

Role of Sec61p in the ER-associated degradation of short-lived transmembrane proteins

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Misfolded proteins in the endoplasmic reticulum (ER) are identified and degraded by the ER-associated degradation pathway (ERAD), a component of ER quality control. In ERAD, misfolded proteins are removed from the ER by retrotranslocation into the cytosol where they are degraded by the ubiquitin-proteasome system. The identity of the specific protein components responsible for retrotranslocation remains controversial, with the potential candidates being Sec61p, Der1p, and Doa10. We show that the cytoplasmic N-terminal

domain of a short-lived transmembrane ERAD substrate is exposed to the lumen of the ER during the degradation process. The addition of N-linked glycan to the N terminus of the substrate is prevented by mutation of a specific cysteine residue of Sec61p, as well as a specific cysteine residue of the substrate protein. We show that the substrate protein forms a disulfide-linked complex to Sec61p, suggesting that at least part of the retrotranslocation process involves Sec61p.

Introduction

The ER provides an environment conducive for the folding and assembly of newly synthesized secretory proteins. To prevent the premature exit of improperly folded proteins from the ER, cells have evolved quality control mechanisms to actively monitor the folding state of proteins (Brodsky and McCracken, 1999; Ellgaard and Helenius, 2003). Proteins that irreversibly misfold are recognized by this quality control system and targeted for destruction through a process termed ER-associated degradation (ERAD; Hampton, 2002). Although initial studies of ERAD implied the action of unidentified ER-localized proteases (Finger et al., 1993), subsequent work clearly defined roles for the cytoplasmically localized enzymes of the ubiquitin pathway and the 26S proteasome, providing the first indication that misfolded proteins must be retrotranslocated back across the membrane of the ER (Jensen et al., 1995; Ward et al., 1995). From a mechanistic standpoint, these results established the need for an ER-localized protein-conducting channel to direct the flow of misfolded protein export from the ER.

Circumstantial evidence suggested that Sec61p, the main component of the protein-conducting channel for translocation

into the ER, participates in retrotranslocation from the ER (Wiertz et al., 1996; Pilon et al., 1997; Plemper et al. 1997, 1998). In mammalian cells, Sec61 α can be coimmunoprecipitated with class I heavy chains that are targeted for ERAD by the human cytomegalovirus-encoded glycoprotein US2 (Wiertz et al., 1996). Subsequently, genetic and biochemical analysis of Sec61p mutants uncovered alleles more prone to defects in protein retrotranslocation than translocation (Pilon et al., 1997). Certain ERAD substrates are stabilized in a partially translocation-defective mutant, *sec61-2* (Plemper et al., 1997, 1998). In an independent approach, Schmitz et al. (2000) showed that blocking Sec61 channels with translation-arrested ribosomes prevented exit of cholera toxin from the ER. However, when the crystallographic structure of SecY/E, an archaeal orthologue of Sec61p, revealed a strict upper size limit for the pore diameter, it was difficult to conceive how such a small pore could accommodate the larger retrotranslocation substrates (Tirosh et al., 2003; Van den Berg et al., 2004). Nonetheless, for certain proteins that are subject to ERAD before the completion of translocation, new evidence suggests a direct participation of Sec61p in the ERAD process (Oyadomari et al., 2006). For the majority of proteins subject to ERAD after membrane assembly, the evidence favors one or more distinct retrotranslocation channels.

Another candidate channel protein, Derlin-1, was identified by virtue of its association with the human cytomegalovirus-encoded glycoprotein US11 in the process of retrotranslocation and degradation of class I heavy chains (Lilley and Ploegh, 2004;

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Abbreviations used in this paper: α f, α -factor; CPY, carboxypeptidase Y; ERAD, ER-associated degradation.

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Ye et al., 2004). The interaction of Derlin-1 with glycosylated class I heavy chains before retrotranslocation and its subsequent association with deglycosylated heavy chains when cells are treated with proteasome inhibitors suggest that it is positioned to interact with substrates before and immediately after they are retrotranslocated (Lilley and Ploegh, 2005). The role of the yeast Derlin-1 homologue Der1p in ERAD is poorly defined but is known to be required for the efficient degradation of misfolded luminal ER proteins (Knop et al., 1996; Hitt and Wolf, 2004). However, numerous ERAD substrates are degraded independent of Der1p (Hill and Cooper, 2000; Vashist and Ng, 2004). A subset of these Der1p-independent substrates is also independent of Sec61p but requires Doa10, an ER-localized E3 ubiquitin ligase. Thus, Doa10 may represent a distinct retrotranslocation channel in the ER (Kreft et al., 2006).

In this paper, we describe our discovery and characterization of a disulfide-linked intermediate complex formed between a short-lived transmembrane ERAD substrate and Sec61p. This complex may accomplish at least an initial stage in the retrotranslocation of unstable membrane proteins.

Results

Cysteine mutants of Sec61p are protein retrotranslocation proficient

To test for defects in the ER export of misfolded membrane proteins, we followed the degradation kinetics of the short-lived transmembrane substrate Deg1:Sec62^{ProtA}. The Deg1 sequence appended to the N terminus of Sec62 (Deshaies and Schekman, 1989) diverts the hybrid protein to the ERAD pathway (Mayer et al., 1998). In wild-type cells, Deg1:Sec62^{ProtA} was rapidly degraded with $t_{1/2}$ of ~13 min (Fig. 1, A and B). Previously, we reported the effect of a substitution at cysteine 150 of the translocation channel (*sec61-32*; C150Y) on the ERAD of an unglycosylated form of α -factor (α f) precursor (Pilon et al., 1997). To explore the role of each of the cysteine residues of Sec61p in the ERAD of Deg1:Sec62^{ProtA}, we created strains in which one or more of these residues was mutated on a CEN plasmid as the sole copy of *SEC61*. The rate of substrate degradation was accelerated nearly twofold in a strain harboring Sec61p C373I ($t_{1/2}$ = 7–8 min; Fig. 1, A and B), whereas Sec61p C121G or C150V had no influence on the degradation rate ($t_{1/2}$ = 13 min; Fig. 1, A and B). As a control, we also monitored the rate of substrate degradation in a yeast strain harboring the retrotranslocation (and translocation)-defective *sec61-2* allele (Plemper et al., 1997). In this background the rate of substrate degradation was not altered, which is consistent with previous reports on the degradation of a misfolded membrane substrate containing a misfolded cytosolic domain (Ste6*; Huyer et al., 2004; see also Discussion). In wild-type and mutant strains, except C373I, we observed two electrophoretically distinct species of substrate. The more rapidly migrating species predominated in C373I (Fig. 1 A).

