Structure of the Lectin Mannose 6-Phosphate Receptor Homology (MRH) Domain of Glucosidase II, an Enzyme That Regulates Glycoprotein Folding Quality Control in the Endoplasmic Reticulum

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Background: Glucosidase II is an endoplasmic reticulum enzyme involved in quality control of glycoprotein folding.

Results: The structure of the lectin domain of GII was determined by NMR spectroscopy.

Conclusion: GII lectin domain structure contains a unique Trp residue critical for GII activity.

Significance: GII MRH domain structure is the first determined of an MRH domain present in a protein with enzymatic activity.

Here we report for the first time the three-dimensional structure of a mannose 6-phosphate receptor homology (MRH) domain present in a protein with enzymatic activity, glucosidase II (GII). GII is involved in glycoprotein folding in the endoplasmic reticulum. GII removes the two innermost glucose residues from the Glc₃Man₇GlcNAc₂ transferred to nascent proteins and the glucose added by UDP-Glc:glycoprotein glucosyltransferase. GII is composed of a catalytic GII subunit and a regulatory GIIB subunit. GIIB participates in the endoplasmic reticulum localization of GIIr and mediates in vivo enhancement of N-glycan trimming by GII through its C-terminal MRH domain. We determined the structure of a functional GII MRH domain by NMR spectroscopy. It adopts a β-barrel fold similar to that of other MRH domains, but its binding pocket is the most shallow known to date as it accommodates a single mannose residue. In addition, we identified a conserved residue outside the binding pocket (Trp-409) present in GIIB but not in other MRHs that influences GII glucose trimming activity.

Mannose 6-phosphate receptor homology (MRH)3 domains were first described in the two receptors responsible for delivering lysosomal enzymes from the trans Golgi network to endosomal compartments in mammalian cells. In these mannose 6-phosphate receptors (MPRs), the MRH domains require the presence of mannose-linked phosphate groups in N-glycans for binding, a structural feature not required by MRH domains present in other proteins of the secretory pathway (1). Of the known MRH domain-containing proteins, three are endoplasmic reticulum (ER)-resident proteins, OS-9 (Yos9p is the yeast homolog of mammalian OS-9), Erlectin (also called XTP3-B), and glucosidase II (GII) (2).

A quality control mechanism reliant on N-glycan structures exists in the ER and ensures the correct folding of glycoproteins within the secretory pathway of eukaryotic cells (see Fig. 1A) (3, 4). OS-9 and Erlectin are apparently involved in driving misfolded glycoproteins to proteasomal degradation. The N-glycan structural requirements for OS-9/Yos9p binding have been studied in detail. Optimal recognition occurs in glycans lacking residues i and k (see Fig. 1B), thus exposing an α(1,6)-linked mannose unit (residue j) (5–7). ER mannose removal (first residue i followed by k) from the transferred glycan (Glc₃Man₇GlcNAc₂) is a relatively slow process compared with deglucosylation and thus occurs mainly in slow folding/misfolded glycoproteins. Thus, the N-glycan binding specificity of OS-9/Yos9p is particularly suited for the known function of this protein. The binding specificity of the C-terminal MRH domain of Erlectin for Man₇ has recently been reported, and it was

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** The MRH domain structure has been deposited in the Biological Magnetic Resonance Data Bank under BMRB accession number 18592.

1 The atomic coordinates and structure factors (code 2LVA) have been deposited in the Protein Data Bank (http://wwpdb.org/).
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3 The abbreviations used are: MRH, mannose 6-phosphate receptor homology; MPR, mannose 6-phosphate receptor; ER, endoplasmic reticulum; GII, glucosidase II; pNPG, p-nitrophenyl α-D-glucopyranoside; SPR, surface plasmon resonance; GAA, acid α-glucosidase; CI-MPR, cation-independent MPR; CD-MPR, cation-dependent MPR; Man-6-P, mannose 6-phosphate; Glc-6-P, glucose 6-phosphate; SUMO, small ubiquitin-like modifier; HSQC, heteronuclear single quantum coherence; r.m.s.d., root mean square deviation; UGGT, UDP-Glc:glycoprotein glucosyltransferase.

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proposed to serve as a negative regulator of ER-associated degradation (8). In contrast to these two proteins, GII is an early player in N-glycan processing as it removes the two inner glucoses from all transferred glycans (residues l and m; see Fig. 1B) irrespective of the folding status of the protein. Removal of the middle glucose (residue m) creates a monoglucosylated epitope that is recognized by two unconventional chaperones, the lectins calnexin and calreticulin. Their interactions with glycoproteins enhance the folding efficiency of nascent polypeptides and prevent the exit of folding intermediates and misfolded glycoproteins from the Golgi. Monoglucosylated N-glycans may be also formed by the action of UDP-Glc:glycoprotein glucosyltransferase (UGGT) on fully deglucosylated glycans linked to protein moieties not displaying their native structures (see Fig. 1A).

GII is a heterodimer composed of a catalytic GIIα subunit and a regulatory GIIβ subunit that contains an MRH domain and in most species a KDEL-like ER retrieval sequence at its C terminus (9, 10) (see Fig. 2A). Contrary to OS-9/Yos9p, maximal N-glycan binding by GIIβ MRH domain requires the presence of mannose residue k of arm C (Fig. 1B). Furthermore, in vivo and cell free assays show that a decrease in N-glycan mannose content results in lower GII enzymatic activity (11–15). Not only does optimal GII deglucosylation activity require the presence of the full complement of mannose units on nascent glycoproteins but also the four conserved amino acids of the GIIβ subunit that are known to be essential for the ability of the MRH domains of MPRs to bind mannose 6-phosphate (Man-6-P)-containing lysosomal enzymes (15, 16) (Fig. 2B).

Why does GII differ from most other glycosidases for which their maximal activity does not require the presence of a lectin-like domain? We have shown previously that a decrease in N-glycan mannose content sharply decreases in vivo GII activity without affecting the activity of UGGT, thereby prolonging the half-life of monoglucosylated glycans produced by UGGT (15). We speculate that ER mannosidase-mediated removal of mannose units would result in an increased interaction of slow folding/misfolded glycoproteins with the lectin chaperones. This in turn would increase the chances for proper folding of glycoproteins displaying an arduous folding process and would result in a more stringent prevention of a surreptitious exit of misfolded glycoproteins to the Golgi. Therefore, the presence of the MRH domain converts GII into a key regulator of the quality control mechanism of glycoprotein folding. As the structure of OS-9 MRH domain was recently determined (7), the significant difference between its N-glycan binding specificity and that of GII prompted us to determine the structure of GIIβ MRH domain. Here we report that GIIβ MRH domain folds into a flattened β-barrel similar to that of the MPRs and OS-9, but its binding pocket is the most shallow solved to date. Moreover, we identified a residue (Trp-409) important for GII activity and propose two models of how it could be influencing GIIβ-mediated enhancement of GII activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Yeast extract and Bacto Peptone were from Difco. Endo-β-N-acetylglucosaminidase H, porcine trypsin, protease inhibitors, p-nitrophenyl α-d-glucopyranoside (pNPG), diithiothreitol (DTT), [15N]ammonium chloride, [13C]glucose, Man-6-P, amino acids and supplements for culture media, and protease inhibitors were from Sigma. [14C]Glucoside (301 Ci/mol) was from PerkinElmer Life Sciences. N-Methyl-1-deoxynojirimycin was from Toronto Biochemicals. Man1,2Man was obtained from Dextra Laboratories (UK).

