Biochemical and Biophysical Research Communications 454 (2014) 436–440

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Association of malectin with ribophorin I is crucial for attenuation of misfolded glycoprotein secretion

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ARTICLE INFO

Article history:
Received 12 October 2014
Available online 27 October 2014

Keywords:
Malectin
Ribophorin I
Glycoprotein quality control
Misfolded protein

ABSTRACT

We previously demonstrated that malectin associates with ribophorin I, which is a subunit of oligosaccharyltransferase in the endoplasmic reticulum (ER). When malectin and ribophorin I are overexpressed in the ER, secretion of an α1-antitrypsin (AT) variant whose folding is defective, termed null Hong Kong (AT^{NHK}), is decreased. To confirm whether the interaction between malectin and ribophorin I is involved in the decreased secretion of misfolded glycoproteins, we constructed an expression vector encoding truncated malectin, which could not associate with ribophorin I and had an Lys-Asp-Glu-Leu ER-retention sequence at its C-terminus. Expression of wild-type malectin abrogated AT^{NHK} secretion, whereas expression of truncated malectin did not affect AT^{NHK} secretion. Both wild-type and truncated malectin bound to AT^{NHK}, and the level of AT^{NHK} was similar in cells expressing wild-type malectin and those expressing truncated malectin. Furthermore, we previously showed that decreased secretion of misfolded AT^{NHK} by malectin overexpression is restored by treatment with a proteasome inhibitor. These results clearly demonstrate that the association of malectin with ribophorin I is required to capture misfolded glycoproteins and direct them to the degradation pathway.

1. Introduction

N-linked glycans attached to nascent proteins play a pivotal role in glycoprotein quality control, which involves the coordination of several endoplasmic reticulum (ER)-resident lectins in association with chaperones [1]. In the ER, *N*-glycosylation is initiated by oligosaccharyltransferase (OST), which catalyzes the transfer of $Glc_3Man_9GlcNAc_2$ (G3M9) from lipid-linked intermediates to asparagine residues with the sequence Asn-X-Ser/Thr in newly synthesized peptides [2]. After the outermost glucose of G3M9 is rapidly removed by the transmembrane protein glucosidase I, $Glc_2Man_9GlcNAc_2$ (G2M9) binds to the membrane-bound lectin, malectin. Malectin was first identified in *Xenopus laevis* and is highly conserved among animals [3]. Nuclear magnetic resonance and frontal affinity chromatography analyses revealed that human malectin specifically binds to G2M9 [4,5]. Expression of malectin is induced under several conditions of ER stress, and malectin preferentially associates with misfolded glycoproteins in the ER [5,6].

Human α1-antitrypsin (AT) is a secreted glycoprotein with three attached *N*-glycans. A carboxyl terminus-truncated AT variant, termed null Hong Kong (AT^{NHK}) [7], exhibits defective folding and has been used to study ER-associated degradation [8,9]. We previously performed a proteomic analysis of proteins co-immunoprecipitated with malectin and showed that malectin is constitutively associated with ribophorin I [10]. Ribophorin I is an ER-resident transmembrane protein and a subunit of the mammalian OST complex [11]. Ribophorin I is thought to deliver nascent proteins to the catalytic core of the OST complex [12,13]. Overexpression of malectin along with ribophorin I attenuates the secretion of AT^{NHK}, but not that of wild-type AT [10]. Galli et al. showed that malectin associates with misfolded glycoproteins and subsequently interferes with their secretion [6]. Furthermore, decreased secretion of AT^{NHK} by malectin overexpression is restored when cells are cultured in the presence of the proteasome inhibitor MG132 [5]. However, it is not clear whether the association of malectin with ribophorin I is required for the decreased secretion of AT^{NHK} from cells.

To evaluate the coordinated function of malectin and ribophorin I in the ER, we expressed truncated malectin, which could not associate with ribophorin I, in cells. Truncated malectin interacted with AT^{NHK} similarly to wild-type malectin; however, it did not

Abbreviations: Ab, antibody; AT, α1-antitrypsin; AT^{NHK}, α1-antitrypsin variant, null Hong Kong; CNX, calnexin; endo H, endo-β-*N*-acetylglucosaminidase H; ER, endoplasmic reticulum; G2M9, $Glc_2Man_9GlcNAc_2$; G3M9, $Glc_3Man_9GlcNAc_2$; KDEL, Lys-Asp-Glu-Leu; OST, oligosaccharyltransferase; PBS, phosphate-buffered saline.

