authors were the first to postulate that there might be two forms of the enzyme. Recently, Arendt and Ostergaard (1997) found glucosidase II associated with CD45, a transmembrane protein-tyrosine phosphatase (PTP) in SAKR mouse T-lymphocyte cell line. In the same study, they cloned a cDNA for the α subunit that encodes an additional 22 amino acids stretch not found in the available human sequence (GenBank accession number D42041). More recently, the same authors reported the existence of several isoforms of the murine α subunit originating from alternative splicing (Arendt *et al.*, 1999). It was suggested that these isoforms differ in their activity, regulation, and possibly in their protein–protein interactions (Arendt *et al.*, 1999).

The β subunit of glucosidase II was originally identified as the protein kinase C substrate 80 K-H (Hirai and Shimizu, 1990). It is characterized by its own ER signal sequence, EF-hand high-affinity calcium binding loops, two glutamic acid repeats in tandem and a putative ER retention motif HDEL (Arendt and Ostergaard, 1997; Arendt *et al.*, 1999; Trombetta *et al.*, 1996). Work by Trombetta *et al.* (1996) has shown that the α and β subunits copurify while attempts to separate the α and β chains under conditions that preserve enzymatic activity were unsuccessful. Alternatively, however, Flura *et al.* (1997) observed a variable increase in glucosidase II activity when they expressed a pig liver cDNA encoding the α subunit alone in CHO cells. Furthermore, Hentges and Bause (1997) had pig liver glucosidase II preparations both with and without a detectable 60 kDa component. Thus the requirement of the β subunit and the functional nature of the heterodimeric glucosidase II complex remained to be established.

The current kinetic model for *p*-nitrophenyl α-D-glucopyranosidase (*p*-NP-Glcase) and maltase activities of glucosidase II, proposed by Alonso *et al.* (1991), 1993b), suggests the existence of both high and low affinity substrate binding sites. This model was supported by the earlier work of Hubbard and Robbins (1979), which reported different rates of glucose hydrolysis from N-linked $Glc_2$- versus $Glc_1Man_9GlcNAc_2$ oligosaccharides. Moreover, bromoconduritol has been shown to only inhibit the hydrolysis of the $Glc_1$ form of the oligosaccharide (Datema *et al.*, 1982; Alonso *et al.*, 1993a). There are two possible explanations for this model, the first being based on the proposed two substrate binding sites. However, with the discovery of α subunit splice variants, the possibility that these activities are properties of distinct isoforms is raised. Previous results could be due to heterogeneous enzyme preparations which are responsible for the differential activities. We can now test this hypothesis with genetically purified isoforms of glucosidase II.

We report here the isolation of human cDNAs encoding for the α1 (short form), α2 (long form), and β subunits of glucosidase II. A role of the β subunit was sought through expression in COS7 cells, while a heterologous expression system was developed to perform a detailed comparison of the recombinant α1/β and α2/β isoforms, based on their kinetic parameters for the *p*-nitrophenyl α-D-glucopyranoside substrate, and their substrate specificities for the physiological oligosaccharides.

## Results

### *Cloning of human α1 and α2 subunits of glucosidase II*

The cDNAs encoding the α and β subunits of glucosidase II were respectively isolated from HeLa cells and human lymphocytes. The full-length cDNA obtained for the human α subunit was essentially identical to the human partial open reading frame (GenBank no. D42041), though it included a start codon and a tyrosine to histidine substitution at position 850. Furthermore, an isoform with a 66 base pair insertion (corresponding to 22 amino acids, GenBank no. AF144074) was isolated. This insertion encodes the amino acid sequence FSDKVNLTLGSIWDKIKNLFSR and has 86% identity to the previously published mouse sequence (GenBank no. U92793) (Arendt and Ostergaard, 1997). Also found in human lymphocytes, it is located in the N-terminal region of the α chain. The insertion contains a potential N-glycosylation site that is not present in the mouse gene.

Based on the human 80K-H cDNA sequence (GenBank J03075), the cDNA encoding the β subunit was amplified from human lymphocyte mRNA. The amplified DNA fragment was identical to the published sequence except for an additional glutamic acid at position 314 (AF144075). Ophoff *et al.* (1996) have shown five polymorphisms for this gene, one of which results from altering the simple trinucleotide repeat length (GAG) encoding glutamic acid stretches. The observed alleles of (GAG)n varied from n = 8 to n =11. The cDNA isolated has 10 GAG repeats (n = 10).

### *α1 and α2 isoforms originate from a single gene*

The 66 nucleotide insertion found in the α2 isoform provides an additional *Xmn*I restriction site. Thus a 220 bp *Xmn*I/*Bam*HI fragment from the human α2 cDNA was used to probe *Xmn*I/*Bam*HI digested genomic DNA, isolated from human lymphocytes or mouse ES cells. If more than one gene had been responsible for the isoforms of this enzyme, a *Bam*HI/*Xmn*I double digestion would have resulted in at least two bands: a higher molecular weight band for the short form and a lower one for the long form. Since only one band could be detected (data not shown), we conclude that both isoforms originate from a single gene. Alternative splicing of the mRNA

is probably responsible for the expression of the isoforms of the α subunit.

*Two α/β complexes of human glucosidase II are expressed in vivo*

Western blot analysis was performed to study differential expression of α and β subunits in various human cell lines. Although the longer α subunit was expressed at lower levels, both isoforms were present in all human cell lines tested (Figure 1A) except for Peer T-cells. PHA$^R$2.7, a mouse glucosidase II deficient cell line was shown to be completely devoid of α subunit whereas only one form of α subunit could be detected in its parental BW5147 cell line. The β subunit was present in all cell lines tested, even in the glucosidase II deficient cell line PHA$^R$2.7, where it appears as an abundant protein of ~80 kDa (Figure 1B).

Immunoprecipitations were performed from [$^{35}$S]-labeled Jurkat, HL-60 and HeLa cell extracts with rabbit antiserum raised against electroeluted β subunit (Figure 1B, lanes 1–6) and on HeLa cell extracts with mouse antiserum raised against recombinant purified human glucosidase II (lanes 7 and 8). Preimmune antiserum was used as a control for both sets of immunoprecipitations (lanes 1, 3, 5, and 7). The α and β subunits were immunoprecipitated together equally well by both antisera, and while the long α subunit isoform was precipitated by both antisera, the doublet at ~110 is more visible upon immunoprecipitation with the mouse anti-recombinant human glucosidase II (lane 8).

*Expression of glucosidase II β subunit promotes expression of soluble α subunit, and yields enzymatically active glucosidase II*

Glucosidase II α and β subunits were transiently transfected individually or together in COS7 cells. Endogenous glucosidase II subunits were visible in the Triton X-100 soluble fraction but barely detectable in the pellet fraction (Figure 2, lane 1). However, when α1 or α2 subunits were overexpressed (Figure 2A lanes 2, 5; Figure 2B, lanes 3, 5), there was a slight increase in the levels of soluble protein but most of the expressed α subunit was found in the Triton X-100 pellet. Overexpressed β subunit (Figure 2A, lane 3) was generally soluble when expressed alone, but traces of insoluble material (pellet) were detected. Co-expression of the β subunit with any of the α subunits (Figure 2A, lanes 4, 6; Figure 2B, lanes 2, 4, 6) significantly reduced the extent of the insoluble α subunits but did not significantly increase the total amount of soluble α subunit.

