Mnl1p, an α-Mannosidase-like Protein in Yeast *Saccharomyces cerevisiae*, Is Required for Endoplasmic Reticulum-associated Degradation of Glycoproteins*

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The endoplasmic reticulum (ER) has a mechanism to block the exit of misfolded or unassembled proteins from the ER for the downstream organelles in the secretory pathway. Misfolded proteins retained in the ER are subjected to proteasome-dependent degradation in the cytosol when they cannot achieve correct folding and/or assembly within an appropriate time window. Although specific mannos trimming of the protein-bound oligosaccharide is essential for the degradation of misfolded glycoproteins, the precise mechanism for this recognition remains obscure. Here we report a new α-mannosidase-like protein, Mnl1p (mannosidase-like protein), in the yeast ER. Mnl1p is unlikely to exhibit α,1,2-mannosidase activity, because it lacks cysteine residues that are essential for α,1,2-mannosidase. However deletion of the *MNL1* gene causes retardation of the degradation of misfolded carboxypeptidase Y, but not of the unglycosylated mutant form of the yeast α-mating pheromone. Possible roles of Mnl1p in the degradation and in the ER-retention of misfolded glycoproteins are discussed.

The endoplasmic reticulum (ER) is the site of entry for proteins destined for the secretory pathway. The ER provides an optimized environment for correct maturation, including correct folding, oligomerization, N- and O-linked glycosylation, and disulfide bond formation of proteins imported into the ER (1, 2). Several components, including molecular chaperones and folding enzymes that mediate these processes, have been identified in the ER. Shortage or defects of these components as well as mutations in secretory proteins and environmental stress tend to result in failure and therefore misfolding of secretory proteins. The misfolded proteins are retained in the ER and are subjected to retrieval for correction maturation with the aid of molecular chaperones and folding enzymes. However if this process is not successful, prolonged retention of the misfolded proteins in the ER eventually leads to their degradation (3, 4). The ER-associated degradation (ERAD) requires the retrotranslocation of misfolded proteins through the Sec61 channel from the ER lumen to the cytosol and subsequent degradation by the 26 S proteasome located in the cytosol (5–9).

For ERAD of aberrant proteins, the question of how proteins that are misfolded and to be degraded are identified and targeted for the retrotranslocation system has not been resolved. In yeast *Saccharomyces cerevisiae*, slow removal of α,1,2-mannose from the middle branch of the protein-bound oligosaccharide, or formation of ManαGlcNAc2, has been shown to be critical for the degradation of misfolded carboxypeptidase Y (CPY), a vacuolar glycoprotein (10). This is consistent with the reports that inhibition of α-mannosidase trimming stabilizes specific misfolded glycoproteins in the mammalian ER (11, 12). On the basis of these observations, it has been proposed that the trimming of the glycoprotein-bound oligosaccharide may well function as the biological timer for the onset of the glycoprotein degradation that prevents permanent residence of misfolded glycoproteins in the ER (2). This naturally suggests the presence of a ManαGlcNAc2-binding lectin involved in ERAD of misfolded glycoproteins, which remains to be identified (10).

In the present study, we identified a new α-mannosidase-like protein, Mnl1p (mannosidase-like protein), in the yeast ER. Although it shows some homology with Masn1p, yeast α,1,2-mannosidase, Mnl1p is unlikely to exhibit the α,1,2-mannosidase activity, because it lacks cysteine residues that are essential for the α,1,2-mannosidase activity (13). However deletion of the *MNL1* gene resulted in retardation of the degradation of misfolded CPY, but not of the unglycosylated mutant form of the yeast α-mating pheromone (ΔGppαF). Possible roles of Mnl1p in ERAD and the ER retention of misfolded glycoproteins will be discussed.

