論文の内容の要旨

Studies on the function of malectin in the quality control of glycoproteins in the ER

（小胞体タンパク質品質管理機構におけるマレクチンの機能解析）

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Introduction

Endoplasmic reticulum (ER) is the major site for the folding and sorting of membrane-bound and secretory proteins, most of which are *N*-glycosylated. There exists a rigorous quality control system consisting of a variety of folding enzymes and molecular chaperones, which assist in properly folding proteins and transporting them through the ER. When misfolded proteins are present in the ER, they are retained in the ER and refolded again, and if this is not possible, they are targeted for degeradation. It has been now commonly accepted that *N*-linked glycans attached to the newly synthesized glycoproteins plays a central role in their quality control, which involves the coordination of several ER-resident lectins and glycosidases.

Malectin is an ER-resident lectin that recognizes the early processing *N*-linked oligosaccharide, $Glc_2Man_9GlcNAc_2$ (G2M9), on newly synthesized glycoproteins. Previous studies by our group and others have demonstrated that malectin preferentially associates with folding-defective glycoproteins and inhibits their secretion. During this process, the sugar-binding activity of malectin is required. However, since G2M9 is generated at the very early stage of processing, which can be typically found not only on misfolded glycoproteins, but also on the glycoproteins undergoing productive folding, therefore, it is highly possible that unknown factors, rather than the sugar-binding activity of malectin, should contribute to this preference. Here, we performed a proteomics study of proteins that were associated with malectin, and found that malectin formed a stable complex with an ER-resident transmembrane protein, ribophorin I. Our present study provides the first evidence of the mechanism by which malectin preferentially associates with misfolded glycoproteins.

Results

1. Identification of malectin binding partners by a proteomics approach

To identify the possible malectin-associated proteins, 293T cells in which malectin with N-terminal FLAG-tag (FLAG-mal) was expressed were lysed and subsequently immunoprecipitated using anti-FLAG antibody. The co-immunoprecipitated proteins were separated by SDS-PAGE and detected by silver staining. Two clearly different bands were observed from the malectin over-expressing cells but not from the mock-transfected cells. The two proteins were excised from the gel, treated with trypsin, and identified using MALDI-TOF mass spectrometry. The protein with a molecular mass approximately 38 kDa was identified to be malectin itself. Another protein with a molecular mass around 68 kDa was demonstrated conclusively as being ribophorin I with the MS/MS spectrometry analysis. Four distinct peptides were identified from MS/MS spectra with 99% confidence (representing 9% sequence coverage) and two peptides were identified with <90% confidence (Fig. 1A). Total coverage (all peptides) was 12.2% by amino acid sequence. As an orthogonal test of the protein identification, the malectin co-immunoprecipitates were analyzed by western blotting using anti-ribophorin I antibody (Fig. 1B). The 68 kDa band corresponding to ribophorin I was clearly and specifically immunoreactive in malectin co-immunoprecipitates from malectin over-expressing cells but not from the mock-transfected cells.

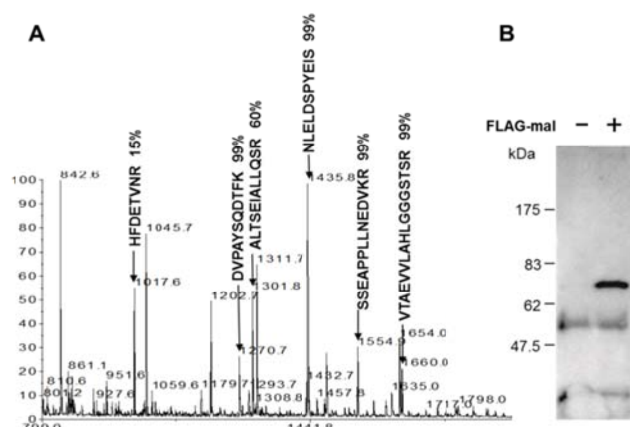

Fig. 1 Identification of ribophorin I as a malectin binding partner. (A) MALDI-TOF-MS analysis of the protein band corresponding to ribophorin I, with amino acid sequences and confidence (%) of identified peptides. (B) the malectin co-immunoprecipitates were analyzed by western blotting using ribophorin I specific antibody.

