Malectin Forms a Complex with Ribophorin I for Enhanced Association with Misfolded Glycoproteins*

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Background: Malectin may play a role in the quality control of glycoproteins, but the underlying molecular mechanism(s) is not known.

Results: Malectin forms a complex with ribophorin I for enhanced association with misfolded glycoproteins.

Conclusion: Malectin functions by forming a complex with ribophorin I.

Significance: This might be the first evidence for the preferential association of malectin with misfolded glycoproteins.

Malectin is an endoplasmic reticulum-resident lectin, which recognizes di-glucosylated Glc2Man9GlcNAc2 (G2M9) N-glycans on newly synthesized glycoproteins. We previously demonstrated that malectin preferentially associates with misfolded glycoproteins and inhibits their secretion (Chen, Y., Hu, D., Yabe, R., Tateno, H., Qin, S. Y., Matsumoto, N., Hirabayashi, J., and Yamamoto, K. (2011) Mol. Biol. Cell 22, 3559–3570). The sugar binding activity of malectin is required for this process. However, because G2M9 N-glycans are generated at the very early stage of processing and are typically found on both misfolded glycoproteins and glycoproteins undergoing folding, other mechanisms must underlie the preference of malectin for misfolded glycoproteins. Here, we searched for proteins that were co-immunoprecipitated with malectin, and we found that malectin formed a stable complex with an endoplasmic reticulum-resident transmembrane protein, ribophorin I. Co-expression of malectin and ribophorin I significantly enhanced the association between malectin and ribophorin I and EDEM1, which progressively remove terminal △1,2-linked mannose residues, thereby reducing the reglucosylation of the resulting oligosaccharide, Glc2Man9GlcNAc2 (G3M9), is rapidly removed by the type II transmembrane protein, glucosidase I. Subsequent trimming of the middle △1,3-linked glucose by glucosidase II is required for the glycoprotein to interact with calnexin/calreticulin (CNX/CRT). The association of newly synthesized glycoproteins with CNX/CRT prevents proteins from aggregating and enhances disulfide bond formation by recruiting ERp57. Cleavage of the innermost △1,3-linked glucose by glucosidase II prevents further association with CNX/CRT, which allows correctly folded glycoproteins to proceed to the Golgi apparatus for further processing to yield hybrid- or complex-type glycoforms. Glycoproteins that have not yet achieved their proper conformation are reglucosylated by UDP-glucose:glycoprotein glucosyltransferase, thereby allowing another folding cycle. Glycoproteins persistently failing to attain a native conformation expose their N-glycans to the ER-resident mannosidases, ER α-mannosidase I and EDEM1, which progressively remove terminal △1,2-bonded mannose residues, thereby reducing the reglucosylation capacity of glucosidase transferase (3, 4). At the same time, mannose trimming also generates ligands for two △1,6-linked mannose-binding lectins, OS-9 and XTP3-B, which facilitate the transport of misfolded glycoproteins across the ER membrane for degradation (5–9).

Recently, an ER-resident protein, malectin, was found to specifically recognize Glc2Man9GlcNAc2 (G2M9) N-glycans (10–12). By analogy with two other ER-resident lectin chaperones, CNX and CRT, which specifically recognize mono-glucosylated Glc2Man9GlcNAc2 (G1M9), malectin is thought to be

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2 The abbreviations used are: ER, endoplasmic reticulum; AT, α1-antitrypsin; ATN, α1-antitrypsin variantnull Hong Kong; DNJ, 1-deoxynojirimycin; ANS, 8-anilino-1-naphthalenesulfonate; OST, oligosaccharyltransferase; CNX, calnexin; CRT, calreticulin.
involved in the quality control of glycoproteins. Indeed, we and others demonstrated that malectin was induced under conditions of ER stress and is preferentially associated with folding-defective glycoproteins (13, 14). However, the molecular mechanisms by which malectin preferentially associates with misfolded glycoproteins are still not clear. Here, we performed a proteomic analysis of proteins co-immunoprecipitated with ribophorin I. The results showed that malectin constitutively co-immunoprecipitated with ribophorin I.

Ribophorin I is an ER-resident transmembrane protein, which serves as a subunit of the mammalian OST complex (15). Recent studies show that ribophorin I regulates the glycosylation of some glycoproteins by selectively associating with, and delivering them to, the catalytic core of the OST complex (16, 17). Association of ribophorin I with newly synthesized membrane proteins was observed after the exit of the nascent chain from the Sec61 translocon, and it was unaffected by the N-glycosylation status (18). The ability of ribophorin I to interact with newly synthesized proteins suggests that it may function as a chaperone in the ER.