We evaluated the degradation of another unstable ERAD substrate, CPY*, in the wild-type and Sec61p cysteine mutant strains. In wild-type cells, CPY* was degraded with a $t_{1/2}$ of ~25 min (Fig. 1, C and D). Mutations at position 121 or 150 of Sec61p retained wild-type degradation kinetics, whereas muta-

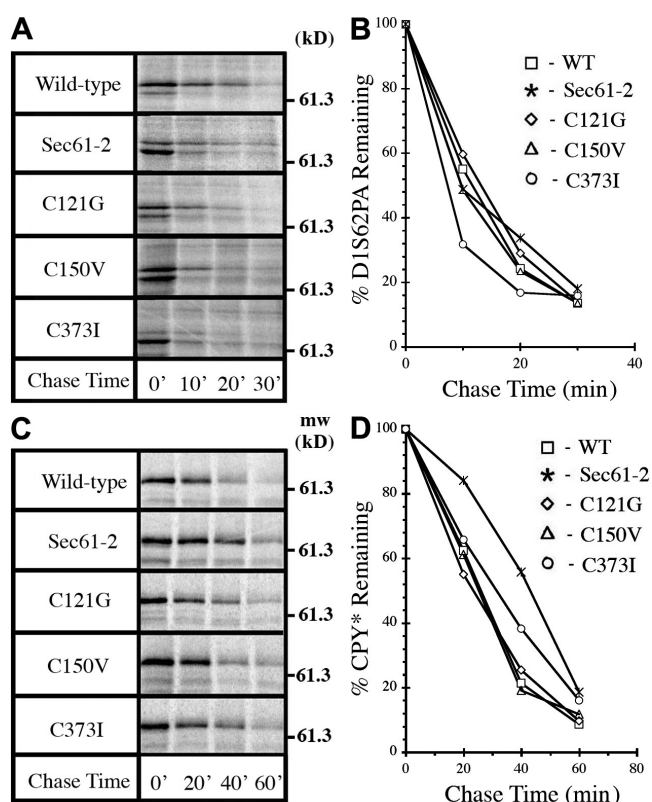


Figure 1. Sec61p cysteine mutants are proficient for ER protein export. (A) Wild-type and Sec61p mutant cells expressing the transmembrane ERAD substrate Deg1:Sec62^{ProtA} were pulse labeled for 5 min and chased for the indicated times. Deg1:Sec62^{ProtA} was immunoprecipitated, resolved by SDS-PAGE, and visualized by autoradiography. (B) Deg1:Sec62^{ProtA} decay was quantified by phosphorimager analysis and plotted as the sum of decay for the two species over time. The data in the plots reflect the mean of three independent experiments. Standard deviations for each time point were typically in the range of 4–8% but are not shown in the plots for ease of viewing. (C) Wild-type and Sec61p mutant cells expressing CPY* were pulse labeled for 15 min and chased for the indicated times. CPY* was immunoprecipitated from detergent-solubilized lysates, resolved by SDS-PAGE, and visualized by autoradiography. (D) CPY* decay was quantified and plotted as described for Deg1:Sec62^{ProtA} in B.

tion of residue 373 resulted in a slight, but reproducible, stabilization of CPY* ($t_{1/2}$ = 28–30 min; Fig. 1, C and D). In contrast, CPY* degradation was delayed around twofold in the *sec61-2* mutant, which is consistent with previous reports for this mutant (Plemper et al., 1997). In all experiments, we observed a single electrophoretic species of CPY* unlike the behavior of Deg1:Sec62^{ProtA}.

Deg1:Sec62^{ProtA} is N glycosylated

The effect of Sec61p C373I on the production of one of two electrophoretic species of Deg1:Sec62^{ProtA} led us to consider the possibility that a covalent modification accompanies the ERAD of this substrate. Although the N and C termini of Sec62p are exposed to the cytosol, the N-terminal Deg signal may influence the disposition of Sec62 during ERAD. Thus, one possibility was that cryptic N-glycosylation sites in the N terminus of Sec62p may become exposed to the ER lumen during ERAD. We found that Deg1:Sec62^{ProtA} was sensitive to endoglycosidase H,

which is consistent with a previous report for Deg1:Sec62^{ProtA} (Fig. 2 A; Kim et al., 2006).

The primary sequence of Deg1:Sec62^{ProtA} possesses two consensus N-glycosylation sites, with the reactive asparagines positioned at residues 90 and 153 (Fig. 2 A). Site-directed mutagenesis of Asn90 resulted in no change in the two Deg-Sec62 species (Fig. 2 B, compare lanes 1 and 2). In contrast, the lower mobility glycosylated form of Deg1:Sec62^{ProtA} disappeared in an Asn153 mutant (Fig. 2 B, compare lanes 1 and 5), indicating that Asn153, but not Asn90, was N glycosylated.

Sec62 is a double pass unglycosylated transmembrane protein with the N and C termini facing the cytosol (Deshaies and Schekman, 1989). Conceivably, the attachment of Deg1 to the N terminus and ProtA to the C terminus altered the topology of the hybrid protein. However, we found that the Deg1:Sec62^{ProtA} hybrid complemented a *sec62* mutant for growth (unpublished data). In addition, we were unable to detect substrate protein with antibodies directed against either the N- or C-terminal tail of the hybrid protein after treatment of intact ER membranes with protease (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200804053/DC1>), which ruled out a potential heterogeneous topological population of the hybrid. Thus, glycosylation of Asn153 suggested that the N terminus became exposed to the ER lumen at some point during the course of synthesis or degradation of the hybrid protein. In an attempt to further define regions of the N- or C-terminal tails that were exposed to the lumen, we introduced additional acceptor sequences throughout the polypeptide chain and evaluated the glycosylation state of these mutants. The introduction of an additional acceptor site within 30 aa of Asn153 led to the production of a doubly glycosylated substrate (Fig. 2 B, lanes 4 and 6). However, addition of sites at positions further removed from this site, as well as multiple sites within the C-terminal tail, maintained a singly glycosylated profile characteristic of the wild-type substrate (Fig. 2 B, lanes 3 and 7–10). These results indicate that a minimal region of ~50 aa within the N-terminal cytosolic tail becomes exposed to the lumen of the ER.

Cysteine mutants of Sec61p are protein translocation proficient

A protein translocation defect could account for the effect of the Sec61p C373I mutant on ERAD of Deg1:Sec62^{ProtA}. We examined the protein import functionality of the Sec61p cysteine mutants by pulse labeling and immunoprecipitation of model secretory proteins (Fig. 3; Pilon et al., 1997, 1998).