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http://dx.doi.org/10.1016/j.bbrc.2014.10.102
0006-291X/

inhibit secretion of AT^{NHK}. These results provide evidence of a role for malectin, in association with ribophorin I, in the quality control of misfolded glycoproteins in cells.

2. Materials and methods

2.1. Cells and antibodies

HeLa cells were obtained from the Cell Resource Center for Biochemical Research (Tohoku University, Miyagi, Japan) and maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen, Carlsbad, CA, USA), 100 μg/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine, 25 mM 4-(2-hydroxyethyl)-1-iperazineethanesulfonic acid (HEPES), and 50 mM 2-mercaptoethanol. Cells were grown at 37 °C in a humid atmosphere containing 5% CO_2. Polyclonal and monoclonal anti-FLAG M2 antibodies (Abs), a polyclonal anti-malectin Ab, and an anti-β-actin Ab were purchased from Sigma–Aldrich (St. Lewis, MO, USA). A monoclonal anti-AT Ab and a monoclonal anti-calnexin (CNX) Ab were purchased from Abcam (Cambridge, MA, USA). An anti-ribophorin I Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and an anti-Myc antibody was purified from the culture supernatant of hybridoma clone 9E10, which was purchased from the American Type Culture Collection (Manassas, VA, USA).

2.2. Expression plasmids

p3xFLAG-CMV9-malectin was used to express FLAG-tagged human malectin [10]. cDNA encoding truncated malectin was prepared using a KOD-Plus-Mutagenesis kit (Toyobo, Osaka, Japan) and the primers 5′-GCTGTTGTCCGAGGCATAGG-3′ and 5′-TGATCT AGAGGATCCCGGGTGG-3′. cDNA encoding the luminal domain of malectin followed by the Lys-Asp-Glu-Leu (KDEL) sequence and a stop codon was amplified using the primers 5′-CGCGAATTCA CCCGGGCTC-3′ and 5′-CGCTCTAGATCACAGCTCGTCCTTGCTGTTG TCCGAGGCATAGGG-3′ (EcoRI and XbaI sites are underlined). Amplified DNA was digested with EcoRI and XbaI and inserted between the EcoRI and XbaI sites of p3xFLAG-CMV9. Myc-tagged ribophorin I, ribophorin II, OST48, AT, and AT variants (AT^{NHK} and AT^{NHK}-Q3) were prepared as described previously [5,10]. Ribophorin I-targeting siRNA and control siRNA (Life Technologies Japan, Tokyo, Japan) were used as previously described [10].

2.3. Immunoprecipitation and Western blotting

HeLa cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After culture for the indicated amount of time, cells and the conditioned medium were collected. Cells were washed twice with phosphate-buffered saline (PBS) and lysed in 50 mM Tris–HCl, pH 7.5, containing 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM PMSF, and 1 μg/ml leupeptin by gently rotating for 1 h at 4 °C. Insoluble material was removed by centrifugation at 13,000*g* for 20 min at 4 °C. The supernatant was mixed with anti-FLAG Ab-coupled protein A beads (GenScript, Piscataway, NJ, USA) or anti-FLAG Ab M2-agarose beads (Sigma–Aldrich) with gentle rotation for 18 h at 4 °C. Precipitated beads were washed three times with 50 mM Tris–HCl, pH 7.5, containing 150 mM NaCl, 0.1% (v/v) Triton X-100, 1 mM PMSF, and 1 μg/ml leupeptin. Immunoprecipitates were eluted by boiling at 95 °C for 5 min in 100 mM Tris–HCl, pH 6.8, 4% (w/v) SDS, 10% (v/v) sucrose, and 0.004% (w/v) bromophenol blue. For endo-β-*N*-acetylglucosaminidase H (endo H) treatment, immunoprecipitates were mixed with endo H (Promega, Heidelberg, Germany) for 16 h at 37 °C according to the manufacturer's instructions. Digests were subjected to SDS–PAGE and Western blotting as previously described [14].