Glucosidase II activity in the COS7 lysates was determined on [$^3$H]G1-AcP as a substrate. Glucosidase II α1 and α2 subunits, with and without β subunit, were expressed and the activity in the lysates determined (Figure 3). Disappearance of radiolabel was monitored over time and reflected the hydrolysis of the terminal glucose from the *N*-linked oligosaccharides on acid phosphatase, by both endogenous and conditionally overexpressed glucosidase II. Control transfections with pcDNA3 plasmid represent the endogenous levels of glucosidase II activity found in COS7 cells. Transfection of the catalytic α1 or α2 subunit alone did not increase the enzymatic activity over that observed in the control. As expected, expression of the β subunit alone did not increase endogenous glucosidase II activity. Increased rates of hydrolysis of radiolabeled substrate occurred only when α1 or α2 were co-transfected together with the β subunit. Glucosidase II activity

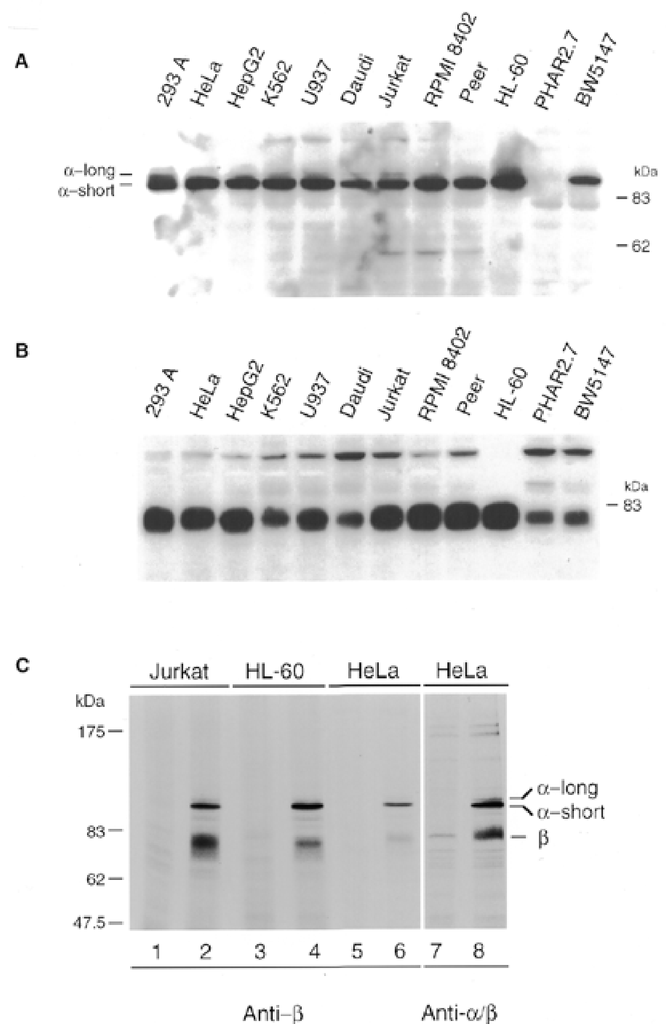


**Fig. 1.** Endogenous expression of two α/β complexes of human glucosidase II. (**A**) Triton X-100 lysates were prepared as described in *Materials and methods*; 50 µg of each sample were resolved by reducing SDS–PAGE, electroblotted and subjected to immunodetection with peptide-derived and affinity-purified anti-α (**A**) or anti-β (**B**) antisera. (**C**) Jurkat, HL-60, and HeLa were labeled with [$^{35}$S]-Promix and immunoprecipitations were performed on cell extracts with pre-immune (lanes 1, 3, 5) and immune (lanes 2, 4, 6) rabbit antiserum raised against electroeluted β subunit or preimmune (lane 7) and immune (lane 8) mouse antiserum raised against purified recombinant glucosidase $II_{α/β}$.

of the lysates did not increase upon transfection of $α1_{mut}$ or $α2_{mut}$ (D542N and D564N, respectively) subunits together with β subunit despite comparable protein yield (see Figure 2A, lanes 4, 6; Figure 2B, lanes 4, 6). This result demonstrates that the glycoside hydrolase Family 31 putative active nucleophile (56), here D542 (α1) and D564 (α2) is required for catalytic activity. Interestingly, expression of either mutant alone had a dominant negative effect possibly competing for endogenous β subunit for heterodimeric assembly.

Deoxynojirimycin (DNJ), a competitive inhibitor of glucosidases I and II was added to the assays at concentrations of 100 µM or 500 µM. Under these conditions, deglucosylation

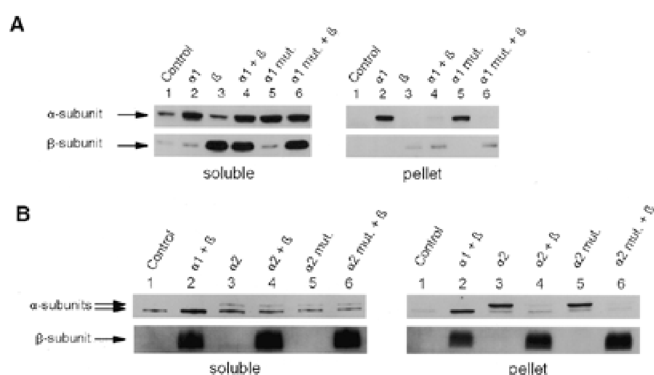


**Fig. 2.** Western blot analysis of transfected subunits. COS7 cells were transfected with glucosidase II subunits as indicated on top of each lane. Expression of α and β subunits was analyzed in the soluble and pellet fractions of 1% Triton X-100 lysates and probed with anti- α or β anti-peptide antiserum. (**A**) and (**B**) represent two sets of experiments.

was inhibited by 70–100% (Figure 3C, F). This residual activity may result from an endo-α-mannosidase present in the crude lysates, which can cleave [$^3$H]Glc$_1$Man$_1$- from [$^3$H]Glc$_1$Man$_9$GlcNAc$_2$-AcP to leave Man$_8$GlcNAc$_2$-AcP. A concentration of 100 μM inhibited most of the glucosidase II activity.

*The β subunit HDEL-ER retrieval motif is responsible for the localization of the heterodimeric glucosidase II*

The β subunit of glucosidase II contains a C-terminal HDEL putative ER retrieval motif. We replaced this amino acid sequence with a (His)$_6$-tag and transfected COS7 cells with an increasing amount of each β subunit, together with a constant level of α1 subunit of glucosidase II. From these cells, but not in the control cells, glucosidase II could be detected extracellularly by immunoprecipitation and by *p*-nitrophenyl α-D-glucopyranosidase (*p*-NP-Glcase) activity (Figure 4). Equal loading of the immunoprecipitations can be assessed by the band at 90 kDa which is the result of nonspecific protein G binding (Figure 4). This data confirms that the C-terminal HDEL/ER-retrieval motif of the β subunits is required to prevent the secretion of the active enzyme into the extracellular space. Optimal activity was observed with 0.4 μg of β subunit DNA, followed by a decline which may indicate deleterious effects of the over-expression of this subunit (data not shown).

*Blue-native electrophoresis separation of glucosidase II shows only heterodimeric structure*

Protein complexes can be electrophoretically separated using blue-native polyacrylamide gel electrophoresis (BN-PAGE) (Schagger and von Jagow, 1991). Upon solubilization, protein complexes are coated with Coomassie brilliant blue G dye