The yeast mating pheromone α f is synthesized in the cytosol as a prepro precursor form (pp α f). Upon translocation into the ER, pp α f becomes signal cleaved (p α f) and modified by the addition of three N-linked glycans (3gp α f). In Sec61p wild-type and cysteine mutant cells, 3gp α f was not observed because transport and proteolytic maturation were too rapid (Fig. 3, top, lanes 1, 7, 9, and 11). Pretreatment of cells with tunicamycin, an inhibitor of N-linked glycosylation, led to the accumulation of signal-cleaved p α f in the ER (Fig. 3, top, lanes 2, 4, 8, 10, and 12). The translocation mutants *sec61-2* and *sec61-32* accumulated pp α f, whereas C121G, C150V, and C373I did not (Fig. 3, top, lanes 5 and 6). The difference between *sec61-32* (C150Y)

and C150V may be attributable to the nature of the substitution. C150Y also produced a cold-sensitive defect, whereas C150V did not. However, other more substantial changes at C121 and C373 led to growth defects (unpublished data).

The vacuolar protease carboxypeptidase Y (CPY) is signal cleaved upon translocation into the ER (proCPY) and modified by N-linked glycosylation (p1 CPY). During its transit through the Golgi complex, outer chain mannose residues are added (p2CPY). Upon arrival at the vacuole, p2CPY is proteolytically cleaved to produce the mature form (mCPY). The p1, p2, and mature forms of CPY were detected in wild-type and C121G, C150V, and C373I mutants (Fig. 3, middle, lanes 1, 7, 9, and 11). Thus, these mutations did not affect proCPY translocation, whereas *sec61-32* did (Fig. 3, lane 5). The precursor form of the luminal chaperone Kar2p was efficiently translocated and processed to the mature form in Sec61p wild-type and C121G, C150V, and C373I mutant backgrounds (Fig. 3, bottom, lanes 1, 7, 9, and 11).

Glycosylation of Deg1:Sec62^{ProtA} occurs during degradation

We considered the possibility that the Deg1 sequence served as a surrogate signal peptide causing the N terminus of Sec62 to be translocated and exposed to the oligosaccharyltransferase in the ER lumen. To test this, we constructed a Deg1:Sec62 deletion hybrid containing residues of Sec62 up to the first transmembrane segment (Deg1:Sec62 1–149). Cells were radiolabeled in the absence and presence of tunicamycin and flag-tagged hybrid proteins were immunoprecipitated from lysates. Unlike the Deg1:Sec62 full-length hybrid, Deg1:Sec62 1–149 accumulated in an unglycosylated form (Fig. 2 C). As a control, we fused the prepro region of α f, which contains a signal peptide and a pro peptide with three N-glycosylation sites, to the N-terminal cytoplasmic tail of Sec62 (proFactor:Sec62 1–149). This hybrid produced multiple glycosylated tunicamycin-sensitive species that accumulated in cells (lysate) and secreted glycosylated forms, which were sensitive to Endo H, into the culture fluid (supernatant; Fig. 2 C). We conclude that the N terminus of Sec62 can be translocated and glycosylated as directed by a standard signal sequence but that the Deg1 sequence does not act as a typical secretory signal.

The lack of a protein translocation defect for the Sec61p cysteine 373 mutant suggested that glycosylation of Deg1:Sec62^{ProtA} may occur during ERAD. We next measured the rate of formation of the glycosylated substrate in backgrounds that stabilized substrate yet did not affect its import. Thus, if glycosylation occurred during the degradation process, the formation of the glycosylated species may be delayed in mutants defective in ERAD. After a short pulse label of 2 min, the glycosylated and unglycosylated forms were labeled in roughly equal amounts in wild-type cells (glycosylated/unglycosylated = 1; Fig. 4, A and B). This ratio increased with labeling time and approached a ratio of four at 8 min of pulse label (Fig. 4, A and B). The ratio was decreased in a mutant lacking the E2 ubiquitin-conjugating enzyme, Ubc7p, with ratios of 0.5 and 2.7 at 2 and 8 min, respectively (Fig. 4, A and B). Although the delay was significant, it was clearly not complete. This observation is likely caused by the

