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Glucosidase II β Subunit Modulates *N*-Glycan Trimming in Fission Yeasts and Mammals

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Glucosidase II (GII) plays a key role in glycoprotein biogenesis in the endoplasmic reticulum (ER). It is responsible for the sequential removal of the two innermost glucose residues from the glycan ($Glc_3Man_9GlcNAc_2$) transferred to Asn residues in proteins. GII participates in the calnexin/calreticulin cycle; it removes the single glucose unit added to folding intermediates and misfolded glycoproteins by the UDP-Glc:glycoprotein glucosyltransferase. GII is a heterodimer whose α subunit (GIIα) bears the glycosyl hydrolase active site, whereas its β subunit (GIIβ) role is controversial and has been reported to be involved in GIIα ER retention and folding. Here, we report that in the absence of GIIβ, the catalytic subunit GIIα of the fission yeast *Schizosaccharomyces pombe* (an organism displaying a glycoprotein folding quality control mechanism similar to that occurring in mammalian cells) folds to an active conformation able to hydrolyze *p*-nitrophenyl α-D-glucopyranoside. However, the heterodimer is required to efficiently deglucosylate the physiological substrates $Glc_2Man_9GlcNAc_2$ (G2M9) and $Glc_1Man_9GlcNAc_2$ (G1M9). The interaction of the mannose 6-phosphate receptor homologous domain present in GIIβ and mannoses in the B and/or C arms of the glycans mediates glycan hydrolysis enhancement. We present evidence that also in mammalian cells GIIβ modulates G2M9 and G1M9 trimming.

INTRODUCTION

The endoplasmic reticulum (ER) of eukaryotic cells discriminates between native and nonnative protein conformations, selectively transporting properly folded proteins to their final destinations through the secretory pathway, or alternatively, retrotranslocating misfolded proteins to the cytosol to be degraded by proteasomes. Protein glycosylation itself and processing of the glycan transferred play a key role in the folding and conformation discrimination of glycoproteins within the ER. The glycan ($Glc_3Man_9GlcNAc_2$) transferred to Asn residues is first deglucosylated by glucosidase I, a type II membrane protein with a lumenal hydrolytic domain, which removes the outermost Glc of the glycan (see Figure 1 for a depiction of all glycan mentioned in text). The $Glc_2Man_9GlcNAc_2$ (G2M9) thus produced is then deglucosylated by glucosidase II (GII) that successively generates $Glc_1Man_9GlcNAc_2$ (G1M9) and $Man_9GlcNAc_2$ (M9) upon cleavage of Glcα1,3Glc (cleavage 1) and Glcα1,3Man (cleavage 2) bonds. Both GII-mediated cleavages play a determining role in the quality control of glycoprotein folding in the ER. Monoglucosylated glycan-bearing glycoproteins may interact with calnexin (CNX) and/or calreticulin (CRT), two ER-resident lectin chaperones that enhance folding efficiency by preventing aggregation and facilitating correct disulfide bond formation through their interaction with ERp57, a protein disulfide isomerase. Furthermore, the interaction of folding intermediates and misfolded glycoproteins and the lectin-chaperones prevent their exit from the ER to the Golgi. The second GII-mediated cleavage that generates M9 abolishes the glycoprotein-lectin-chaperone interaction, thus allowing glycoproteins to pursue their transit through the secretory pathway. However, if not yet properly folded, glycoproteins may be reglucosylated by the uridine 5′-diphosphate (UDP)-Glc:glycoprotein glucosyltransferase (GT), an enzyme that specifically glucosylates nonnative conformers and regenerates monoglucosylated glycans. These, in turn, interact again with the lectin chaperones. Cycles of reglucosylation and deglucosylation catalyzed by the opposing activity of GT and GII continue until species acquire their proper tertiary structure (for reviews, see Parodi, 2000; Trombetta and Parodi, 2003).

GII is a soluble ER-resident heterodimer composed of two tightly but noncovalently bound α and β chains (GIIα and GIIβ) (Trombetta *et al*., 1996, 2001). GIIα is a 95-110 kDa protein that contains the consensus sequence (G/F)(L/I/V/M)WXDMNE) of the active site of family 31 glycosyl hydrolases and lacks the ER retention signal –XDEL at its C-terminus (Trombetta *et al*., 1996; D'Alessio *et al*., 1999). This subunit has a single active site but it has been proposed to have different kinetics for the first and second cleavages (Alonso *et al*., 1993), although recent work suggests that the differential trimming rates of both Glc units is not operative

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Abbreviations used: CHX, cycloheximide; CNX, calnexin; CRT, calreticulin; DTT, dithiothreitol; Endo H, endo-β-*N*-acetylglucosaminidase H; ER, endoplasmic reticulum; GII, glucosidase II; G1M4-5, $Glc_1Man_{4-5}GlcNAc$; G2M4-5, $Glc_2Man_{4-5}GlcNAc$; G1M9, $Glc_1Man_9GlcNAc$; G2M9, $Glc_2Man_9GlcNAc$; GT, UDP-Glc:glycoprotein glucosyltransferase; M9, $Man_9GlcNAc$; MRH, mannose 6-phosphate receptor homologous; NMDNJ, *N*-methyl 1-deoxynojirimycin; PMSF, phenylmethyl sulfonyl fluoride; pNPG, *p*-nitrophenyl α-D-glucopyranoside; YFP, yellow fluorescent protein.

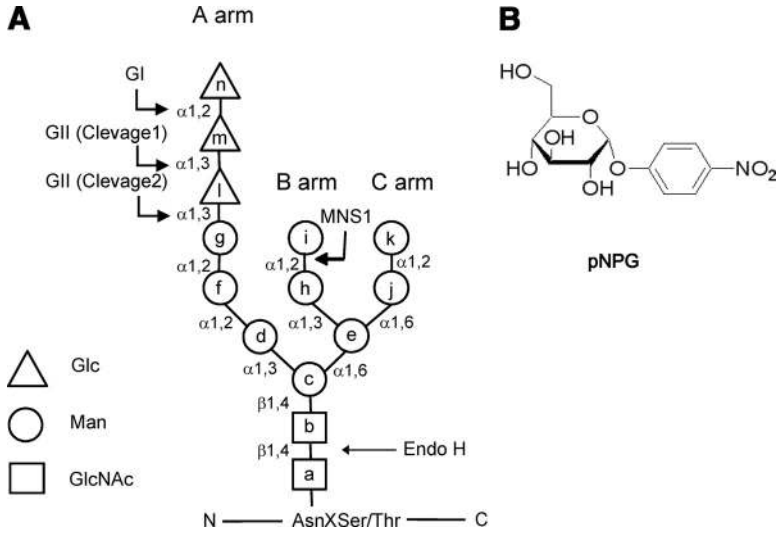

Figure 1. Glycan structures. (A) The structure depicted is that of the glycan transferred to Asn residues in *N*-glycosylation. Lettering (a, b, c …) follows the order of addition of monosaccharides in the synthesis of the dolichol-P-P derivatives. Glucosidase I removes residue n and GII, residues l and m. GT re-adds residue l. ER mannosidase cleaves residue i. G2M9, G1M9, and M9 comprise residues a–m, a–l, and a–k, respectively, except when the glycans had been previously treated with Endo H. Because this enzyme cleaves the bond between residues a and b, those glycans are devoid of residue a. Jack bean α-mannosidase removes residues h–k and partially also residue e from the glucosylated glycans. Therefore, G2M4-5 comprise residues b–d, f, g, l, and m and partially also residue e. G1M4-5 is similar to G2M4-5 but lacks residue m. Arm A comprises residues d, f, g, and l–n; arm B comprises residues h and I; and arm C comprises residues j and k. (B) Structure of pNPG.

at the high protein concentrations occurring within the ER lumen (Totani *et al*., 2008).

The roles of GIIβ are controversial and have been object of growing interest in the last years, as autosomal dominant polycystic liver disease may develop in individuals carrying mutations in GIIβ gene (Drenth *et al*., 2003, 2005; Davila *et al*., 2004) and GIIβ is induced in differentiating neuritic rat progenitor cells and in response to the glial cell-derived neurotrophic factor (Hoffrogge *et al*., 2007). GIIβ is a 50- to 60-kDa subunit that has been suggested to be responsible for GIIα maturation to an active conformation in mammals (Pelletier *et al*., 2000; Treml *et al*., 2000) as well as for its presence in the ER as GIIβ displays an ER retention/retrieval consensus sequence at its C-terminus (Trombetta *et al*., 1996; D'Alessio *et al*., 1999). In contrast, the GIIβ subunit of the budding yeast *Saccharomyces cerevisiae* does not display a consensus ER retention/retrieval sequence. Furthermore, GIIα was also retained in the ER of GIIβ null mutants and G1M9 was the *N*-glycan formed in these last cells, indicating that GIIβ is required for the second but not for the first cleavage (Wilkinson *et al*., 2006). It should be mentioned that this microorganism lacks a classical CNX/CRT cycle because it does not express an active GT (Fernández *et al*., 1994).