**Strains and Media**—Escherichia coli DH5α was used for cloning purposes. Recombinant GIIβ expression was performed in SHuffle T7 Express lysY competent E. coli cells (BL21 C3030, New England Biolabs). Bacteria were grown at 37 °C in LB medium (0.5% NaCl, 1% tryptone, 0.5% yeast extract) supplemented with 200 μg/ml ampicillin or 50 μg/ml kanamycin as needed. Schizosaccharomyces pombe cells were grown at 28 °C in rich YES medium (0.5% yeast extract, 3% glucose, and 75 μg/liter adenine) or Edinburgh minimal medium (17, 18) supplemented with adenine (75 μg/liter), uracil (75 μg/liter), and/or leucine (250 μg/liter) for selective growth. Thiamine (0.5 μl/l) was added to pREP3x-GIIαVDEL-transformed strains to control expression levels from nmt1 promoter. S. pombe strains used were Sp611α (ΔGIIα: h +, leu1–32, ade6-M210, ade1, ura4-D18, Δgls2::ura4Δ), ADmllβ (ΔGIIβ::h +, leu1–32, ade6-M210, ura4-D18, Δgls2β::ura4Δ), and SpADIIαβ (ΔGIIαβ::h +, leu1–32, ade6-M216, ura4-D18 Δgls2α::ura4Δ, Δgls2β::ura4Δ) as described (14).

**Cloning and Expression of Recombinant Mature S. pombe GIIβ**—Total RNA from S. pombe was obtained from exponentially growing S. pombe wild-type cultures as described (19). GIIβ-coding DNA lacking the signal peptide (first 23 amino acids) sequence was obtained by RT-PCR using the primers B2sNde1 (5′-GGGATTTATGGCAATGCCTCGGTTGTTGGA -3′) and B2aNotI (5′-ATAGTTTGCCGGCGTTCATCGACAGATGTTGCG-3′) that include Ndel and NotI restriction sites (underlined) and a Superscript II reverse transcriptase kit (Invitrogen). The PCR product was cloned in Ndel/NotI sites of pET22b(-) vector as a C-terminal His6 fusion protein. The construction was transformed into BL21 expression was performed in Bacto T7 Express lysY competent E. coli cells (BL21 C3030, New England Biolabs). Bacteria were grown at 37 °C in LB medium (0.5% NaCl, 1% tryptone, 0.5% yeast extract) supplemented with 200 μg/ml ampicillin or 50 μg/ml kanamycin as needed. Schizosaccharomyces pombe cells were grown at 28 °C in rich YES medium (0.5% yeast extract, 3% glucose, and 75 μg/liter adenine) or Edinburgh minimal medium (17, 18) supplemented with adenine (75 μg/liter), uracil (75 μg/liter), and/or leucine (250 μg/liter) for selective growth. Thiamine (0.5 μl/l) was added to pREP3x-GIIαVDEL-transformed strains to control expression levels from nmt1 promoter. S. pombe strains used were Sp611α (ΔGIIα: h +, leu1–32, ade6-M210, ade1, ura4-D18, Δgls2::ura4Δ), ADmllβ (ΔGIIβ::h +, leu1–32, ade6-M210, ura4-D18, Δgls2β::ura4Δ), and SpADIIαβ (ΔGIIαβ::h +, leu1–32, ade6-M216, ura4-D18 Δgls2α::ura4Δ, Δgls2β::ura4Δ) as described (14).

**Structure of Glucosidase II β MRH Domain**

**Materials**—Yeast extract and Bacto Peptone were from Difco. Endo-β-N-acetylglucosaminidase H, porcine trypsin, protease inhibitors, p-nitrophenyl α-d-glucopyranoside (pNPG), diithiothreitol (DTT), [15N]ammonium chloride, [13C]glucose, Man-6-P, amino acids and supplements for culture media, and protease inhibitors were from Sigma. [14C]Glucoside (301 Ci/mol) was from PerkinElmer Life Sciences. N-Methyl-1-deoxynojirimycin was from Toronto Biochemicals. Man1,2Man was obtained from Dextra Laboratories (UK).
S. pombe Microsomal Fraction Preparations—S. pombe microsomes were prepared from 250 ml of exponentially growing cultures at an $A_{600}$ of 2. Cells were harvested, washed with 5 mM NaN₃, and broken by 10 repetitive cycles of 1-min vortexing on ice with glass beads in 0.25M sucrose, 20 mM imidazole, 5 mM EDTA with protease inhibitors (100 μM PMSF, 10 μM 1,1'-tosylamido-2-phenylethyl chloromethyl ketone, 10 μM N-p-tosyl-l-lysine chloromethyl ketone, 10 μM leupeptin, 10 μM pepstatin, and 10 μM E64), and the microsomal fraction was obtained as described (10). Protein concentrations were determined using a Bio-Rad protein assay as described by the manufacturer.

Protein Expression and Purification of S. pombe GIIβ MRH Domain—MRH GIIβ cDNA encoding residues 357–450 was cloned into a pQE30 vector modified to express the small ubiqui-
uitin-like modifier (SUMO) protein with an N-terminal hexa-His tag and transformed into BL21(pREP4) cells. Constructs containing the amino acid substitutions W409A and W409F were generated using the QuikChange® Lightning site-directed mutagenesis kit (Stratagene). Cells were grown at 37 °C until an OD of ~0.7 at which time protein expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactoside. Cells were harvested after 18 h. Protein was purified from insoluble inclusion bodies using metal affinity chromatography. Refolding was performed by diluting the protein into 100 mM Tris, pH 8.0, 10 mM cysteine, 0.5 mM cystine and stirring for 2 h at 22 °C. Following concentration, the SUMO-specific peptidase ULP-1 was added to remove the SUMO domain. The protein was dialyzed and subjected to metal affinity chromatography to separate the cleaved SUMO peptide from the MRH domain. Protein for NMR analyses was then buffer-exchanged into a solution containing concentration, the SUMO-specific peptidase ULP-1 was added to remove the SUMO domain. The protein was dialyzed and subjected to metal affinity chromatography to separate the cleaved SUMO peptide from the MRH domain. Protein for NMR analyses was then buffer-exchanged into a solution containing 10 mM deuterated imidazole (pH 7.1) and 150 mM NaCl. The protein was further purified by size exclusion chromatography using a Superdex 75 column. The concentration of the purified protein was determined using the Bradford assay (Bio-Rad) with bovine serum albumin (BSA) as the standard.