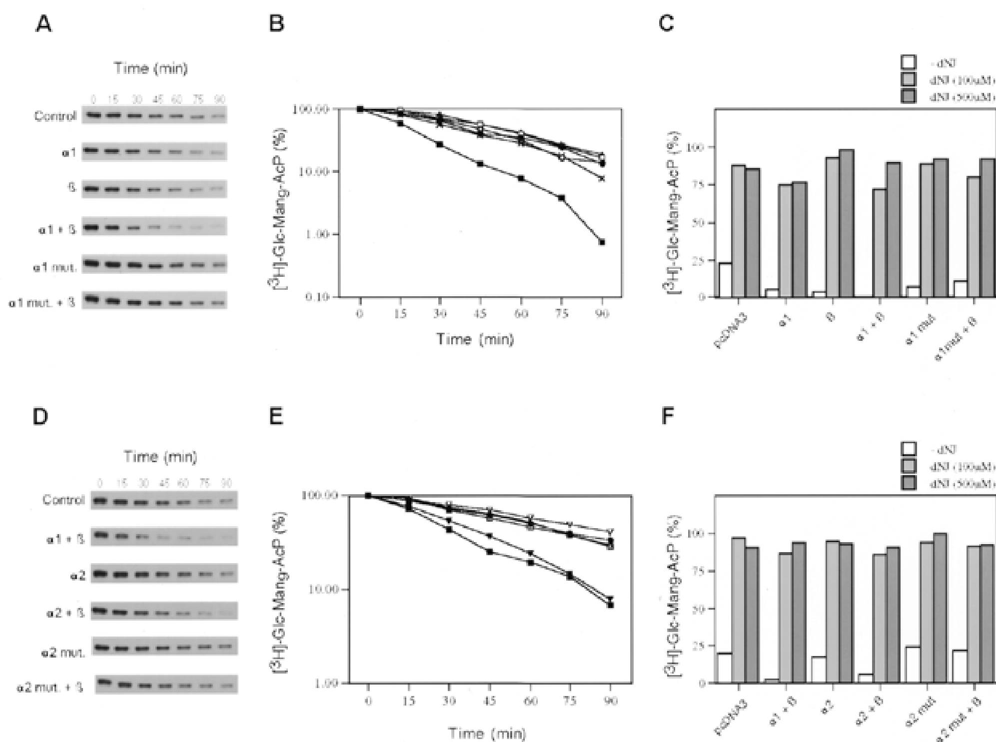


**Fig. 3.** Glucosidase II activity in COS7 cell lysates with transfected subunits. (**A–C**) illustrate the activities of the α1 or α1 mutant (mut.) subunits with or without co-expression of the β subunit. (**D–F**) illustrate the activities of α2 or α2 mutant (mut.) subunits co-expressed or not with the β subunit and the comparative activity of α1 and α2 co-expressed with the β subunit. Lysates were prepared as described in *Materials and methods* and then incubated at 37°C in presence of [$^3$H]G1-AcP substrate. Samples were removed at the indicated time and separated by reducing SDS–PAGE, amplified, and autoradiographed. Films were scanned (**A** and **D**), analyzed using NIH image software and plotted (**B** and **E**) as a percentage of initial substrate (time 0). Control (solid circles), α1 (open circles), β (multiplication sign), α1 + β (solid squares), α1mut. (open squares), α1mut. + β (solid triangles), α2 (open triangles), α2 + β (solid inverted triangles), α2mut. (open inverted triangles), α2mut. + β (crosshatched squares). (**C**) and (**F**) represent inhibition of specific deglucosylation activity by DNJ. Columns indicate percentage of residual substrate after 120 min of incubation at 37°C of lysates without or with 100 μM or 500 μM DNJ.

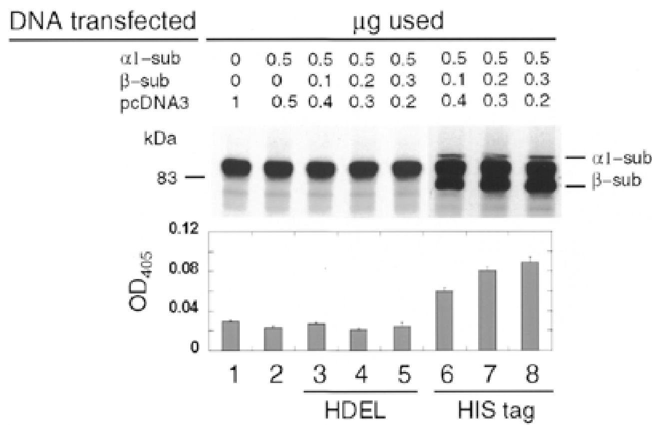


**Fig. 4.** The HDEL/ER-retrieval motif found in the β subunit is responsible for glucosidase II localization. Immunoprecipitations were performed from the medium of [$^{35}$S]-labeled COS7 cells cultures upon transfections with equal amounts of α subunit DNA and a range of 0 to 0.3 μg of β subunit DNA, with the C-terminal HDEL (lanes 3–5) and with this sequence replaced by a $(His)_6$-motif (lanes 6–8). Medium from COS7 cultures also was tested for *p*-nitrophenyl α-D-glucopyranosidase activity. Empty vector (pcDNA3) was used in both experiments to bring the total amount of DNA used in the transfections to 1 μg.

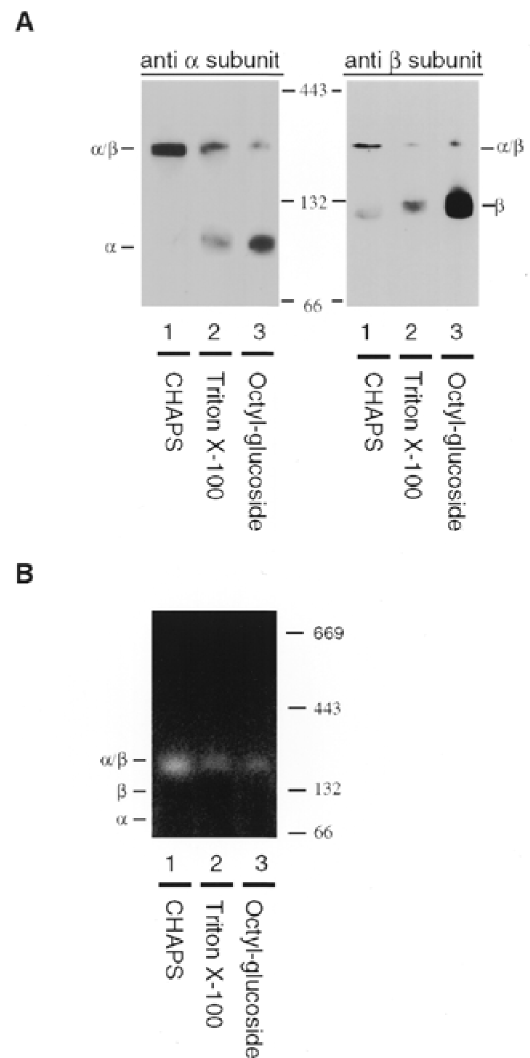


**Fig. 5.** Structural analysis of human glucosidase II by blue native-polyacrylamide electrophoresis. (**A**) Western analysis was performed on HeLa cell extracts made with 2% CHAPS, 1% Triton X-100, or 2% octyl-glucoside (lanes 1, 2, and 3, respectively). Protein samples were then separated by blue-native PAGE (see *Materials and methods*) and transferred to Immobilon-P membrane. Peptide-derived antisera against either the α or β subunit were used as indicated. (**B**) An *in gel* glucosidase II assay using the fluorescent substrate 4-methylumbelliferyl α-D-glucopyranoside was performed with HeLa extracts prepared and separated as described above.

which confers negative charge without disrupting protein–protein interactions therein, allowing for their migration and separation in a polyacrylamide gel matrix. Proteins from HeLa cell lysates were solubilized with 2% CHAPS, 1% Triton X-100, or 2% octyl-glucoside, subjected to BN-PAGE and transferred to Immobilon-P membrane for Western blot analysis. Using specific antisera for the α and β subunits of glucosidase II, we observed that the α/β heterodimer complex migrated at ~250 kDa (Figure 5A) while no higher molecular weight complexes were found. This suggests that glucosidase II is not permanently or strongly associated with other components of the ER lumen. Varying amounts of non-complexed α or β subunits were detected depending on the detergent employed. Octyl-glucoside appeared to solubilize the individual subunits most efficiently. Moreover, an *in gel* glucosidase II assay using the fluorescent substrate 4-methylumbelliferyl α-D-glucopyranoside confirmed that only the heterodimeric complex exhibited enzymatic activity (Figure 5B). Slow migration of the β subunit might be explained by poor binding of the Coomassie blue dye described in the work of Trombetta *et al.* (1996).

### *Expression of the human α and β subunits of glucosidase II in insect cells, and purification of the recombinant protein complex*

The baculovirus/insect cell expression system was used to produce the α1/β and α2/β isoforms of human recombinant glucosidase II. Initially, we expressed $(His)_6$-tagged subunits individually in *Sf*9 insect cells using recombinant baculovirus. Although the α1 and α2 subunits were expressed at high levels, they were present as inactive insoluble aggregates that could only be partially solubilized in 8 M urea (data not shown). The minor portion of α subunit (<1%) that was soluble and active, remarkably copurified with β subunit, which is presumably the endogenous one present in *Sf*9 cells. While the recombinant human β subunit was soluble, it copurified with a putative α subunit from insect cells and the resulting hetero-enzyme exhibited *p*-NP-Glcase activity (data not shown). This confirmed that the β subunit is essential for solubility of glucosidase II and demonstrated a surprising functional complementation of the human α and β subunits of glucosidase II by their insect homologues.

