論文内容の要旨

Study on the sugar-binding activity and function of glucosidase II β subunit and malectin

（グルコシダーゼ II β サブユニットとマレクチンの糖結合活性及び機能解析）

氏名：胡丹

Introduction:

Endoplasmic reticulum (ER) is the organelle where membrane-bound and secretory proteins are synthesized. To ensure the proper folding and assembly of newly synthesized proteins, there exists a rigorous quality-control system consisting of a variety of folding enzymes and molecular chaperons. Calnexin (CNX) and calreticulin (CRT) are two homologous chaperones having sugar-binding ability. They comprise the so-called CNX/CRT cycle (Fig. 1), together with glucosidase I (GI) and glucosidase II (GII), both of which remove glucose from *N*-glycans, and UDP-glucose:glycoprotein glucosyltransferase (GT), which adds back glucose. GII mediated deglucosylation and GT-catalyzed reglucosylation cycle continues until proper folding of ptotein is achieved.

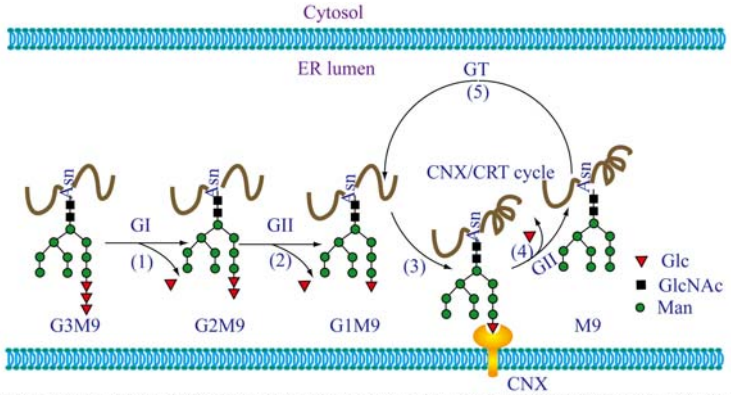

Fig. 1 Scheme of the CNX/CRT cycle. There are 5 main steps in the CNX/CRT cycle. (1) trimming of the outermost glucose by GI, (2) trimming of the middle glucose by GII allowing the entry of glycoproteins to CNX/CRT cycle, (3) association of glycoproteins with CNX/CRT, (4) trimming of the innermost glucose by GII leads to release of glycoproteins from CNX/CRT, (5) reglucosylation of incompletely folded glycoproteins by GT allows entry to another cycle.

GII is a heterodimeric complex consisting of a catalytic α subunit (GIIα) and a β subunit (GIIβ). It controls the import and export of newly synthesized glycoproteins within CNX/CRT cycle by trimming two α1,3-linked glucose residues from *N*-glycans (Fig. 1). Though past studies have made great progress in understanding this glucose trimming process, there are still some issues

that need to be addressed. *First*, GIIβ contains a domain (MRH) with homology to mannose 6-phosphate receptors that specifically bind to phosphorylated mannose residues on acid hydrolases. This raises a possibility that GIIβ may possess a sugar-binding activity. However, whether GIIβ possesses sugar-binding activity and, if so, what role this activity plays in the function of GII has not been demonstrated. *Second*, recently, an ER resident protein, malectin, is found to selectively recognize Glc2-*N*-linked glycans in *Xenopus laevis*. The capacity of malectin to bind to G2M9, one of the substrates of GII, suggests malectin may have some effects on the trimming of G2M9. To provide further insights into the glucose-trimming process by GII, my present study focuses on the investigation of the sugar-binding ability of human GIIβ and malectin, and the functions mediated by their sugar-binding activity.