Using a genetic approach, we have demonstrated previously the heterodimeric nature of GII in the fission yeast *Schizosaccharomyces pombe* and that microsomes from ΔGIIα and ΔGIIβ mutant cells are devoid of GII activity when using G1M9 as substrate in the assays. Nevertheless, whereas *N*-glycans formed in intact ΔGIIα cells were identified as G2M9, ΔGIIβ cells formed, in addition, small amounts of G1M9 (D'Alessio *et al*., 1999). It was suggested then that this last compound was formed either by GIIα transiently present in the ER in its way to secretion and/or by low amounts of GIIα that folded successfully in the absence of GIIβ. Moreover, ΔGIIβ cells presented the unfolding protein response as the BiP gene was induced in these mutant cells, thus showing that the subunit plays a key role in the efficient folding of glycoproteins. *S. pombe* constitutes an ideal organism to study the role of GIIβ because it has a glycoprotein folding quality control mechanism similar to that occurring in mammalian cells and it expresses an active GT (Fernández *et al*., 1994; D'Alessio *et al*., 1999). Furthermore, its GIIβ presents a high homology to its mammalian counterpart, including the presence of a consensus ER retention/retrieval sequence at its C terminus.

An efficient *N*-glycan deglucosylation is not only highly relevant to a proper glycoprotein folding quality control but also to the numerous *N*-glycan processing reactions occurring as glycoproteins migrate through the Golgi and that determine the final glycan structures that in many cases play key roles in cellular processes. In the present work, we studied whether GIIβ is solely involved in GIIα retention and folding or if, additionally, it also participates in the regulation of *N*-glycan hydrolysis.

MATERIALS AND METHODS

Materials

Yeast extract, bactopeptone, and yeast nitrogen base with or without ammonium sulfate were from Difco (Detroit, MI). *p*-Nitrophenyl α-D-glucopyranoside (pNPG), endo-β-*N*-acetylglucosaminidase H (Endo H), Jack bean α-mannosidase, protease inhibitors, lysing enzyme, dithiothreitol (DTT), amino acids, and supplements for culture media were from Sigma-Aldrich (St. Louis, MO). Zymolyase 100T was from Seikagaku Kogyo (Tokyo, Japan), and [^{14}C]Glc (301 Ci/mol) was from PerkinElmer Life and Analytical Sciences (Boston, MA). *N*-Methyl-1-deoxynojirimycin (NMDNJ) was from Research Chemicals (North York, ON, Canada). Enzymes used for DNA procedures were from New England Biolabs (Ipswich, MA). Gateway LR clonase mix was purchased from Invitrogen (Carlsbad, CA).

Strains and Media

Escherichia coli DH5α and JA226 were used for cloning purposes, whereas recombinant protein expression was carried out using BL26 cells. Bacteria were grown at 37°C in Luria broth medium (0.5% NaCl, 1% tryptone, and 0.5% yeast extract), supplemented with 100 mg/l ampicillin or 35 mg/l kanamycin as needed. *S. pombe* cells were grown at 28°C in rich YES medium (0.5% yeast extract, 3% glucose, and 75 mg/l adenine) or Edinburgh minimal medium (EMM) (Moreno *et al*., 1991; Alfa *et al*., 1993), supplemented with adenine (75 mg/l), uracil (75 mg/l), and/or leucine (250 mg/l) for selective growth. *S. cerevisiae* strains were grown at 28°C in rich media (YPDA, 1% yeast extract, 2% bactopeptone, 2% glucose, and 20 mg/l adenine) or selective minimal media SD (0.67% yeast nitrogen base without amino acids and 2% glucose) plus appropriate supplements for selective growth. Geneticin was added to media at 200 mg/l for *kanMX4* marker selection. When double selection for *kanMX4* and auxotrophic markers was needed, yeast nitrogen base was replaced in SD by 1.7% yeast nitrogen base without ammonium sulfate, and 0.1% monosodium glutamate was added. *S. cerevisiae* mutant MK1-11B 9.16a and BY4741Δgls2 were kindly provided by A. Herscovics (McGill Cancer Centre, Montreal, QC, Canada) and A. Colman-Lerner (School of Sciences, University of Buenos Aires, Buenos Aires, Argentina), respectively. The *S. pombe* and *S. cerevisiae* strains used are summarized in Table 1.

Table 1. Yeast strains used in this study

Strains (nickname)	Genotype	Source
S. pombe		
Sp61 (WT)	*h*$^-$, *leu1-32, ade6-M210, ura4-D18, ade1*	Our stock (D'Alessio *et al*., 1999)
Sp61IIα (ΔGIIα)	*h*$^-$, *gls2α::ura4*$^+$, *leu1-32, ade6-M210, ura4-D18, ade1*	Our stock (Soussilane *et al*., 2009)
SpADIIβ (ΔGIIβ)	*h*$^-$, *gls2β::ura4*$^+$, *leu1-32, ade6-M210, ura4-D18*	Our stock (D'Alessio *et al*., 1999)
SpADIIαβ (ΔGIIαβ)	*h*$^-$, *gls2α::ura4*$^+$, *gls2β::ura4*$^+$, *leu1-32, ade6-M216, ura4-D18*	Our stock (Soussilane *et al*., 2009)
S. cerevisiae		
MK1-11B 9.16a	*MATα, his4-619, mns1::URA3, ams1::LEU2*	Puccia *et al.* (1993)
BY4741Δgls2	*MATa, ura3Δ0, leu2, his3Δ1, met15Δ0, Δgls2α::KanMX4*	Winzeler *et al*. (1999)
GIIM-36	*MATα, Δgls2α::KanMX4, mns1::URA3, leu2Δ0*	This study

Genetic and DNA Procedures

DNA procedures were as described previously (Sambrook and Russell, 2001). Yeast DNA extraction was done as described previously (Hoffman and Winston, 1987). Yeast transformations were performed by electroporation, by using the following conditions: *S. pombe* electrocompetent cells were prepared by extensively washing them when exponentially growing, first twice with water and then twice with 1 M sorbitol and finally resuspending cells at a 100× initial concentration. 0.5 μg of plasmidic DNA was electroporated at 1.5 kV, 200 ohm, 25 μF with a Gene Pulser II (Bio-Rad Laboratories, Hercules, CA). Cells were recovered in 0.5 M sorbitol in YES for 1 h at 28°C. For *S. cerevisiae*, sorbitol was replaced by 10% glycerol, and the electroporator was set at 2.5 kV and 200 ng of DNA was used. The *S. cerevisiae* mutant for *N*-glycan production was obtained using standard techniques of mating, sporulation, dissection, and tetrad analysis as described previously (D'Alessio *et al*., 2005). Strain GIIM-36 was obtained by crossing strain MK1-11B 9.16a with BY4741Δgls2, respectively. Relevant genotypes were determined by auxotophic growth in the appropriate media and also by colony polymerase chain reaction (PCR) using the following primers: for Δ*gls2::KanMX4* insertion gls2s 5′-AATAACATCCTTTCACACACTCACA-3′ and kanB 5′-CTGCAGCGAGGAGCCGTAAT-3′ were used, yielding a 615-base pair PCR product; and for *mns1::URA3* disruption MNS1s 5′-ATGAAGAACTCTGTCGGTATTTC-3′ and MNS1a 5′-GTTTGGATTGTGCTAATAAATGC-3′ were used, yielding a 1940-base pair fragment.

Cloning and Expression of GIIα

The GIIα gene was amplified by PCR using genomic *S. pombe* DNA as a template, with the following primers: GIIα forward 5′-AAACCGCTCGAGATGAGATATCATGGCATATG-3′ and GIIα reverse 5′-CGCGGATCCTTAAACCAAAAAAAGTTGTGG-3′ or GIIαVDEL reverse 5′-CGCGGATCCTTATAACTCATCGACAACCAAAAAAAGTTGTGG-3′ to obtain wild-type or GIIα with the C-terminal VDEL ER retention signal, respectively. PCR products were subsequently cloned into *S. pombe* nmt promoter-driven expression vector pREP3x, kindly provided by Dr. Susan Forsburg (Department of Biological Sciences at the University of Southern California, Los Angeles, CA), to obtain pREP3x-GIIα and pREP3x-GIIαVDEL. The constructs were electroporated into *S. pombe* and expression levels were regulated with thiamine added to the media.

Yeast Total Protein Extract and Microsomal Fraction Preparations

Yeast whole cell extracts were prepared from 20 ml of exponentially growing cultures (A_{600} = 2). Cells were disrupted by 10 repetitive cycles of 1-min vortexing 1-min on ice with glass beads in 1% Triton X-100, 0.1 M HEPES, pH 7.2, and 5 mM EDTA with protease inhibitors (100 μM phenylmethylsulfonyl fluoride (PMSF), 10 μM L-1-tosylamido-2-phenylethyl chloromethyl ketone, 10 μM *N*α-p-tosyl-L-lysine chloromethyl ketone, 10 μM leupeptin, 10 μM pepstatin, and 10 μM E64) and cleared by centrifugation at 20,000 × *g* for 20 min. Microsomes were prepared from 250 ml of cultures at A_{600} = 2. Cells were broken as described above but in 0.25 M sucrose, 20 mM imidazole, pH 7.2, and 5 mM EDTA buffer with protease inhibitors and the microsomal fraction was obtained as described previously (D'Alessio *et al*., 1999). Protein concentrations were determined by Bio-Rad Protein Assay as described by the manufacturer.