Next, either α1 or α2 subunits were simultaneously coexpressed with the β subunit. This coexpression lead to a significant increase in soluble human recombinant α subunit (data not shown). In this case, only the α subunit was $(His)_6$-tagged to allow for separation of the human recombinant glucosidase II from the endogenous insect enzyme. The first step of purification consisted of $Ni^{2+}$-NTA chromatography. This separation was followed by anion exchange chromatography and contributed to a significant improvement in purification factor (Figure 6A) thus increasing the specific activity of recombinant gluco-

sidase II (Figure 6B). The relative specific activity levels obtained for each isoform throughout purification correlated with the expression levels which were consistently higher for α1/β than α2/β (Figure 6B).

The α1 subunit (110 kDa) (Figure 6C, lane 4) displayed a slightly higher electrophoretic mobility than the α2 subunit due to the additional 22 amino acid insertion of the latter (Figure 6C, lane 3). Furthermore, the β subunit (80 kDa) (Figure 6C), later determined as human by Western analysis (Figure 6D), copurified equally well with both isoforms. Neither the α nor β subunits of *Sf*9 glucosidase II cross-reacted with either antisera (Figure 6D, lanes 1 and 5 respectively). This demonstrates the specificity of the peptide antibodies for the human recombinant subunits of glucosidase II and confirms that the human recombinant β subunit was indeed co-purified from co-infected insect cells.

*Comparison of the kinetic parameters for p-nitrophenyl α-D-glucopyranosidase activity for recombinant glucosidase II isoforms*

Reaction rates of glucosidase II with the substrate *p*-NP-Glc did not obey the simple hyperbolic form of the Michaelis-Menten equation (Figure 7A), but can be described by a double hyperbolic form corresponding to a model that takes into account the two postulated binding sites (Figure 7B) (Alonso *et al.*, 1991). This can also be clearly visualized using an Eadie-Hofstee plot (Figure 7C). These findings agreed with the results of Alonso *et al.*, 1991 for rat liver glucosidase II activity on *p*-NP-Glc (1991) and for maltase activities (1993b). The $K_{M1\text{-}2}$ values for both the high and low affinity sites were indistinguishable for both isoforms using the Student's *t*-test (Table I). The observed $K_{M1}$ for the high affinity sites of α1/β and α2/β were slightly lower at 0.50 and 0.52 mM, than for the $K_{M1}$ of the high affinity site of the rat liver enzyme at 0.78 mM (Alonso *et al.*, 1991). They also approach the rat liver glucosidase II high affinity $K_{M1}$ for maltose at 0.43 mM (Alonso *et al.*, 1993b). The $K_{M2}$ for the low affinity sites of α1/β and α2/β were 61.2 and 68.5 mM, which are lower than the reported $K_{M2}$ for rat liver glucosidase II for *p*-NP-Glc at 481 mM (Alonso *et al.*, 1991) but approaches, however, the $K_{M2}$ for maltose, at 57.7 mM (Alonso *et al.*, 1993b).

The velocities for both isoforms may reflect the different active enzyme concentrations found in our preparations. With higher specific activity, α1/β yielded $V_{max1\text{-}2}$ of 112 and 744 mU/mg versus 49 and 466 mU/mg for α2/β (Table I). A difference in enzyme concentration would also translate to a downward shift on the Eadie-Hofstee plot with the shape of the curve remaining unaltered (Figure 7C). Alternatively, the isoforms may have different $k_{cat}$ values which could also lead to a similar downward shift. Nevertheless $V_{max}/K_M$ ratios, which represent the efficiency of the active sites, suggested differences of 18.3- and 13.6-fold for site 1 versus site 2 (Table I), for α1/β and α2/β, respectively, and agreed well with previous results for rat liver glucosidase II which gave an

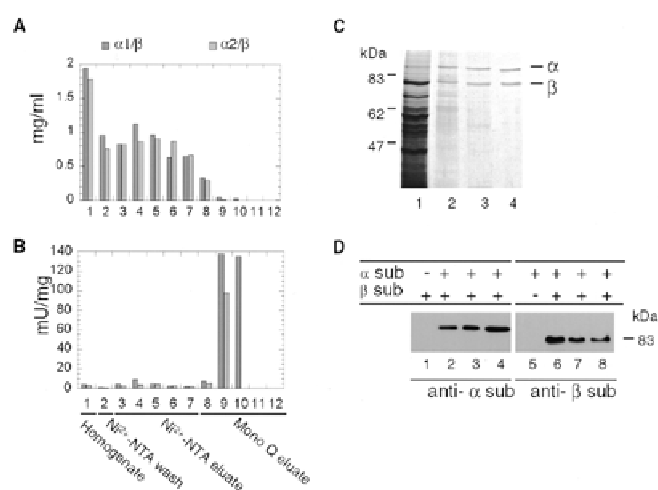


**Fig. 6.** Purification profiles of α1/β and α2/β recombinant isoforms of human glucosidase II from insect cells. (**A**) Protein concentrations (mg/ml) of the α1/β (dark) and α2/β (light) isoforms are described for the cell homogenate (lane 1), $Ni^{2+}$-NTA wash (lane 2) and eluate (lanes 3–7) and fractions near the Mono Q activity peak (lanes 8–12). (**B**) *p*-Nitrophenyl α-D-glucopyranosidase specific activity (mU/mg) for the α1/β and (dark) α2/β (light) isoforms at 5 mM *p*-NP-Glc, in 50 mM PIPES pH 6.5, for 30 min at 37°C. One unit of *p*-NP-Glcase activity is defined as the production of 1 µmol *p*-nitrophenol/minute at 37°C. (**C**) Samples were separated on an 8% SDS–PAGE. The α2/β isoform in the cell homogenate (lane 1) and from the peaks of activity of the $Ni^{2+}$-NTA (lane 2) and in the Mono Q fractions (lane 3) is shown. Lane 4 shows the Mono Q activity peak from the purification of the α1/β isoform. (**D**) Western analysis was performed with peptide-derived antisera specific for the human α (lanes 1–4) and β subunits (lanes 5–8). Extracts were made from cells expressing only the β subunit and only the α subunit (lanes 1, 5). Represented are cell extracts from coexpression of the α2 subunit with the β subunit (lanes 2, 6), activity peaks of the $Ni^{2+}$-NTA (lanes 3, 7) and the mono Q fractions (lanes 4, 8), respectively.

**Table I.** Kinetic parameters and inhibition of *p*-nitrophenyl α-D-glucopyranosidase activity for the α1/β and α2/β recombinant isoforms of human glucosidase II

| Isoform | Binding site | $K_M$ (mM) | $V_{max}$ (mU[a]/mg) | $V_{max}/K_M$ (ml/min/g) | Site 1/Site 2 | DNJ[b] (µM app. $K_i$) | CST (µM app. $K_i$) |
|---|---|---|---|---|---|---|---|
| α1/β | 1 (high affinity) | 0.50 ± 0.05 | 112 ± 4 | 222 ± 24 | 18.3 ± 5.0 | 3.0 ± 0.3 | 17.0 ± 0.8 |
| | 2 (low affinity) | 61.2 ± 12.8 | 744 ± 113 | 12.1 ± 3.1 | | 18.8 ± 1.3 | 45.3 ± 3.5 |
| α2/β | 1 (high affinity) | 0.52 ± 0.06 | 49 ± 2 | 92 ± 11 | 13.6 ± 3.5 | 2.9 ± 0.7 | 15.0 ± 1.5 |
| | 2 (low affinity) | 68.5 ± 12.2 | 466 ± 65 | 6.8 ± 1.5 | | 22.0 ± 1.4 | 39.8 ± 1.2 |

Curve fit was performed to the Michaelis-Menten equation modified for a two binding site model. App. $K_i$ were determined from plots of reciprocal activity versus inhibitor concentration, for the high and low affinity binding sites, at substrate concentration ranges of 0.25–1.0 mM and 8.0–14 mM *p*-NP-Glc, respectively. Values are means ± SEM.

[a]One unit of activity is defined as 1 µmol of nitrophenol formed/min, at 37°C.

[b]Abbreviations: DNJ, deoxynojirimycin; CST, castanospermine.