**Experimental Procedures**

Plasmids, Strains, and Culturing Conditions—Standard recombinant techniques were employed using an Escherichia coli strain TG1 (supE hsdSΔ5 thiΔ [lac-proAB] F’ [tra36 proAB’ lacIq lacZAM15]). Yeast strains used in this study were SEY6210 (MATα ura3 leu2 trpl his3 lys2 suc2) (14) and KYSC1 (MATα trpl-1 ura3 leu2 trpl his3 lys2 suc2).2 Yeast strain BJ3505 (MATα pep4::HIS3 prb1 lys2 trpl ural3 gal2 can1) was used for preparation of the cytosol (9). Cells were grown in YPD medium containing 1% yeast extract, 2% polypeptone, and 2% glucose. A sulfate-free synthetic minimal medium (15) was used for metabolic labeling of yeast cells. The gene for the C-terminally HA (influenza hemagglutinin) epitope-tagged version of Mnl1p was cloned by PCR from the yeast genomic DNA. The amplified DNA was sub-

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1 The abbreviations used are: ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; CPY, carboxypeptidase Y; CPY*, a mutated version of carboxypeptidase Y; pαF, pro-α factor; pαpF, prepro-α factor; ΔGppαF, an unglycosylated mutant form of prepro-α factor; HA, hemagglutinin; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

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cloned into a yeast multicopy plasmid pYO326 (16) to generate pKNM1, which expresses the Mnl1p-HA fusion protein. A null allele of MNL1 was constructed by the PCR-based gene disruption using the HIS3 gene of Candida glabrata (a gift from Dr. Satoshi Harashima) as described previously (17, 18). The ∆mnl1 strains derived from SEY6210 and KYSC1 were named SNY1079 and SNY1080, respectively.

ERAD Assays—Metabolic labeling of yeast cells with [35S]S-label (ICN) and preparation of cell extracts were performed as described previously (19). Immunoprecipitation was performed as described by Nishikawa et al. (20). Yeast microsome fractions were prepared from wild-type cells and ∆mnl1 cells (9) and the yeast cytosol S100 fraction from yeast BJ3505 cells. 35S-Labeled mutant prepro-α factor lacking the three consensus glycosylation sites (∆Gpp(F)) (9) was synthesized in vitro using a yeast cell-free translation. The in vitro ERAD assay was performed as described previously (9).

Fluorescence Microscopy—Immunofluorescent staining of yeast cells was performed as described previously (20) with minor modifications. Incubation with the primary and the secondary antibodies was performed as follows: 1) 1:250 dilution of the rabbit anti-BiP polyclonal antibodies (21) and 1:100 dilution of the rhodamine-conjugated goat anti-rabbit IgG antibody, 1:100 dilution of the fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody.

RESULTS

Mnl1p Is an ER Protein—MNL1 (YHR204W) on yeast chromosome VIII encodes an α-mannosidase-like protein, which is 796 amino acids long, with a calculated molecular weight of 92,206. The predicted amino acid sequence of Mnl1p shows 25% identity with Mns1p, yeast α,1,2-mannosidase that converts glycosyl residues from ManαGlcNAc2 to ManαGlcNAc2 in the ER (Fig. 1A). Mns1p is a type II ER transmembrane glycoprotein with its N-terminal hydrophobic segment functioning as a signal-anchor sequence. The question of whether the segment of hydrophobic residues 1–21 (Fig. 1A) functions as a signal-anchor sequence to make Mnl1p a type-II membrane protein should await future studies. The amino acid residues involved in the substrate binding, Ca2+ binding, and catalytic activity in Mns1p (22) are conserved in Mnl1p except for the two cysteine residues (Cys440 and Cys435 in Mns1p) that form a disulfide bridge and are essential for the α-mannosidase activity (13, 22). The lack of the essential cysteine residues in Mnl1p suggests that Mnl1p is unlikely to have the α-mannosidase activity. The C-terminal part of Mnl1p shows no homology with any proteins deposited in the database.

To assess the functions of Mnl1p, we first analyzed its subcellular location by indirect immunofluorescent microscopy. Mnl1p was tagged at the C terminus with the HA epitope for recognition by the monoclonal antibody 16B12. Cells that express the HA-tagged version of Mnl1p from a multicopy plasmid were fixed, permethylized, and subjected to staining with the anti-HA antibody. Staining with the anti-HA antibody showed perinuclear staining with tubular extensions in the cytoplasm (Fig. 1B, panel b). This staining is typical for yeast ER proteins, and nearly identical staining was observed with the anti-BiP antibodies (Fig. 1B, panel a). Cells that contain a multicopy plasmid without expression of the HA-tagged Mnl1p did not show such staining (data not shown). These results indicate that Mnl1p resides exclusively in the ER.