2.4. Immunofluorescence microscopy

Transfected HeLa cells were cultured on cover glasses (Matsunami, Osaka, Japan) coated with 1% gelatin. Cells were fixed for 20 min with 4% paraformaldehyde prepared in PBS, and aldehyde groups were quenched with 30 mM glycine prepared in PBS. After permeabilization for 1 h with 0.1% Triton X-100 prepared in PBS, samples were blocked with 5% goat serum (Sigma–Aldrich) for 60 min. Permeabilized cells were incubated with 5 μg/ml anti-CNX Ab and 2 μg/ml polyclonal anti-FLAG Ab for 2 h, and then stained with 5 μg/ml Alexa Fluor 488-labeled anti-mouse IgG and 5 μg/ml Alexa Fluor 532-labeled anti-rabbit IgG for 1 h. Finally, cover glasses were mounted on slides in a drop of Vectashield mounting media (Vector laboratories, Burlingame, CA) containing 4′,6-diamidino-2-phenylindole. Cells were observed under a confocal laser scanning microscope (LSM510; Carl Zeiss, Gottingen, Germany) using LSM image browser software (Carl Zeiss).

3. Results

3.1. Malectin mutants cannot interact with ribophorin I

Malectin forms a complex with ribophorin I, which may enhance the ability of malectin to retain misfolded proteins in the ER [10]. To clarify whether malectin functions as a chaperone in association with ribophorin I in cells, we constructed an expression vector encoding a malectin mutant that could not associate with ribophorin I (Fig. 1). FLAG-tagged malectin and Myc-tagged ribophorin I were co-expressed in HeLa cells and the cell lysate was immunoprecipitated with an anti-FLAG Ab. Wild-type malectin co-precipitated with Myc-tagged ribophorin I, as described previously [10]. By contrast, the truncated malectin mutant, in which the transmembrane domain and short cytoplasmic tail were deleted (MalΔTM in Fig. 1), did not co-precipitate with ribophorin I (Fig. 2A). It is possible that MalΔTM did not localize in the ER, which would abrogate its interaction with ER-localized ribophorin I. Therefore, we analyzed the distribution of FLAG-tagged wild-type malectin and MalΔTM in cells by immunostaining with an anti-FLAG Ab. Wild-type malectin colocalized with CNX, indicating that wild-type malectin localized in the ER (Fig. 3, upper), which is in good agreement with previous reports [3,6]. By contrast, FLAG-tagged MalΔTM did not colocalize with CNX and seemed to be mainly localized in the Golgi (Fig. 3, middle). Therefore, we constructed a plasmid encoding MalΔTM with a KDEL sequence at its C-terminus, termed MalΔTM-KDEL, and expressed it in cells. The KDEL sequence is an ER-retention/retrieval signal of soluble proteins because KDEL receptors are localized in the ER [15]. When

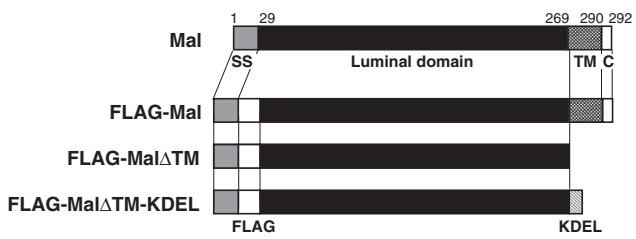

Fig. 1. A schematic illustration of the domain structures of human wild-type malectin, FLAG-tagged malectin, and FLAG-tagged truncated malectin mutants used in this study. SS, signal sequence; TM, transmembrane domain; C, cytoplasmic domain; FLAG, FLAG-tag; KDEL, Lys-Asp-Glu-Leu.