This study showed that malectin forms a stable complex with ribophorin I. Co-expression of malectin and ribophorin I significantly enhanced the association of malectin with a folding-defective α1-antitrypsin variant, null Hong Kong (AT\textsuperscript{N\textsubscript{HK}}), and reduced its secretion; however, it did not affect secretion of wild-type α1-antitrypsin (AT). Down-regulation of ribophorin I expression by a specific siRNA impaired the association of malectin with AT\textsuperscript{N\textsubscript{HK}} and augmented AT\textsuperscript{N\textsubscript{HK}} secretion. Additionally, ribophorin I preferentially interacted with misfolded ribonuclease A, but not with the native form, suggesting that ribophorin I functions as a chaperone inside the cell. These findings identify a possible role for malectin in the quality control of glycoproteins via formation of a complex with ribophorin I.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**—293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen), 2 mM glutamine, and 25 mM HEPES-NaOH, pH 7.4. BWZ.36 cells, kindly provided by N. Shastri (University of California Berkeley, CA), were cultured in RPMI 1640 medium (Invitrogen) supplemented as described for DMEM. The retroviral vector, pMXs-ires-EF1 (pMXs-1G), and the retrovirus packaging cell line, Plat-E, were provided by T. Kitamura (University of Tokyo, Tokyo, Japan) and were used for retroviral transduction throughout this study (19). Plat-E cells were maintained in DMEM containing 10 μg/ml blasticidin S (Invitrogen) and 1 μg/ml puromycin (Sigma). All cell lines were cultured at 37 °C in a humid atmosphere containing 5% CO\textsubscript{2}. Ribonuclease A (RNase A) was obtained from Sigma. Scrambled RNase A was prepared as described previously (20). Anti-Myc antibody was purified from the culture supernatant of hydridoma clone, 9E10, which was purchased from the American Type Culture Collection (Manassas, VA). Polyclonal anti-FLAG antibody, monoclonal anti-FLAG antibody (M2), and monoclonal anti-β-actin antibody were purchased from Sigma. Anti-ribophorin I antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Monoclonal anti-α1-antitrypsin antibody was purchased from Abcam (Cambridge, UK).

**Construction of Expression Plasmids**—The coding sequence for human ribophorin I, ribophorin II, and OST48 (lacking its signal sequence) was amplified by PCR using the primers 5’-GAGTTAACGGCTCTCCCGACGACGACGGC-3’ and 5’-AAGGAAAAAGCGGCCGCTAACAGGGCATCCAGGAT-3’ for ribophorin I; the primers 5’-GAGTTAACGGCTCTCCCGACGACGACGGC-3’ and 5’-AAGGAAAAAGCGGCCGCTAACAGGGCATCCAGGAT-3’ for ribophorin II, and the primers 5’-GAGTTAACGGCTCTCCCGACGACGACGGC-3’ and 5’-AAGGAAAAAGCGGCCGCTAACAGGGCATCCAGGAT-3’ for OST48. (Hpal and NotI sites are underlined). The amplified DNA was digested with Hpal and NotI and then inserted into the Hpal and NotI sites of a pRcCMV-based vector containing an N-terminal CD8\textsubscript{α} signal sequence followed by a Myc tag sequence as described previously (21). Plasmids for the expression of human malectin and its mutants and AT and its variant, AT\textsuperscript{N\textsubscript{HK}}, were prepared as described previously (14).

**Protein Identification by MALDI-TOF MS**—Coomassie Brilliant Blue-stained gel bands were excised and destained in 25 mM NH\textsubscript{4}HCO\textsubscript{3}, 50% (v/v) acetonitrile for 10 min. Destained gels were then reduced in 50 mM NH\textsubscript{4}HCO\textsubscript{3}, 10 mM DTT for 1 h at 56 °C before alkylation with 55 mM iodoacetamide in 50 mM NH\textsubscript{4}HCO\textsubscript{3} for 45 min at room temperature. Samples were washed with 50 mM NH\textsubscript{4}HCO\textsubscript{3} for 10 min, equilibrated with 25 mM NH\textsubscript{4}HCO\textsubscript{3}/50% (v/v) acetonitrile for 10 min, and shrink in 100% acetonitrile for 5 min and dried. Dried gel samples were rehydrated in 20 ng/ml trypsin (Sigma) in 50 mM NH\textsubscript{4}HCO\textsubscript{3} on ice for 30 min and incubated at 37 °C overnight. Trypsized peptide fragments were extracted by sonication of the gel pieces first in 40 μl of 50% (v/v) acetonitrile, 0.1% TFA for 15 min and then in 40 μl of 75% (v/v) acetonitrile, 0.1% TFA for another 15 min. The supernatants were collected and dried to a final volume of 10 μl. The samples were then analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) using a TOF/TOF 5800 system (AB Sciei, Framingham, MA). Proteins comprising one or more peptides with a high confidence score (>95%) determined by ProteinPilot (AB Sciei) were considered positively identified.