Analysis of Glycans Synthesized In Vivo

For assessing ER *N*-glycan composition, *S. pombe* cells in the exponential growth phase were harvested, extensively washed with EMM without glucose and then resuspended in 2 volumes (vol/wt) of the same medium. Cells were then preincubated for 5 min in 5 mM DTT and pulsed for 15 min in 5 mM Glc with 300 μCi/ml [^{14}C]Glc. In pulse-and-chase experiments, DTT concentration was then raised to 10 mM, and cycloheximide (CHX) (50 μg/ml) was added. Incubation was stopped after 30 min at 28°C. Further details on the labeling procedure and preparation of whole cell Endo H-sensitive *N*-glycans have been described previously (Fernández *et al*., 1994). Glycans were run on paper chromatography by using Whatman 1 papers (Whatman, Maidstone, United Kingdom) and 1-propanol/nitromethane/water (5:2:4) as solvent.

Synthesis of labeled N-Glycans

Glucose- and mannose-labeled [^{14}C]$Glc_2Man_9GlcNAc$ was obtained by in vivo labeling and *N*-glycan purification of *S. cerevisiae* strain GIIM-36 (*gls2α*, *mns1*, GII, and ER mannosidase minus) cells (Figure 1 and Table 1). The strain was obtained as described under Genetic and DNA Procedures, and Endo H-sensitive *N*-glycans were obtained as described previously (Fernández *et al*., 1994). [^{14}C-*glucose*]$Glc_1Man_9GlcNAc$ was obtained by glucosylation of denatured bovine thyroglobulin in the presence of UDP-[^{14}C]Glc and rat liver microsomes followed by glycan purification as described previously (Trombetta *et al*., 1989) [^{14}C]$Glc_2Man_{4\text{-}5}GlcNAc$ and [^{14}C]$Glc_1Man_{4\text{-}5}GlcNAc$ were obtained by Jack bean α-mannosidase treatment of the above-mentioned glycans as described previously (Engel and Parodi, 1985).

GII Activity Assays

GII activity was assayed in total cell *S. pombe* extracts, microsomal fractions, or rat purified GII preparations using pNPG or labeled *N*-glycans as substrates as described previously (Trombetta and Parodi, 2005), with the following modifications. For the former substrate, a 96-well plate assay was developed, in which the reactions were performed in a final volume of 50 μl of 5 mM pNPG, 0.1 M HEPES buffer, pH 7.2, for 20 min at 37°C. An amount of 125 μg of protein from *S. pombe* total cell extracts or microsomal fractions, or 0.5 μg of pure rat GII was used. Reactions were stopped by the addition of 20 μl of 10% SDS and 50 μl of 2 M Tris base. Hydrolysis was quantified by measuring absorbance at 405 nm in a multimode detector DTX 880 (Beckman Coulter, Fullerton, CA). We determined the K_m value for the *S. pombe* GII for pNPG to be ~4 mM. GII activity in yeast microsomes as well as that of purified rat liver GII toward labeled glycans was measured by incubating the indicated concentrations of protein with substrates in 40 mM sodium phosphate buffer, pH 7.2, for 10 or 15 min at 30°C. In this case, 125 μg of *S. pombe* microsomal fraction proteins, or 0.1 μg of pure rat GII were used. The reactions were stopped by the addition of 50 μl of methanol, incubated for 5 min at 60°C, centrifuged for 5 min at 15,000 × *g*, and the cleaved Glc contained in the supernatant was separated from the remaining substrate by ascending paper chromatography using 2-propanol:acetic acid:H_2O (25:4:9) as solvent and small (2.5 × 12 cm) Whatman 1 paper strips. Activity represents the percentage of total glucose released. When only the Glc residue in glycans was labeled (G1M9 and G1M4-5), 100% radioactivity corresponds to total label. For glucose- and mannose-labeled glycans (G2M9 and G2M4-5), 100% radioactivity corresponds to total label in Glc units as determined by strong acid hydrolysis of glycans followed by paper chromatography in *n*-butanol: pyridine:water (10:3:3). Based on the specific radioactivity of UDP-[^{14}C]Glc (300 Ci/mol) used for G1M9 synthesis it was calculated that the concentration of this glycan used in the assays was 0.06 μM. The concentration of G2M9 used in the assay was 10 μM as determined by carbohydrate chemical assays. These values are below the K_m values of GII for glycans (~80 μM for mammalian GII).

Antibodies and Immunodetection

Microsomal *S. pombe* proteins (250 μg) were electroblotted to Immobilon P membranes (Millipore, Billerica, MA) after 9% SDS-polyacrylamide gel electrophoresis (PAGE), and incubated with mouse anti *S. pombe* GIIα or rabbit anti-CNX antibodies. Immunodetection was carried out using enhanced chemiluminescence (West Pico SuperSignal Chemiluminescent Substrate; Thermo Fisher Scientific, Waltham, MA) with horseradish peroxidase-conjugated immunoglobulin Gs (IgGs) (Sigma-Aldrich). Mouse polyclonal serum raised against *S. pombe* GIIα protein was obtained as follows: A 1030-base pair DNA of GIIα DNA fragment was PCR amplified from genomic DNA by using primers GIIα-Nde1

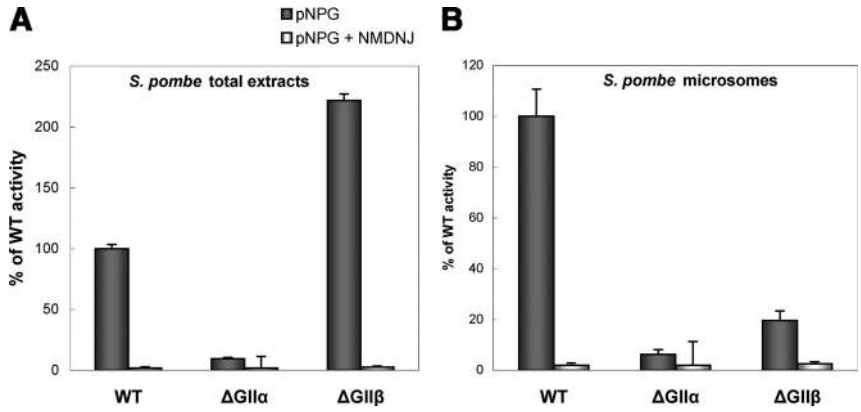

Figure 2. GIIβ is not necessary for GIIα activity toward pNPG. GII activity was measured in total cell extracts and in microsomal fractions. We incubated 125 μg of either total *S. pombe* cell extract (A) or microsomal proteins (B) of wild-type, ΔGIIα, or ΔGIIβ *S. pombe* cells with 5 mM pNPG for 20 min at 37°C. The reactions were stopped with 20 μl of 10% SDS and 50 μl of 2 M Tris base. Absorbance was measured at 405 nm.

forward 5′-GAATTCCCCATATGCAAGCTCATAAACC-3′ and GIIα-Xho1 reverse 5′-CGGCGGCTCGAGTTCAGCGTCAGGGTTGCC-3′, cloned in pET22b+ and expressed as a C-terminal His_6 fusion protein in *Escherichia coli* BL26 cells. After induction for 4 h with 1 mM isopropyl β-D-thiogalactoside, the protein was purified using an immobilized metal ion affinity chromatography chelating Sepharose (GE Healthcare, Chalfont St. Giles, Buckinghamshire, United Kingdom). Then, 7.5 μg of protein was injected intradermically to BALb/C mice, and two boosters of 5 μg were given after 15 and 30 d. The sera of 10 mice were analyzed for specificity, pooled, and used at a 1:500 dilution as a primary antibody for *S. pombe* GIIα immunodetection. For CNX rabbit polyclonal antibody production a soluble *S. pombe* CNX protein (without signal peptide and transmembrane fragment) was cloned, expressed, and purified as described above using primers CNXsNdeI 5′-GGAATTCCATATGCCTGAATCCGAACAAGAACC-3′ and CNXaXhoI 5′-TCCGCTCGAGCTCGATGATGGTTTCAAACTAG-3′. A rabbit immunization was done with 100 μg of recombinant Cnx1p, and three boosts of 50 μg each were performed after 2, 4, and 6 wk as described previously (Jannatipour *et al*., 1998). Total rabbit serum was obtained and used at a 1:100,000 dilution.

Confocal Microscopy

Images were obtained using an LSM510 Meta confocal microscope (Carl Zeiss, Oberkochen, Germany) with a plan Apochromat 63×/1.4 objective. Images were acquired with LSM software (Carl Zeiss).