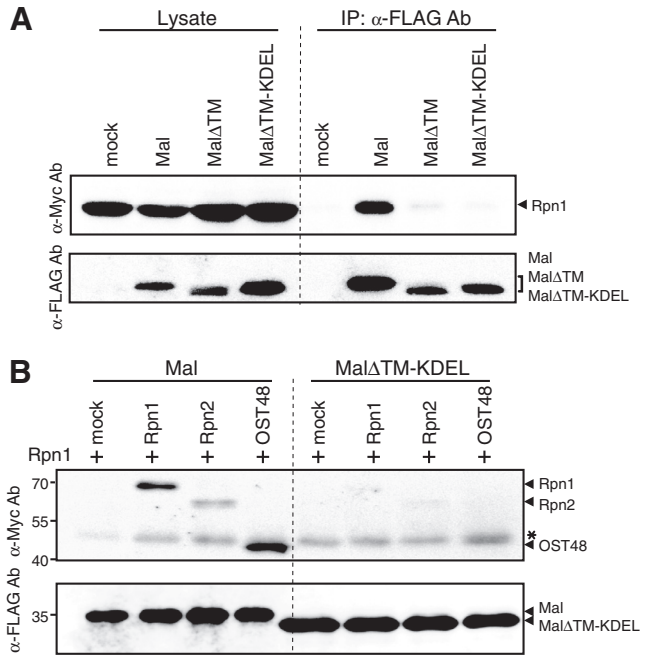

Fig. 2. Transmembrane domain-deleted malectin cannot interact with ribophorin I or other oligosaccharyltransferase subunits. (A) HeLa cells were transfected with expression vectors encoding FLAG-tagged wild-type malectin (Mal), or truncated malectin with or without a Lys-Asp-Glu-Leu sequence at the C-terminus (MalΔTM-KDEL and MalΔTM, respectively), together with Myc-tagged ribophorin I (Rpn1) and cultured for 48 h. Cell lysates were immunoprecipitated with an anti-FLAG antibody (Ab). Cell lysates and immune complexes (IP: αFLAG Ab) were separated by SDS–PAGE under reducing conditions. (B) HeLa cells were transfected with expression vectors encoding Myc-tagged ribophorin I (Rpn1), ribophorin II (Rpn2), or OST48, together with FLAG-tagged malectin (Mal) or MalΔTM-KDEL, and cultured for 48 h. Cell lysates were immunoprecipitated with an anti-FLAG Ab and immune complexes were separated by SDS–PAGE under reducing conditions. The asterisk indicates the heavy chain of the anti-FLAG Ab used for precipitation.

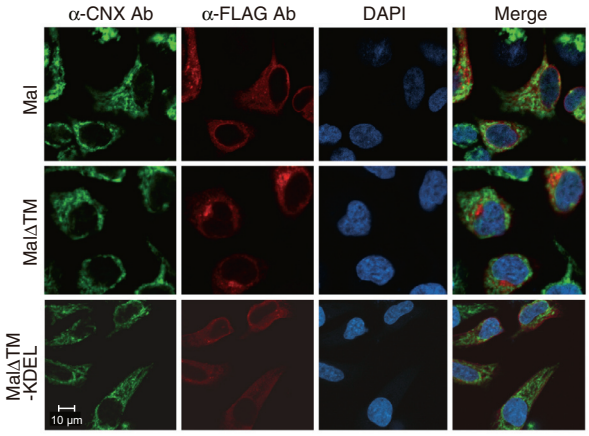

Fig. 3. Subcellular localizations of wild-type malectin and transmembrane domain-deleted malectin with or without a Lys-Asp-Glu-Leu (KDEL) sequence. HeLa cells were transfected with an expression vector encoding FLAG-tagged wild-type malectin (Mal) or truncated malectin with or without a KDEL sequence at the C-terminus (MalΔTM-KDEL and MalΔTM, respectively) and cultured for 48 h. Thereafter, cells were fixed and stained with an anti-calnexin (CNX) antibody (Ab), an anti-FLAG Ab, and 4′,6-diamidino-2-phenylindole (DAPI). Merged images are shown in the right-hand panels.

MalΔTM-KDEL (FLAG-MalΔTM-KDEL in Fig. 1) was expressed in cells, it had a similar distribution to wild-type malectin and colocalized with ER-resident CNX (Fig. 3, lower). These results indicate that MalΔTM-KDEL localizes in the ER.

Next, we expressed FLAG-tagged MalΔTM-KDEL and Myc-tagged ribophorin I in HeLa cells, and the cell lysates were immunoprecipitated with an anti-FLAG Ab. Ribophorin I did not co-precipitate with MalΔTM-KDEL, although MalΔTM-KDEL colocalized with ribophorin I in the ER (Fig. 2A; IP: α-FLAG Ab, MalΔTM-KDEL). This result indicates that the transmembrane and cytoplasmic segments of malectin are required for its interaction with ribophorin I.