**Immunoprecipitation and Western Blotting**—HEK293T cells were transfected with p3\times\textsubscript{FLAG}/CMV9/malectin using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. After 24 h, both the cells and conditioned medium were collected. For deoxynojirimycin (DNJ) treatment (24 h after transfection), cells were treated with 1 mM DNJ for a further 3 h. For siRNA treatment, HEK293T cells were transfected with p3\times\textsubscript{FLAG}/CMV9/malectin, pRCMV/Myc/ribophorinI, pcDNA3.1(+)/α1AT or pcDNA3.1(+)/AT\textsuperscript{N\textsubscript{HK}}, and 10 nm ribophorin I-specific siRNA (sense, 5’-GCAUGACCGAGUCAUUGATT-3’; antisense, 5’-UCAAUGACUGCGGCUCAUGCTG-3’) (Ambion, Carlsbad, CA) or control siRNA (negative control 1 siRNA, Ambion) using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol. After 24 h, cultured media were changed, and the culture was continued for another 24 h. Both cells and the conditioned medium were collected and then used for each assay. The cells were washed with
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phosphate-buffered saline (PBS) and lysed for 30 min with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% (w/v) CHAPS containing protease inhibitors (1 mM PMSF, and 1 μg/ml leupeptin)) on ice. The lysate was centrifuged for 20 min at 12,000 × g at 4 °C, and the supernatant was collected and subjected to immunoprecipitation. The cell lysates were first pre-cleared with protein G-Sepharose 4 Fast Flow by gently rotating at 4 °C for 1 h. The pre-cleared lysates were then pre-cleared with antibody-conjugated beads by gently rotating at 4 °C for overnight. The beads were washed three times with 20 mM Tris-HCl, pH 8.0, containing 6M guanidine-HCl, 1 mM DTT, and 0.1 mM EDTA, and then with 30 ml of a linear gradient of NaCl from 25 to 500 mM in the same buffer. Elution was performed at flow rate of 1 ml/min, and fractions of 1 ml were collected. For thermal shift assay of purified soluble ribophorin I, fluorescence intensity (Iex = 475–500 nm and Iem = 520–550 nm) of SYPRO Orange (Molecular Probes) was monitored using PikoReal 24 (Thermo Scientific) in the presence of recombinant ribophorin I (0.01 mg/ml) in 25 mM Tris-HCl, pH 8.5, 400 mM NaCl, 1 mM CaCl2) under increasing temperatures. Fluorescence spectra of ANS were recorded on a Hitachi F-4500 spectrofluorometer using a 3-mm cuvette at 25 °C. A solution of soluble recombinant ribophorin I (0.1 mg/ml) was mixed with a concentrated solution of ANS in 25 mM Tris-HCl, pH 8.5, containing 400 mM NaCl and 1 mM CaCl2 and then incubated for 10 min. The excitation wavelength was set to 390 nm. The final concentration of ANS was 140 μM. An RNase A solution (0.1 mg/ml) was used as a control. The hydrophobicity of ribophorin I was monitored by measuring tryptophan fluorescence as described previously (22).

RESULTS

Glycan Binding Activity of Malectin Is Not Sufficient for Its Selective Recognition of Misfolded Glycoproteins—Our previous study demonstrated that the G2M9 binding activity of malectin was required for its association with misfolded glycoproteins (14). However, considering that G2M9 is generated during the very early stages of glycoprotein processing, regardless of their folding status (i.e. folded or misfolded), the glycan binding activity of malectin does not explain why malectin preferentially associates with misfolded glycoproteins. To examine this further, we investigated the interaction between malectin and normally folded AT and misfolded ATNHK both before and after treatment with 1 mM DNJ (which causes the accumulation of G2M9 glycans on glycoproteins) (14). As shown in Fig. 1, before treatment with DNJ, only misfolded variant ATNHK co-precipitated with FLAG-tagged malectin (FLAG-mal). However, when cells were treated with DNJ to increase the G2M9 glycans on the glycoproteins, both ATNHK and AT co-precipitated with malectin, indicating that the G2M9 binding activity of malectin is important for its association with glycoproteins but not for its selective association with misfolded glycoproteins. Therefore, other mechanisms must be responsible for selectivity.