**GIIβ Mutagenesis and Expression in S. pombe**—The Gateway pDONR201 plasmid containing clone 26/D11 (S. pombe GIIβ subunit, which was obtained from RIKEN DNA Bank (20)) was used as the template for single amino acid PCR mutagenesis of the GIIβ MRH domain (W409A or W409F). The amplified mutant DNA containing both GIIβ and vector sequences was phosphorylated, religated, and electroporated into DH5α cells. The following primers were used (mutagenic codons are underlined): W409F forward mutagenic primer 5′-TTCAATGG-TCCCTCATAGATCTGCC-3′ or W409A forward mutagenic primer 5′-GCTAATGGTCCTCATAGATCTGCC-3′ was used with W409 reverse primer 5′-GCAACTTTGACCATTT-TTCATAC-3′. Wild-type and mutant GIIβ DNA clones were transferred to the pREP1-cdc2 Gateway-compatible S. pombe destination expression vector (RIKEN DNA Bank) by the LR recombination reaction (Invitrogen). S. pombe competent ΔGIIβ cells were electroporated with the pREP1-GIIβ episomal constructs. The MRH binding pocket mutant constructs used in this study (pREP1-GIIβ Y462F and pREP1-GIIβ E456Q) were described in Stiglano et al. (14). Please note that the numbering in that case starts with the initial methionine, although throughout this work the numbering corresponds to the mature protein sequence.

**Synthesis of Labeled N-Glycan Substrates**—[14C-Glc]-Glc₃Man₃GlcNac was obtained by glycosylation of denatured bovine thyroglobulin in the presence of UDP-[14C]glucose (obtained as described (21)) and rat liver microsomes followed by purification of endoglycosidase H-sensitive N-glycans as described previously (22). Monoglucosylated glycans were separated from the unglucosylated compounds by two successive paper chromatographic separations performed in 1-propanol/nitromethane/H₂O (5:2:4).

**GII Activity Assays**—GII activities using the small substrate analog pNPG and labeled N-glycans as substrates were assayed in S. pombe microsomal fractions as described (14). Briefly GII activity in ΔGIIβ mutants transformed with wild-type or mutant regulatory subunits was assayed using 5 mM pNPG as the substrate in 0.1 M HEPES buffer, pH 7.2 for 20 min at 37 °C, and absorbance at 405 nm was measured, or [14C-Glc]-Glc₃Man₃GlcNac was used as the substrate in 40 mM sodium phosphate buffer, pH 7.2 for 15 min at 30 °C. In the latter case, glucosidase activity was determined as the percentage of total glucose released. Complementation assays between GIIα and endogenous GIIβ were performed using a mixture of 125 μg of proteins from microsomes containing GIIα but not GIIβ (ΔGIIαβ cells transformed with pREP3x-GIIαVDEL as described (14)) with 125 μg of proteins from microsomes containing GIIβ but not GIIα (ΔGIIα S. pombe cells expressing only endogenous GIIβ). When assaying GIIβ enhancement of GIIα activity toward N-glycans of recombinant GIIβ or MRH, the microsomal source of GIIβ subunit was replaced with 125 ng of pure recombinant GIIβ (this is in the linear range of activation; data not shown) or with the indicated MRH/GIIβ or BSA/GIIβ mass ratios. The mixtures were preincubated for 30 min at 4 °C in the presence of 1% Triton X-100 and then assayed for GII activity with [14C-Glc]-Glc₃Man₃GlcNac as described above.

**Purification of Man₃GlcNac**—Man₃GlcNac was obtained from endo-β-N-acetylglucosaminidase H-digested denatured soybean agglutinin purified from untoasted soybean meal by affinity chromatography on acid-treated Sepharose 6B (23). Soybean agglutinin (10 mg) was denatured in 8M urea for 5 h at 65 °C, dialyzed against H₂O:preincubated for 10 min at 95 °C in 0.5% SDS, 0.1 M β-mercaptoethanol in 50 mM triethylamine acetate buffer, pH 5.5; and treated with 20 milliunits of endo-β-N-acetylglucosaminidase H (Sigma) for 16 h at 37 °C. Proteins were precipitated with 66% methanol. To separate Man₃ from remaining proteins, the supernatant was dried, resuspended in 10% ethanol, and resolved in a Superdex Peptide gel filtration column (GE Healthcare) in 10% ethanol at 1 ml/min. The glycan-containing fractions were detected using the phenol-sulfuric acid assay (24), and protein elution was monitored by absorbance at 280 nm.

**Analysis of Glycans Synthesized in Vivo**—To assess ER N-glycan composition, S. pombe cells in the exponential growth phase expressing GIIβ mutations were harvested, extensively washed with 1% yeast nitrogen base medium without glucose, and resuspended in 2 volumes (v/w) of the same medium. Cells (0.5 ml) were then preincubated for 5 min in 5 mM DTT and pulsed for 15 min with 5 mM glucose containing 150 μCi of [14C]glucose (300 Ci/mol). Further details on the labeling procedure and the preparation of whole cell endo-β-N-acetylglucosaminidase H-sensitive N-glycans have been described previously (25). Glycans were separated by paper chromatography using Whatman 1 papers and 1-propanol/nitromethane/H₂O (5:2:4) as solvent, and the peaks were identified by standards run in parallel. To improve the resolution, the identified glycans were eluted from paper and resolved by HPLC using a TSKgel Amide-80 column (4.6-mm inner diameter × 25 cm; Tosoh) with a mobile phase of H₂O/CH₃CN in a linear gradient from 35:65 to 55:45 over 65 min and a flow rate of 0.75 ml/min at room temperature. Because of slight variations in retention times among runs, the positions of the peaks in paper chromatography and not the retention times from HPLC were used to identify glycans.
Structure of Glucosidase II β MRH Domain

**Surface Plasmon Resonance (SPR) Analyses**—Biosensor studies were carried out as described previously (26). All SPR measurements were performed at 25 °C using a BIACore 3000 instrument (GE Healthcare). Purified protein (acid α-glucosidase (GAA), GAA phosphomonoester, or GAA phosphodiester) was immobilized on a CM5 sensor chip (GE Healthcare) following activation of the surface using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and N-hydroxysuccinimide. The proteins were injected onto the activated dextran surface at a concentration of 10–20 μg/ml in 10 mM sodium acetate buffer, pH 5.0 (GAA phosphomonoester), pH 4.5 (GAA phosphodiester), or pH 5.0 (GAA) using immobilization buffer (10 mM MES, pH 6.5, 150 mM NaCl, 0.005% (v/v) P20) as the running buffer. After coupling, unreacted N-hydroxysuccinimide ester groups were blocked with ethanolamine. The reference surface was treated in the same way except that protein was omitted. To assess binding affinities, samples of purified proteins were prepared in a running buffer (50 mM imidazole, pH 7.4, 150 mM NaCl) and injected in a volume of 80 μl over the coupled and reference flow cells at a flow rate of 40 μl/min. After 2 min, the solutions containing the purified proteins were replaced with buffer, and the complexes were allowed to dissociate for 3 min. The sensor chip surface was regenerated with a 10-μl injection of 10 mM HCl at a flow rate of 10 μl/min. The surface was allowed to re-equilibrate in running buffer for 1 min prior to subsequent injections. The response at equilibrium (R eq) for each concentration of protein was determined by averaging the response over a 10-s period within the steady state region of the sensorgram using the BIASevaluation software package (version 4.0.1). The response at equilibrium (R eq) was plotted versus the concentration of protein and fitted by nonlinear regression to a one-site saturation binding model using the equation

\[
R_{eq} = \frac{R_{max} \times [MPR]}{K_d + [MPR]}\]

where controls for the contribution of the change in refractive index were performed in parallel with flow cells derivatized in the absence of protein and subtracted from all binding sensorsgrams.