Ribophorin I is a subunit of OST, along with various other subunits such as ribophorin II and OST48 [11]. When wild-type malectin was expressed with ribophorin I and either Myc-tagged ribophorin II or Myc-tagged OST48, both ribophorin II and OST48 co-precipitated with wild-type malectin. By contrast, neither ribophorin II nor OST48 co-precipitated with FLAG-tagged MalΔTM-KDEL (Fig. 2B). These data clearly demonstrate that MalΔTM-KDEL localizes in the ER but does not interact with ribophorin I.

3.2. Secretion of misfolded AT^{NHK} is abrogated by expression of wild-type malectin, but not by expression of MalΔTM-KDEL

Next, we examined the effect of the interaction between malectin and ribophorin I on the secretion of AT^{NHK}. When wild-type malectin and AT^{NHK} were co-expressed in HeLa cells, AT^{NHK} dimers were particularly co-precipitated with malectin (Fig. 4B). When FLAG-tagged MalΔTM-KDEL and AT^{NHK} were co-expressed in cells and the cell lysate was immunoprecipitated with an anti-FLAG Ab, AT^{NHK} co-precipitated with MalΔTM-KDEL (Fig. 4B). Endo H treatment of precipitated AT^{NHK} decreased its size, such that it electrophoresed almost at the same size as an *N*-glycosylation-defective AT^{NHK} mutant (AT^{NHK}-Q3) (Fig. 4C). High mannose-type glycans are susceptible to endo H treatment and are found in the ER lumen. Therefore, these data suggest that wild-type malectin and MalΔTM-KDEL interact with AT^{NHK} in the ER.

Secretion of AT^{NHK} was markedly decreased to 33% by expression of wild-type malectin (level in control cells set to 100%). By contrast, AT^{NHK} secretion was not affected (96%) by expression of MalΔTM-KDEL (Fig. 4D). Decreased secretion of AT^{NHK} by wild-type malectin expression was restored when cells were treated with ribophorin I-targeting siRNA (data not shown). However, the level of intracellular AT^{NHK} was not higher in cells expressing wild-type malectin than in cells expressing MalΔTM-KDEL (Fig. 4A), despite AT^{NHK} secretion being blocked in the former cells (Fig. 4B). Our previous data using the proteasome inhibitor MG132 suggest that malectin guides AT^{NHK} to the proteasome-mediated degradation pathway [5]. Taken together, these results strongly indicate that malectin, in association with ribophorin I, efficiently inhibits secretion of AT^{NHK} via guiding it to the degradation pathway.

4. Discussion

In our previous proteomic analysis, malectin co-immunoprecipitated with ribophorin I [10]. We expressed several domain-deleted malectin mutants together with ribophorin I in cells, and cell lysates were immunoprecipitated. Truncated malectin lacking the transmembrane and short cytoplasmic segments did not co-precipitate with ribophorin I (MalΔTM, Fig. 2). This suggests that the interaction of malectin with ribophorin I is mediated by its transmembrane domain and/or cytoplasmic tail. The transmembrane domains of proteins sometimes participate in their associations with specific target molecules such as paired immune receptors, Fc receptor γ, DAP12 [16], EDEM, and CNX [17]. MalΔTM was mainly distributed in the Golgi, in addition to the ER (Fig. 3), which indicates that the association of malectin with an OST subunit, namely, ribophorin I, via its transmembrane domain (and/or cytoplasmic tail) underlies the retention of malectin in the ER.