Identification of Malectin Binding Partners—Based on the fact that some ER-resident lectins rely on their association with molecular chaperones and foldases to recognize and modify incompletely folded glycoproteins (23, 24), we hypothesized that selective recognition of misfolded substrates by malectin may operate in a similar way. To identify candidate proteins...
associated with malectin, 293T cells overexpressing FLAG-mal were lysed and then subjected to immunoprecipitation with anti-FLAG antibodies. The co-immunoprecipitated proteins were separated by SDS-PAGE and detected by silver staining. Two clearly different bands were precipitated from FLAG-mal-overexpressing cells but not from mock-transfected cells (Fig. 2, lanes 1 and 2). The two proteins were excised from the gel, digested with trypsin, and identified using MALDI-TOF mass spectrometry. The protein with a molecular mass of ~38 kDa was identified as FLAG-mal. Another protein corresponding to 68 kDa was clearly identified as ribophorin I by MS/MS spectrometry analysis (Fig. 2B). Four distinct peptides were identified from the MS/MS spectra with 99% confidence (representing 9% sequence coverage), and two peptides were identified with 60 and 15% confidence scores. Total coverage (all peptides) was 12.2% by amino acid sequence (Table 1). As an orthogonal test of protein identification, the co-immunoprecipitates were analyzed by Western blotting using a specific antibody against ribophorin I (Fig. 2A, lanes 3 and 4). A 68-kDa band corresponding to ribophorin I was clearly and specifically detected in malectin co-immunoprecipitates from FLAG-mal-overexpressing cells but not from the mock-transfected cells. In HeLa cells, we confirmed also that ribophorin I was co-precipitated with FLAG-tagged malectin, which was demonstrated by immunostaining using anti-ribophorin I antibody (data not shown).

Malectin and Ribophorin I Form a Stable Complex Independent of the Glycan Binding Activity of Malectin—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 followed by immunoprecipitation. Expression of FLAG-mal and Myc-rpn1 in these cells was confirmed by Western blotting of total cell lysates with anti-FLAG and anti-Myc antibodies, respectively (Fig. 3A, panels a and b). When the lysates of cells expressing FLAG-mal plus Myc-rpn1 were precipitated with the anti-FLAG antibody, Myc-rpn1 was co-precipitated (Fig. 3A, panel d, lane 4). However, no signal corresponding to Myc-rpn1 was detected in cells expressing FLAG-mal alone (Fig. 3A, panel d, lane 2) or cells expressing Myc-rpn1 alone (Fig. 3A, panel d, lane 3). Similarly, when lysates of cells expressing FLAG-mal plus Myc-rpn1 were precipitated with the anti-Myc antibody, FLAG-mal was co-precipitated (Fig. 3A, panel f, lane 4). However, no signal corresponding to FLAG-

![FIGURE 1. Glycan binding activity of malectin is not sufficient for its selective recognition of misfolded glycoproteins. 293T cells were transiently co-transfected with plasmids coding for FLAG-malectin (FLAG-mal) and either AT (lanes 2 and 5) or its misfolded variant, ATNHK (lanes 3 and 6), for 24 h and then treated with or without 1 mM DNJ for another 3 h. After that, cells were lysed and subjected to immunoprecipitation (IP) with an anti-FLAG antibody (Ab). The immunoprecipitates were separated by SDS-PAGE under a nonreducing condition followed by immunoblotting (IB) with an anti-AT antibody. An upper band (ATNHK) represents the dimeric form of ATNHK.](image1)

![FIGURE 2. Identification of proteins associated with malectin. 293T cells were transfected with a plasmid coding for human FLAG-malectin (FLAG-mal). After 24 h, cells were lysed and subjected to immunoprecipitation (IP) with an anti-FLAG antibody (Ab). A, immunoprecipitates were separated by SDS-PAGE followed by silver staining or by immunoblotting (IB) with an anti-ribophorin I antibody. B, MALDI-TOF mass spectra of the 68-kDa band. Arrowheads represent the peptides that were identified as being ribophorin I by MS/MS spectrometry analysis.](image2)

| Peptide no. | Amino acid sequence | Molecular mass | confidence % |
|-------------|---------------------|---------------|--------------|
| 1 | DVPAYSQDTFK | 1270.34 | 99 |
| 2 | NIEIDSPYEISR | 1435.54 | 99 |
| 3 | SSEAPPLINEDVKR | 1554.70 | 99 |
| 4 | VTAEVYLAHLGGGSTSR | 1653.84 | 99 |
| 5 | ALTSEIALLOQSR | 1301.51 | 60 |
| 6 | HFDETVNR | 1017.05 | 15 |
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A

|   | cell lysate |   |   |
|---|-------------|---|---|
| a | Myc-rpn1    | b | Myc-rpn1 |
| IP: α-FLAG Ab |   | FLAG-mal |   |
| c | Myc-rpn1 | d | Myc-rpn1 |
| IP: α-Myc Ab |   | FLAG-mal |   |
| e | Myc-rpn1 | f | Myc-rpn1 |
| FLAG-mal |   | FLAG-mal |