**Data Collection and Structure Determination by Heteronuclear NMR Spectroscopy**—NMR spectra were acquired at 25 °C on a Bruker 600-MHz spectrometer equipped with a triple resonance CryoProbe™. Spectral data were processed using NMRPipe (28) and visualized using XEASY (29). Complete 1H, 13N, and 15C resonance assignments for GIIβ MRH domain were obtained in a semi-automated manner using peak lists from three-dimensional HNCO, HNCOCA, HNOCACB, HNCA, HNCAACB, HNCACO, CCONH, HBHAACONH, HCCONH, and HCCH total correlation spectroscopy and 13C (aromatic)-edited NOESY-HSQC spectra and the assignment program GARANT (30). Automated assignments were verified and completed by manual inspection. Structures of the GIIβ MRH domain were calculated using distance constraints derived from three-dimensional 15N-edited NOESY-HSQC and 13C-edited NOESY-HSQC spectra (τ mix = 80 ms). Backbone φ and ψ dihedral angle constraints were generated using TALOS+ (31) and the secondary shifts of the 1H, 13Cα, 13Cβ, 13C, and 15N nuclei. Initial structures were generated in an automated manner using the NOEASSIGN module of the torsion angle dynamics program CYANA (32). Initial structures underwent iterative rounds of manual refinement in CYANA to eliminate constraint violations. The top 20 CYANA conformers with the lowest target function were further refined using an Xplor-NIH (33) molecular dynamics protocol in explicit solvent (34). The structural statistics are listed in Table 1.

**Determination of Binding Affinities by Heteronuclear NMR Spectroscopy**—NMR studies in which 15N-labeled GIIβ MRH domain was titrated with increasing concentrations of ligand (Man-6-P (Sigma), Manα1,2Man (Dextra Laboratories), or Man,GlcnAc purified from soybean agglutinin) were carried out as described previously (35).

**Antibodies and Immunodetection**—Microsomal S. pombe proteins (250 μg) were resolved in a 10% SDS-PAGE, electroblotted to ImmobilonP membranes (Millipore), and incubated with mouse anti-S. pombe GIIα or mouse anti-S. pombe GIIβ antibodies. Immunodetection was carried out using enhanced chemiluminescence (West Pico SuperSignal Chemiluminescent Substrate, Thermo Scientific) with horseradish peroxidase-conjugated IgGs (Sigma) as described (15).

**RESULTS AND DISCUSSION**

**Full Functionality of the E. coli-expressed S. pombe GIIβ MRH Domain**—We have previously shown that, in the absence of GIIβ, GIIα folds into an active subunit that can hydrolyze the small substrate analog pNPG but is unable to efficiently hydrolyze glucose from N-glycans in vivo or in vitro (14). We developed a complementation assay and showed that a mixture of microsomes derived from mutant S. pombe cells devoid of GIIα or GIIβ subunits could partially restore the level of GII deglucosylation of Glc,Manα,GlcnAc observed in microsomes from wild-type cells (14). Using this assay, addition of recombinant GIIβ dramatically increased the Glc trimming from Glc,Manα,GlcnAc of S. pombe microsomes lacking GIIβ (Fig. 3A). To determine whether the isolated MRH domain of S. pombe GIIβ (amino acids 357–450; Fig. 2) was functional when expressed in E. coli, we used the same assay. Addition of up to 50 times more molar amounts of the recombinant GIIβ MRH domain (compared with full-length GIIβ) to microsomes containing only GIIα subunits did not enhance GII enzymatic activity (Fig. 3A). This result was expected as the MRH domain lacks the portion of the GIIβ subunit responsible for the interaction between GIIα and GIIβ subunits (GIIβ G2B domain; Fig. 2A) (15, 16). However, addition of GIIβ MRH domain inhibited GII activity in mixtures of microsomes containing GIIα plus GIIβ subunits in a dose-dependent manner (Fig. 3B), thus strongly suggesting that the recombinant GIIβ MRH domain was sequestering the high mannose glycan portion of the substrate. In contrast, BSA had no effect (Fig. 3B).

To directly assess the ability of GIIβ MRH domain to bind high mannose-type glycans, SPR experiments were conducted in which a glycoprotein, GAA, was immobilized and GIIβ MRH domain was flowed over the surface of the sensor chip. The immobilized recombinant GAA contains exclusively high mannose-type glycans with 8.35 mannose residues on average per glycan (36). In these studies, we used a robust E. coli expression system in which recombinant GIIβ MRH domain was purified from insoluble inclusion bodies and refolded. The results dem-
Lectin Domain Adopts the Conserved MRH Fold

GIILβ lectin domain adopts the conserved MRH fold. The MRH domain of GIILβ and CD-MPR share significant sequence similarity and are predicted to adopt the MRH fold. The structural MRH domain of GIILβ is shown in green, and that of CD-MPR is shown in yellow. The corresponding tyrosine residue in GIILβ, Trp-118 of OS-9 that is within hydrogen-bonding distance of the mannose ring as determined by the crystal structure of OS-9 (Protein Data Bank code 3AIH), domains 3, 5, and 9 of the MRH domain of GIILβ are shaded in red. The MRH domain is functional in binding high mannose N-glycans and thus is suitable for structural analyses (13).

The MRH domain binds high mannose type glycans with an estimated $K_d$ of 120 μM (Fig. 4A). The affinity measured by SPR, although lower, is comparable with the affinities ($K_d = 20–80 \mu M$) determined for a tetrameric form of the human GIILβ MRH domain using frontal affinity chromatography and 2-aminopyridine-labeled glycans (13). Differences may be due to differences between species, between substrates, in the analytical methods used for measurements, and between monomeric and tetrameric avidities of the protein preparations. Taken together, these results demonstrate that the refolded recombinant *S. pombe* GIILβ MRH domain is functional in binding high mannose N-glycans and thus is suitable for structural analyses (13).