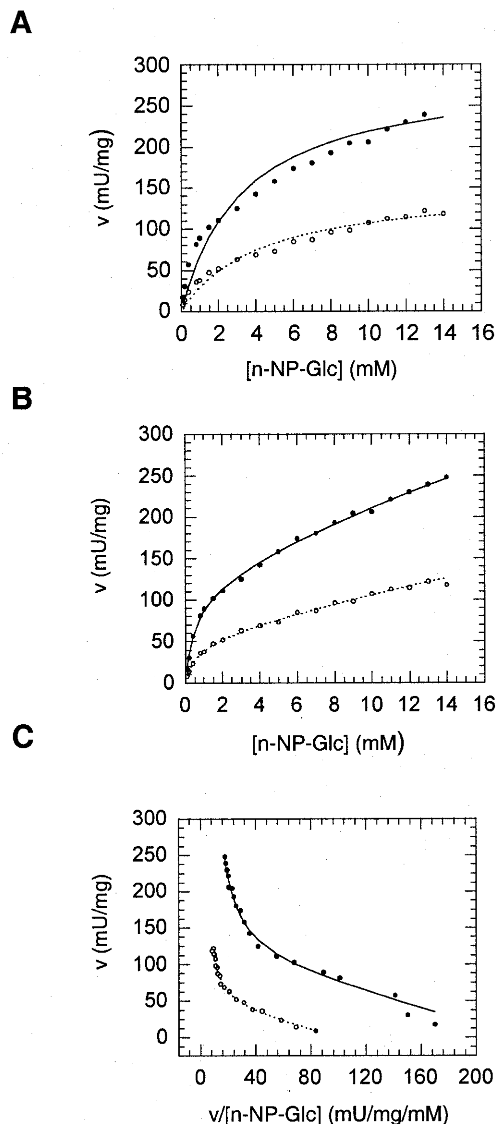


**Fig. 7.** Kinetic profiles for α1/β and α2/β recombinant isoforms of human glucosidase II. Data for the α1/β (solid circles) and α2/β (open circles) is presented. (**A**) Curve fit obtained using a simple single hyperbolic Michaelis-Menten model. (**B**) Velocity (v, mU/mg) plotted against [*p*-NP-Glc] demonstrates fitting to the double hyberbolic equation for both isoforms. (**C**) Eadie-Hofstee plots (v×v/[*p*-NP-Glc]) also support the two binding site model for *p*-NP-Glcase activity (Alonso *et al.*, 1991).

~20-fold difference in efficiency between sites (Alonso *et al.*, 1991).

### *Inhibition of p-NP-Glcase activity*

Inhibition at both the high and low affinity sites was tested for the two ranges of substrate concentration, 0.25–1.0 mM and 8.0–14 mM *p*-NP-Glc, respectively. Plots of reciprocal activity versus inhibitor concentration at these ranges suggested similar inhibition of *p*-NP-Glcase activity for both isoforms. We report here $K_{i1\text{-}2}$ values for deoxynojirimycin of 3.0 and 18.8 μM (α1/β), and 2.9 and 22.0 μM (α2/β) for the high and low affinity sites respectively (Table I). Similarly with castanospermine we measured $K_{i1\text{-}2}$ values of 17.0 and 45.3 μM (α1/β), and 15.0 and 39.8 μM (α2/β) for the high/low affinity sites (Table I). These values were indistinguishable between isoforms demonstrating similar inhibition by deoxynojirimycin and castanospermine of *p*-NP-Glcase activity for both isoforms. It should be noted that neither α1/β nor α2/β were affected by EDTA nor EGTA at concentrations up to 10 mM (data not shown). Hence, recombinant glucosidase II activity was not affected by the depletion of cations, including calcium.

### *Glucose hydrolysis from $Glc_{1\text{-}3}Man_9GlcNAc_1$ oligosaccharides by α1/β and α2/β forms of glucosidase II*

Glucosidase II assays were performed for both α1/β and α2/β forms of recombinant enzyme after normalizing for *p*-NP-Glcase activity. [$^3$H]-Mannose labeled lipid-linked $Glc_{1\text{-}3}Man_9GlcNAc_1$ oligosaccharides were extracted, purified and pooled from *S. cerevisiae* Δ*alg8*Δ*gls2*, Δ*alg10*Δ*gls2*, and Δ*gls2* mutants (Jakob *et al.*, 1998) and used as substrates. The extent of hydrolysis was determined by following changes in HPLC elution profiles (Figure 8). While the $Glc_3Man_9GlcNAc_1$ oligosaccharide remained uncleaved after 30 min, glucose hydrolysis from both $Glc_2$- and $Glc_1Man_9GlcNAc_1$ was shown for both enzyme isoforms. Hydrolysis from the $Glc_2Man_9GlcNAc_1$ was observed as a downward shift for the $Glc_2$ peak with a concomitant rise in the $Glc_1$ peak. Moreover, the rise in the $Man_9GlcNAc_1$ demonstrated the subsequent cleavage of the $Glc_1Man_9GlcNAc_1$ oligosaccharides. The profiles for both forms of recombinant glucosidase II not only suggested that they share substrate specificities, but with similar vectorial changes in peak height confirm that they are catalytically indistinguishable for the substrates examined.

## Discussion

Our results demonstrated the expression of two forms of human glucosidase II *in vivo.* We have identified two α/β complexes through Western analysis and immunoprecipitations and also isolated two forms of the α subunit that differed by the inclusion of a 66 bp stretch. Brada and co-workers (1990) were first to suggest that there might be two forms of glucosidase II based on different subcellular localization and also different enzyme species carrying Endo H-sensitive high-mannose, as

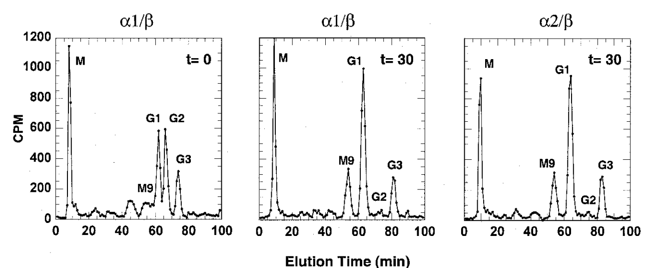


**Fig. 8.** Comparison of α1/β and α2/β glucosidase II activity on physiological substrates. Glucosidase II assays were performed on [$^3$H]$Glc_{1\text{-}3}Man_9GlcNAc_1$ oligosaccharides as described in the *Materials and methods*. HPLC profiles of oligosaccharides after hydrolysis for 0 and 30 min are shown for the α1/β glucosidase II, and 30 min for the α2/β form. The positions of mannose (M), $Man_9GlcNAc_1$ (M9), $Glc_1Man_9GlcNAc_1$ (G1), $Glc_2Man_9GlcNAc_1$ (G2), $Glc_3Man_9GlcNAc_1$ (G3), are indicated.

well as sialylated Endo H-resistant oligosaccharide chains in pig kidney epithelial cells. Later, Hentges and Bause (1997) demonstrated the purification of a tight glucosidase II α subunit doublet estimated at 107/112 kDa, with the 107 kDa more abundant form ascribed as a cross-reacting degradation product. However, it was Arendt and Ostergaard (1997) that identified a murine α subunit cDNA highly conserved with the human cDNA (D42041) that also encoded for an additional 22 amino acids. In more recent studies, the same authors ascribed the domain as a splice variant termed box A1 (66 bp) and also identified a second domain by PCR, referred to as A2 (27 bp) (Arendt *et al.*, 1999). Complementary DNAs with three of four possible combinations were isolated; i.e. $A1^+$ A2- (form1), A1- $A2^+$ (form 2), and A1- A2- (form 3) while the existence of a cDNA encoding an isoform with both A1 and A2 has yet been confirmed (Arendt *et al.*, 1999). It is possible, based on a comparison with mouse splice variants, that the longer α subunit observed by Western analysis and by immunoprecipitation corresponds to the cloned α2 subunit which could be of the $A1^+$ A2- form. This assumption rests on the similar migration of the observed long α subunit with the recombinant α2 subunit (data not shown) and the respective difficulty of resolving the alternative A1- $A2^+$ splice variant on a SDS–PAGE. Nevertheless, other splice variants may exist that have not yet been identified that encode for proteins with molecular weights similar to the α2 subunit.