Results

1. GIIβ-MRH binds to cell surface glycans

MRH domain of GIIβ (GIIβ-MRH) with a C-terminal biotinylation sequence was expressed in *E.coli* BL21(DE3)pLysS, refolded and purified. After biotinylation with biotin ligase BirA, purified GIIβ-MRH was incubated with *R*-phycoerythrin-labeled streptavidin (PE-SA) to form PE-labeled GIIβ-MRH tetramer, which was then used to investigate the capacity of GIIβ-MRH to bind sugars on the cell surface. GIIβ-MRH did not bind to the HeLaS3 cells, but treatment of the cells with either of the two α-mannosidase I inhibitors, kifunensine (KIF) and deoxymannojirimycin (DMJ), but not the Golgi α-mannosidase II inhibitor, swainsonine (SW), caused the binding of GIIβ-MRH (Fig. 2A). High-mannose type glycans accumulate on the cell surface in KIF- or DMJ-treated cells, which can be cleaved by endo-β-*N*-acetylglucosaminidase H (endo H). I treated the DMJ-treated HeLaS3 cells with endo H and examined the binding of GIIβ-MRH (Fig. 2B). Endo H treatment of the cells abolished the binding of GIIβ-MRH to the cells almost completely, confirming the binding of GIIβ-MRH to DMJ-treated HeLaS3 cells is through the cell surface high mannose-type glycans. Given that KIF or DMJ specifically inhibit the cleavage of α1,2-linked mannose residues from high mannose-type glycans (Fig. 3A), these results suggest that the α1,2-linked mannose may be important for recognition by GIIβ-MRH.

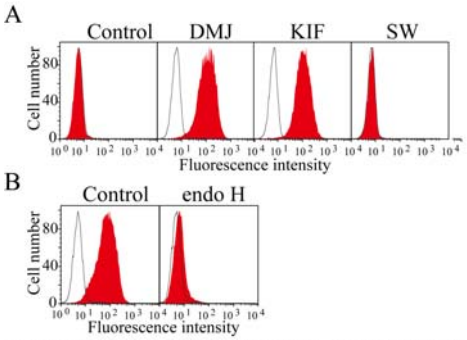

Fig. 2. GIIβ-MRH binds to cell suface glycans (A) the binding of GIIβ-MRH tetramer (filled histogram) or PE-SA as a control (thin line) to HeLaS3 cells that were treated with DMJ, KIF or SW. (B) the binding of GIIβ-MRH tetramer (filled histogram) or PE-SA (thin line) to DMJ-treated HeLaS3 cells that were pretreated with endo H.

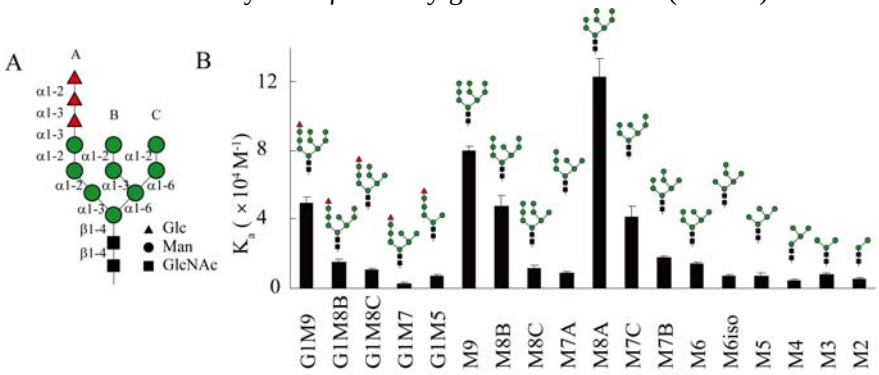

Fig. 3 Sugar-binding specificity of GIIβ-MRH analyzed by FAC (A) Structure of the *N*-linked glycan precursor, G3M9. (B)The affinity of each PA-labeled oligosaccharide to GIIβ-MRH was determined by the FAC analysis.

2. The terminal α1,2 linked mannose, especially that on the C-arm, of high mannose-type glycans, is required for the binding of GIIβ-MRH

The sugar-binding specificity of GIIβ-MRH was investigated by frontal affinity chromatography (FAC). As shown in Fig. 3B, M9 and M8A, both of which contain three terminal α1,2-linked mannose, exhibited the highest affinity to GIIβ-MRH. G1M9, M8B, M8C, M7C, and M7B, each of which possesses two terminal α1,2-linked mannose residues, showed lower affinities, and the affinities of the oligosaccharides with a single terminal α1,2-linked mannose were further impaired. These results suggest that the terminal α1,2-linked mannose of *N*-glycan is the major determinant for the binding of GIIβ-MRH. Interestingly, although each of G1M9, M8B, M8C, M7C, and M7B possesses two terminal α1,2-linked mannose residues, the affinity of M8C and M7B is quite lower than those of the other three. Comparing their structures indicates that M8C and M7B are distinguished from G1M9, M8B, M7C by the absence of the α1,2-linked mannose on the C-arm. These data suggest that the α1,2-linked mannose on the C-arm is essential for the strong binding of GIIβ-MRH.