**GIILβ Lectin Domain Adopts the Conserved MRH Fold**—The 300-kDa cation-independent MPR (CI-MPR) contains 15 extracellular MRH domains, three of which bind phosphomannosyl residues (domains 3, 5, and 9), whereas the smaller cation-dependent MPR (CD-MPR) contains a single MRH domain. Our crystal/solution structures of the CD-MPR and domains 1–3 and domain 5 of the CI-MPR have been determined in the
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FIGURE 4. SPR analyses of GIIβ MRH domain binding to a glycoprotein, GAA. Purified GIIβ MRH domain was flowed over the surface of a CMS sensor chip immobilized with GAA containing high mannose N-glycans (A), high mannose N-glycans with phosphomonoesters (B), or high mannose N-glycans with phosphodiester surfaces (C). The response at equilibrium (R_eq) to the GAA surfaces is plotted versus the concentration of protein (0, 5, 10, 20, 40, 80, 120, and 160 μM) and fitted as described previously (35). GAA contains exclusively high mannose-type glycans with 8.35 mannose residues on average per glycan and either 0.25 mol of Man-6-P/mol of glycan (phosphomonoester) or GlcNAc-modified glycan (phosphodiester) (36). The mean and S.D. from three independent analyses are as follows for the high mannose, phosphomonoester, and phosphodiester surfaces, respectively: wild type, 121 ± 35, 115 ± 16, and 111 ± 28 μM; W409A, 294 ± 219, 203 ± 44, and 395 ± 44 μM; W409F, 193 ± 72, 176 ± 35, and 212 ± 73 μM.

Table 1

| r.m.s., root mean square. |  |
|--------------------------|--|
| Experimental constraints |  |
| Distance constraints     |  |
| Long                      | 737 |
| Medium (1 < (i - j) ≤ 5)  | 260 |
| Sequential (i - j) = 1    | 465 |
| Intraresidue (i = j)      | 338 |
| Total                     | 1,800 |
| Dihedral angle constraints (φ and ψ) | 130 |
| Average atomic r.m.s.d. to the mean structure (Å) |  |
| Residues                  | 358–450 |
| Backbone (Ca, C', N)      | 0.39 ± 0.06 |
| Heavy atoms               | 0.83 ± 0.09 |
| Deviations from idealized covalent geometry |  |
| Bond length r.m.s.d. (Å)  | 0.019 |
| Torsion angle violations  | 1.4 |
| Constraint violations     |  |
| NOE distance              |  |
| Number >0.5 Å             | 0.0 ± 0 |
| r.m.s.d. (Å)              | 0.020 ± 0.001 |
| Torsion angle violations  |  |
| Number >5°                | 0.0 ± 0 |
| r.m.s.d. (°)              | 0.734 ± 0.089 |
| WHATCHECK quality indicators |          |
| Z-score                   | 0.34 ± 0.15 |
| r.m.s. Z-score            |  |
| Bond lengths              | 0.85 ± 0.02 |
| Bond angles               | 0.67 ± 0.02 |
| Bumps                     | 0.0 ± 0 |
| Lennard-Jones energy* (kJ mol⁻¹) | −2,261 ± 47 |
| Ramachandran statistics (% of all residues) |  |
| Most favored              | 83.5 ± 1.8 |
| Additionally allowed      | 13.7 ± 1.7 |
| Generously allowed        | 1.3 ± 0.9 |
| Disallowed                | 1.5 ± 0.5 |

*Nonbonded energy was calculated in Xplo-NIH.

Presence of a carbohydrate ligand (35, 37). These structures allowed us to identify four conserved residues (Gln, Arg, Glu, and Tyr), which interact with the 2-, 3-, and 4-hydroxyl groups of the mannose ring, that are essential for Man-6-P recognition by the MRH domains of MPRs. These four residues, which form the binding pocket, are also conserved in the GIIβ MRH domain (Fig. 2B). Mutagenesis of the analogous residues in S. pombe GIIβ MRH domain abolished the ability of GIIβ to enhance glycan trimming by GII in live cells (14). Previous studies showed that the GIIβ MRH domain recognizes mannose residues in the B and/or C arms of N-glycans as the absence of these mannose residues in the glycan prevents trimming of the glucose in arm A by GII both in vitro and in living cells (11–13, 15, 38). The differences in substrate specificity between this ER glycoprotein folding quality control enzyme and OS-9, a receptor involved in ER-associated degradation, prompted us to obtain the solution structure of the isolated GIIβ MRH domain from S. pombe using three-dimensional NMR techniques. Sample conditions were optimized as described under “Experimental Procedures” to obtain high quality spectra of 15N/13C-labeled recombinant GIIβ MRH domain at pH 7.2 (Table 1).

S. pombe GIIβ is the shortest (89 residues; Fig. 2) of the MRH domain structures that have been determined to date (eight of the 15 MRH domains of CI-MPR (35, 39–41), CD-MPR (42), and OS-9 (7)). GIIβ MRH domain folds into a flattened stranded β-barrel comprising two antiparallel β-sheets oriented orthogonally over one another (Fig. 5, A–C). This structure is similar to the structures reported for the MRH domains of the MPRs and OS-9 with the overall fold of GIIβ MRH domain being most similar to that of OS-9 (r.m.s.d. = 1.32 Å; 85 Ca atoms) (Fig. 5D). Both OS-9 and GIIβ adopt a compact β-barrel structure due to their lack of ~20 amino acids present at the N terminus of the CD-MPR and CI-MPR MRH domains that form either an α-helix or two to three short β-strands, respectively. However, S. pombe GIIβ MRH domain loops A and B as well as β-strands 4/5 and 6 are significantly shorter than those in OS-9 (Fig. 5D). Additionally, S. pombe GIIβ MRH domain contains only four cysteine residues in contrast to the six (CD-MPR, CI-MPR domain 5, and OS-9) or eight (domains 3 and 9 of the CI-MPR) cysteine residues found in the other glycan-binding MRH domains. The absence of the N-terminal disulfide bond in S. pombe GIIβ MRH domain that is found in other MRH domains as well as other species of GIIβ does not appear to affect the overall fold of the domain. The structural stability provided by the N-terminal disulfide bond may be
replaced in *S. pombe* GIIβ MRH domain with electrostatic interactions between Glu-364 and Lys-366 and Lys-375 located on strands 1 and 2, respectively (Fig. 5).

The dynamic nature of the GIIβ MRH domain was evaluated by comparison of r.m.s.d. values calculated between corresponding Cα atoms for each member of the structure ensemble and $^{15}$N–H heteronuclear NOE experiments. Unlike domain 5 of the CI-MPR, which showed overall greater mobility as assessed by $^{15}$N–H heteronuclear NOE values (35), the compact nature of GIIβ MRH domain with shortened loops between strands 1 and 2 and strands 4/5 and 6 coupled with the presence of a disulfide tethering loops C and D together results in comparably lower flexibility in the molecule in the absence of ligand and r.m.s.d. values more similar to domain 5 of CI-MPR.
in the ligand-bound state. These results demonstrate that the structure of GII/ H9252 MRH domain is relatively constrained with the exception of residues in loops C and D (Fig. 5, A, C, and E).

