論文の内容の要旨

論文題名：Role of ER-lectins in protein folding and early secretory pathways

(新生タンパク質品質管理における小胞体レクチンの機能解析)

氏名：　陳 阳

Prologue

The vast majority of proteins synthesized in eukaryotic cells are glycosylated. The glycosylation, especially *N*-linked glycosyaltion, is an inevitable step in eukaryotic protein synthesis pathway, which maintains homeostasis in protein biosynthesis. In the endoplasmic reticulum (ER), *N*-linked glycans are cotranslationally attached to consensus asparagine residues on newly synthesized polypeptide and subsequently processed. This processing generates specialized oligosaccaride structures recognized by carbohydrate-binding proteins, ER-lectins. The interplaying between the ER-lectins and their *N*-glycan ligands plays pivotal roles in the synthesis of nascent proteins that ensures their proper folding and targeting for correct destination. A growing list of ER-lectins have been found and reported to be involved in the regulation of protein synthesis. In this study, I investigated the sugar-binding ability and distinct functions of a novel ER-lectin, malectin and a L-type ER-lectin, ERGIC-53 to reveal their involvement in homeostasis of protein biosynthesis.

Role of malectin in $Glc_2Man_9GlcNAc_2$-dependent quality control of glycoprotein folding

Introduction

Malectin was recently discovered as a novel ER-resident lectin from *Xenopus laevis* that exhibits structural similarity to bacterial glycosylhydrolases. Like other intracellular lectins involved in glycoprotein synthesis, malectin was found to be highly conserved in animals. However, the sugar-binding specificity and the function of this novel lectin is still yet to be clarified. Our previous studies have revealed that human malectin specifically binds with $Glc_2Man_9GlcNAc_2$ (G2M9) *N*-glycan, with a *Ka* of 1.97×10^5 M^{-1}. In this study, I focused on clarifying the biological role of malectin in quality control of glycoprotein synthesis. I investigated the function of malectin in regulating the folding of α1-antitrypsin (AT), a model glycoprotein which was well used in research on protein folding. This study provided the first evidence that malectin functions as a chaperone to ensure the correct folding of newly synthesized protein via its sugar-binding ability.

Results

Malectin preferentially associates with misfolded AT^{NHK} via G2M9 *N*-glycans

To understand the biological role of malectin in the ER, we investigated the function of malectin on folding of α1-antitrypsin (AT). AT is a highly glycosylated secretory protein. A mutant, AT^{NHK} is a well-characterized misfolded variant of the human AT, and a good substrate for endoplasmic reticulum-associated degradation (ERAD). We also generated a variant of AT^{NHK}, AT^{NHK}-Q3, in which the three *N*-glycosylation site Asn residues were substituted with Gln as an approach to evaluate whether protein folding or ERAD was dependent on the presence of *N*-glycans (Fig. 1A). AT, AT^{NHK} or AT^{NHK}-Q3 was expressed in HeLa cells along with FLAG-tagged malectin. Newly synthesized glycoproteins were metabolically labeled with ^{35}S-methionine and -cysteine. To accumulate the G2M9 glycans in cells, we treated the cells with the glucosidase II inhibitor, deoxynojirimycin (DNJ). Lysates of DNJ-treated cells were subjected to immunoprecipitation using an anti-FLAG Ab. The amount of AT^{NHK} (dimers and monomers) that coprecipitated with malectin was markedly increased, and AT weakly coprecipitated (Fig. 1B, lanes 6-7). The band corresponding to AT^{NHK}-Q3

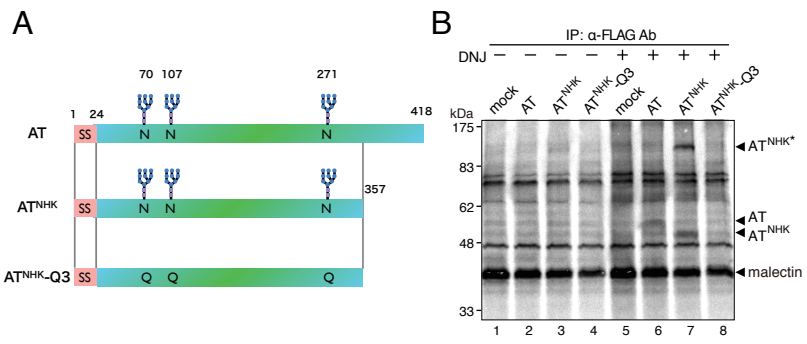

Figure 1. Significant interaction between malectin and AT^{NHK}.
A) structure of α1-antotrypsin and its mutants. B) The interaction of malectin with AT and its varinets was monitered by immunoprecipitaion in ^{35}S-labeled HeLa cells in the presence or absence of DNJ. Asterisk represents the dimmer.