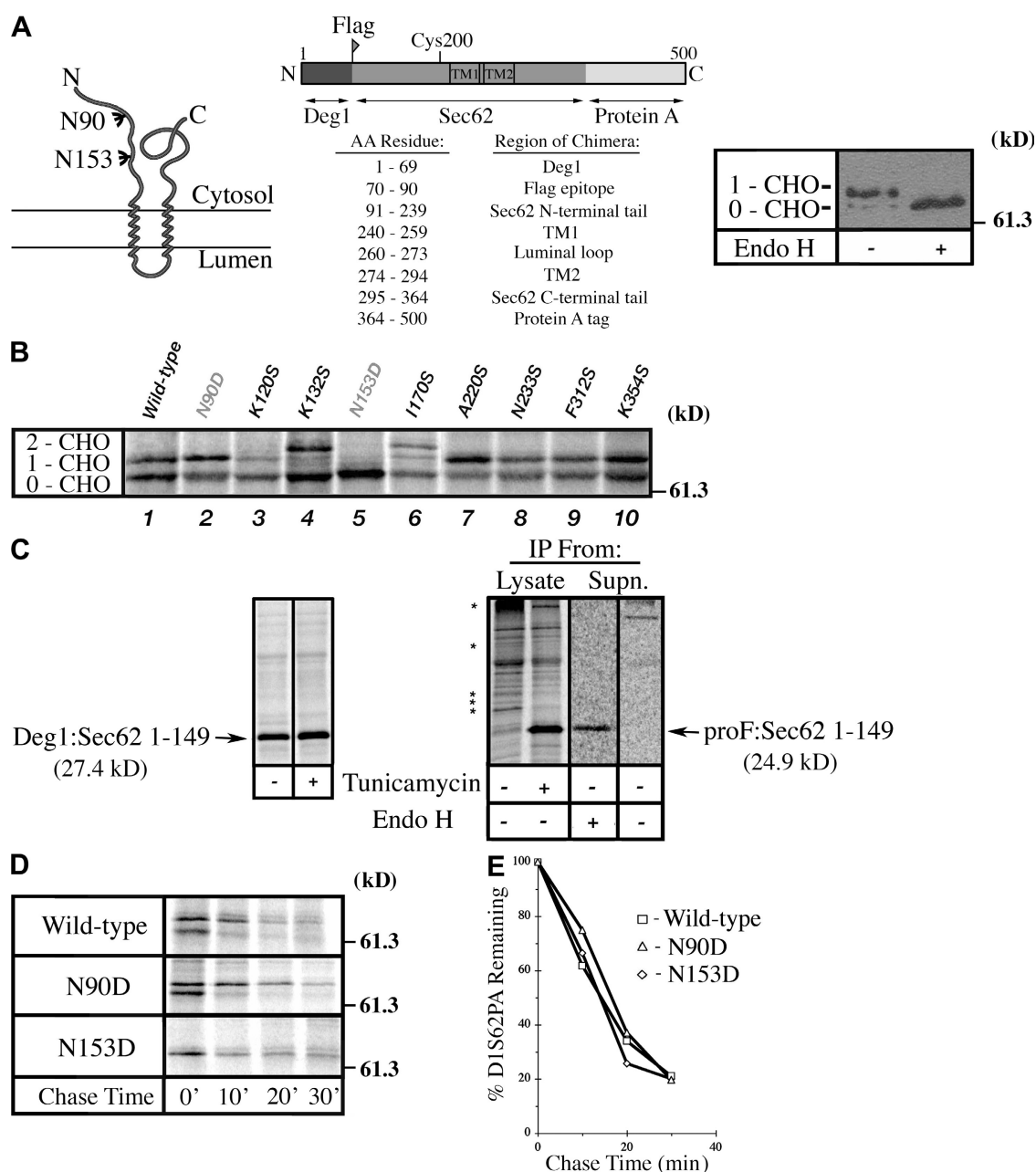


Figure 2. Deg1:Sec62^{ProA} is modified by N-linked glycosylation. (A, left) Schematic representation of Deg1:Sec62^{ProA} and its presumed membrane topology. The relative positions of the two cryptic N-glycan acceptor sites [Asn90 and Asn153] are depicted. (A, middle) Schematic representation of Deg1:Sec62^{ProA} highlighting the amino acid boundaries of the individual segments of the hybrid protein. (A, right) Steady-state levels of Deg1:Sec62^{ProA} from wild-type cells were immunoprecipitated with IgG Sepharose and either mock treated or treated with the deglycosylating enzyme endoglycosidase H. Samples were separated on SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-Flag M2 monoclonal antibodies to visualize the substrate protein. (B) Yeast cells carrying wild-type or the indicated Deg1:Sec62^{ProA} mutants were pulse labeled for 5 min and hybrid protein was immunoprecipitated from cell lysates. Samples were separated on SDS-PAGE and visualized by autoradiography. The mobility of substrate forms without (0-CHO), with 1 (1-CHO), and with 2 (2-CHO) N-glycans is indicated. (C, left) BY4742 harboring Deg1:Sec62 1–149 was pulse labeled in the presence or absence of 10 µg/ml tunicamycin. Deg1:Sec62 1–149 was immunoprecipitated from detergent-solubilized lysates with anti-Flag antibodies. (C, right) BY4742 harboring proFactor:Sec62 1–149 were pulse labeled in the presence or absence of tunicamycin. After centrifugation, the supernatant and cell pellet fraction were subjected to detergent-solubilized immunoprecipitation with anti-Flag antibodies. Samples were separated on SDS-PAGE and visualized by autoradiography. Asterisks indicate the migration of glycosylated forms of proFactor:Sec62 from whole cell lysates. (D) Wild-type and glycosylation acceptor site mutants of Deg1:Sec62^{ProA} were pulse labeled for 5 min and chased for the indicated times. Deg1:Sec62^{ProA} was immunoprecipitated, resolved on SDS-PAGE, and visualized by autoradiography. (E) Wild-type and mutant Deg1:Sec62^{ProA} decay were quantified and plotted as described in Fig. 1 B.

independence of ubiquitination for the initial stages of retrotranslocation (Flierman et al., 2003). We then introduced site-specific mutations in the Deg1 signal itself in an effort to disrupt an early step in the retrotranslocation event. A systematic study of the

Deg1 signal localized its degradation determinant to a relatively short peptide segment spanning residues 13–32. We selected mutations that significantly stabilize a Deg1:βgal fusion protein (Johnson et al., 1998; $t_{1/2}$ s for deg1 mutant hybrids: F18S ≈ 40 min,

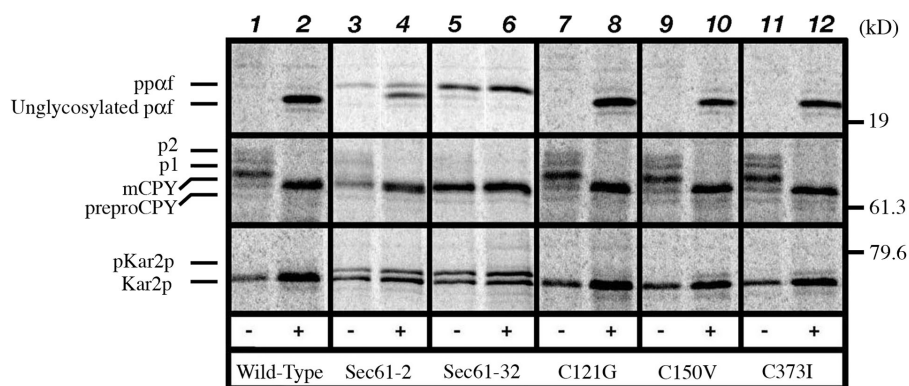


Figure 3. Sec61p cysteine mutants are proficient for ER protein import. Wild-type and Sec61p mutant cells were pulse labeled at 30°C for 15 min and model secretory proteins were immunoprecipitated from detergent-solubilized lysates. The immunoprecipitated proteins were resolved on SDS-PAGE and visualized by autoradiography. Where indicated, tunicamycin was present at 10 µg/ml. The positions of precursor forms (ppaf and pKar2), signal-cleaved unglycosylated proteins (paf, proCPY, and Kar2), and signal-cleaved glycosylated forms (p1, p2, and mCPY) are indicated.

F18S/L29H \approx 120 min, Δ 13–32 \geq 200 min [unpublished data]). As shown in Fig. 4 (C and D), substitution of serine for phenylalanine at position 18 of the signal resulted in a pronounced delay in the formation of glycosylated substrate (ratios of 0.2 and 1.5 at 2 and 8 min, respectively). The rate was further reduced in the double mutant, F18S/L29H, with ratios of 0.1 and 0.8, respectively (Fig. 4, C and D). In the most extreme case, deletion of the entire Deg1 determinant prevented glycosylation of the substrate entirely (Δ 13–32, ratios at 2 and 8 min = 0; Fig. 4, C and D). Thus, the delay in the appearance of glycosylated substrate in Deg1 mutants supports the notion that ER penetration of the N terminus of Deg1:Sec62^{ProtA} occurs during degradation.