Different levels of expression have previously been observed for the α subunit isoforms (Arendt *et al.*, 1999; Hentges and Bause, 1997). Hentges and Bause (1997), in the purification of pig liver glucosidase II, found lower yields for the slower mobility form in their 107/112 kDa α subunit doublet. Also, cloning experiments by Arendt *et al.* (1999) suggest a 5-fold lower expression of the form 1 ($A1^+$ A2-) than form 3 (A1- A2-) using a PCR cloning strategy. We also found lower yields for α2 subunit, when expressed in COS7 cells (Figure 2) and *Sf*9 insect cells (Figure 7B). While factors that lead to differential expression of the α subunits remain unknown, protein complex stability may be ruled out since purified recombinant α1/β and α2/β display equal stability in continuous enzymatic assays, with linear progress curves proceeding for close to one h (data not shown).

These studies also sought to clarify the role of the β subunit in the expression of active human glucosidase II in mammalian cells. Trombetta *et al.* (1996) first characterized glucosidase II as a heterodimer. They noted that the lower molecular weight subunit could not be removed while maintaining glucosidase II activity. More recently, D'Alessio *et al.* (1999) genetically demonstrated the heterodimeric structure of *Schizosaccharomyces pombe* glucosidase II through the loss of endogenous glucosidase II activity in cells disrupted for GIIβ. It was postulated in both studies that the β subunit was involved in maintaining the integrity of catalytically active glucosidase II and the ER localization though its HDEL-ER retrieval signal. Flura *et al.* (1997) on the other hand observed a slight increase in glucosidase II activity in CHO cells upon the lone expression of a pig liver derived cDNA encoding the α subunit. Moreover, while Hentges and Bause (1997) identified a 60 kDa component, it was undetectable by Coomassie staining in some of their preparations. We found however that the α1 or α2 subunits were largely insoluble when expressed alone but this could be counteracted through the coexpression of the β subunit, which concomitantly produced active glucosidase II. As well, we demonstrate here the requirement of heterodimeric structure for enzyme activity. Thus, the β subunit contributes to the solubility/stability of the α/β complex and is a prerequisite for the formation of the active enzyme. This rules out the hypothesis that the β subunit is simply required for α subunit folding and that the α subunit then remains active upon dissociation. Moreover, the detection of active glucosidase II in the culture medium upon the replacement of the C-terminal HDEL sequon with a $(His)_6$-tag, establishes a role of the β subunit in retaining the α/β glucosidase II dimer in the ER. It is possible however that the variable-fold increase in enzyme activity observed by Flura *et al.* (1997) may be attributable to heterodimer assembly with endogenously expressed β subunit. While abundantly expressed, the level of endogenous β subunit does in fact vary according to cell line, as observed in our study (Figure 1B).

Heterologous expression of the α subunit in *Sf*9 insect cells confirmed these functions of the β subunit but also by the formation of active human/insect heteroenzymes remarkably demonstrates the wide conservation of its properties. While the yields of α subunit were high when expressed alone, most of the recombinant protein was insoluble. The small fraction of α subunit that was soluble always copurified with an insect protein with a molecular weight similar to that of the human β subunit. Conversely, purified recombinant human β subunit, when expressed alone, had an associated insect protein at ~110 kDa. These preparations also displayed *p*-NP-Glcase activity characteristic of glucosidase II. Protein alignments of the human β subunit with homologues in *Caenorhabditis elegans* (Z47356) and *S.pombe* (D89245) suggest that it is a highly conserved protein. Both have putative ER targeting and retrieval signals, and overall homologies of 36% and 22%, respectively, particularly concentrated in the N-terminal region (Q50-E105). This region of high conservation, with 71% and 79% for *C.elegans* and *S.pombe*, respectively, may play a role in mediating α/β interactions. Interestingly, the open reading frame YDR221w in *S.cerevisiae* (S59428) with an homology of 21% has both a putative ER targeting signal peptide and a transmembrane domain. Moreover, most of its homology is found within the conserved domain (61%). This open reading frame probably encodes a likely candidate for the glucosidase II β subunit for this species. While it remains to be demonstrated, we propose that this highly conserved region acts as the major α/β protein–protein interaction domain.

One hypothesis for the existence of the α1and α2 forms was that they each may display different specificities for the $Glc_2Man_9GlcNAc_1$ and $Glc_1Man_9GlcNAc_1$ substrates. The current kinetic model for *p*-NP-Glc and maltose, which predicts both high and low affinity substrate binding sites (Alonso *et al.*, 1991) could have easily been derived from the copurification of two isoforms of glucosidase II with such substrate specificities. The first evidence of this differential activity was demonstrated by Hubbard and Robbins (1979). They showed that the sequential hydrolysis of glucose from the N-linked oligosaccharides occurred at different rates. Alonso *et al.* (1993a) later assigned the cleavage of the innermost α-1,3-linked glucose ($Glc_1Man_9GlcNAc_2$) to the low affinity site since bromoconduritol, which selectively inhibited both *p*-NP-Glc and maltose binding to this site, had also been shown to inhibit glucose hydrolysis from $Glc_1Man_9GlcNAc_2$ (Datema *et al.*, 1982;

Alonso *et al.*, 1993a). However, we have found, through the expression and purification of both recombinant α1/β and α2/β complexes of glucosidase II and through the determination of their kinetic parameters for the *p*-NP-Glc substrate, that both forms displayed activity that fit well to the two binding site model proposed by Alonso *et al.* (1991). Hence, the kinetics displayed are truly representative of an intricate active site and not the result of copurified isoforms with different substrate affinities. Furthermore, we have shown that the α1/β and α2/β forms of glucosidase II share similar properties of *p*-NP-Glcase hydrolysis and inhibition while exhibiting similar substrate specificities for the physiological oligosaccharide substrates.

A role for two or more isoforms of glucosidase II still remains to be determined. However, as an early step in glycan processing of nascent glycoproteins and the entry point into the calnexin cycle, glucosidase II is widely used by most, if not all secreted glycoproteins that may or may not require ER quality control. It is not without precedent to find biological complexity in such a pivotal step. And while glucosidase II was not found complexed with other ER constituents, transient interactions with the translocon, ER chaperones or even proteins of the ER matrix may exist that are isoform specific. Differential regulation cannot be ruled out and may be mediated by yet unknown interactions. While it remains to be determined, any new found complexity in the early steps of glycan trimming may provide us with novel and very specific antiviral strategies, as inhibitors of the ER α-glucosidases have already shown great promise against viruses that require the host ER lectin-like chaperone protein folding apparatus (Zitzmann *et al.*, 1999).

## Materials and methods

### *Reagents*

All reagents were purchased from Sigma (St. Louis, MO) except where otherwise mentioned. Dulbecco's modified Eagle's medium (DMEM), Opti MEM I, Dulbecco's PBS (D-PBS) and lipofectamine were purchased from Gibco/Life Technologies Inc. (Rockville, MD). Fetal bovine serum was purchased from Hy-Clone (Logan, UT), deoxynojirimycin from Calbiochem (San Diego, CA) and octyl glucoside from Boehringer Mannheim Canada (Laval, QC). Restriction and modification enzymes were from Amersham Pharmacia Biotech Inc. (Uppsala, Sweden) and New England Biolabs (Beverly, MA).

### *Cell lines*

RPMI8402 cells were obtained from Dr. C.Milstein (Cambridge, UK), Peer cell lines from Fujisaki Cell Center (Tokyo, Japan), mouse ES cells from Genome Systems Inc. (St. Louis, MO) and PHA$^R$2.7 from Dr. I.Trowbridge (La Jolla, CA). All other cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA).