GIIβ Mutagenesis

The gateway pDONR201 plasmid containing clone 26/D11 (*S. pombe* GIIβ subunit, SPCC825.02) was obtained from RIKEN DNA Bank (Matsuyama *et al*., 2006). GIIβ DNA mutants containing a single amino acid substitution (Y463F, E457Q, R438K, and Q408E) were generated by PCR using the above-mentioned plasmid as a template. The amplified sequence was phosphorylated, religated, and transferred into JA226 cells. Primers were as follows (mutagenic codons are underlined): Y463F forward mutagenic primer 5′-GAGTTTTTAATCAAAATGAAGAGC-3′, reverse primer 5′-ACATTTCTGAGCCTCCAGTAC-3′; E457Q forward mutagenic primer 5′-CAGGCTCAGAAATGTGAG-3′, reverse primer 5′-CAGTACACTAACAATCTC-3′; R438K forward mutagenic primer 5′-AAATCTGCCATTGTTACTG-3′, reverse primer 5′-ATGAGGACCATTCCAGCAAC-3′; and Q408E forward mutagenic primer 5′-GAAGATTCCATTCTATTAGG-3′, reverse primer 5′-AAAAACATTTTCGTAAAACACC-3′. A double mutant was also constructed using the following primers: Y463F/E457Q forward mutagenic primer 5′-GTGTACTGCAGGCTCAGAAATGTGAGTTTTTAATCAAAATG-3′ and Y463F/E457Q reverse primer 5′-CATTTTGATTAAAAACTCACATTTCTGAGCCTGCAGTACAC-3′. Wild-type and mutated GIIβ DNA clones were transferred to the pREP1-ccdb2 and pDUAL-YFH1c-ccdb2 Gateway-compatible *S. pombe* destination expression vectors (RIKEN) by the LR recombination reaction (Invitrogen). It should be noted that 10 amino acids beyond the VDEL sequence were present at the C terminus of GIIβ in the above-named constructions. *S. pombe*-competent ΔGIIβ cells were electroporated with either 3 μl of the pREP1-GIIβ episomal constructs or with 10 μl of dialyzed NotI-digested pDUAL-GIIβ-YFH1c integrating clones as described previously (Matsuyama *et al*., 2006). Correct plasmid integration was confirmed by PCR.

Rat Liver GII Purification and Proteolysis of GIIβ Subunit

GII purification was performed as described previously (Trombetta *et al*., 1996). Purified rat GIIβ was proteolyzed as described previously (Trombetta *et al*., 2001), with minor modifications. In brief, 10 μg of GII holoenzyme protein was incubated for 5 min at 25°C with chymotrypsin at a protease:GII ratio of 1:500 in 10 mM Tris-HCl, pH 8.0. The reaction was stopped by the addition of 1 mM PMSF. As controls, no protease or PMSF alone was added. Proteolysis was monitored by 9% SDS-PAGE.

RESULTS

GIIβ Is Not Required for GIIα Activity toward pNPG

As reported previously, *S. pombe* GII is composed of two subunits GIIα (geneID SPAC1002.03c) and GIIβ (geneID SPCC825.02) (D'Alessio *et al*., 1999). Disruption of the former (ΔGIIα cells) resulted in a total loss of activity as assayed both in vivo and in vitro, whereas formation of small amounts of G1M9 was detected in vivo in cells carrying a disrupted GIIβ-encoding gene (ΔGIIβ cells). It was assumed then that GIIα in transit through the ER was responsible for the low activity detected. However, only partial misfolding of the catalytic subunit could not be discarded. Mammalian GIIα could not be expressed in an active and soluble conformation in COS cells or in a baculovirus system without simultaneously coexpressing GIIβ (Pelletier *et al*., 2000; Treml *et al*., 2000). In contrast, Feng *et al*. (2004) succeeded in expressing an active GIIα alone, but coexpression of GIIβ resulted in an increase of GII activity. It was later assumed that GIIβ participated in heterodimer maturation but not in GII activity as GIIβ could be specifically proteolyzed without affecting the enzymatic activity (Trombetta *et al*., 2001).

To study whether *S. pombe* GIIβ was involved in GIIα folding, we assayed GII activity in microsomes and in total cell extracts from wild-type, ΔGIIα, and ΔGIIβ knockout mutants. As depicted in Figure 2, A and B, ΔGIIα cells showed a nearly undetectable activity both in cell extracts and in microsomes, whereas cells lacking GIIβ displayed a high activity in the first case and a very low activity in the second case. Experimental conditions used ensured that the activity detected was essentially that of GII because 1) the substrate used (pNPG) is not hydrolyzed by GI; 2) the activity was totally abolished upon addition of 5 mM NMDNJ, a GII activity inhibitor; 3) similar results were obtained when assays were conducted under conditions in which vacuolar (lysosomal) glycosidases are inactive and/or unstable (pH 8.0) (Trombetta and Parodi, 2005); and finally, 4) almost no activity was detected in total extracts or microsomes of ΔGIIα cells. It may be concluded, therefore, that GIIβ is not required for GIIα folding or maturation to an active conformation. However, GII activity is significantly reduced in the microsomal fraction of ΔGIIβ cells (Figure 2B), suggesting that GIIβ is involved in ER localization of GIIα. A rather unexpected result was the increase in GIIα activity observed in total extracts of ΔGIIβ cells. We have no explanation for this result and are currently studying it.

GIIβ VDEL Retention Signal Participates in GII ER Retention

To gain further insight on the role of GIIβ in GIIα ER localization, we expressed GIIα with (GIIαVDEL) or without (GIIα) the GIIβ ER retention/retrieval sequence (VDEL) at their C termini in ΔGIIα and in GIIα/GIIβ minus double knockout mutants (ΔGIIαβ). We used the expression vector pREP3x that drives the expression under the control of the nmt promoter, which is repressed in the presence of thiamine. Because total absence of thiamine resulted in a very high expression level that resulted toxic for cell growth, we determined that a 0.5 μM thiamine concentration was optimal for expression levels compatible with normal cell growth. This concentration resulted also in similar expression levels for all transforming plasmids used (data not shown).

Similar activity levels were observed in microsomes of ΔGIIα cells (with endogenous GIIβ) expressing exogenous GIIα or GIIαVDEL (Figure 3A). Alternatively, although lower activity levels were observed in Δ*GIIαβ* cells expressing both GIIα forms, a higher activity was detected in the case of GIIαVDEL (Figure 3, A and C) reaching levels similar to those observed in wild type cells. In all cases GII activity was measured using pNPG as substrate. No activity was detected when measured in the presence of NMDNJ (data not shown). GII activity observed on transformation with the different constructions roughly correlated with the GIIα protein levels present in microsomes as shown by Western blot analysis (Figure 3B). The reaction kinetics of GII toward pNPG of both GIIα and GIIα-VDEL expressed in Δ*GIIαβ* cells is displayed in Figure 3C, the reaction being faster for the latter. It may be concluded that GIIβ subunit participates in the ER subcellular localization of GII by means of its VDEL retention motif as its addition to GIIα improves the ER retention of the catalytic subunit.

GIIβ Is Required for an Efficient In Vitro Glucose Trimming from G2M9 and G1M9

It was reported that in *S. cerevisiae* GIIβ is required for G1M9 trimming but not for that of G2M9 (Wilkinson *et al*., 2006). Our next aim was to determine the role of GIIβ on the trimming of physiological substrates by *S. pombe* GIIα. Microsomes from the same cells used in experiments shown in Figure 3A were now assayed with labeled G2M9 and G1M9 as substrates (Figure 4A). Although pNPG was efficiently cleaved when microsomes derived from ΔGIIαβ cells expressing GIIαVDEL were used as enzyme source, no activity toward G2M9 and G1M9 was detected (compare Figures 3A and 4A). These results clearly demonstrate that GIIβ is required in vitro for the *N*-glycan first and second Glc cleavages but that it is not necessary to hydrolyze the small analogue pNPG. To further confirm that GIIβ assists GIIα in glucose trimming from *N*-glycans, we incubated G1M9 with microsomes from ΔGIIαβ cells transformed with the GIIαVDEL construction, with microsomes from ΔGIIα cells that contain endogenous GIIβ subunit or with a mixture of both preincubated in the presence of 1% Triton X-100. As depicted in Figure 4B, a substantially higher G1M9 hydrolysis was observed with the microsomal mixture, showing that GIIβ corrects GIIα inability to efficiently trim *N*-glycans.