2. Malectin and ribophorin I forms a stable complex

To further confirm the interaction between malectin and ribophorin I, we co-expressed FLAG-mal and Myc-tagged ribophorin I (Myc-rpn1) in 293T cells and carried out co-immunoprecipitation studies. Expressions of FLAG-mal and Myc-rpn1 were confirmed by western analyses of the total cell lysates (Fig. 2A). Interaction of ribophorin I with malectin was determined by immunoprecipitation with anti-FLAG antibody, followed by immunoblotting with anti-Myc antibody (Fig. 2B). Myc-rpn1 was co-precipitated with FLAG-mal when cells were co-transfected with FLAG-mal and Myc-rpn1. However, ribophorin I was not detected in

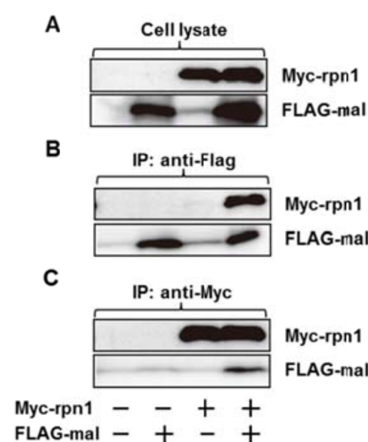

Fig. 2 Malectin and ribophorin I formed a stable complex (A) Cell lysates were immunoblotted with anti-FLAG and anti-Myc antibodies, respectively. (B) Cell lysates were subjected to immunoprectipitation using anti-FLAG antibody (C) Cell lysates were subjected to immunoprectipitation using anti-Myc antibody.

immunoprecipitates using anti-FLAG antibody from cells transfected with either FLAG-mal or Myc-rpn1 alone. Similar results were also confirmed by immunoprecipitation of the cell lysates using anti-Myc antibody, followed by immunoblotting with anti-FLAG antibody (Fig. 2C). These results clearly indicated that malectin could interact with ribophorin I in cells.

3. Ribophorin I enhances the association of malectin with misfolded glycoproteins

To determine the functional significance of the interaction between ribophorin I and malectin, a co-immunoprecipitation experiment was conducted to examine whether the association of malectin with misfolded glycoproteins might be affected when ribophorin I was over-expressed. In FLAG-mal and Myc-rpn1 co-expressing cells or FLAG-mal over-expressing cells, α1-antitrypsin (AT) or its misfolded form AT^{NHK} was expressed. After the expression of individual proteins in cells was confirmed by western blotting (Fig. 3A), cell lysates were immunoprecipitated by anti-FLAG antibody and then blotted with anti-FLAG, anti-AT, and anti-Myc antibodies, respectively (Fig. 3B). In accordance with previous results indicating that malectin preferentially interacted with misfolded glycoproteins, we observed that only the misfolded AT^{NHK}, but not the wild type AT, was co-precipitated with malectin. Interestingly, the association of malectin with AT^{NHK} was significantly enhanced when ribophorin I was over-expressed.

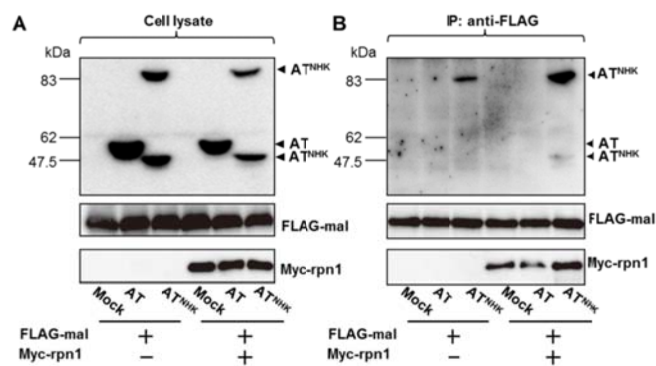

Fig. 3 Ribophorin I over-expression enhanced association of malectin with misfolded glycoproteins. (A) Cell lysates were immunoblotted with anti-AT, anti-FLAG and anti-Myc antibodies, respectively. (B) Cell lysates were subjected to immunoprectipitation using anti-FLAG antibody. Upper bands represent dimeric form.

When these cells were treated with glucosidase II inhibitor, deoxynojirimycin (DNJ) that cause the accumulation of G2M9 on glycoproteins, both AT and AT^{NHK} became co-precipitated with malectin. This confirmed that the G2M9 is required for the binding with malectin, but not a key factor in the selective association of malectin with misfolded glycoproteins. Interestingly, the interaction of AT with malectin under DNJ treatment was significantly impaired when ribophorin I was over-expressed. These lines of evidence indicated that ribophorin I plays an important role in the selective association of malectin with misfolded glycoproteins.