3. The sugar-binding activity of GIIβ is important for GII to trim glucose efficiently from *N*-glycans.

Glucosylated high mannose-type oligosaccharides (G1M9, G2M9) are known substrates of GII. The capacity of GIIβ-MRH to bind high mannose-type glycans suggests that GIIβ is possibly involved in glucose-trimming process. Two GIIβ mutants myc-GIIβ(Y410A) and myc-GIIβ(Q420E) that do not have the ability to bind sugars were expressed in 293T cells. We found that these point mutations have no effects on the ability of GIIβ to make complex with GIIα and that endogenous GIIβ can be substituted with over-expressed myc-tagged GIIβ mutants. To investigate possible dominant negative effects of the GIIβ mutations on enzymatic activity of GII, the myc-GIIβ or its mutants were over-expressed in 293T cells and the glucosidase activity in the cell lysates was measured using *p*-nitrophenyl-α-glucopyranoside (pNP-αGlc) or methotrexate (MTX)-derivatized G1M9 and G2M9 as substrates. As shown in Fig. 4A, similar amounts of the wild type GIIβ and the GIIβ mutants were expressed. Over-expression of either the wild type GIIβ or the GIIβ mutants slightly increased the hydrolysis of pNP-αGlc to the similar extent (Fig. 4B). However, when MTX-derivatized G1M9 (Fig. 4C) or G2M9 (Fig. 4D) were used as substrates, expression of the wild type GIIβ and that of the GIIβ mutants showed opposite effects on the glucosidase activity. Over-expression of the wild type GIIβ slightly increased the removal of glucose from G1M9 and G2M9, while over-expression of the either of the two GIIβ mutants significantly decreased the removal of glucose from G1M9 and G2M9 compared to mock transfected cells. These data suggest that the sugar-binding activity of GIIβ-MRH is not required for the hydrolysis of pNP-αGlc, but important for hydrolysis of glucosylated high mannose-type glycans.

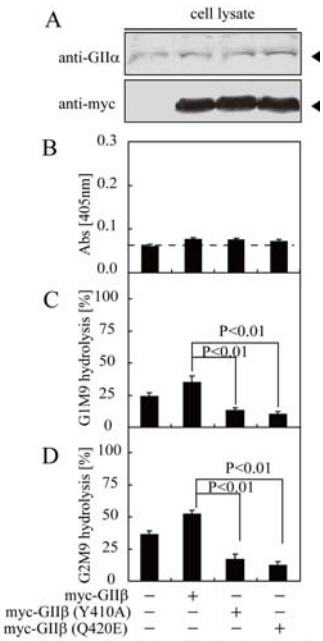

Fig. 4 Enzymatic activity assay of GII.(A) Expression of GIIα and GIIβ by Western blotting. (B) The activity of GII in the cell lysates prepared in (B) using pNP-αGlc as substrate. (C) the activity of GII in the cell lysates prepared in (B) using G1M9-MTX (D) and G2M9-MTX (E) as subtrates

4. Human malectin selectively binds to G2M9

The sugar-binding activity of human malectin was investigated using malectin tetramer prepared as similar to that of GIIβ-MRH. Human malectin did not bind to the cells treated with castanospermine (CST), KIF, or SW, but selectively bind to deoxynojirimycin (DNJ)-treated cells

(Fig. 5A). Given that DNJ preferentially inhibits the activity of GII and causes the accumulation of Glc2-*N*-glycans, these results suggest human malectin binds to Glc2-*N*-linked glycan. The detailed sugar-binding specificity of human malectin was analyzed by FAC using a series of high mannose-type glycans resident in the ER. Among these oligosaccharides, G2M9 was found to be the only oligosaccharide recognized by malectin (Fig. 5B). Given that M9, G1M9 and G3M9 could not bind to malectin, these results indicate that terminal Glcα1,3Glc is the major determinant for the binding of malectin.