GIIβ Is Necessary for In Vivo Processing of Both Middle and Innermost Glucoses

To check whether also in vivo GIIβ was required for an efficient conversion of G2M9 to G1M9 and from the latter to M9 we incubated live cells with [^{14}C]Glc for 15 min in the presence of DTT. As reported previously, within the time

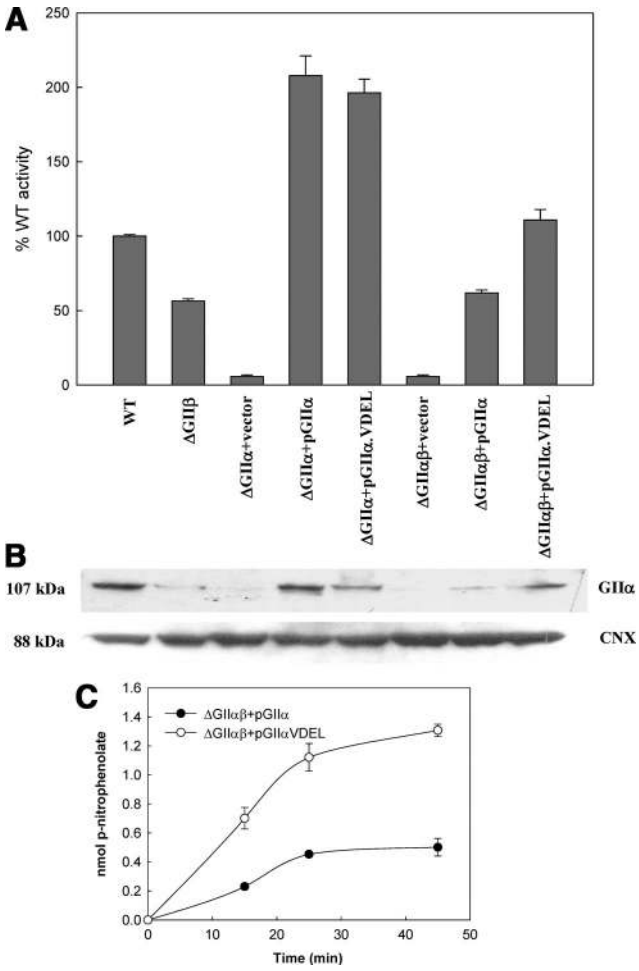

Figure 3. GIIβ VDEL retention signal participates in GII ER localization. (A and C) GII activity was measured by incubating pNPG for 20 min at 37°C with 125 μg of microsomal proteins of wild-type, ΔGIIα, ΔGIIβ, or ΔGIIαβ *S. pombe* mutant cells transformed with vector pREP3X alone or with cloned wild-type GIIα or with the same but having a C-terminal-fused VDEL (GIIαVDEL). (B) Immunodetection of GIIα in microsomal fractions. We loaded 250 μg of microsomal proteins in each lane. The membrane was blotted using mouse polyclonal anti GIIα subunit (1:500) and rabbit polyclonal anti CNX (1:100000) primary antibodies obtained as described under *Materials and Methods*. Goat horseradish peroxidase anti-mouse or rabbit IgG (1:5000 and 1:30,000, respectively) were used as secondary antibodies. Reactions were detected by chemiluminescence.

periods of the experiment, the drug impedes disulfide bond formation, thereby preventing exit of glycoproteins from the ER to the Golgi (Fernández *et al*., 1998). The 15-min sample from wild-type cells revealed the presence of M9 only, showing that total deglucosylation is extremely rapid in *S. pombe* (Figure 5A). On the contrary, the 15-min samples produced by either ΔGIIβ cells expressing endogenous GIIα or ΔGIIαβ double mutants expressing GIIαVDEL showed a limited conversion of G2M9 to G1M9 (Figure 5, B and D). We then added CHX to inhibit protein synthesis and therefore further *N*-glycosylation and incubated cells for additional 30 min. The chase samples from both cell types showed the conversion of G1M9 to M9 (Figure 5, C and E). These patterns reveal that GIIα is indeed able to degrade G2M9 and G1M9, in apparent contradiction with results obtained in vitro. The extremely low rates of deglucosylation observed cannot be ascribed to a lower GIIα content

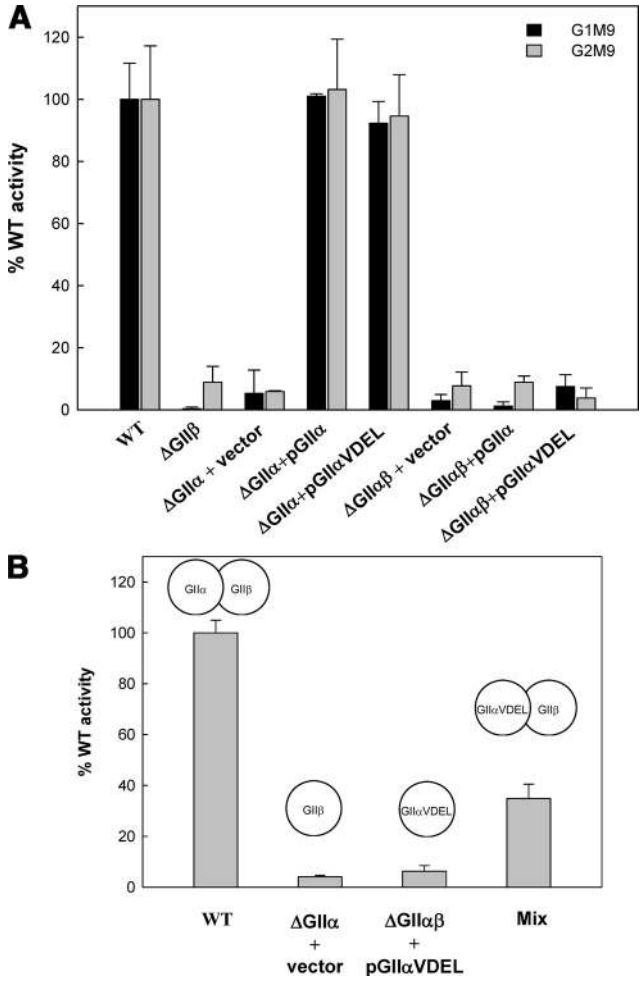

Figure 4. GIIβ is required for an efficient in vitro G2M9 and G1M9 trimming. (A) We incubated 125 μg of microsomal fraction proteins from indicated cells with 6000 cpm of G2M9 or 2000 cpm of G1M9 (see *Materials and Methods* for percentages of labeled Glc in each substrate) for 15 min at 30°C in 40 mM sodium phosphate buffer, pH 7.2. Reactions were stopped and cleaved glucoses were separated on paper chromatography. (B) Complementation between GIIα and GIIβ. We incubated 125 μg of proteins from microsomes containing only GIIβ (ΔGIIα mutants) or 125 μg of proteins from microsomes containing only GIIα (ΔGIIαβ cells transformed with pGIIαVDEL) or the mixture of both (Mix: ΔGIIα mutants + ΔGIIαβ cells transformed with pGIIαVDEL) for 30 min at 4°C in 0.1 M HEPES buffer, pH 7.2, 5 mM EDTA, and 1% Triton X-100, and then assayed for GII activity by using 2000 cpm of [^{14}C-*glucose*]G1M9 as described in Figure 4A.

because experiments shown in Figure 3A revealed that the rate of pNPG degradation was similar in microsomes derived from wild-type and GIIαVDEL-expressing cells. Furthermore, patterns depicted in Figure 5, C and E, indicate that G2M9 and G1M9 were deglucosylated by GIIα at roughly the same rates: a preferential G2M9 deglucosylation would have yielded a G1M9 proportion much higher than that of M9, whereas a preferential second cleavage would have resulted in the opposite proportion of glycans. The roughly similar deglucosylation rates of both substrates agrees with previous results obtained in cell free assays containing protein concentrations resembling those occurring within the ER lumen (Totani *et al*., 2008). Evidence that GIIβ was responsible for accelerating of both deglucosylation reactions was provided by the fact that expression of exogenous GIIβ in ΔGIIβ cells restored the rapid deglucosylation rates of both substrates (Figure 5, F and G).

The Mannose 6-Phosphate (man 6-P) Receptor Homologous (MRH) Domain in GIIβ Is Involved in Glycan Hydrolysis Rate Enhancement

The GIIβ C-terminal region has a domain highly homologous to the MRH lectin domain of the mammalian man 6-P receptor, involved in the transport of glycoprotein enzymes from the Golgi to lysosomes (Munro, 2001). All amino acids responsible for interaction with the mannose residues are also present in GIIβ, with the notable exception of those interacting with the phosphate (Figure 6A). The presence of the MRH domain strongly suggests that this domain might be involved in the GIIβ-mediated enhancement of G2M9 and G1M9 hydrolysis. Reported work on the bovine man 6-P receptor showed that the following four amino acids were essential for binding the sugar moiety: Q66, R111, E133, and Y143 (Olson *et al*., 1999; Sun *et al*., 2005). In the alignment shown in Figure 6A, *S. pombe* GIIβ residues R438, E457, and Y463 but not the Q align perfectly with identical residues in the four proteins shown. We mutated Y463F, E457Q, and R438K and also Q408E, because it was the only Q present in the upstream vicinity of the other three residues. Wild-type GIIβ and subunits bearing individual or a double (Y463F-E457Q) mutation were expressed in a ΔGIIβ strain. Expressed wild-type but not GIIβ mutant subunits corrected the defect of the ΔGIIβ strain in G2M9 and G1M9 deglucosylation (Figure 6B). However, all GII mutant heterodimers were capable of hydrolyzing pNPG at an optimal rate. Wild-type GIIβ and the R438K mutant both fused to yellow fluorescent protein (YFP) showed an ER subcellular localization by fluorescent confocal microscopy when expressed in ΔGIIβ cells (Figure 6C). These results demonstrate first that the GIIβ MRH domain is responsible for GIIα efficient trimming of its physiological substrates but not of the substrate analogue pNPG; and finally, that the GIIβ MRH domain is neither responsible of the GIIα–GIIβ interaction nor of GII ER localization.

