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

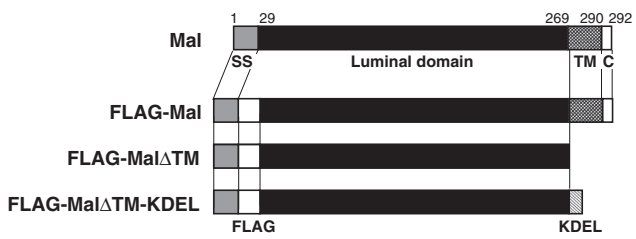

Fig. 1. A schematic illustration of the domain structures of human wild-type malectin, FLAG-tagged malectin, and FLAG-tagged truncated malectin mutants used in this study. SS, signal sequence; TM, transmembrane domain; C, cytoplasmic domain; FLAG, FLAG-tag; KDEL, Lys-Asp-Glu-Leu.