### *Cloning of human glucosidase II cDNAs*

*α-Subunit.* Rat glucosidase II was purified as described previously (Zapun *et al.*, 1997) electroblotted onto a PVDF membrane and the major 120 kDa protein was N-terminally sequenced by using an Applied Biosystems 470A protein sequencer equipped with an on-line phenylthiohydantoin amino acid analyzer. The N-terminal sequence was determined as VDRSNFKTCEESSFCKRQRS. A partial ORF sequence of human immature myeloid cell line KG1 (accession number D42041) displayed homology to the N-terminal amino acid sequence. mRNA was isolated from HeLa cells using the Quick Prep mRNA Purification Kit (Amersham Pharmarcia Biotech,) and cDNA amplified using the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA). Oligonucleotides 5′ CAT GCT CAG GGA TCC CAT AGA CAT GCT (anti-sense) and 5′ AAG CAT ACG GCC AGG CCT CTC TCC ATA CCG (sense) were used in conjunction with the adapter primer AP1 supplied with the kit (underlined nucleotides indicate *Bam*HI and *Stu*I restriction sites, respectively). PCR conditions were as described by the manufacturer (all PCR reactions were performed with a Perkin Elmer 9600 instrument). The amplicons of ~0.8 kb (doublet) and ~3.6 kb, respectively, were mixed and reamplified using AP2 primer and Expand Long Template PCR (Boehringer Mannheim Canada). The resulting ~3.9 kb fragment was digested with *Not*I and ligated in the mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA). The *Eco*RV/*Hind*III fragment of the polylinker was deleted to remove a hairpin structure to enhance translation and protein production. Full-length cDNAs for human glucosidase II were sequenced. The point-mutated α subunit was generated using the primer pairs 5′ GTC TGG AAT **AAC** ATG AAC GAA CC (sense) (boldface indicates the mutated codon) / 5′ GCT GAA GCT TAT CGC AGG TGA ATA CTC CAA TC (anti-sense) and 5′-GGT TCG TTC AT**G TT**A TTC CAG AC (anti-sense) (bold face indicates the mutated codon) / 5′-AAG CAT ACG GCC AGG CCT CTC TCC ATA CCG (sense) (underlined nucleotides indicate a *Stu*I restriction site). The fragments obtained were purified and reamplified using the flanking primers 5′ GCT GAA GCT TAT CGC AGG TGA ATA CTC CAA TC (anti-sense) and 5′-AAG CAT ACG GCC AGG CCT CTC TCC ATA CCG (sense). An *Aat*II/*Kpn*I fragment (901 bp) was replaced in the original full-length clones and resequenced. The final 3.9 kb amplicon contains original 5′ and 3′ untranslated regions.

*β-Subunit.* The β subunit of human glucosidase II sequence was previously identified (GenBank accession #J03075) (Tabaczewski and Stroynowski, 1994; Trombetta *et al.*, 1996). This gene was amplified using normal human lymphocytes mRNA as template. cDNA was obtained using the TITAN RT-PCR kit (Boehringer Mannheim) using oligos 5′ GCT CGA GAA TTC GGT GAG ATG CTG TTG CCG CTG (sense) (underlined nucleotides indicate the *Eco*RI restriction site used for cloning) and 5′ CGC GGT CTA GAT TAC TAG AGC TCG TCA TGG TCG TCT TC (anti-sense) (underlined nucleotides indicate the *Xba*I restriction site used for cloning). The 1.6 kb amplicon obtained was digested with *Eco*RI/*Xba*I and cloned in pcDNA3. Both DNA strands were sequenced. A 135 bp *Nco*I/*Xba*I fragment of sequence encoding the C-terminal HDEL was removed and replaced by a mutagenic PCR fragment amplified with the primer 5′ CG CGG TCT AGA TTA CTA **ATG GTG ATG GTG ATG ATG** GTC GTC TTC GGT GGG TGC (anti-sense) (underlined nucleotides indicate the *Xba*I restriction site used for cloning, and bold represents the $(His)_6$-tag) and 5′ GTC CGA GAA TTC GGT GAG ATG CTG TTG CCG CTG (sense).

*Southern blot analysis of glucosidase II*

Human lymphocyte or mouse ES cell genomic DNA (10µg) was digested overnight at 37°C with 100U of *BamH*I and/or *Xmn*I, separated on a 0.7% agarose gel and transferred to Hybond N+ membrane (Amersham Pharmacia Biotech). Blotted DNA was probed with a [$^{32}$p]-labeled *Xmn*I/*BamH*I cDNA fragment (220 bp). The blot was washed twice in 2× SSC, 0.1% SDS at 65°C for 30 min and exposed with Kodak XAR-5 film.

*Transfections*

COS7 African green monkey kidney cells were cultured in D-10 (DMEM supplemented with 10% FBS inactivated at 56 °C for 30 min). Cell monolayers at 40% confluency were transfected with 9.5 µg DNA and 40 µl lipofectamine in 100 mm plates or 1.2 µg DNA and 5 µl lipofectamine in 35 mm plates in Opti-MEM I and incubated for 16 h. Medium was replaced with fresh D-10 medium and cells were collected 24 h later. Large-scale cotransfections were performed with 4 µg pcDNA3 and 4 µg α subunit, with 4 µg pcDNA3 and 4 µg β subunit, or with 4 µg α and 4 µg β subunit. Small-scale transfections were performed with combinations of DNA as described in Figure 8. Transfections included either 1.5 µg (large-scale) or 0.2 µg (small-scale) of the vector pQBI25 (Quantum Biotechnologies Inc., Montreal, Quebec) coding for the Green Fluorescent Protein in order to evaluate transfection efficiency.

*Mammalian cell lysate preparation*

Cells were scraped and washed three times in D-PBS. They were then lysed on ice for 20 min in 10 mM Tris–HCl pH 6.8, 300 mM NaCl, 2 mM $CaCl_2$ and 1% Triton X-100 supplemented with Complete™ EDTA-free Protease Inhibitors (Boehringer Mannheim). Samples were homogenized using a Potter Elvejheim for 30 s on ice. The lysates were diluted 10-fold (0.1% Triton X-100, final concentration) and cleared by centrifugation at 13000 × *g* for 5 min. The supernatant was collected and protein concentration measured using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA).

*Glucosidase II Assays on N-linked [$^3$H]$Glc_1Man_9GlcNAc_2$*

Acid phosphatase (AcP) was purified from *Saccharomyces cerevisiae* (DT111 strain that secretes glycoproteins with asparagine-linked $Man_9GlcNAc_2$ glycans) and glucosylated *in vitro* with recombinant rat liver UDP-glucose glycoprotein:glucosyltransferase (UGGT) (Tessier *et al.*, 2000). Briefly, 10 µg of purified $Man_9GlcNAc_2$-acid phosphatase (G0-AcP) in 20mM Tris–HCl pH 7.5, 10 mM $CaCl_2$ was labeled with 50 µCi UDP-[$^3$H]glucose (30 Ci/mmol, Amersham Pharmacia Biotech) using 100 nM of purified recombinant UGGT for 4 h at 37°C. Unincorporated UDP-[$^3$H]glucose was removed using G-50 Sepharose spin columns (Amersham Pharmacia Biotech). Typical labeling obtained was 2–4 × $10^5$ c.p.m./µg AcP. Cell lysates adjusted to a protein concentration of 500 µg/ml were incubated at 37 °C with 5 × $10^5$ c.p.m. [$^3$H]Glc1-AcP/ml, with or without 100 µM or 500 µM DNJ. Aliquots were removed every 15 min and immediately diluted in 3× SDS sample buffer with DTT (New England Biolabs) and boiled for 5 min. Half of each of the sample was resolved on 8% SDS–PAGE, followed by fluorography (Amersham Pharmacia Biotech). Dry gels were exposed with Kodak XAR-5 film for 60 h at –80°C. Films were scanned and image analyzed using the NIH Image Software (public domain).

*Blue native-polyacrylamide gel electrophoresis*

HeLa cell extracts were solubilized in 2% CHAPS, 1% Triton X-100, or 2% octyl-glucoside for 30 min on ice. Unsolubilized proteins were removed by centrifugation for 30 min at 100,000 × *g*. Samples were mixed with Serva blue and E-amino-caproic acid and separated on a linear 5 to 10 % acrylamide gradient as previously described (Schagger and von Jagow, 1991). Separated proteins were transferred to Immobilon-P membrane and detected by Western blot analysis.

*In gel 4-methylumbellifyl α-D-glucopyranosidase assay*

Glucosidase II assays using the fluorescent substrate 4-methylumbelliferyl α-D-glucopyranoside were performed as described elsewhere (Brada and Dubach, 1984).

*Antibodies*

Map-peptide antibodies were raised in New Zealand rabbits. They correspond to amino acid sequence SFQHDPETSVLVLRK (α subunit) and DHDKSFAMKYEQGTG (β subunit). The antisera also recognized the mouse glucosidase II homologue. Affinity-purified antibodies were prepared as described in *The Xenopus Molecular Marker Resource* (http://vize222.zo.utexas.edu) using the appropriate immobilized peptide Sepharose. Anti-β antiserum was raised by injecting human β subunit, electroeluted from SDS–PAGE, into New Zealand rabbits. Mouse anti-human glucosidase $II_{\alpha/\beta}$ antiserum was raised by injecting BALB/c mouse with purified recombinant glucosidase II.