Glycosylation of Deg1:Sec62^{ProtA} is not required for its recognition or targeting

The ERAD of soluble glycoproteins requires glycan trimming and recognition by lectin-like receptors (Liu et al., 1999; Spear and Ng, 2005). We asked if the addition of N-linked glycan to Deg1:Sec62^{ProtA} played a role in its degradation. Two independent lines of evidence suggested that this was not the case. Pulse-chase analysis of the mutant substrate protein carrying a disruption of the acceptor site asparagine (Asn153; Fig. 2 C) revealed degradation rates identical to those of its wild-type counterpart ($t_{1/2}$ = 13 min; Fig. 2, D and E). Furthermore, the degradation rate of wild-type substrate was independent of the lectin-like receptors Htm1p/Mnl1p and Yos9p (Jakob et al., 2001; Kim et al., 2005; unpublished data). We conclude that the addition of oligosaccharide to Deg1:Sec62^{ProtA} plays no mechanistic role in the ERAD of this substrate. Rather, it appears as a byproduct of the process by which the substrate is degraded.

A single cysteine residue in Deg1:Sec62^{ProtA} is also required for glycosylation

We next considered the possibility that cysteine 373 of Sec61p may form a transient disulfide bond to Deg1:Sec62^{ProtA} to promote or stabilize the insertion of the N-terminal domain of the hybrid protein in the lumen of the ER. The primary sequence of Deg1:Sec62^{ProtA} contains five cysteine residues, three of which (Cys33, Cys34, and Cys200) are localized within the N-terminal cytosolic tail, one (Cys255) within transmembrane domain one, and the last (Cys315) to the C-terminal cytosolic tail. Cysteine to serine mutation of residues 33, 34, 255, or 315 had no effect on the formation of the glycosylated substrate species (Fig. 5 A). However, muta-

genesis of cysteine 200 produced an unglycosylated form of the hybrid protein (Fig. 5 A). The inability of this substrate cysteine mutant to become glycosylated resembled the effect of Sec61p C373I on wild-type hybrid protein (compare Fig. 1 A to Fig. 5 A). Thus, a disulfide bond may form between the hybrid protein and the translocation channel (or with other proteins) as an intermediate in the ERAD process. The formation of this intermediate may promote the formation of the glycosylated hybrid but impede the ERAD process. ERAD was accelerated in the C373I Sec61p mutant (Fig. 1, A and B) and in the C200S mutant Deg1 hybrid

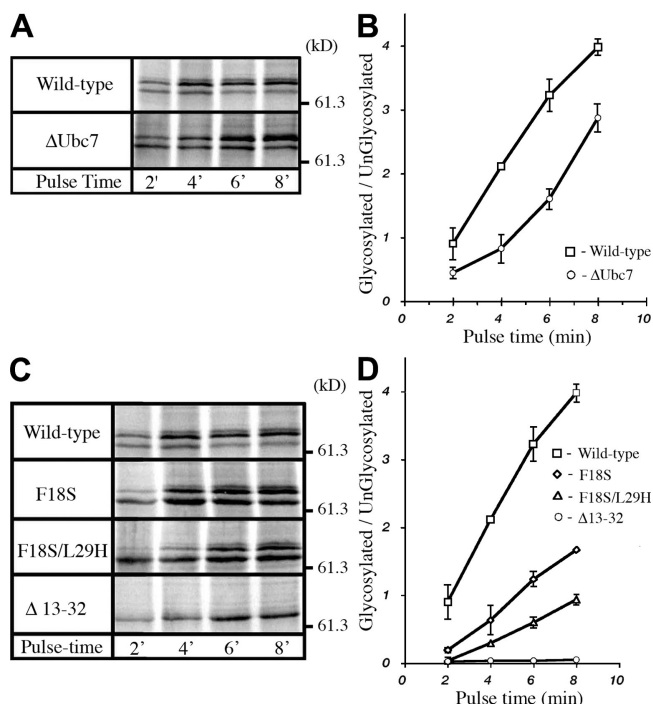


Figure 4. Glycosylation of Deg1:Sec62^{ProtA} occurs during degradation. (A) Wild-type and Δ ubc7 cells expressing Deg1:Sec62^{ProtA} were pulse-labeled for 2, 4, 6, or 8 min. Deg1:Sec62^{ProtA} was immunoprecipitated, resolved on SDS-PAGE, and visualized by autoradiography. (B) Phosphorimager quantitation of the results depicted in A. The data reflect the mean of at least three independent experiments with the SD of the mean indicated. The data are plotted as the ratio of the glycosylated to unglycosylated substrate protein present at the indicated time of the pulse label. (C) Wild-type and Deg1 region mutants of Deg1:Sec62^{ProtA} were pulse labeled for the indicated times. Deg1:Sec62^{ProtA} was immunoprecipitated, resolved on SDS-PAGE, and visualized by autoradiography. (D) Phosphorimager quantitation of lanes in C. Results are plotted as in B.

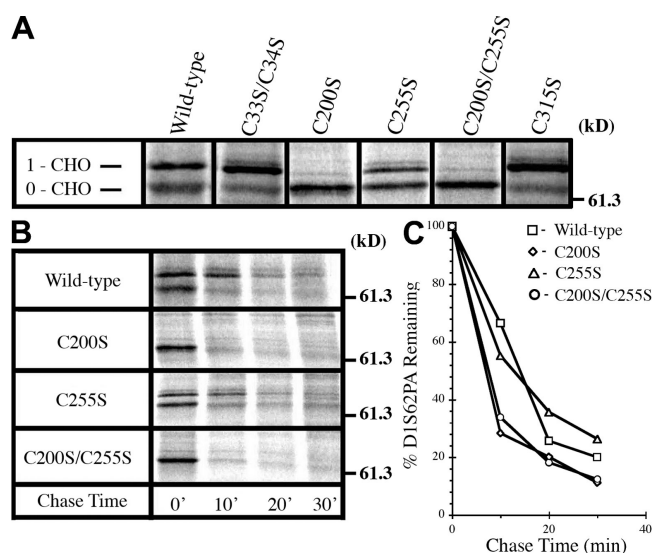


Figure 5. Glycosylation of Deg1:Sec62^{ProtA} is dependent on a cysteine residue within the substrate protein. (A) Wild-type and cysteine mutant alleles of Deg1:Sec62^{ProtA} were pulse labeled for 5 min. Deg1:Sec62^{ProtA} was immunoprecipitated, resolved by SDS-PAGE, and visualized by autoradiography. The position of unglycosylated (0-CHO) and glycosylated (1-CHO) substrate is indicated. (B) Degradation kinetics of Deg1:Sec62^{ProtA} cysteine mutants in a wild-type Sec61p background. Deg1:Sec62^{ProtA} and cysteine mutant derivatives were subjected to a pulse-chase regimen and lysates were immunoprecipitated as described in Fig. 1 A. (C) Wild-type Deg1:Sec62^{ProtA} cysteine mutant decay was quantified and plotted as described in Fig. 1 B.

protein ($t_{1/2}$ = 8 min for C200S vs. $t_{1/2}$ = 13 min for wild type; Fig. 5, B and C). Reduction of a disulfide-linked intermediate may become rate limiting for ERAD of this substrate.