Binding Pocket and Specificity of GII/H9252 MRH Domain—To map the binding pocket of GII/H9252 MRH domain with carbohydrates, increasing amounts of the disaccharide Man/H92511,2Man, the monosaccharide Man-6-P or glucose 6-phosphate (Glc-6-P), or the glycan Man9GlcNAc were added to 13C/15N-labeled sample, and changes in chemical shifts of amide groups located in either backbone or side chains were monitored. These ligand titrations were also used to (i) determine whether pure glycan and simple carbohydrate molecules bound specifically to the conserved MRH domain recognition site and (ii) test the hypothesis that the GII/H9252 MRH domain selectively recognizes mannose in contrast to glucose. An overlay of spectra acquired in the presence of increasing concentrations of Manα1,2Man, Man-6-P, or Man9GlcNAc demonstrated progressive chemical shift changes for residues either within or adjacent to the proposed binding pocket (Fig. 6A). The weighted changes in combined 1H/15N chemical shift perturbations were used to determine relative binding affinities for Manα1,2Man (Kd = 65 ± 3 mM), Man-6-P (Kd = 77 ± 7 mM), and Man9GlcNAc (Kd = 2.6 ± 0.4 mM) (Figs. 7, A–C). These results show a significantly higher relative affinity (~25-fold) for the larger glycan than for the disaccharide. The ability to recognize both mannose and Man-6-P suggests that substitution at the 6-position (i.e. hydroxyl or phosphate) does not constitute a major recognition determinant for GII/H9252 MRH domain. This hypothesis is further validated by SPR analyses show that Kd values for GII/H9252 MRH domain interacting with an immobilized glycoprotein bearing high mannose (Fig. 4A), phosphomonoesters (Fig. 4B), or phosphodiesters with a GlcNAc on the 6'-OH group of the mannose (Fig. 4C) are similar. Thus, the GII/H9252 MRH domain differs from the P-type lectin MRH domains of the MPRs that exhibit a preference to bind either phosphate on the 6-hydroxyl of mannose (domain 3 of the CI-MPR and CD-MPR) or a phosphodiester (domain 5 of the CI-MPR) (36) and from human OS-9, which binds mannose but not Man-6-P (43).

To probe whether the GII/H9252 MRH domain can recognize glucose, 15N-1H HSQC collected in the presence of Glc-6-P, which differs from Man-6-P only in the positioning of the 2'-OH group, produced chemical shift perturbations localized to the same regions as Man-6-P although to a significantly lower extent (Figs. 6B and 7D). These weak interactions observed with Glc-6-P demonstrate that the axial 2'-OH present in mannose is a key recognition determinant for the GII/H9252 MRH
domain. Although $K_d$ values of 65–77 mM for the monosaccharide Man-6-P and disaccharide Man$_6$/H$_9$25$_1$,2Man may not equate to the binding interactions occurring between GII and its heterogeneous population of N-glycan-containing substrates in the ER, the results demonstrate that GII MRH domain interacts preferentially with mannose. Taken together, the observation that the affinity for the monosaccharide Man-6-P and the disaccharide Man$_6$/H$_9$25$_1$,2Man are similar along with the ability of Glc-6-P to cause specific but very low chemical shift perturbations supports the structural findings that GII MRH domain has a shallow binding pocket (see below) that accommodates preferentially a single mannose residue. From a functional standpoint, the inability of GII MRH domain to efficiently recognize glucose eliminates the potential for the β-subunit to compete with the catalytic α-subunit of GII for glucose present in arm A of the N-glycan.

Mannose Binding Pocket—GII MRH domain contains the four residues (Gln-384, Arg-414, Glu-433, and Tyr-439) known to be essential for Man-6-P binding by the MPRs (Fig. 2B), and mutational studies support their involvement in GII activity (13, 14). To evaluate the role these residues play in carbohydrate binding, measurements of weighted differences in chemical shift perturbations for Man$_6$/H$_9$25$_1$,2Man (A), Man-6-P (B), Man$_6$/GlcNAc (C), or Glc-6-P (D) were plotted against the residue number of GII MRH domain (left panels). Secondary structural elements are listed below the sequence numbers. Missing values correspond to proline residues or residues not observed in the $^{15}$N-$^1$H HSQC spectra. Middle panels depict chemical shift mapping on the GII MRH domain structure. The molecular surface highlighted in red corresponds to amino acids with the largest chemical shift perturbations ($> 1$), whereas those colored in cyan represent moderate perturbations ($0.5$). Right panels, chemical shift differences for the eight selected residues (labeled in A) were calculated as $[\Delta \delta = (\delta_{\text{NH}} - \delta_{\text{NN}}) / \sqrt{\delta_{\text{NN}}^2 + \delta_{\text{NN}}^2}]$. Representative curves for four of the eight residues monitored for the Man$_6$/H$_9$25$_1$,2Man and Man-6-P titrations and curves for the three residues monitored for the Man$_6$/GlcNAc titration are shown. Dissociation constants ($K_d$) were determined from monitoring changes in the chemical shift of the eight selected residues as described under "Experimental Procedures."
bined $^1$H/$^{15}$N chemical shift perturbations with increasing amounts of ligand were used to map the location of the Man$_{1,2}$Man, Man$_6$-P, and Man$_{9}$GlcNAc (Fig. 7, A–C) binding site to the surface of GIIB MRH domain. The greatest chemical shift perturbations of the ligands map to an area (loops C and D) corresponding to the known carbohydrate binding sites of CD-MPR, of domains 3 and 5 of the CI-MPR, and of OS-9. Although chemical shift perturbations were observed for residues in the proposed binding pocket, two of the essential ligand binding residues, Gln-384 and Tyr-439, did not show significant changes in chemical shift in the presence of ligand (Fig. 7). We postulate from the structure that the amide protons are unable to shift due to extensive hydrogen bonding with neighboring atoms due to their location in $\beta$-strands that are part of the two $\beta$-sheets. Residues in loop A experienced slightly greater chemical shift differences in the presence of the larger ligands: a stronger chemical shift perturbation at Gly-370 and additional perturbations for Tyr-372 and Thr-373 were observed (Fig. 7, A–C). However, the striking similarity of the chemical shift mapping between the disaccharide and the monosaccharide coupled with their similar affinities supports the lack of an extended binding pocket. This notion is further supported by the observation that the branched Man$_9$GlcNAc glycan, which exhibited ~25-fold higher affinity than Man$_6$-P or Man$_{1,2}$Man, failed to produce additional measurable chemical shift perturbations at the highest concentration used (Fig. 7). Although these structural analyses failed to identify additional protein contacts to explain the increased affinity observed for the Man$_9$GlcNAc glycan compared with the smaller ligands, the longer glycan may have constrained the terminal and prepenultimate mannose into an optimal conformation for binding. Furthermore, the observed ~20-fold higher affinity of GIIB MRH domain for a glycoprotein bearing high mannose-type $N$-glycans compared with a glycan free in solution ($K_D = \sim 120 \ \mu M$ (Fig. 4A) versus $K_D = 2.6 \pm 0.4 \ \mu M$ (Fig. 7C); SPR data were obtained with glycoproteins, whereas NMR data were obtained using free glycans) may be the result of protein-induced restrictions that increase the percentage of $N$-linked glycans in the optimal conformation for binding and/or of differences in the principles of the methods used to estimate affinities.