*Western blot analysis*

Protein samples were resolved on 8% SDS–PAGE and transferred on Immobilon-P membrane (Millipore). The membranes were first probed with α subunit anti-peptide antiserum or affinity-purified antibodies, incubated with anti-rabbit HRP (Bio-Rad or Santa-Cruz) and developed using Lumilight (for overexpressed proteins) or Lumilight Plus (endogenously expressed proteins) chemiluminescent substrate (Boehringer Mannheim). They were stripped according to the manufacturer's instructions and reprobed with anti-β subunit antibodies.

*Immunoprecipitations*

HeLa cells were labeled with [$^{35}$S] methionine ([$^{35}$S] Promix Protein Labeling Mix, Amersham Pharmacia Biotech), and immunoprecipitations were performed with either protein A (Figure 1B) or protein G Sepharose (Figure 4C) as described in *Current Protocols in Immunology* (Coligan *et al.*, 1997) using rabbit anti-electroeluted β subunit or mouse anti-glucosidase $II_{\alpha/\beta}$.

*Baculovirus production and protein expression*

Human cDNAs encoding both α1 (D42041 with Y850H) and α2 subunits (AF144074) with $(His)_6$-tags at the C-terminus and the β subunit of glucosidase II, both with and without a $(His)_6$-tag (AF144075) were subcloned into pFastBac1 and transformed into *E.coli* DH10Bac to produce recombinant bacmids. *Sf*9 cells were transfected with bacmid DNAs and the virus stocks amplified as described by the manufacturer

(Gibco/Life Technologies). Cells were grown in Sf900 II SFM serum-free medium at 27°C for 3 days to a cell density of 1.5–$2.5 \times 10^6$/ml and infected with either one or both virus for an α subunit and β subunit virus at an M.O.I. of 2–5 pfu/cell. Only the β subunit without a $(His)_6$-tag was used in α/β coinfections. Infected cells were incubated for 3 days, harvested by centrifugation at $3000 \times g$, and stored at –80 °C.

*Recombinant glucosidase II purification*

Infected cells (~$7.5 \times 10^8$ ) were lysed in a Dounce homogenizer in 20 ml of Buffer A (250 mM NaCl, 20 mM Tris–HCl pH 7.5) supplemented with 1% Triton X-100 and Complete$^{TM}$ EDTA-free protease inhibitors (Boehringer Mannheim Canada). The homogenate was treated with bovine pancreas deoxyribonuclease I (Amersham Pharmacia Biotech) for 30 min on ice, and centrifuged at $10,000 \times g$ for 10 min at 4°C. The supernatant was loaded by gravity flow onto a 1 ml $Ni^{2+}$-NTA Superflow column (Qiagen Inc., Valencia, CA). 2× Buffer A with 1% Triton X-100 and 20 mM imidazole was added to the flow through and was loaded again onto the $Ni^{2+}$-NTA column. The column was then washed with 50 ml of 2× Buffer A with 10 mM imidazole, followed by 4 ml each of 2× Buffer A with 25 mM imidazole, and 50 mM imidazole. Glucosidase II was then eluted in 5× 1ml fractions of 2× Buffer A with 200 mM imidazole. The eluate was desalted on a Sephadex G-25M column (PD-10; Amersham Pharmacia Biotech) in Buffer B (20 mM Tris–HCl pH 7.5, 5 mM β-mercaptoethanol).

*Anion exchange chromatography*

The desalted $Ni^{2+}$-NTA fractions were loaded onto a Mono Q HR5/5 column (Amersham Pharmacia Biotech) equilibrated in Buffer B, on a BioCAD Perfusion Chromatography Workstation (PerSeptive BioSystems Inc., Framingham, MA). A linear gradient of 50 ml from 0 to 0.5 M NaCl in Buffer B was performed and the eluate collected in 1 ml fractions. The fractions were tested for activity and protein concentrations determined using a Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). All fractions for each step of purification were analyzed by SDS–PAGE and Western blot.

*p-Nitrophenyl α-D-glucopyranosidase assays and analysis of kinetic parameters*

The activity for *p*-nitrophenyl α-D-glucopyranosidase (*p*-NP-Glcase) was tested in a 100 µl reaction mixture consisting of 5 mM *p*-NP-Glc (Sigma) in 50 mM PIPES pH 6.5 for 30 min at 37°C. Assayed culture medium (COS7 cells) (Figure 4C) was precleared with activated charcoal (BDH, Ville St. Laurent, Canada) to remove traces of phenol red. The reactions were stopped with an equal volume of 1 M glycine/NaOH and the $OD_{405}$ read immediately. One unit is the production of 1 µmol/minute of *p*-nitrophenol at 37°C. Progress curves were monitored for both isoforms by continuous assay ($n = 5$) for 30 min at 37°C in a SpectraMax 250 spectrophotometer (Molecular Devices Corp., Sunnyvale, CA). Continuous assay reactions were performed in 200 µl of 50 mM PIPES pH6.5 with a substrate concentration ranging from 0.1 to 14 mM *p*-NP-Glc. The slopes of the progress curves were determined by linear regression analysis using SoftMax Pro software version 1.1.1 (Molecular Devices, Corp), and kinetic parameters determined using ENZFITTER version 1.05 (Biosoft, Cambridge, UK). The two-binding site adaptations of the Michaelis-Menten and the Eadie-Hofstee equations described by Alonso *et al.* (1991) were used to analyze the data.

*p-Nitrophenyl α-D-glucopyranosidase inhibition*

Inhibition experiments were also performed by continuous measurement of *p*-NP-Glcase activity. Deoxynojirimycin (DNJ) and castanospermine (CST) (Calbiochem) were tested between 1.25–10 µM and 20–80 µM, respectively. The velocity was measured for the substrate concentration range of the high affinity site (0.25–1 mM) and the low affinity site (8–14 mM). Apparent $K_i$ values were determined from plots of reciprocal activity versus inhibitor concentration.

*Purification and glucosidase II hydrolysis of $[^3H]Glc_{1\text{-}3}Man_9GlcNAc_1$*

Metabolic labeling with $[^3H]$glucose and extraction of lipid-linked oligosaccharides was performed as described by Zufferey *et al.* (1995) from the following strains of *Saccharomyces cerevisiae:* YG424 (MATa, ade2-101, his3Δ200, lys2-801, Δalg8::HIS3, Δgls2::KanMX) (Jakob *et al.*, 1998); YG491 (MATa, ade2-101, his3Δ200, Δalg10::KanMX, Δgls2::KanMX) (Jakob *et al.*, 1998); SS328 (MATα, ade2-101, his3Δ200, lys2-801, ura3-52) (Vijayraghavan *et al.*, 1989), to yield $[^3H]Glc_1Man_9GlcNAc_2$, $[^3H]Glc_2Man_9GlcNAc_2$, and $[^3H]Glc_3Man_9GlcNAc_2$, respectively. The oligosaccharides were quantified by liquid scintillation counting, pooled, and treated overnight with $5 \times 10^3$ units Endo H (New England Biolabs). Glucosidase II assays were performed with both purified recombinant isoforms with enzyme concentrations normalized for *p*-NP-Glcase activity. The reactions were in 840 µl with 210 µl of enzyme, 50 mM PIPES pH 6.5 and $2.0 \times 10^4$ c.p.m. of $[^3H]Glc_{1–3}Man_9GlcNAc_2$ pooled oligosaccharides. The time points used for the assays were 0, 15, 30, and 60 min, and the reactions were terminated by boiling for 2 min. Glucose hydrolysis from the oligosaccharides was monitored by HPLC as described by Romero and Herscovics (1986).

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## Abbreviations

ER, endoplasmic reticulum; UGGT, UDP-glucose glycoprotein:glucosyltransferase; AcP, acid phosphatase**;** *p*-NP-Glc, *p*-nitrophenyl α-D-glucopyranoside; *p*-NP-Glcase, *p*-nitrophenyl α-D-glucopyranosidase; DNJ, deoxynojirimycin; CST, castanospermine; TCA, trichloroacetic acid; PAGE, polyacrylamide gel electrophoresis; G0-AcP, acid phosphatase with N-linked $Man_9GlcNAc_2$; G1-AcP, acid phosphatase with N-linked $Glc_1Man_9GlcNAc_2$.

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