Isolation of a disulfide-bonded substrate-Sec61p intermediate

To obtain direct evidence for the presence of a disulfide complex, we used a two-dimensional nonreducing/reducing electrophoresis strategy. To limit nonspecific disulfide-bonded species, we created a version of the substrate protein that contained cysteine-to-serine substitutions at the four residues not required for formation of the glycosylated substrate (Cys33, 34, 255, and 315; Fig. 5 A). In addition, the Protein A tag was removed to prevent cross-reactivity of substrate with antibodies directed against Sec61p. These modifications were without phenotypic consequence, as the modified substrate retained wild-type glycosylation and degradation kinetics (unpublished data).

Two-dimensional gels probed for substrate revealed three species. One (spot A) represented the monomeric substrate protein because it migrated at the predicted molecular weight for monomer in both dimensions (Fig. 6 A, spot A, molecular mass 41 kD). Another (spot C) migrated in the nonreducing dimension at a position of roughly 100 kD and likely represents a disulfide-bonded homodimer of the substrate protein (see subsequent paragraph and Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200804053/DC1>). This species migrated at a position consistent with monomer hybrid protein in the reducing second dimension of electrophoresis (Fig. 6 A, spot C). Moreover, this disulfide complex persists

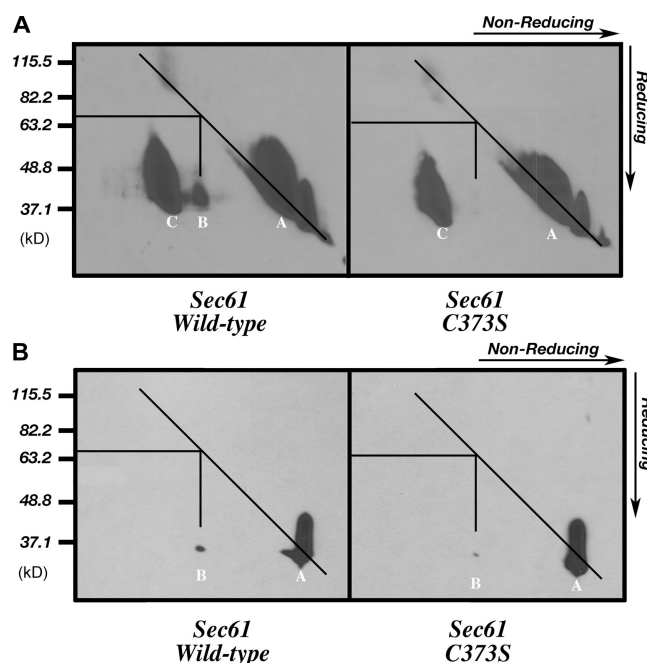


Figure 6. Two dimensional nonreducing/reducing electrophoresis. (A) Membranes from wild-type or Sec61p C373S cells expressing Δ Cys4: Deg1:Sec62^{ProtA} were prepared for two-dimensional electrophoresis as described in Materials and methods. After electrophoresis, proteins were transferred to nitrocellulose and probed with anti-Flag M2 monoclonal antibodies to detect substrate protein. The migration of three spots representing the monomeric substrate and two disulfide-bonded complexes containing the substrate are marked A, B, and C, respectively. (B) Duplicate blots from the samples in A. In this case, the nitrocellulose membrane was probed with anti-Myc 9B11 monoclonal antibodies to detect Sec61p-myc. The migration of two spots representing monomeric Sec61p and a disulfide-bonded complex containing Sec61p are marked A and B, respectively.

in the Sec61p C373S mutant (Fig. 6 A, spot C). A final species (spot B) migrated at a position consistent with an apparent molecular mass of \sim 70 kD (Fig. 5 A, spot B). The size of this complex in the nonreducing dimension conformed to the range of molecular masses expected for a Sec61p-Deg1: Sec62 disulfide complex (35 kD + 41 kD = 76-kD complex). Furthermore, this species was not detected in a strain harboring the Sec61p C373S mutation (Fig. 6 A, compare wild type and C373S). We confirmed the presence of Sec61p within the 70-kD complex by probing the two-dimensional blots with antibodies directed against a myc epitope located at the extreme C terminus of Sec61p. Two species were evident: one represented monomeric Sec61p, which migrated at a position of roughly 35 kD in both dimensions (Fig. 6 B, spot A), and another that migrated at a position corresponding to an \sim 70-kD species in the nonreducing dimension, which collapsed to 35 kD upon reduction (Fig. 6 B, spot B).

A Sec61p species that migrated in the two-dimensional gels at a position similar to Spot B persisted in the C373S mutant background (Fig. 6 B). This form was observed in membranes from a strain in which all three Sec61p cysteine residues were mutated (unpublished data). This species may represent a stable nondisulfide dimer of Sec61p that resists complete denaturation at the high protein concentration used in sample preparation for this analysis. Nevertheless, we reproducibly observed

a sixfold difference in the abundance of Spot B in wild-type versus the C373S Sec61p mutant background (wild type/C373S = 5.8 ± 0.45 ; see Materials and methods). We conclude that the majority of Spot B is a disulfide-bonded heterocomplex between residue 373 of Sec61p and residue 200 of Deg1:Sec62.

To determine if the Sec61p–Deg1:Sec62 disulfide complex forms as a result of the degradation of Deg1:Sec62^{ProtA}, we subjected the deg1 determinant deletion mutant $\Delta 13$ –32 to the two-dimensional analysis. Deletion of the deg1 degradation signal confers to the hybrid protein stability and an inability to become modified with glycan (Fig. 4, C and D), presumably because it can no longer enter the ERAD pathway. When this mutant is analyzed by two-dimensional nonreducing/reducing electrophoresis only monomeric substrate protein is observed (Fig. S2, spot A). The absence of the Sec61p–Deg1:Sec62 disulfide complex, spot B, confirms that the substrate protein must be degradation competent to form a disulfide complex with Sec61. Disappearance of the higher molecular mass complex, spot C, suggests that this spot represents a disulfide-bonded homodimer of the substrate protein, which is consistent with removal of a large segment of the region shown to mediate MAT α 2 homodimerization (α 2A residues 18–43; Ho et al., 2002).

Discussion

Improperly folded secretory proteins are recognized and targeted for degradation by a quality control system in the ER. Misfolded substrates are degraded by the cytosolic 26S proteasome. Hence, they must first be retrotranslocated back across the membrane of the ER. Despite significant progress in understanding the events of misfolded protein recognition, targeting, and degradation, the retrotranslocation process remains poorly defined. A significant obstacle to further defining this event is the observation that different substrate molecules display distinct genetic and biochemical requirements for their degradation.