To check whether the GIIβ MRH domain was also required for an efficient in vivo deglucosylation of G2M9 and G1M9 we expressed Y463F and E457Q GIIβ mutants in ΔGIIβ cells and pulsed-chased them for 15 and 30 min as above. Results obtained with the first mutant (the other one gave almost identical results) are depicted in Figure 6, D and E. The 15-min pulse sample showed the presence of G2M9, G1M9, and a shoulder at the position of M9. This last compound was a major compound after the 30-min chase. Comparison of Figures 6, D and E, and 5A confirmed that a functional MRH domain in GIIβ was required in vivo for an efficient total deglucosylation of G2M9.

To study the possibility that the MRH domain might be interacting with mannose units in the *N*-glycans we incubated microsomes derived from wild-type or ΔGIIαβ cells expressing GIIαVDEL with either G2M4-5 or G1M4-5, that is, with glycans in which an α-mannosidase treatment had removed mannoses in B and C arms (see Figure 1 for identification of glycan arms). As shown in Figure 6F, removal of mannose units drastically decreased glycan trimming rates, the same as mutating the MRH domain. These results strongly suggest that GIIβ recognizes mannoses in those arms and not in arm A.

Mammalian GIIβ Is Required for an Efficient Trimming of Both Internal Glucoses

To analyze whether also in mammalian cells the GIIβ subunit assists GIIα in *N*-glycan trimming, purified mammalian

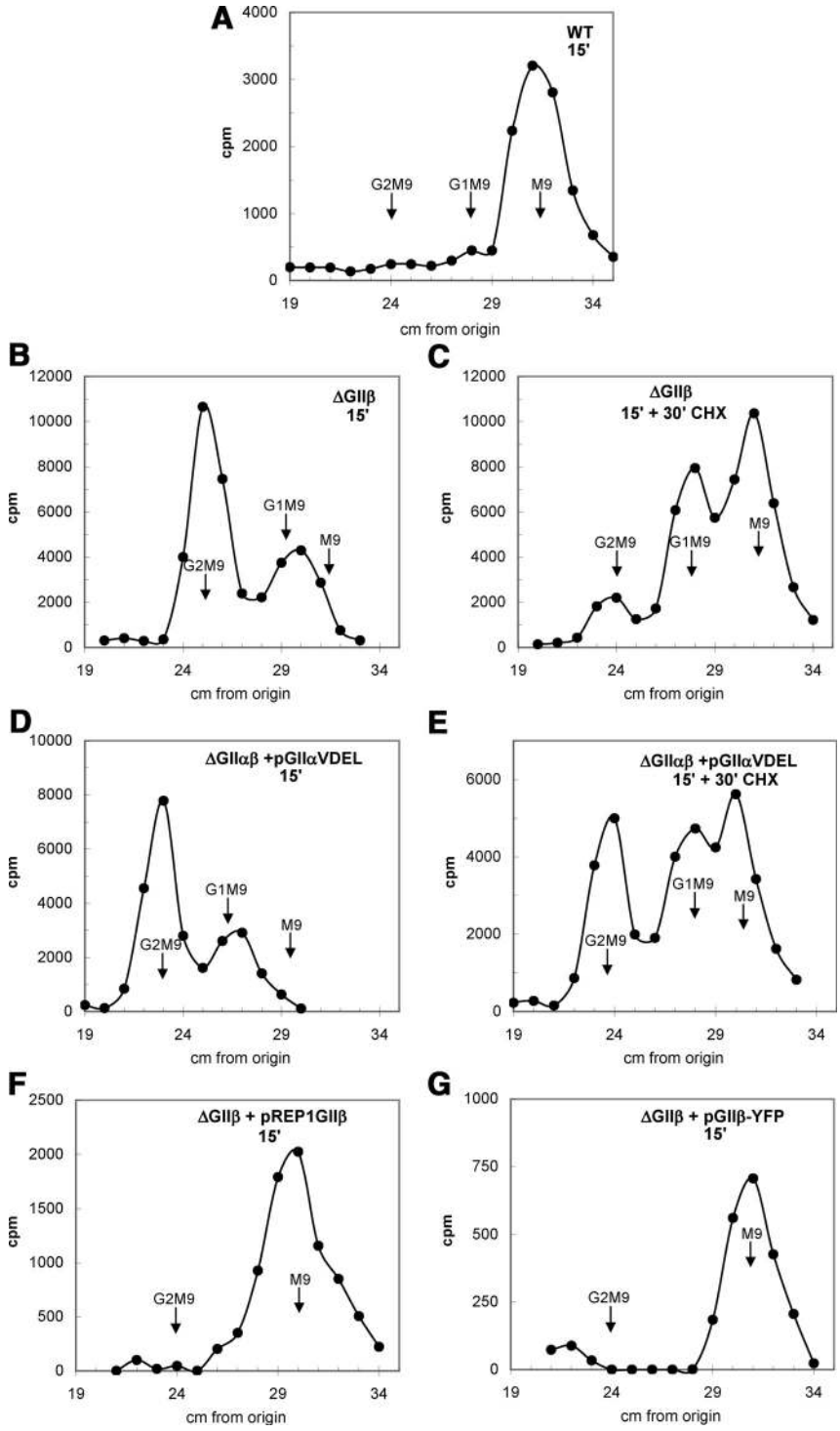

Figure 5. GIIβ is required for an efficient in vivo G2M9 and G1M9 trimming. *S. pombe* wild type (A), ΔGIIβ mutant cells expressing endogenous GIIα (B and C), ΔGII$\alpha\beta$ mutants expressing ΔGIIαVDEL (D and E), or ΔGIIβ mutants expressing exogenous GIIβ (F) or YFP-fused GIIβ (G) were incubated for 15 min with [^{14}C]Glc (A, B, D, F, and G), chased with CHX and further incubated for 30 min (D and E). Migration of standards is indicated.

GII heterodimer was subjected to regulated proteolysis with chymotrypsin. GII has a highly asymmetric structure: although GIIα has a globular shape, GIIβ is not globular and extended (Trombetta *et al*., 2001). This makes GIIβ much more sensitive to protease treatment than GIIα. As communicated by Trombetta *et al*. (2001), controlled proteolysis of pure rat liver GII leads to a preferential degradation of GIIβ that, at least in its first stages, does not affect glucose removal from pNPG. We repeated the experiment, confirmed the partial degradation of GIIβ but not of GIIα by SDS-PAGE (Figure 7A, inset) and compared pNPG hydrolysis rate by native or proteolyzed preparations. As depicted in Figure 7A the activity toward pNPG was not modified by the treatment. In contrast, GIIβ proteolysis resulted in a decrease of enzymatic activity when G2M9 and G1M9 were used as substrates (Figure 7B). Results presented show that, as in *S. pombe*, mammalian GIIβ subunit also modulates G2M9 and G1M9 trimming.