B

|   | cell lysate |   |   |
|---|-------------|---|---|
| a | Myc-rpn1 | b | FLAG-mal |
| IP: α-FLAG Ab |   |   |
| c | Myc-rpn1 | d | FLAG-mal |
| IP: α-Myc Ab |   |   |
| e | Myc-rpn1 | f | FLAG-mal |
| FLAG-mal |   |   |

C

|   | cell lysate |   |   |
|---|-------------|---|---|
| a | FLAG-mal | b | FLAG-mal |
| IP: α-FLAG Ab |   |   |
| c | FLAG-mal | d | FLAG-mal |
| IP: α-Myc Ab |   |   |
| e | FLAG-mal | f | endo-rpn1 |

FIGURE 3. Malectin and ribophorin I form a stable complex independent of the glycan binding activity of malectin. 293T cells were transfected with plasmids coding for Myc-ribophorin I (Myc-rpn1) and/or FLAG-malectin (FLAG-mal). After 24 h, the cells were harvested and lysed. A, cell lysates were separated by SDS-PAGE followed by immunoblotting with anti-Myc (panel a) and anti-FLAG antibodies (panel b). Cell lysates were subjected to immunoprecipitation (IP) with an anti-FLAG antibody and immunoblotted with anti-FLAG (panel c) and anti-Myc antibodies (panel d). Cell lysates were subjected to immunoprecipitation with an anti-Myc antibody and immunoblotted with anti-Myc (panel e) and anti-FLAG antibodies (panel f). B, 293T cells expressing either Myc-rpn1 or FLAG-mal were mixed together before lysate preparation (lane 2, mix). 293T cells transfected with a plasmid for FLAG-mal (lane 1) or co-transfected with plasmids for both proteins (lane 2, co-transfect) were used as negative and positive controls, respectively. Expression of Myc-rpn1 (panel a) or FLAG-mal (panel b) in cell lysates, or after immunoprecipitation with anti-FLAG antibodies, was analyzed by immunoblotting using anti-Myc (panel c) and anti-FLAG antibodies (panel d). C, 293T cells were transfected with plasmids for FLAG-mal or its sugar binding-deficient mutants (Y104A, Y131A, F132A, and D201A) for 24 h. The expression of FLAG-mal and its mutants was confirmed by immunoblotting with an anti-FLAG antibody (panel a). Cell lysates were subjected to immunoprecipitation with an anti-FLAG antibody and immunoblotted with an anti-FLAG antibody (panel b) and anti-ribo- phorin I antibodies (panel c). Anti-ribophorin I antibody detected endo- genous ribophorin I (endo-rpn1) that was co-precipitated with FLAG-mal or its mutants.

These results clearly indicate that malectin specifically interacts with ribophorin I in cells.

To rule out the possibility that the interaction between ribophorin I and malectin occurred as a consequence of detergent extraction during immunoprecipitation studies, 293T cells were transfected with plasmids coding for either FLAG-mal or Myc-rpn1 and mixed together before lysate preparation. The expression of individual proteins in mixed cells or co-transfected cells (positive control) was confirmed by Western blotting (Fig. 3B, panels a and b). Co-immunoprecipitation of FLAG-mal with Myc-rpn1 did not occur from mixed cells expressing FLAG-mal or Myc-rpn1 individually (Fig. 3B, panel c, lane 2), but it was clearly observed in cells co-transfected with plasmids for both proteins (Fig. 3B, panel c, lane 3). These results confirm that the interaction between malectin and ribophorin I was not an artifact caused during lysate preparation.

Ribophorin I is a glycoprotein containing an N-glycosylation site within its luminal domain; thus, it is possible that malectin recognizes the glycans on ribophorin I to form a complex. To examine this possibility, immunoprecipitation was performed using cell lysates expressing sugar binding-deficient malectin mutants (Y104A, Y131A, F132A, or D201A) (14). Western blotting of total cell lysates revealed that both wild-type and mutant malectin showed similar expression in cells (Fig. 3C, panel a). The lysates of these cells were immunoprecipitated with anti-FLAG antibodies and immunoblotted with anti-ribophorin I antibodies. As shown in Fig. 3C, panel c, both malectin and its mutants could equally precipitate endogenous ribophorin I (endo-rpn1), suggesting that the interaction between malectin and ribophorin I was not mediated via the sugar binding activity of malectin.

Ribophorin I is one of the subunits of the oligomeric complex, OST (15). Because ribophorin I associates with other OST subunits, ribophorin II, OST48, and so on, there might be the possibility that malectin was co-precipitated with ribophorin I via association with other OST subunit(s). To test such a possibility, we performed immunoprecipitation of FLAG-mal from the lysate of the cells expressing Myc-tagged ribophorin II (Myc-rpn2) or Myc-tagged OST48 (Myc-OST48) (Fig. 4). Both Myc-rpn2 and Myc-OST48 were co-precipitated with FLAG-mal and ribophorin I (Fig. 4B, panel a, lanes 1 and 3). However, knockdown of ribophorin I using specific siRNA dramatically abrogated co-precipitation of both Myc-rpn2 and Myc-OST48 (Fig. 4B, panel a, lanes 2 and 4) indicating that malectin interacts with ribophorin I and that ribophorin II and OST48, which form a complex with ribophorin I, were also precipitated along with ribophorin I.