Our discovery of a disulfide intermediate complex between Sec61p and the short-lived transmembrane ERAD substrate Deg1:Sec62^{ProtA} provides direct evidence for the participation of Sec61p in the retrotranslocation process, possibly serving as a retrotranslocation channel. In support of this view is the recent characterization of proteasome binding sites within Sec61p (Ng et al., 2007) and the ER stress-induced induction of subunits of the TRAP complex (Nagasawa et al., 2007). Although we cannot rule out the action of alternative channels in the ERAD of Deg1:Sec62^{ProtA}, it is degraded independently of Der1p and its yeast orthologue Dfm1p (Hitt and Wolf, 2004; unpublished data). In contrast, yeast cells lacking the E3 ubiquitin ligase Doa10p stabilize the hybrid twofold (unpublished data). However, because Doa10p functions in the degradation of ER-associated and cytosolic substrates, we cannot discriminate roles in the recognition of the deg signal or as an ERAD channel (Swanson et al., 2001).

The byproduct of disulfide complex formation is the addition of oligosaccharide to a short segment of the N-terminal tail of Deg1:Sec62^{ProtA}. This tail is otherwise cytoplasmically localized, indicating that during the retrotranslocation of membrane substrates, cytosolic loops and/or tails pass through the lumen

of the ER. The formation of a glycoprotein intermediate may require the action of Sec61p and may become apparent only when substrate extraction is delayed by formation of the disulfide complex. Given the small number of misfolded transmembrane substrates available for study and the specific requirements for an appropriately placed cysteine residue and glycosylation acceptor site, this unusual intermediate may have escaped detection in other ERAD substrates. As an example, a recent study on the degradation of a Deg1:Vma12^{ProtA} hybrid protein failed to reveal a glycoprotein intermediate (Ravid et al., 2006). Vma12p is similar in size and membrane topology to Sec62p (Jackson and Stevens, 1997), and the Deg-Vma12 hybrid is degraded with a half-life of 7 min, which is identical to the degradation rate of the Deg-Sec62 cysteine mutant hybrid (Fig. 1, A and B; and Fig. 5, B and C). Degradation of Deg-Vma12 proceeds without apparent covalent modification, perhaps because the coding sequence contains no glycosylation acceptor sites and the only cysteine present in the N-terminal cytosolic domain is 75 residues away from the first transmembrane domain. In contrast, the Sec62 coding sequence contains two potential glycosylation sites (N90 and N153; Fig. 2), and the cysteine involved in disulfide bonding to Sec61p is only 40 residues removed from the first transmembrane domain.

How are cytoplasmic regions of misfolded transmembrane proteins reoriented during the retrotranslocation process so that they become exposed to the lumen of the ER? Our results suggest that the Deg signal recruits the N-terminal domain of the hybrid protein, possibly through the first transmembrane anchor domain, into the Sec61p channel where at least a portion of the polar N terminus is imported into the ER lumen (Fig. 7, A [II–IV] or B [III–IV]). This event may be preceded by the retrotranslocation of the C-terminal domain of the hybrid, as depicted in Fig. 7 B (I–III), or by the prior action of a distinct channel.

A fortuitous apposition of cysteine residues in Sec61p and the Deg hybrid permits the formation of a transient disulfide that stabilizes the N terminus of Deg-Sec62 in the ER lumen long enough to make it available for N glycosylation (Fig. 7, A [IV] or B [V]). Of importance to this model is the relative position of cysteine 373 of Sec61p in the crystal structure of the SecY complex (Van den Berg et al., 2004). C373 resides in the cytoplasmic half of TM8 (transmembrane segment eight) with the –SH side chain pointing to the channel interior. Interestingly, the cytoplasmic half of the lateral gate is thought to form at the interface between TM8 and TM2b and between TM2b and TM7. Thus, this residue may contact a retrotranslocating polypeptide chain.

Derlin (yeast Der1p) and Doa10p represent other potential retrotranslocation channels that may act alone or in concert with Sec61p (Wahlman et al., 2007). The task of assigning a direct role for Sec61p in this process is confounded by the effect of *sec61* mutations on the translocation process (Pilon et al., 1997; Plemper et al., 1997). Sec61p and Der1p have been assigned roles in the ERAD of the soluble misfolded protein CPY* (Knop et al., 1996; Plemper et al., 1997). However, the role of Sec61p and Der1p in degradation of membrane-bound substrates remains unclear. Der1p, despite being tested against a large panel of membrane-bound ERAD substrates (e.g., Vph1p,