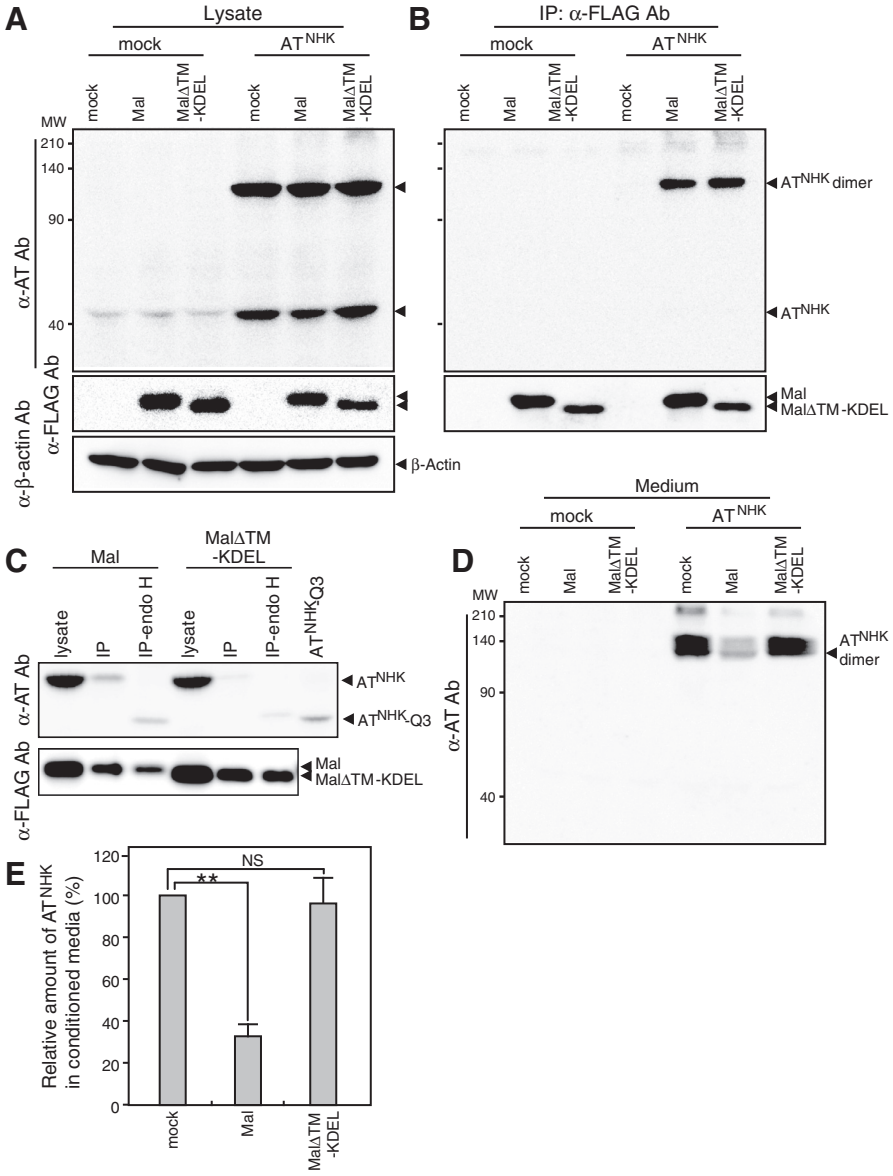

Fig. 4. Effects of wild-type malectin and transmembrane domain-deleted malectin overexpression on secretion of the α1-antitrypsin variant null Hong Kong (AT^{NHK}). HeLa cells were transfected with expression vectors encoding FLAG-tagged malectin (Mal), or transmembrane domain-deleted malectin with a Lys-Asp-Glu-Leu sequence (MalΔTM-KDEL), together with AT^{NHK} and cultured for 24 h. Thereafter, the cell medium was replaced, and cells were cultured for another 48 h, and then cell lysates and medium were collected. (A) To confirm the expression levels of Mal, MalΔTM-KDEL, and AT^{NHK}, cell lysates were separated by SDS-PAGE under non-reducing conditions and immunoblotted with anti-FLAG, anti-α1 antitrypsin (AT), and anti-β-actin antibodies (Abs). (B) To examine the interactions of Mal and MalΔTM-KDEL with AT^{NHK} in cells, immunoprecipitates of cell lysates from (A) (using anti-FLAG Ab) were immunoblotted with anti-FLAG and anti-AT Abs. (C) AT^{NHK} precipitated with Mal or MalΔTM-KDEL (IP) was treated with endo-β-*N*-acetylglucosaminidase H (IP-endo H) and analyzed by Western blotting using an anti-AT Ab under reducing conditions. AT^{NHK}-Q3 is a mutant of AT^{NHK} in which three *N*-glycosylated asparagine residues are mutated to glutamine. (D) To monitor AT^{NHK} secretion from HeLa cells transfected with expression vectors encoding Mal or MalΔTM-KDEL together with AT^{NHK}, conditioned media of the cells were analyzed by immunoblotting with an anti-AT Ab. (E) The data in (D) were quantified by scanning densitometry. Results represent the mean of triplicate experiments, and error bars indicate the standard deviation. $^{**}p < 0.01$; NS, not significant (Student's *t*-test).