Deletion of MNL1 Causes a Defect in ERAD of CPY*—The trimming of N-linked oligosaccharides is critical for proteasome-dependent ERAD of misfolded glycoproteins. For example in yeast, removal of α,1,2-glucosyl by glucosidase I and glucosidase II and that of α,1,2-mannosyl by α,1,2-mannosidase, Mns1p, to yield ManαGlcNAc2 are essential for the efficient degradation of CPY*, mutated and therefore misfolded carboxypeptidase Y (10, 23). Although Mnl1p is not expected to have the α-mannosidase activity, its homology with Mns1p raises the possibility that Mnl1p is involved in the recognition of specific oligosaccharide structures and/or ERAD of misfolded glycoproteins. We thus analyzed the effects of deletion of the MNL1 gene on ERAD of a model misfolded protein, CPY*, in vitro. The Δmnl1 null mutant strain did not show any detectable growth phenotypes as reported previously (24).

CPY is known to receive distinct posttranslational modifications in different cellular compartments on its transport pathway to the vacuole (25, 26). On translocation across the ER membrane, a prepro form of CPY (prepro-CPY) receives proteolytic cleavage of the signal sequence and addition of four N-linked oligosaccharide chains to generate a 67-kDa ER form, p1CPY. In the Golgi complex, p1CPY is converted to a 69-kDa form, p2CPY, by addition of mannose to the N-linked oligosaccharides.
The deletion of MNL1 causes retardation of the degradation of CPY*. A, wild-type cells (KYSC1, wt) and mnl1 cells (SNY1080, mnl1) were pulse-labeled with 35S-containing amino acids for 5 min at 30 °C and chased for the indicated times. Cell extracts were prepared from the labeled cells and subjected to immunoprecipitation with the anti-CPY antibodies. The immunoprecipitated proteins were incubated with (+) or without (−) 0.17 unit/ml endoglycosidase H (EndoH) at 37 °C for 22 h and analyzed by SDS-PAGE and radioimaging with a Storm 860 image analyzer (Molecular Dynamics). p1, p1CPY; p1', p1CPY/ΔGp1, a deglycosylated form of p1CPY* and p1CPY*. B, quantification of the results shown in A (− EndoH). The amount of CPY* at 0-min chase is set to 100%. Data represent the average of the results of two independent assays.

We also noticed that p1CPY* in Δmnl1 null mutant cells was converted to a higher molecular weight form, which was designated as p1' CPY* and was observed as a smear on the SDS-PAGE gel, during the chase period (Fig. 2A). When p1' CPY* was treated with endoglycosidase H, it was converted to a deglycosylated form with the same electrophoretic mobility as that for p1CPY* (Fig. 2A). This means that p1' CPY* arose from glycosyl modifications of p1CPY*.

A possible explanation for the glycosyl modifications of p1CPY* in Δmnl1 cells is that a part of misfolded p1CPY* escaped the ER and received addition of α-1→6- and/or α-1→3-linked mannose to core oligosaccharides in the Golgi complex (25, 28). To test if p1' CPY* had been modified by the Golgi enzymes, reactivity of p1' CPY* to the anti-α-1→6 mannose and the anti-α-1→3 mannose antibodies was examined. Wild-type cells expressing CPY or CPY* and Δmnl1 cells expressing CPY* were metabolically labeled for 5 min and chased, and the CPY or CPY* species were immunoprecipitated with the anti-CPY antibodies from the cell extracts. The immunoprecipitated proteins were subjected to the second-round immunoprecipitation with the antibodies against CPY, α1→6 mannose, or α1→3 mannose. As shown in Fig. 4, p1' CPY* in Δmnl1 cells was immunoprecipitated with the anti-α-1→6 mannose antibodies but not with the anti-α-1→3 mannose antibodies (Fig. 3, lanes 14 and 15), whereas p1CPY* was not recognized by these antibodies (Fig. 3, lane 13). p1CPY* in wild-type cells, which was retained in the ER and therefore not glycosylated by the Golgi enzymes, was not precipitated with the anti-α-1→6 mannose or anti-α-1→3 mannose antibodies (Fig. 3, lanes 9 and 10).

As a control, it was confirmed that mCPY in wild-type cells, which had received carbohydrate modification in the Golgi complex, were precipitated with these antibodies (Fig. 3, lanes 4 and 5). These results indicate that p1' CPY* was modified by the early Golgi enzymes in Δmnl1 cells and that it is the intermediate form of the mannose addition in the Golgi complex.