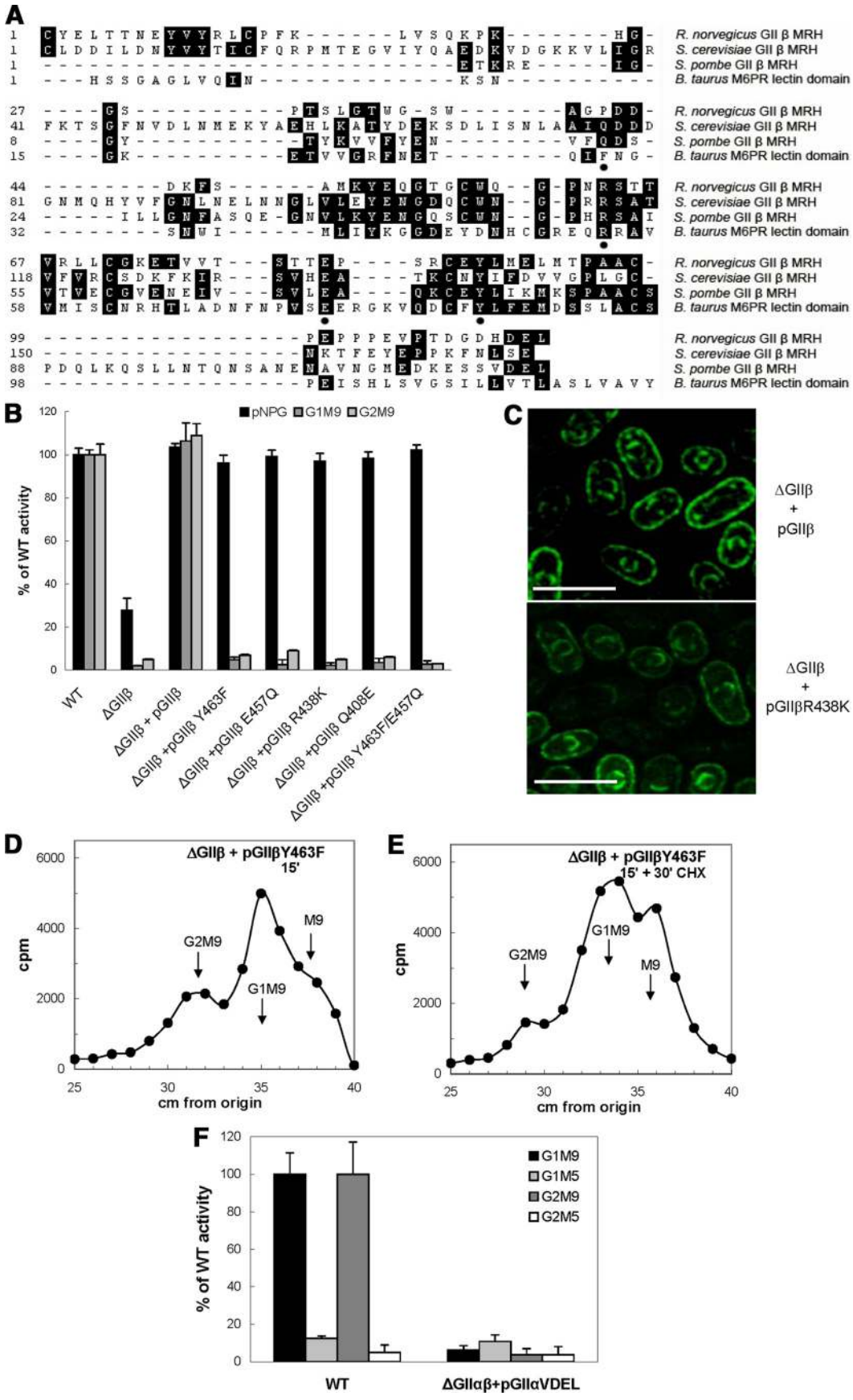
A
R. norvegicus GII β MRH
S. cerevisiae GII β MRH
S. pombe GII β MRH
B. taurus M6PR lectin domain
B
pNPG G1M9 G2M9
% of WT activity
WT
ΔGIIβ
ΔGIIβ + pGIIβ
ΔGIIβ +pGIIβ Y463F
ΔGIIβ +pGIIβ E457Q
ΔGIIβ +pGIIβ R438K
ΔGIIβ +pGIIβ Q408E
ΔGIIβ +pGIIβ Y463F/E457Q
C
ΔGIIβ + pGIIβ
ΔGIIβ + pGIIβR438K
D
ΔGIIβ + pGIIβY463F 15'
G2M9
G1M9
M9
cpm
cm from origin
E
ΔGIIβ + pGIIβY463F 15' + 30' CHX
F
G1M9
G1M5
G2M9
G2M5
% of WT activity
WT
ΔGIIαβ+pGIIαVDEL

Figure 6.

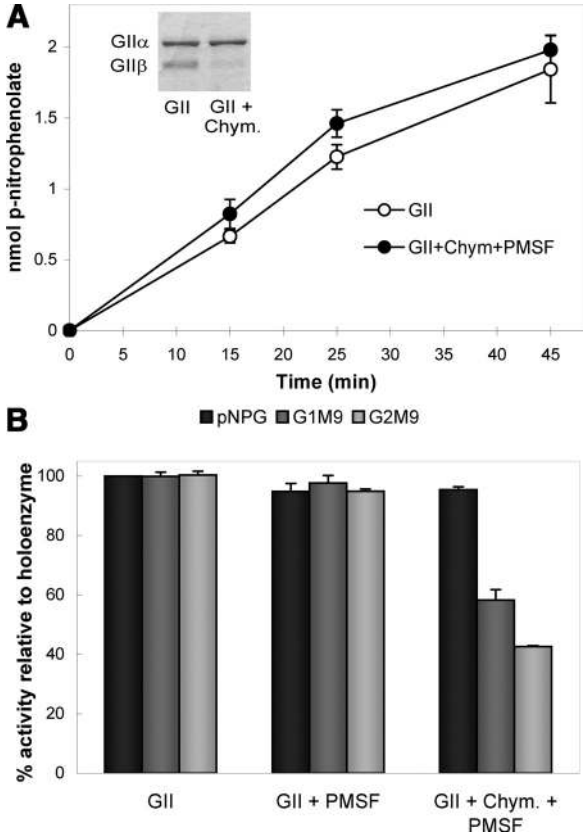

Figure 7. Proteolysis of rat liver GIIβ decreases G2M9 and G1M9 trimming rates. Ten micrograms of rat liver purified GII was proteolyzed with chymotrypsin at a 1/500 ratio for 5 min at 25°C. Reactions were stopped with 1 mM PMSF, and partial GIIβ specific proteolysis was checked by 9% SDS-PAGE (inset). (A) pNPG hydrolysis by purified native and partially proteolyzed rat liver GII was measured at indicated times. (B) GII activity toward pNPG, G1M9 and G2M9 was measured with 0.5 μg (pNPG) or 0.1 μg (*N*-glycans) of protein from native or proteolyzed preparations. Activity in chymotrypsin treated samples (GII + Chym + PMSF) or PMSF control samples (GII + PMSF) are referred relative to the activity of untreated GII.

Figure 6. (cont) Involvement of the GIIβ MRH domain in *N*-glycan trimming. (A) Sequence alignment of MRH domains from GIIβ from *S. pombe*, *S. cerevisiae* (NP_010507), rat (NP_001100276) ,and from the bovine 46-kDa mannose 6-P receptor (NP_786973). Identical amino acids are boxed in black. Dots indicate amino acids mutated in *S. pombe* GIIβ. (B) Microsomes from *S. pombe* ΔGIIβ cells, transformed with pREP1 in which wild-type GIIβ or the same subunit displaying indicated mutations had been cloned, were incubated with pNPG, G2M9, or G1M9. Glc release was determined as described in legends to Figures 3 and 4. (C) Fluorescence confocal microscopy of ΔGIIβ *S. pombe* mutants complemented with wild-type and R438K GIIβ mutant coupled to YFP. Bar, 10 μm. (D and E) In vivo *N*-glycan processing in ΔGIIβ cells transformed with the Y463F GIIβ mutant. Cells were incubated for 15 min with [^{14}C]Glc (D) and chased for 30 min after CHX addition (E). Endo H-sensitive *N*-glycans were isolated and run on paper chromatography. (F) GII processing of truncated glycans. Assays were conducted as indicated in Figure 4A with microsomal proteins derived from wild-type or ΔGII$\alpha\beta$ mutants transformed with GIIαVDEL and G2M9, G2M4-5, G1M9, or G1M4-5 as substrates.

DISCUSSION

When the dimeric structure of GII was first reported, it was assumed that the sole role of the β subunit was that of providing an element (the -XDEL C-terminal sequence) for the ER localization of GIIα (Trombetta *et al*., 1996; D'Alessio *et al*., 1999). Further work ascribed additional roles to GIIβ, as for example in mammalian cells it was described to be required for an efficient folding of the catalytic subunit (Pelletier *et al*., 2000; Treml *et al*., 2000). These two roles (ER localization and efficient folding) proved not to be absolute as *S. cerevisiae* GIIα reached an active conformation and an ER localization in the absence of GIIβ. Moreover, in this yeast GIIβ lacked the ER retention/retrieval sequence. Rather surprisingly, GIIβ in *S. cerevisiae* proved to be required for removal of the innermost glucose unit but not that of the middle unit (Wilkinson *et al*., 2006).

Results reported here show that in *S. pombe* GIIβ is not required for GIIα proper folding because cells completely lacking GIIβ displayed a total cell GII activity when measured with pNPG as substrate, which was even higher than that found in wild-type cells (Figure 2).

Here, we show that GIIβ is indeed involved in GII ER localization, because disruption of its encoding gene decreased the ER GIIα content and furthermore, VDEL addition to GIIα C-terminus expressed in ΔGIIβ cells significantly improved the ER retention of the catalytic subunit (Figure 3). However, although the ER retention/retrieval sequence proved to partially replace GIIβ role in GIIα ER retention, it is unknown for the moment whether this element is the main determinant in GII localization. Results depicted in Figure 5, F and G, indicated that a normal glycan processing rate occurred in cells in which the GIIβ VDEL sequence was occluded by either 10 amino acids or YFP (see *Materials and Methods*), suggesting the presence of additional retention mechanisms besides the above mentioned sequence. It has been reported previously that either removing or occluding ER retention/retrieval sequences have similar effects on protein localization (Munro and Pelham, 1987). It may be concluded, therefore, that GIIβ and its ER retention/retrieval sequence at its C terminus certainly play a role in GII ER localization, but not an absolute role because other retention mechanisms seem to be operative as well. Moreover, cells lacking GIIβ had an ER GIIα content that varied between 20 and 50% of that found in wild-type cells in different preparations (Figures 2, 3, and 6), suggesting that the GIIα subunit itself may bear another yet unknown ER localization signal. Redundant subcellular localization mechanisms have been described for another soluble ER-resident protein as CRT (Sönnichsen *et al*., 1994). In that study, two independently operating retention/retrieval mechanisms were proposed to occur: one mechanism provided retention in the ER in a Ca^{2+}-dependent manner, whereas the other mechanism was a KDEL-based retrieval system.