Ribophorin I Overexpression Enhances the Association of Malectin with Misfolded Glycoproteins and Inhibits Their Secretion—To determine the functional significance of the interaction between ribophorin I and malectin, a co-immunoprecipitation experiment was performed to ascertain whether...
the association between malectin and misfolded glycoproteins might be affected by overexpression of ribophorin I. In cells co-expressing FLAG-mal and Myc-rpn1, or overexpressing FLAG-mal alone, AT or its misfolded variant ATNHK was expressed. After the expression of individual proteins in cells was confirmed by Western blotting (Fig. 5A), cell lysates were immunoprecipitated with anti-FLAG antibodies and blotted with anti-FLAG, anti-Myc, or anti-AT antibodies (Fig. 5B). In accordance with a previous study (14), only the misfolded ATNHK (AT♯NHK in Fig. 5B), but not the wild-type AT, was co-precipitated by FLAG-mal (Fig. 5B, lane 3). This was particularly true for the dimer. The co-precipitation of malectin with AT♯NHK, but not AT, was significantly enhanced when ribophorin I was overexpressed (Fig. 5B, lane 6), suggesting that ribophorin I did enhance the association between malectin and misfolded glycoproteins. The secretion of AT and AT♯NHK was also examined. In accordance with the enhanced association between malectin and AT♯NHK upon co-expression with ribophorin I, the decreased secretion of AT♯NHK induced by overexpression of FLAG-mal (Fig. 5C, lane 2) was further inhibited by ribophorin I co-expression (Fig. 5C, lane 3), whereas the secretion of wild-type AT was not markedly affected (Fig. 5C, lanes 4–6). We also investigated the effects of ribophorin I overexpression upon DNJ treatment (Fig. 5D). DNJ, an inhibitor of glucosidase I and II, preferentially inhibits the activity of glucosidase II when low concentrations (1 mM) are used (14). Interestingly, although further enhancement of AT♯NHK and malectin co-precipitation was not observed by overexpression of ribophorin I upon DNJ treatment (Fig. 5D, lanes 3 and 4), the interaction between AT and malectin induced by DNJ treatment (Fig. 5D, lane 7) was inhibited by overexpression of ribophorin I (Fig. 5D, lane 8), suggesting that ribophorin I may prevent the association of correctly folded glycoproteins with malectin. These lines of evidence indicate that ribophorin I may play a central role in the selective association between malectin and misfolded glycoproteins.

**Down-regulation of Ribophorin I Impairs the Association of Malectin with Misfolded Glycoproteins**—To further confirm the effects of ribophorin I on the association of malectin with misfolded glycoproteins, we used siRNA to down-regulate the expression of ribophorin I before subjecting cells to immunoprecipitation. When 293T cells were treated with ribophorin I-specific siRNA for 48 h, expression of both endogenous and exogenous ribophorin I was significantly inhibited (Fig. 6A, panel a, lanes 3, 4, 7, and 8). These cells were then subjected to immunoprecipitation with the anti-FLAG antibody followed by blotting with anti-FLAG (Fig. 6B, panel c), anti-ribophorin I (Fig. 6B, panel b), or anti-AT antibodies (Fig. 6B, panel a). Along with siRNA-mediated knockdown of ribophorin I, co-precipitation of ribophorin I and malectin was evidently reduced (Fig. 6B, panel b, lanes 3, 4, 7, and 8). Concomitantly, the enhanced co-precipitation of AT♯NHK and malectin induced by ribophorin I overexpression (Fig. 6B, panel a, lane 2) was counteracted by treatment with siRNA (Fig. 6B, panel a, lane 4). Along with this change, decreased secretion of AT♯NHK (Fig. 6C, lane 2) induced by overexpression of ribophorin I was also counteracted by siRNA treatment (Fig. 6C, lane 4). However, siRNA treatment affected neither the association of malectin with wild-type AT nor its secretion (Fig. 6, B, panel d, and C, panel b). These results further confirm the role of ribophorin I in the selective association of malectin with misfolded glycoproteins. Treatment of siRNA significantly decreased expression of endogenous ribophorin I; however, AT♯NHK co-precipitated with FLAG-malectin was not so significantly decreased (~70% of control, Fig. 6B, panel d, lanes 1 and 3). This result may be explained by assuming that a part of AT♯NHK remains bound to malectin via N-glycans.