4. Ribophorin I down-regulation impairs the association of malectin with misfolded glycoproteins

To further characterize the effects of ribophorin I on the preferential association of malectin with

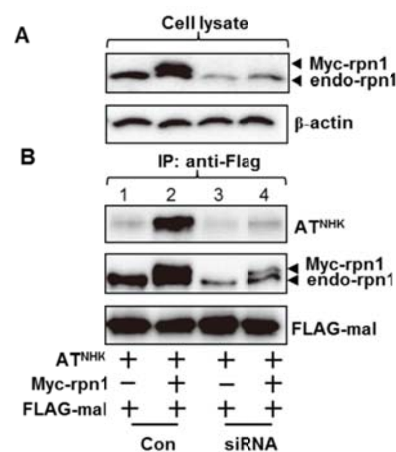

Fig. 4 Ribophorin I down-regulation impairs the association of malectin with misfolded glycoproteins (A) Cell lysates were immunoblotted with anti-ribophorin I and anti-β-actin antibodies, respectively. (B) Cell lysates were subjected to immunoprectipitation using anti-FLAG antibody, and then immunoprecipitates were immunoblotted with anti-AT, anti-ribophorin I and anti-FLAG antibodies, respectively.

misfolded glycoproteins, we used siRNA to down-regulate the expression of ribophorin I before an immunoprecipitation experiment. The expression of ribophorin I was analyzed using anti-ribophorin I antibody after 48 h. As shown in Fig. 4A, both the endogenous and exogenous ribophorin I were significantly decreased by approximately 60% in siRNA-treated cells. These cells were then subjected to immunoprecipitatiton by anti-FLAG antibody and blotted with anti-AT, anti-ribophorin I, and anti-FLAG antibodies, respectively (Fig. 4B). Along with siRNA-mediated knock-down of ribophorin I, the co-precipitated ribophorin I was obviously decreased. Concomitantly, the enhanced association of AT^{NHK} with malectin by over-expression of ribophorin I was not observed in the presence of siRNA. These results clearly confirm the important role of ribophorin I in the association of malectin with misfolded glycoproteins.

5. Ribophorin I recognizes the misfolded proteins

To clarify wheather ribophorin I recognizes misfolded proteins, we generated the ribophorin I reporter cell line BWZ.Rib I, which enabled us to detect interaction of the ER-lumenal part of ribophorin I with misfolded proteins. In this study using disulfide-scrambled ribonuclease A (RNase A) as a ligand, we found that BWZ.Rib I, but not BWZ.Myc reporter cells, interacted with the scrambled RNase A, while neither BWZ.Rib I or BWZ.Myc reporter cells interacted with the native RNase A.

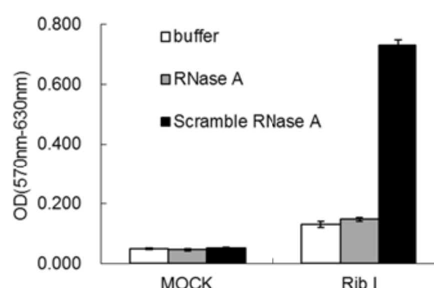

Fig. 5 Ribophorin I recognizes misfolded proteins BWZ.Rib I or BWZ.Myc (control) cells were incubated on the ELISA plate coated with 20 μg/mL of RNase A (native form) or disulfide-scrambled RNase A (misfolded form) for 16 h and assayed for β-galactosidase activity.

Conclusions

Based on these results, we propose the potential mechanism for explaining how malectin preferentially associates with misfolded glycoproteins. In our model, malectin specifically recognizes the newly synthesized glycoproteins via G2M9 generated by glucosidase I, while ribophorin I functions as a chaperone to recognize the unfolded protein backbone and facilitate the folding process. At this step, there is no significant difference between normally foldable glycoproteins and terminally misfolded ones in their association with malectin. According to the progress toward protein folding, the interaction of partially folded protein with ribophorin I is gradually decreased, which will lead to the release of these glycopteins followed with the trimming of the second glucose by glucosidase II to prevent further association with malectin. Actually, we observed that the co-precipitation of malectin with AT was significantly reduced by over-expression of ribophorin I. However, the improperly folded glycoproteins, such as AT^{NHK}, will continue to be assocated with ribophorin I, which may significantly increase the half-time of their association with malectin. Therefore, the preferential association of malectin with misfolded glycprotiens can be accounted for by both the lectin property of malectin and chaperone nature of ribophorin I.

In conclusion, our studies demonstrate that malectin forms a complex with ribophorin I, which is important for malectin to recognize misfolded glycoproteins.