The most significant finding reported here is that GIIβ is an absolute requirement for efficient hydrolysis of the physiological substrates G2M9 and G1M9 but not of that of pNPG, a much smaller molecule. This proved to be valid both in vitro and in vivo (Figures 4 and 5). Results obtained when pulse-chasing intact cells showed that GIIα alone was indeed able to cleave both glycans but at much reduced rates. This was not due to a lower GIIα content because ΔGII$\alpha\beta$ cells expressing GIIαVDEL, which displayed a GIIα content similar to that of wild-type cells, also hydrolyzed substrates at reduced rates.

The old observation of Grinna and Robbins (1980) that the glycan deglucosylation rates by mammalian dimeric GII decreased upon demannosylation of G2M9 and G1M9

proved to be valid also for the *S. pombe* enzyme. That is, both removal of mannose units from the glycan B and C arms or disruption of the GIIβ-encoding gene reduced the rates of *N*-glycan hydrolysis. Because GIIβ displays a sequence highly homologous to the bovine MRH domain in the man 6-P receptor involved in transporting glycoprotein enzymes from the Golgi to lysosomes, we tested the possibility that the efficient GIIα hydrolysis of glycans could be mediated by the interaction of the MRH domain and mannoses in arms B and/or C of the glycans. This happened to be the case because mutations in amino acids conserved in several MRH domain-containing proteins and believed to be involved in the interaction of the man 6-P receptor with mannose units in lysosomal glycoprotein enzymes sharply decreased the GIIβ enhancing capacity of G2M9 and G1M9 hydrolysis by the catalytic subunit (Figure 6). Moreover, that selective proteolysis of GIIβ reduced the capacity of G2M9 and G1M9 hydrolysis but not that of pNPG by rat liver GII indicates that also in the mammalian enzyme the noncatalytic subunit is required for an efficient *N*-glycan processing (Figure 7).

At least two mechanisms may be envisaged for the MRH domain-mediated enhancement of *N*-glycan deglucosylation rate. In the first mechanism, upon binding mannose units in the B and/or C arms of the glycan, the MRH domain presents bonds to be cleaved to the catalytic site in GIIα (Figure 8A). This possibility is apparently at odds with the known three-dimensional structure of $Glc_3Man_9GlcNAc_2$. As determined by NMR, the bond to be cleaved first (Glcα1,3Glc) is exposed to the external face of the A arm (residues d, f, and g, Figure 1), whereas the second bond (Glcα1,3Man) faces the internal side (i.e., it faces the B and C arms, residues e and h–k in Figure 1) (Petrescu *et al*., 1997). The need to reorient the substrate would make the mechanism suggested above highly improbable because both bonds to be cleaved lie far apart in space and cannot be conceivably be reached by the single GIIα catalytic site without such reorientation. However, this mechanism cannot be ruled out altogether as the reported flexibility of bonds joining mannoses c, d, f, and g (Figure 1) may allow the successive presentation of both bonds in the MRH domain-bound glycan to the catalytic site (Woods *et al*., 1998). It is probably the required reorientation that prevents the consecutive trimming of both residues, thereby allowing the glycoprotein to enter the CNX/CRT cycle when in the monoglucosylated form.

The second mechanism was proposed by A. Helenius and coworkers to explain the apparent mammalian GII requirement of two glycans in the same glycoprotein to efficiently perform the first cleavage (Deprez *et al*., 2005). According to this mechanism (Figure 8B and figure 7 in Deprez *et al*., 2005), binding of the GIIβ MRH domain to a glycan would not result in the presentation of the glycan to the catalytic site but in a conformational change in the GIIβ subunit that in turn would modify GIIα structure thus activating the catalytic site. Due to the structural constraints of both bonds to be cleaved mentioned above, it was proposed that binding of the GIIβ MRH domain to a glycan would activate the first cleavage in a neighboring glycan. The second cleavage in this last glycan would be activated by the binding of its own mannoses in arms B and C to the MRH domain as the arms and the Glcα1,3Man bond would be on the same face. The necessary arm rotation in the first mechanism is replaced in the second one by the successive binding of two different glycans to the MRH domain. Nevertheless, there are exceptions and cases in which glycoproteins bearing a single *N*-glycan interact with CNX/CRT are known. According to the proposed second mechanism, these cases may be due to transactivation of the first cleavage by an *N*-glycan in different glycoprotein molecules in the crowded ER environment or, for glycoproteins that have a rather long folding process, to a GII basal, GIIβ-independent activity. It is interesting to note that no experimental evidence for all assumptions in the proposed second mechanism were provided at that time, particularly the occurrence of two different GII activities, one of them independent (basal) and the other dependent on the presence of GIIβ and the activation of *N*-glycan deglucosylation by the interaction of the MRH domain in GIIβ and mannose units in the glycan B and/or C arms. Present work demonstrates that GII has indeed a basal activity that is revealed when pNPG is used as substrate in the absence of the GIIβ subunit and that in *S. pombe* and mammalian cells the GIIβ subunit sustains an efficient glycan processing by the interaction of its MRH domain and mannoses in the glycans.

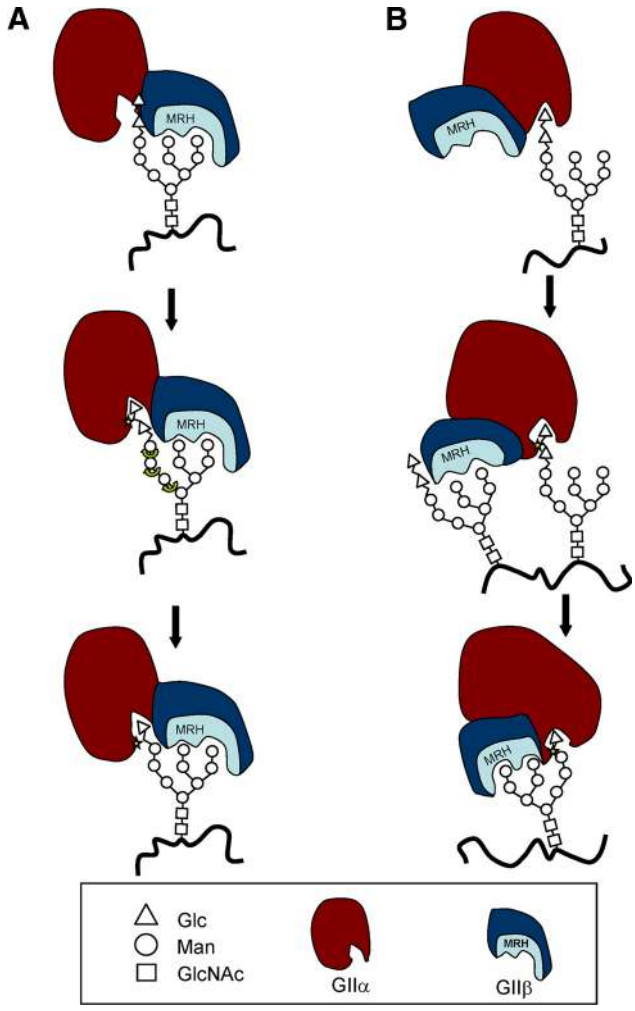

Figure 8. Models proposed for GIIβ-mediated enhancement of *N*-glycan deglucosylation. (A) On binding mannose units in the B and/or C arms of the glycan, the GIIβ MRH domain presents bonds to be cleaved to the GIIα catalytic site (star). Please note that in this model a rotation of A arm is required for both cleavages to proceed. (B) Binding of a glycan to the GIIβ MRH domain would result in a conformational change in the GIIβ subunit, which in turn would modify GIIα structure thus activating the catalytic site and allowing hydrolysis of the middle Glc residue in another glycan. Cleavage of the inner Glc in this last glycan would be enhanced by the interaction of Man units in its own B and/or C arms with the MRH domain as the bond to be cleaved and the arms lie on the same face.

Glycan structure in glycoproteins determines whether a glycoprotein is retained in the ER, sorted to its final normal destination or retrotranslocated to the cytosol to be degraded by proteasomes. It is, therefore, highly important to understand the underlying mechanisms that participate in glycan processing. GIIβ malfunction may change ubiquitous processes within the eukaryotic cell, and this may explain why mutations in this subunit affect different cellular functions. The recent observation that GIIβ is involved in neural development and RNA binding indicates that GIIβ may not only be involved in quality control of glycoprotein folding but also in novel but still poorly defined cellular processes.

ACKNOWLEDGMENTS

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