The lumen of the ER is a crowded environment with protein concentrations up to 200–300 mg/ml (44). Given the high substrate concentration for the ER quality control machinery in which transient interactions occur, it is reasonable to expect that the affinities of ER MRH domains for glycoproteins in this environment would not be as high as those of the Golgi MRH domains in which the Man$_6$-P receptors must capture the very low abundance of lysosomal enzymes for transportation to late endosomes. The limited interactions with mannose and the failure of either Man$_9$GlcNAc or a disaccharide to produce submillimolar binding affinities are likely a direct consequence of the shallowness of this binding pocket relative to the other MRH binding pockets solved to date (CD-MPR, domains 3 and 5 of CI-MPR, and OS-9 (Fig. 8). In S. pombe, this shallow mannose binding region is due to the dramatically shortened loop B relative to other species and the positioning of loop C more distally from the essential residues in the pocket, thereby allowing the binding site to accommodate mannose modified at the 6-hydroxyl (i.e. phosphate and GlcNAc) (Figs. 5 and 8). Notably, the four residues considered essential for mannose binding (Gln-384, Arg-414, Glu-433, and Tyr-439) overlay well with those in the other four MRH domains (Fig. 8). Because the ligand-bound crystal structures of domain 3 of the CI-MPR, OS-9, and the CD-MPR demonstrate directly that the common positioning of these four essential residues results in a nearly identical positioning of the mannose ring within the binding pocket, we predict that the mode of mannose binding by the GIIB MRH domain to these four residues will be essentially the same.

Although the four mannose-interacting residues are conserved among these MRH domains, other structural features must exist to explain the different preferences displayed by these proteins for high mannose-type glycans (45). For example, OS-9 contains a double tryptophan motif that is proposed to determine the $\alpha$1,6-linkage specificity of OS-9 (7). These residues form an extended surface to accommodate the penultimate and prepenultimate mannose residues of one arm of the glycan (see Fig. 8) (7). In contrast, GIIB MRH domain contains a conserved tyrosine residue (Tyr-372; strand $\beta_3$) as observed in the MPRs (Figs. 2B, cyan, and 8). This tyrosine has been proposed by Satoh et al. (7) to be the residue responsible for conveying $\alpha$1,2-linkage specificity by the MPRs. However, we have crystallized domain 3 of the CI-MPR with a Man$_9$Man in the binding pocket (39), suggesting that this tyrosine (Tyr-324) in domain 3 is not involved in specifying a linkage preference for $\alpha$1,2-containing ligands. Furthermore, the side chain of Tyr-372 of the GIIB MRH domain, unlike that of the MPRs, is oriented $90^\circ$ away from the binding pocket such that the hydroxyl group is unable to interact with the glycosidic linkage of the ligand (Fig. 8). The significant number of NOE constraints for this residue along with the rest of the molecule (average of 21 NOE constraints per residue) further validates the positioning of the side chain of Tyr-372 in the GIIB MRH domain structure determined in the absence of ligand. However, we cannot exclude the possibility that in the presence of ligand the side chain of Tyr-372 may reposition. Thus, additional studies are needed to evaluate the role of this conserved residue in the MPRs and GIIB.

Role of a Conserved Trp-409 among GIIB MRH Domains—A comparative analysis of the residues within and surrounding the proposed binding pocket revealed another residue that is highly conserved and unique to the GIIB MRH domain. Trp-409, which is located in loop C, is absolutely conserved among 77 of the 79 homologs analyzed (Fig. 8) and is not present in the other MRH domains (Fig. 2B). Our initial assessment of the role of the conserved Trp-409 was performed in the context of the isolated MRH domain. Mutants containing conservative (W409F) or non-conservative (W409A) substitution of Trp-409 were generated, and $^{15}$N-labeled proteins were evaluated. $^{15}$N-$^1$H HSQC experiments show that both constructs are folded, which is reflected in their similar $^{15}$N-$^1$H HSQC spectra as the wild-type protein (Fig. 9A). Furthermore, addition of 50 mm Man$_9$1,2Man disaccharide to the W409A $^{15}$N-labeled sample resulted in chemical shift perturbations on the order of those found for similar concentrations of the wild-type protein.
In addition, SPR experiments show that W409A and W409F bound immobilized glycoproteins bearing high mannose (Fig. 4A), phosphomonoesters (Fig. 4B), or phosphodiesters bearing a GlcNAc on the 6′-OH group of the mannose (Fig. 4C) with a slight decrease in affinity (within 3.5-fold) compared with the wild-type protein. Thus, substitution of Trp-409 does not dramatically lower binding affinity toward glycans in the context of the isolated MRH domain.

To study the influence of Trp-409 of GIIβ MRH domain in GII activity, we mutated Trp-409 in the full-length GIIβ (W409A or W409F), expressed the wild-type or mutated GIIβ in S. pombe cells lacking endogenous GIIβ (ΔGIIβ), and measured GII activity toward both the small substrate analog pNPG and [14C-Glc]GlcαManαGlcNAc in microsomal fractions obtained from the mutant cells. Results presented in Fig. 10A show that although the influence of Trp-409 in GIIβ-mediated GIIα activity enhancement was not as strong as mutation of the conserved residues (Gln, Arg, Glu, and Tyr) that interact with the 2-, 3-, and 4-hydroxyl groups of the mannose ring (Fig. 2B), mutation of Trp-409 precluded full GII activity toward N-glycans in vitro. We have reported previously that microsomal GII activity using pNPG reflects ER GIIβ content (14). Reduced GII activity observed in cells expressing Trp-409-mutated GIIβ is not due to a reduced ER GIIα content as the activity toward pNPG was not reduced in mutant GIIβ W409A or GIIβ W409F (Fig. 10A). Moreover, Western blot analyses show normal GIIα...
Structure of Glucosidase II β MRH Domain

**A**

![Comparison of two-dimensional HSQC spectra of wild-type and mutant recombinant GII β MRH domain. A, overlay of $^{15}$N-$^1$H HSQC spectra from wild-type (black) or mutant (W409A, red; W409F, cyan) GII β MRH domain. B, overlay of $^{15}$N-$^1$H HSQC spectra from wild-type (black) or mutant (W409A, red) GII β MRH domain in the absence of ligand. The $^{15}$N-$^1$H HSQC spectrum of W409A collected in the presence of 50 mM Man$_1$2Man disaccharide is also shown (green).](image)

To determine whether substitution of Trp-409 in the GII β MRH domain affects GII glucose trimming activity in vivo, we analyzed the N-glycan pattern produced by ΔGIIB cells expressing Trp-409 GIIB mutants (W409A and W409F) and compared it with the N-glycan patterns of cells expressing wild-type GIIB or GIIB mutated in amino acids predicted to interact with the mannose ring (Y439F and E433Q). Cells were incubated with $^{14}$C-Glc for 15 min in the presence of 5 mM DTT, and whole cell N-glycans were isolated and analyzed as described under “Experimental Procedures.” The total incorporation of the label into the N-glycans was linear during the incubation time, and the presence of DTT prevented the passage of glycoproteins to the Golgi, hindering further extension of the recently synthesized N-glycans (46). The N-glycan patterns obtained from *S. pombe* cells expressing wild-type GIIB revealed that whereas deglucosylation of the transferred glycan Glc$_3$Man$_9$GlcNAc$_2$ was so rapid that almost no glucose-containing glycans were detected (Fig. 10C) the amount of glucosylated glycans increased in cells expressing GIIB Trp-409 mutants, although it was not as much of an increase as observed in the GIIB Y439F mutant (Fig. 10, D–F) and as observed previously in ΔGIIB mutants (14). Taken together, the results demonstrate that substitution of Trp-409 in the context of the full-length GIIB has a moderate to high (60% inhibitory) effect on GII activity.