**Ribophorin I Recognizes Misfolded Proteins**—The enhanced association of malectin with misfolded glycoproteins induced by ribophorin I led us to hypothesize that ribophorin I might function as a chaperone that recognizes misfolded proteins. To test this, we investigated the direct interaction between ribophorin I and scrambled RNase A, a misfolded protein caused by a mismatched cysteinyl disulfide, using the ribophorin I reporter cell line, BWZ.Rpn1. BWZ.Rpn1 cells expressed ribophorin I/CD3ζ fusion protein on the cell surfaces and transduced cross-linking of ribophorin I on the cell surface into β-galactosidase expression. As shown in Fig. 7A, neither BWZ.Rpn1 cells nor control reporter cells (BWZ.Myc) interacted with native RNase A. However, we observed a specific interaction between BWZ.Rpn1 and disulfide-scrambled RNase A (Fig. 7A). Because chaperones often bind their substrates via hydrophobic interactions, the hydrophobicity of the ribophorin I surface was further examined using a widely used hydrophobic probe, ANS, which becomes fluorescent when bound to the nonpolar sites of proteins. The luminal part of human ribophorin I (Ala-24 to Glu-438) was expressed in
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E. coli and purified. Heat denaturation of the soluble recombinant ribophorin I was observed around 55 °C only by thermal shift assay, which suggested that the recombinant ribophorin I was correctly folded (data not shown). The recombinant ribophorin I induced a marked enhancement of ANS fluorescence, with maximum fluorescence observed at 483 nm (Fig. 7B). By contrast, ANS showed weak fluorescence (with maximum fluorescence at 521 nm) in the presence of a control hydrophilic protein, RNase A. These observations clearly show that the luminal part of human ribophorin I possesses hydrophobic surfaces. Taken together, these results reveal that ribophorin I may function as a chaperone that recognizes misfolded proteins inside cells.

**DISCUSSION**

Because malectin, a novel ER-resident protein, was first reported by the *Xenopus laevis* proteomic study, its selective binding to di-glucosylated high mannose-type N-glycans has led to much speculation regarding its function (12, 13). By analogy with the mono-glucosylated N-glycan-binding lectins, CNX and CRT, which play an essential role in the folding of glycoproteins, malectin is also suggested to be involved in the

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**FIGURE 5.** Ribophorin I overexpression enhances the association of malectin with misfolded glycoproteins and inhibits their secretion. 293T cells were transfected with plasmids coding for human AT, ATNHK, Myc-rpn1, or FLAG-mal as indicated. After 24 h, cells and conditioned medium were collected. A, cell lysates were separated by SDS-PAGE under nonreducing conditions followed by immunoblotting (IB) with anti-AT (panel a), anti-FLAG (panel b), or anti-Myc antibodies (panel c) (panel d). B, cell lysates were subjected to immunoprecipitation (IP) with an anti-FLAG antibody, and the precipitates were separated by SDS-PAGE under nonreducing conditions followed by immunoblotting with anti-AT (panel a), anti-FLAG (panel b), or anti-Myc antibodies (panel c). Upper band (85 kDa) represents the dimeric form of ATNHK (ATNHK*). The precipitated ATNHK and AT in panel a was quantified by scanning densitometry (panel d). C, medium collected from the transfected cells was separated by SDS-PAGE under nonreducing conditions followed by immunoblotting with an anti-AT antibody (panel a). The amount of transfected cell lysate was confirmed by immunoblotting with an anti-β-actin antibody (panel b). The secretion of ATNHK and AT in the upper panel was quantified by scanning densitometry (panel c). D, 293T cells were transfected with expression vectors for human AT, ATNHK, Myc-rpn1, or FLAG-mal as indicated for 24 h and then treated with 1 mM DNJ for another 3 h. Cell lysates were subjected to immunoprecipitation with an anti-FLAG antibody, and the precipitates were separated by SDS-PAGE under nonreducing conditions followed by immunoblotting with anti-AT antibody (panel a) and anti-FLAG antibodies (panel b). All the experiments were performed three times independently (n = 3). Data represent the mean ± S.D. from three independent experiments. **, p < 0.01 and *, p < 0.05 (Student’s t test).
Role of the Malectin-Ribophorin I Complex