was not detected in the precipitates (Fig. 1, lanes 8). These results indicated that malectin preferentially interacted with misfolded AT^{NHK} rather than native AT in G2M9 glycan dependent manner.

Overexpression of malectin abrogated the secretion of misfolded AT^{NHK}

The secretion and intracellular levels of AT, AT^{NHK} and AT^{NHK}-Q3 were examined in cells that overexpressed malectin. The amounts of secreted and intracellular wild-type AT were similar with or without malectin overexpression (Fig. 2, left). By contrast, the amount of secreted AT^{NHK} was markedly decreased in malectin-overexpressing cells compared with mock-transfected cells, although the levels of intracellular AT^{NHK} were similar (Fig. 2, middle). Deletion of the *N*-glycans from AT^{NHK} canceled this decreased secretion by malectin overexpression (Fig. 2, right). Taken together with the data suggesting that folding-defective AT^{NHK} is recognized by malectin in a sugar-dependent manner (Fig. 1), these results indicated that malectin prevents the secretion of AT^{NHK} through retention of the misfolded glycoprotein in the ER mediated by binding to G2M9 glycan.

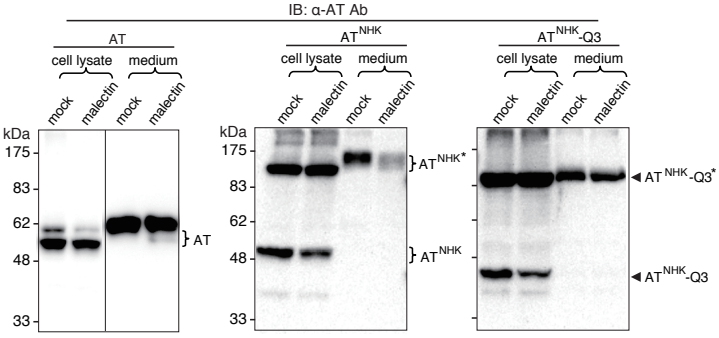

Figure 2. Overexpression of malectin dramatically abrogated the secretion of AT^{NHK}. The protein expression of AT or its varients from intracellular and culture supernatant was monitered by immunoblotting in malctin overexpressed HeLa cells.

Attenuated secretion of misfolded AT^{NHK} by malectin overexpression is due to enhancement of ERAD

The previous results indicated that AT^{NHK} was retained in the ER by malectin. However, the level of intracellular AT^{NHK} was not increased in malectin overexpressing cells, despite the fact that secretion of AT^{NHK} was blocked. The observations imply that the decrease of secretion is possibly due to the ERAD of misfolding AT^{NHK}. To test this postulation, we examined the effect of treatment with the proteasome inhibitor MG132 on the secretion of AT^{NHK} under the condition of malectin overexpression. Overexpression of malectin decreased the secretion of AT^{NHK} in untreated cells, whereas it had no effect on the secretion of AT^{NHK} when proteasome was blocked by MG132 (Fig. 3A). The result indicates that malectin may guide misfolded AT^{NHK} into the proteasome-mediated degradation pathway. To further confirm this result, we examined the association of AT^{NHK} with OS-9, an ER-chaperone that guides misfolded proteins to ERAD. The amount of AT^{NHK} coprecipitated with OS-9 was increased by malectin overexpression, whereas there were no significant differences in the amount of AT^{NHK}-Q3 associated with OS-9 (Fig. 3B). Based on the sugar-binding specificity of malectin and OS-9, these results indicated that malectin traps misfolded *N*-glycosylated proteins and guides them to the ERAD pathway via OS-9 in a sugar-dependent manner to block their secretion.