**How Does GIIB MRH Domain Interact with High Mannose-type Glycans?**—Studies both in vitro and in vivo indicate that residue $k$ in arm C plays a more significant role than residue $i$ in arm B of the glycan in the enhancement of GII catalytic activity by GIIB (12–15). To envision how GIIB enhances the catalytic activity of GII toward glycans, we propose two models that are consistent with the existing biochemical and current structural studies. In the first model, the terminal mannose (residue $k$) of arm C is captured by the shallow binding pocket of the MRH domain of GIIB defined by the four conserved residues (Gln-384, Arg-414, Glu-433, and Tyr-439). Interaction of the terminal mannose (residue $i$) of arm B with Trp-409 would further constrain the N-glycan and facilitate optimal positioning of the glucose-containing arm A near the catalytic site of Gillα (Fig. 11A). Visualization of this model was obtained by positioning the terminal mannose residue of arm C of a Man$_9$ glycan into the MRH binding pocket from the superimposition of the four mannose-binding essential residues (Gln-384, Arg-414, Glu-433, and Tyr-439) with that of domain 3 of CI-MPR containing bound Man$_6$-P (47). This overlay indicated that the terminal mannose of arm B of the glycan is near loop C and that Trp-409 in loop C could stack against the terminal mannose ring of arm B, thereby providing a bidentate mode of interaction between the MRH domain and two arms of the glycan. Although a previous molecular dynamics simulation of a Man$_9$GlcNAc$_2$ glycan and NOE analyses indicate that significant flexibility of arms A, B, and C of the glycan (48) complicates such analyses, CH-$^\pi$-mediated stacking interactions between aromatic residues and carbohydrates are commonly observed in lectins (49, 50), including another ER-resident lectin, malectin, that recognizes the Glc$_3$1,3Glc moiety on Glc$_2$-N-glycans (51). It is intriguing to speculate that Trp-409 is located in a relatively dynamic region of the GIIB MRH domain (Fig. 5, A and C) to capture one of the many and related conformers of an N-glycan.

In the second model (Fig. 11B), Trp-409 interacts with other regions of the β-subunit. Although the GIIB MRH domain alone is sufficient for binding glycans, its affinity for ligand may be augmented by the presence of the remaining portion of the β-subunit and/or the α-subunit. This enhancement in ligand binding affinity has been reported for both domain 3 (52) and domain 5 (26) of the CI-MPR. Trp-409 of GIIB could be positioned to interact with residues on adjacent parts of the holoenzyme and act to either stabilize the position of loops C and/or D much like the linker region between domains 1 and 2 does for domain 3 of CI-MPR, resulting in increased affinity for Man$_6$-P (39, 47), or orient the MRH domain closer to Gillα, resulting...
**FIGURE 10.** Influence of Trp-409 in GlIβ-mediated GlI activity enhancement toward N-glycans. 

A. GlI activity toward pNPG or Glc, Man, GlcNAc was measured in 125 μg of microsomes from *S. pombe* cells lacking endogenous GlIβ (ΔGlIβ) and expressing GlIβ or the same subunit displaying the indicated mutations. The activity of ΔGlIβ cells transformed with wild-type GlIβ was taken as 100%. Error bars represent S.D. 

B. GlI ER content was evaluated. Microsomal proteins (250 μg) of *S. pombe* ΔGlIβ, ΔGlIβ, or ΔGlIβ expressing exogenous variants of GlIβ were resolved by 8% SDS-PAGE, transferred to a PVDF membrane, and blotted using mouse polyclonal anti-GlI (1:500) or anti-GlI (1:5000) subunit primary antibodies. Goat HRP anti-mouse IgG (1:5000) was used as the secondary antibody. Reactions were detected by chemiluminescence. Because endogenous GlI levels are significantly lower than exogenously expressed levels, the GlI band was detected in the GlI mutant (middle lane; ΔGlIβ) only upon overexposing the blot. 

C–F, glycan patterns synthesized by *S. pombe* ΔGlIβ mutant cells expressing GlI (C) or mutated GlIβ versions (D and E). Endoplasmic reticulum N-glycans produced by ΔGlIβ cells expressing GlIβ W409A were indistinguishable from those produced by cells expressing GlIβ W409F. The N-glycan pattern produced by ΔGlIβ cells expressing GlIβ E433Q was indistinguishable from that of the same cells expressing GlIβ Y439F. Quantification of the relative amounts of the di-, mono-, and unglycosylated species in each mutant is shown in F. The label of Man₃ species was added to that in Man₃ species to account for unglycosylated glycans.
Structure of Glucosidase II β MRH Domain

**FIGURE 11.** Possible models for the influence of Trp-409 in GII activity. In A, mannose-binding essential residues Glc-384, Arg-414, Glu-433, and Tyr-354 form a pocket that binds arm C of the glycan, whereas residue Trp-409 interacts with arm B. This bidentate interaction allows the glucose-containing arm A to be juxtaposed to the catalytic site of GII. In B, Trp-409 interacts with other regions of the β-subunit and influences its affinity for N-glycans. Note that in both models binding of the C arm of the glycan to the binding pocket of GII β MRH domain is required for an efficient presentation of the C arm to the GII catalytic subunit. This implies that removal of mannoses by ER mannosidases will reduce binding of the glycan and GII activity as has been shown in the model presented in Ref. 15.

in arm A of the glycan becoming optimally positioned into the catalytic site. Both models indicate that the active site of GIIX must be very close to that of the binding pocket of the MRH domain of GII. In comparison with the other MRH domains, the shorter loops between strands 2 and 3 and strands 4/5 and 6 along with shorter strands 4/5 and 6 (Fig. 5D) may allow a close juxtapositioning of the catalytic site with the MRH domain.

**Concluding Remarks**—In the current study, we have determined the solution structure of the S. pombe GII β MRH domain, and its function was characterized alone and in the context of the full-length GII catalytic subunit. This is the first time that the structure of an MRH domain of an enzyme has been determined. The structure reveals the conserved MRH fold observed in the MPRs (35, 39–42) and OS-9 (7), but in contrast to these proteins, GII β MRH domain contains a shallow binding pocket that accommodates only a single mannose residue. In addition, we have identified a conserved residue, Trp-409, that plays an important role in GII glucose trimming activity in vivo. Additional structural studies are needed to fully understand how this heterodimeric enzyme interacts with N-glycans and thus regulates the quality control of glycoprotein folding in the ER.

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