Malectin, a member of the Cys(6)-Cys(3)-Cys(2) type II transmembrane lectin family, recognizes and binds misfolded glycoproteins, leading to their enhanced degradation through the ER-associated degradation (ERAD) machinery. Ribophorin I is a membrane protein that interacts with malectin and facilitates the folding of glycoproteins. In this study, we investigated the role of the malectin-ribophorin I complex in quality control of glycoproteins in the ER. In fact, Galli et al. (13) recently showed that malectin associates with newly synthesized glycoproteins, especially those with misfolded conformations, and subsequently interferes with their secretion. These results were confirmed by this study, which showed that malectin preferentially co-precipitated with misfolded AT\(^{\text{NHK}}\) but not with wild-type AT (Fig. 1). However, the mechanism by which malectin preferentially recognizes the misfolded glycoproteins remains unknown. Although the sugar binding activity of malectin is important for its association with misfolded glycoproteins (14), di-glucosylated G2M9 glycans are a common intermediate on the pathway to newly synthesized glycoproteins, regardless of whether they are properly folded or terminally misfolded. In accordance with this, we observed that both AT and AT\(^{\text{NHK}}\) were co-precipitated with malectin after treatment of cells with DNJ, which causes the accumulation of G2M9 on glycoproteins (Fig. 1). Therefore, another as-yet unknown mechanism(s) responsible for the preferential association of malectin with misfolded glycoproteins must exist.

Some ER-resident lectins facilitate the folding of glycoproteins, or the degradation of misfolded glycoproteins, by forming a complex with other foldases or molecular chaperones. For example, CNX forms a complex with ERP57 to facilitate disulfide formation in folding intermediates and with EDEM to enhance the degradation of misfolded glycoproteins (23, 24); thus, it is possible that malectin forms a complex with other chaperones to recognize misfolded glycoproteins. Based on this assumption, we searched for proteins that co-precipitated with malectin, and found that an ER-resident transmembrane protein, ribophorin I, was stably associated with malectin (Figs. 2 and 3). Malectin does not have an ER localization signal in its molecule, although it localizes in the ER. Schallus et al. (12) proposed that malectin would associate with other ER-resident protein(s) to keep its localization in the ER. Our finding may explain malectin localization in the ER because ribophorin I has ER retention signal in its cytoplasmic domain.

Recent studies show that ribophorin I is involved in the N-glycosylation of some specific membrane glycoproteins by selecting them and subsequently delivering them to the catalytic core of OST (16). Ribophorin I interacts with newly synthesized incompletely folded glycoproteins, suggesting that it may function as a molecular chaperone in the ER (18). Consistent with this, we showed a significant interaction between ribophorin I-expressing reporter cells and misfolded RNase A but not with correctly folded RNase A (Fig. 7A).

First, we observed that DNJ treatment of the cell resulted in the co-precipitation of both wild-type AT and misfolded variant AT\(^{\text{NHK}}\) with malectin, which indicated that the binding of G2M9 glycan is important in the complex formation of glycoproteins with malectin, but it cannot be responsible for the selective association of malectin with misfolded variant AT\(^{\text{NHK}}\) (Fig. 1). Second, we found that malectin formed a stable complex with ribophorin I (Fig. 2), whose expression (both overexpression and down-regulation) greatly affected the association of malectin with misfolded AT\(^{\text{NHK}}\) but not wild-type AT (Figs. 3, 5, and 6). Third, ribophorin I had hydrophobic properties reminiscent of chaperones and selectively recognizes misfolded proteins (Fig. 7). These findings suggest a potential mechanism...
whereby malectin preferentially associates with misfolded glycoproteins (Fig. 8).

In our hypothesis, the initial interaction of nascent glycoproteins with malectin occurs through the binding of G2M9 glycans generated by glucosidase I. As shown in Fig. 8(i), unfolded glycoproteins then bind to chaperone-like ribophorin I through their hydrophobic segment or patches. At this time, there is no significant difference between normally foldable glycoproteins and terminally misfolded ones in terms of their association with malectin, which was confirmed in a previous study (i.e. both AT and ATNHK were co-precipitated with malectin in a short time pulse-chase experiment) (13). Unfolded glycoproteins may enhance the association between malectin and ribophorin I and form a stable ternary complex, because co-precipitation of ribophorin I with malectin in the wild-type AT-expressing cells was reduced compared with ATNHK-expressing cells (Fig. 6B). Subsequently, the polypeptide folding proceeds and the hydrophobic area on the surface of proteins may decrease, which causes decreased interaction of polypeptide chain with ribophorin I. When partially folded glycoproteins are released from malectin, G2M9 glycan becomes susceptible to glucosidase II followed by trimming of the second glucose to prevent further association with malectin (14). As shown in Fig. 8(ii), it appears the release of misfolded glycoproteins such as ATNHK from the malectin-ribophorin I complex is difficult. This is because the hydrophobic interactions with ribophorin I are maintained as a result of the glycoproteins not folding correctly. The persistence of hydrophobic interactions with ribophorin I significantly increase the half-life of the association of misfolded glycoproteins with the malectin-ribophorin I complex.

To clarify the regulated interaction between malectin and ribophorin I in the cell, further analysis should be required.

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