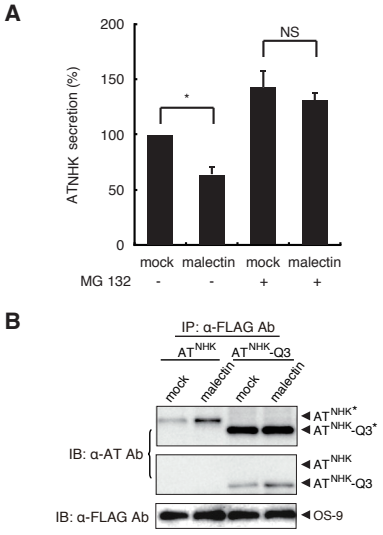

Figure 3. Overexpression of malectin enhanced the ERAD of AT^{NHK}. (A) The evaluation of secreted AT^{NHK} in malectin overexpressed cells in the presence or absence of MG132. (B) The interaction of OS-9 with AT^{NHK} in malectin overexpressed HeLa cells.

Conclusions

From the results of the sugar-binding ability and regulation of α1-antitrypsin folding, we clarified the possible roles of human malectin in the protein synthesis pathway. In cells, malectin stably interacted with misfolded nascent protein rather than native one, via G2M9 glycans. This stable interaction resulted in enhanced ERAD of misfolded glycoproteins and prevented their secretion. These findings provided a possible role of malectin in regulating glycoprotein folding via G2M9-binding. The data also suggested that malectin may function as an ER chaperone to eliminate misfolded substrate, thereby assisting in the quality control of newly synthesized glycoproteins.

Sugar-binding of ERGIC-53 regulates early secretion of newly synthesized glycoprotein

Introduction

Leguminous-type (L-type) lectin ERGIC-53 is a homo-oligomeric ER-Golgi recycling protein, which functions as a transport receptor for newly synthesized glycoproteins in the early secretory pathway. A limited subset of cargo glycoproteins transported by ERGIC-53 such as coagulation factors V and VIII, cathepsin C and Z, and α1-antitrypsin were identified, while the exact roles of *N*-glycan-binding of ERGIC-53 in transport of secretory glycoproteins for ER-exit are yet to be clarified. By screening a cDNA library from HepG2 cells based on green fluorescent protein (GFP) based protein fragment complementation assay (PCA), we found some possible candidates for luminal ERGIC-53 interacting partners. In this study, I focused on one of these candidates, Mac-2BP and identified this molecule as a novel member of ERGIC-53 transported cargo glycoproteins. My study proved that *N*-glycan-binding of ERGIC-53 is essential for ER-Golgi transport of Mac-2BP at early secretion stage.

Results

Identification of Mac-2BP as a novel ERGIC-53-interacting cargo glycoprotein

To confirm the interaction of ERGIC-53 with Mac-2BP, I constructed the ERGIC-53 which was N-terminally fused with N-fragment of GFP (nGFP-ERGIC-53), and Mac-2BP which was C-terminally fused with C-fragment of GFP (Mac-2BP-cGFP). Compared with transfection of nGFP-ERGIC-53 alone, cotransfection of nGFP-ERGIC-53 and Mac-2BP-cGFP induced 20.8% of total cells expressed GFP fluorescence (Fig. 4A), which indicated the complementation of ERGIC-53 to Mac-2BP. Next, I evaluated this complementation by immunoprecipitation. FLAG-tagged Mac-2BP was transfected into HeLa cells, and the lysates were subjected to immunoprecipitation using anti-ERGIC-53 antibody. FLAG-tagged Mac-2BP was coprecipitated with endogenous ERGIC-53 even ERGIC-53 was not over-expressed (Fig. 4B, lane 3). Moreover, the amount of precipitated Mac-2BP was increased when ERGIC-53 was overexpressed (Fig. 4B, lane 4). The results confirmed the interaction between two molecules and identified Mac-2BP as a novel ERGIC-53 interacting cargo glycoprotein.