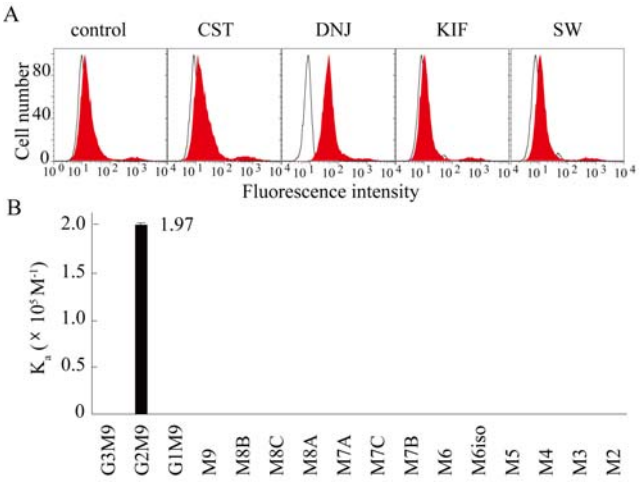

Fig. 5 Sugar-binding specificity of Malectin (A) Binding of Malectin tetramer to cells treated with CST, DNJ, KIF, or SW (B) The affinity of each PA-labeled oligosaccharide to Malectin was determined by the FAC analysis.

5. The expression of malectin was significantly induced during tunicamycin-induced ER stress

The remarkable selectivity of malectin to Glc2-*N*-linked glycan points to a role for malectin in the early glucose trimming process (Fig. 1). By analogy with CNX/CRT, malectin may function as a lectin chaperon that specifically recognizes G2M9 on folding glycoproteins, slowing the trimming of G2M9 (Fig. 1, step 2) to increase protein folding efficiency. To address this possibility, I first analyzed the effects of malectin on the trimming of G2M9. Unexpectedly, the trimming of G2M9 in vitro was not influenced by malectin. Though the effect of malectin on the glucose trimming process is still not clear, I found that the expression of malectin was significantly induced by tunicaymcin-induced ER stress (Fig. 6). Tunicamycin is the inhibitor of GlcNAc-phosphotransferase. It blocks the synthesis of all *N*-linked glycoproteins and is well-known inducer of ER stress. These results suggest that malectin is likely to involve in the folding of glycoproteins, though its detailed function remains unknown.

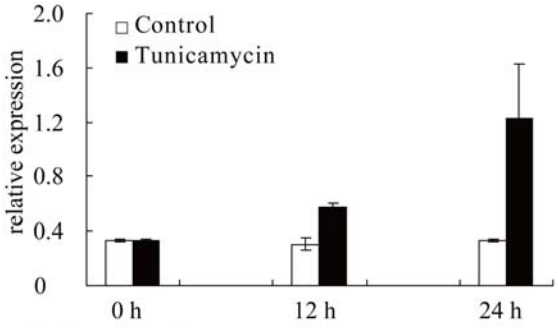

Fig. 6. Increased expression of malectin during tunicamycin-induced ER stress. HEK293 cells were treated with 5 μg/ml tunciamycin for 12 and 24 hours. The expressin of malectin was anlyzed by real time PCR

Conclusions

To provide further insights into the glucose trimming process in the ER, I have investigated the sugar-binding activity and function of GIIβ and malectin. My present study demonstrated the sugar-binding activity of GIIβ, and the importance of the activity for efficient glucose trimming. In addition to previous known roles of GIIβ, ER localization and assisting folding of GIIα, our study revealed that GIIβ also participates in the glucose trimming process. My study also showed that human malectin selectively binds to G2M9. Though the effects of malectin on the trimming of G2M9 are not clear, I found the induced expression of malectin during tunicamycin-induce ER stress. These studies suggest that malectin may functions as a lectin chaperon in the ER to promote the folding of glycoproteins