In other words, deletion of the MNL1 gene allowed exit of a fraction of p1CPY*, which is strictly retained in the ER in wild-type cells, from the ER for at least the early Golgi cisternae (29).

Mnl1p Is Not Involved in ERAD of ∆GpαF—We next asked if Mnl1p is involved in ERAD of misfolded but unglycosylated proteins. For this purpose, we performed in vitro export/degradation assays with an unglycosylated mutant form (∆GpαF) of the yeast α-mating pheromone, pro-α-factor (paF), as a substrate (8, 9, 30, 31). A radiolabeled precursor form of ∆GpαF (∆GpαFa) was translocated into microsomes prepared from the Δmnl1 strain or from the isogenic wild-type strain. Upon signal sequence cleavage, the resulting product, ∆GpαF, becomes an ERAD substrate when the washed vesicles are incubated in the presence of the cytosol and ATP (9). When this assay was performed using microsomes prepared from Δmnl1 cells, degradation of ∆GpαF was as efficient as that with wild-type microsomes at 30 °C (Fig. 4A).

In parallel with these in vitro degradation assays, we followed the degradation of paF in vivo by pulse-chase experiments. The previous study showed that tunicamycin, an inhibitor of the N-linked glycosylation in the ER, causes formation of the unglycosylated and therefore misfolded paF precursor (32). The unglycosylated paF is rapidly degraded in a Sec61p-dependent manner, suggesting that it is a substrate for ERAD in vivo (53); Fig. 4C). Wild-type cells and Δmnl1 cells were metabolically labeled for 5 min in the presence of tunicamycin, and paF was recovered as pellets by immunoprecipitation by the anti-α-factor antibodies. As shown in Fig. 4B, unglycosylated paF was rapidly degraded in Δmnl1 cells with nearly the same kinetics as that for wild-type cells. Taken together, these results suggest that Mnl1p is not involved in ERAD of ∆GpαF.
ERAD of misfolded proteins with the translated protein, suggesting that Mnl1p plays important roles in the cysteine residues strongly suggests that Mnl1p does not have then what is the role of Mnl1p in ERAD? The lack of two C
radiolabeled crosomes prepared from wild-type (SEY6210, wt) and ∆mnl1(SNY1079, Δmnl1) cells at 20 °C for 50 min. Microsomes containing ∆GppF were collected by centrifugation, washed once with buffer 88 (20 mM HEPES-KOH, pH 6.8, 250 mM sorbitol, 150 mM KOAc, 5 mM Mg(OAc)2 (9), and resuspended in buffer 88. The microsomes were further incubated in buffer 88 containing an ATP-regenerating system (1 mM ATP, 50 μM GDP-mannose, 40 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase) and the yeast cytosol (5 mg of protein/ml) at 30 °C. At the indicated time points, proteins were precipitated with trichloroacetic acid and subjected to SDS-PAGE in the presence of 4 M urea. The upper band is ∆GppF, which is bound to the cytosolic surface of the microsomes and escaped ERAD. B, quantification of the results shown in A. The amount of ∆GppF at 0-min incubation was set to 100%. Data represent the average of the results of two independent assays. C, in vivo ERAD assays for radiolabeled pF. Wild-type (SEY6210, wt) cells and ∆mnl1(SNY1079, Δmnl1) cells were pulse-labeled with 35S-containing amino acids for 5 min at 30 °C in the presence of 10 μg/ml of tunicamycin, chased for the indicated times, and subjected to immunoprecipitation with the anti-pF antibodies. D, quantification of the results shown in C. The amount of ∆GppF at 0-min chase was set to 100%. Data represent the average of the results of two independent assays. The fate of CPY* was followed in detail when its degradation was impaired by the deletion of the MNL1 gene. Whereas CPY* was partly retained as p1CPY*, the ER form to be degraded, a part of p1CPY* molecules were converted to the early-Golgi form, p1 CPY*, containing α1–6 mannose. This indicates that at least some p1CPY* was released from the ER to the Golgi complex. However, since α1–3 mannose was not attached to p1 CPY* even after the prolonged chase, CPY* most likely undergoes recycling through the early-Golgi cisternae into the ER. Deletion of DER1 encoding an ER protein involved in ERAD also leads to escape of some CPY* from the ER for the early-Golgi compartment (33). Therefore ER-resident proteins that are involved in ERAD are also responsible for retaining their substrates in the ER probably by their affinity for misfolded proteins.

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