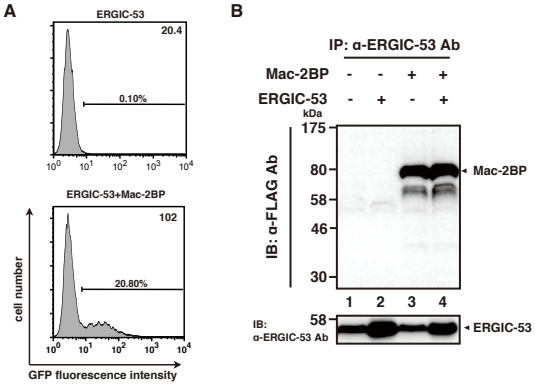

Figure 4. ERGIC-53 constitutively associates with Mac-2BP. The interaction of ERGIC-53 with Mac-2BP was confirmed by GFP-based PCA (A) and immunoprecipitaion (B).

N-glycan-binding of ERGIC-53 is essential for the interaction with Mac-2BP, which regulates its early secretion.

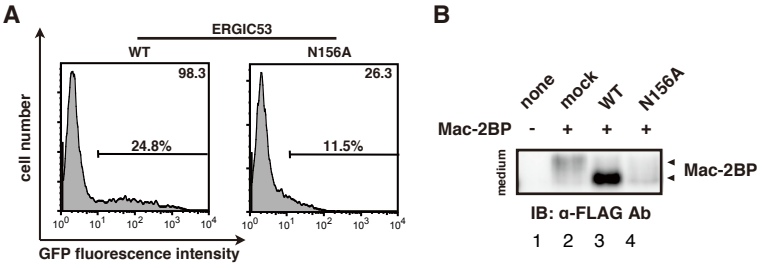

Figure 5. N-glycan-binding of ERGIC-53 mediates the interaction with Mac2-BP which reglutates its secretion. (A) The interaction of wild type ERGIC-53 (WT), N-glycan binding deficeent mutant (N156A), with Mac-2BP was evaluated by PCA. (B) The protein expression of secreted Mac-2BP in culture supernatant from ERGIC-53 (WT) or N156A overexpressed HeLa cells was montered by immunoblotting.

ERGIC-53 functions as the cargo receptor with a lectin domain, and Mac-2BP is a highly glycosylated secretory protein. Based on these evidence, we postulated that sugar-binding activity of ERGIC-53 is essential for the interaction with Mac-2BP. To verify this hypothesis, a *N*-glycan binding-deficient mutant of ERGIC-53 (N156A) was expressed in the cells and compared the interaction of ERGIC-53 with Mac-2BP by PCA. N156A mutant significantly abolished the complementation of nGFP-ERGIC-53 to Mac-2BP-cGFP (Fig. 5A), which implies binding to Mac-2BP is *N*-glycan dependent. Since ERGIC-53 was reported to function as a cargo receptor for ER-Golgi transport of some secretory glycoproteins, I tried to clarify whether *N*-glycan-binding of ERGIC-53 mediates the transport of Mac-2BP from the ER to the Golgi and regulates its secretion. The secretion and intracellular levels of FLAG-tagged Mac-2BP were evaluated in HeLa cells in which ERGIC-53 was overexpressed. Overexpression of ERGIC-53 significantly increased the secretion level of Mac-2BP (Fig. 5B, lane 3), which indicates that exogenously expressed ERGIC-53 facilitated the secretion of Mac-2BP. By contrast, overexpression of *N*-glycans binding deficient mutant, N156A, significantly abolished the secretion of Mac-2BP less than mock vector transfection (Fig. 5B, lane 4), which indicates that *N*-glycans-binding of ERGIC-53 plays a key role in secretion of Mac-2BP.

Secretion of Mac-2BP is dependent on ERGIC-53-mediated COPII vesicles budding from the ER.

ERGIC-53 forms budding vesicle by binding to COPII coat protein via C-terminal double phenylalanine residues. To confirm ERGIC-53 induces the secretion of Mac-2BP via COPII budding vesicles, a distinct mutant of ERGIC-53, in which the C-terminal double phenylalanine was substituted with double alanine (termed KKAA), was overexpressed in HeLa cells. KKAA mutant resulted in the mistargeting of ERGIC-53 in the ER due to the deficient binding with COPII. Compared with wild-type ERGIC-53, overexpression of KKAA mutant dramatically diminished the secretion of Mac-2BP (Fig. 6). This result indicated that ERGIC-53 was involved in COPII vesicles budding from the ER, which are essential for secretion of Mac-2BP.