We performed immunoprecipitations to investigate whether FLAG-tagged malectin associated with the OST complex or with ribophorin I alone in another compartment. FLAG-tagged malectin co-precipitated with ribophorin II and OST48 in the presence of ribophorin I (Fig. 2B), indicating that FLAG-tagged malectin associated with the OST complex. This is in good agreement with proteomic analysis of canine OST, which showed that a malectin homologue (KIAA0152) associates with OST subunits such as STT3A, DAD1, ribophorin I, ribophorin II, and OST48 [18].

MalΔTM was a powerful tool to examine the association between malectin and ribophorin I in the ER. We added KDEL, an ER-retention/retrieval signal, to the C-terminus of MalΔTM [15]. MalΔTM-KDEL had a similar localization to wild-type malectin (Fig. 3). When we expressed wild-type malectin or MalΔTM-KDEL together with AT^{NHK}, MalΔTM-KDEL and wild-type malectin co-precipitated with AT^{NHK} similarly (Fig. 4B). AT^{NHK} has three *N*-glycans, and all the *N*-glycans on co-precipitated AT^{NHK} were susceptible to endo H digestion (Fig. 4C). Malectin preferentially binds to high mannose-type G2M9 glycans [4,5], indicating that AT^{NHK} binds to malectin and MalΔTM-KDEL in the ER. Wild-type malectin and MalΔTM-KDEL both bound to AT^{NHK} in the ER. The level of AT^{NHK} was similar in cells expressing wild-type malectin and cells expressing MalΔTM-KDEL; however, the secretion of AT^{NHK} differed (Fig. 4D). Expression of wild-type malectin significantly abrogated the secretion of AT^{NHK} (Fig. 4E), as reported previously [5,10]. By contrast, expression of MalΔTM-KDEL did not affect AT^{NHK}

secretion. We previously reported that decreased secretion of AT^{NHK} upon malectin overexpression is restored by treatment with the proteasome inhibitor MG132 [5]. Based on this, wild-type malectin in association with OST might guide AT^{NHK} to the proteasome-mediated degradation pathway, leading to decreased secretion of AT^{NHK}. Furthermore, MalΔTM-KDEL might not be able to guide AT^{NHK} to this pathway owing to its defective interaction with ribophorin I.

In Western blot analysis, AT^{NHK} secreted into the medium was detected as multiple bands, which correspond to the AT^{NHK} dimer and higher molecular weight complexes (Fig. 4D). The intracellular AT^{NHK} dimer showed a similar mobility to AT^{NHK} co-precipitated with malectin, indicating that AT^{NHK} has high mannose-type glycans and localizes in the ER. Prior to secretion, AT^{NHK} is transported via the Golgi apparatus to the cell surface. During this transport, high mannose-type glycans are processed to complex-type glycans, meaning secreted AT^{NHK} migrates at a higher molecular weight [5]. All glycans are complex-type in correctly folded AT, where as misfolded AT^{NHK} may be processed partially, resulting in multiple bands in Western blot analysis.

In summary, we expressed truncated malectin, which could not associate with ribophorin I, in cells. Expression of wild-type malectin, but not of truncated malectin, in the ER attenuated secretion of AT^{NHK}, although wild-type and truncated malectin interacted similarly with AT^{NHK}. We previously showed that proteasome inhibitor treatment restores the decrease in AT^{NHK} secretion upon malectin overexpression. Taken together, these data provide the first evidence that malectin, in association with ribophorin I, is involved in misfolded glycoprotein quality control via guiding misfolded proteins to the degradation pathway.

Author contributions

KY and NM conceived and designed the experiments, KT and S-YQ performed the experiments, KT analyzed the data, KT and S-YQ contributed reagents, materials, and analysis tools, and KY wrote the manuscript.

Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (24390015, 24659026, and 25117710 to KY) and by a Grant-in-Aid from The Naito Foundation (to KY). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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