Conclusion

By the screening of a cDNA library from HepG2 cells, we identified Mac-2BP as a novel ERGIC-53 cargo molecule. The data that *N*-glycan-binding was essential for ERGIC-53 binding with Mac-2BP in ER-Golgi transport, suggested the function of ERGIC-53 in the early secretion of newly synthesized glycoproteins. In the ER, the correctly folded nascent glycoprotein is captured by ERGIC-53 via its sugar-binding ability. Cooperated by vesicle coat protein COPII, the nascent glycoprotein is then transported by ERGIC-53 from the ER to the Golgi for further maturation and final secretion. The observations provided the evidence that *N*-glycan-binding of ERGIC-53 plays a key role in ER-Golgi transport of newly synthesized glycoproteins at early secretion stage.

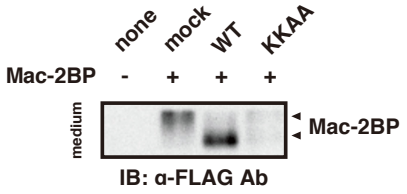

Figure 6. ERGIC-53 mediates the ER-exit of Mac-2BP in early secretion stage. The secretion of Mac-2BP in ERGIC-53 or KKAA mutant overexpresing cells was monitored by immunoblotting.

Epilogue

The elucidation of functional roles of malectin and ERGIC-53 illustrated a detailed pathway of protein biosynthesis (Fig. 7). Nascent polypeptides are synthesized in ribosomes and immediately glycosylated with G3M9 *N*-glycan in the ER. The outermost glucose residue of G3M9 is trimmed by glucosidase I, and the diglucosylated G2M9 is subsequently trapped by malectin. G2M9 trapping by malectin initiates the conformational validation of nascent polypeptide. Folding intermediates without any mistake rapidly escape and subsequently enter into calnexin-cycle for further folding, while misfolded ones are retained by malectin and subsequently degraded via ERAD pathway. After exit from calnexin-cylce, α-mannosidase trimming of *N*-glycans on correctly folded polypeptide produces high-mannose-type *N*-glycan which is recognized by ERGIC-53. Binding of ERGIC-53 to high-mannose glycans mediates the ER-Golgi transport of correctly folded nascent proteins for further maturation and final secretion. In summary, the observations from functional studies on ER-lectins revealed their distinct roles in regulating protein synthesis step-by-step for maintaining the entire biosynthetic homeostasis.

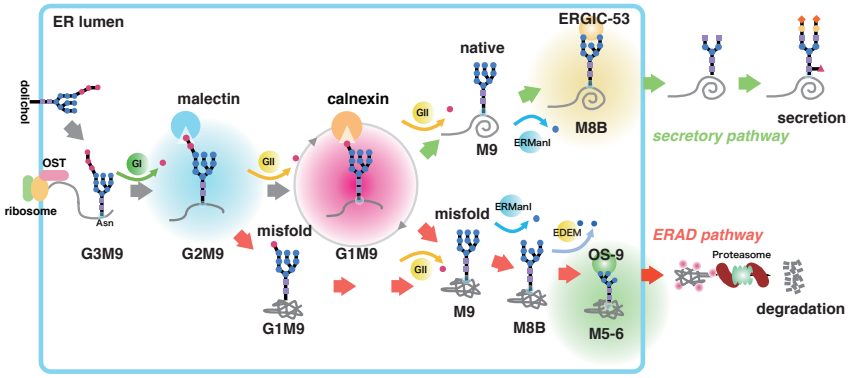

Figure 7. The role of ER-lectins in protein biosynthetic biosynthesis.

Publications

Chen Y, Hu D, Yabe R, Tateno H, Qin SY, Matsumoto N, Hirabayashi J, Yamamoto K. Role of malectin in Glc(2)Man(9)GlcNAc(2)-dependent quality control of α1-antitrypsin. *Mol Biol Cell*. 2011 Oct;22(19): 3559-70.

Satoh T, Chen Y, Hu D, Hanashima S, Yamamoto K, Yamaguchi Y. Structural basis for oligosaccharide recognition of misfolded glycoproteins by OS-9 in ER-associated degradation. *Mol Cell*. 2010 Dec 22;40(6): 905-16.

Chen Y, Hojo S, Matsumoto N, Yamamoto K. *N*-glycan-binding of ERGIC-53 to a novel ligand Mac-2BP regulates its early secretion. *Glycobiology*, in submission.