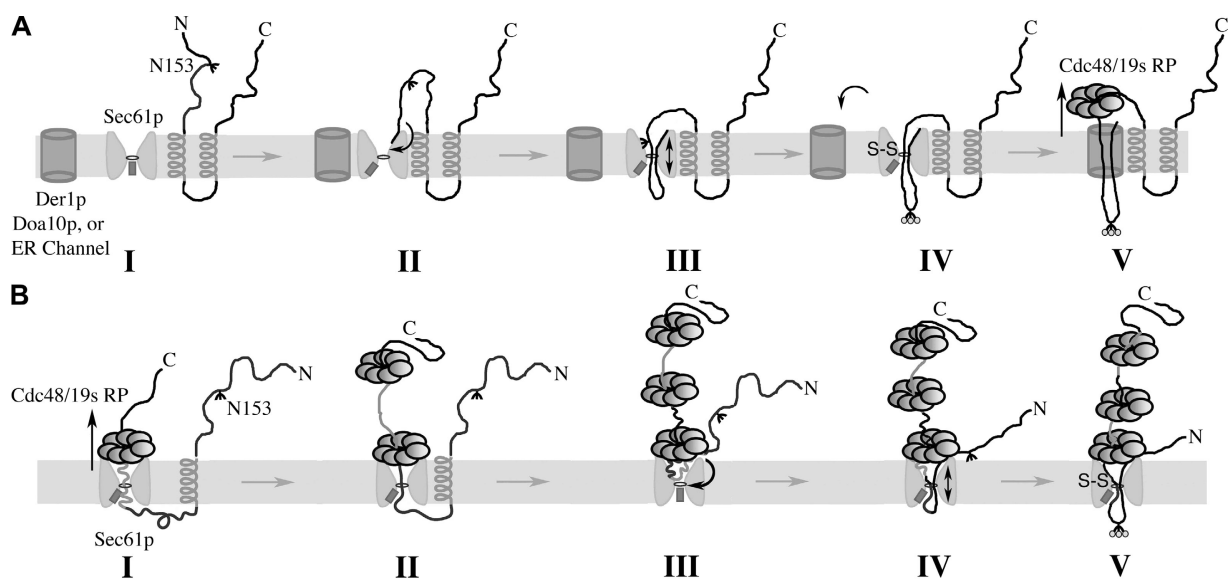


Figure 7. Models for disulfide complex formation and substrate glycosylation. (A) A model depicting a role for Sec61p in the initial state of Deg1 translocation into the ER. In this model, the amphipathic Deg1 region mimicks a signal peptide and is recruited to the Sec61p channel (I and II). After inversion of this segment, the N-terminal cytosolic region is looped back through the channel and may either be actively translocated or passively backslide through the channel (III). Next, the cysteine residues of the substrate protein and Sec61p complex form a disulfide intermediate that provides for the extended exposure and glycosylation at Asn153 within the N-terminal tail of the substrate protein (IV and V). After this initial translocation step, the misfolded Deg-Sec62 may be transferred to another channel (cylindrical symbol) to execute retrotranslocation. (B) A model depicting a role for Sec61p in membrane protein retrotranslocation from the ER. Substrate is initially targeted to Sec61p, and its C-terminal transmembrane helix partitions into the channel through a lateral gate (I). Ubiquitination and the Cdc48p and/or 19S RP complex assist in membrane extraction of the substrate protein (II). The substrate's N-terminal transmembrane partitions into the channel where the extraction force at the C terminus results in the reorientation of the N-terminal transmembrane segment within the channel (III). The N-terminal segment inverts, resulting in a reorientation of the N-terminal cytoplasmic tail with regions exposed to the lumen of the ER. Next, the looped segment may enter the lumen further by backsliding within the channel (IV). Finally, the disulfide complex formation between Sec61p and substrate stalls the N terminus in the channel, permitting glycosylation of Asn153 (V).

Pdr5*, Ste6-6*, CT*, and CTG*; Plemper et al., 1998; Hill and Cooper, 2000; Taxis et al., 2003; Vashist and Ng, 2004), is only known to be partially required for the chimeric ERAD substrate KWW (Vashist and Ng, 2004). Sec61p, although not as extensively tested as Der1p, is required for the degradation of Pdr5* that contains a misfolded luminal domain (Plemper et al., 1998; Walter et al., 2001; Huyer et al., 2004). Sec61-2, which is partially defective in the ERAD of CPY* but not in the ERAD of the Deg-Sec62 hybrid, possesses a mutant residue predicted to face the ER lumen (G213D; Vashist and Ng, 2004). Work from Vashist and Ng (2004) supports a view of multiple ERAD pathways requiring a distinct set of gene products that is dictated by the location of the misfolded domain. Hence, the effect of sec61-2 on the degradation of membrane proteins with luminal misfolded domains and its independence for membrane proteins with cytosolic misfolded domains may result from a lesion in the assembly of the ERAD machinery.

The interaction profiles for the Derlin family of proteins suggest they might act as adaptor proteins to provide a direct link between events on the luminal side of the ER membrane (association with misfolded proteins) with those on the cytoplasmic side (association with the ubiquitin ligase Hrd1 and the p97 AAA-ATPase; Lilley and Ploegh 2005; Ye et al., 2005; Nagasawa et al., 2007). Because Hrd1 and Der1p display overlapping substrate preferences, it is tempting to propose that Der1p organizes the machinery necessary for ubiquitination and extraction of misfolded luminal proteins. In contrast, substrates requiring the Doa10p ubiquitin ligase show Der1p independence

because they require other factors for assembly of the degradation machinery. Clearly, much remains to be done to understand exactly how Der1p, Doa10p, and Sec61p participate in the retrotranslocation event.

Materials and methods

Strains and plasmids

All strains used in this study were derived from the strains listed in Table I. Strains that were used to evaluate the functionality of Sec61p mutants were constructed by transformation of RSY633 with Sec61p alleles (CEN/LEU) followed by plasmid selection on 5-FOA plates. These strains were used directly for protein import assays (Fig. 3) or transformed with a CEN/URA plasmid for expression of Deg1:Sec62^{ProtA} to evaluate degradation kinetics in Sec61p mutant backgrounds. To analyze ERAD of CPY* in the Sec61p mutant backgrounds, we created transformants in which the sec61 mutant alleles (CEN/LEU) replaced the wild type. After selection on 5-FOA, these strains were transformed with a CEN/URA plasmid for the expression of CPY* (pHIT341). Pulse-time experiments were performed in the EUROscarf background (BY4742) with strains carrying the indicated chromosomal knockouts (Δ ubc7) or Deg1:Sec62^{ProtA} variants. For studies to identify the mixed disulfide complex, we generated strains by transformation of YDS174 with the Sec61p-myc alleles (CEN/TRP). After plasmid selection on 5-FOA, these strains were transformed with the Δ Cys4:Deg1:Sec62^{ProtA} plasmid.

Plasmids pDF40 (Pilon et al., 1997), pHIT341 (Norgaard et al., 2001), and pSec61-32 (Pilon et al., 1997) have been previously described. A CEN/URA vector for the expression of Deg1:Sec62^{ProtA} was constructed by transfer of an EcoRI-HindIII fragment, containing the Deg1:Sec62^{ProtA} coding region under control of the Gal1-10 promoter, from a ylpac128 (Mayer et al., 1998)-based vector to pRS316. Site-directed mutants of Sec61p and Deg1:Sec62^{ProtA} were constructed using Quick-Change Mutagenesis according to the manufacturer's recommendations (Stratagene) and mutations were confirmed by cycle sequencing.

Table I. Strains used in this study

Strain	Genotype	Source/reference
BY4742	MATa <i>his3-Δ1 leu2-Δ0 lys2-Δ0 ura3-Δ0</i>	Brachmann et al. (1998)
YDS060	Same as BY4742 but <i>ubc6::His3</i>	This study
YDS061	Same as BY4742 but <i>ubc7::leu2</i>	This study
YDS062	Same as BY4742 but <i>ubc6::His3 ubc7::leu2</i>	This study
RSY633	MATα <i>can1-100 leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 s61::HIS3</i> [pDF40]	Schekman laboratory
YDS174	MATα <i>can1-100 leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 ubc7::leu2 s61::his3::[loxP-KanMX]</i> [pDF40]	This study
RSY2962	MATα <i>can1-100 leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 prc1::HIS3 s61::his3::[loxP-KanMX]</i> [pDF40]	Schekman laboratory
RSY524	MATα <i>leu2-3,-112, ade2 ura3-52 pep4-3 SUC2 s61-2</i>	Schekman laboratory

A CEN/LEU-based vector for the expression of *sec61-2* was constructed by PCR amplification of RSY524 genomic DNA with the primers 5' TTGA-GCCTGCTGAAAGCTTG 3' and 5' AGAAGTTAGCCCGGGACCAT 3'. The resulting fragment was digested with *Sma*I–*Hind*III and cloned into pRS315. Additional details of strain construction and cloning are available upon request.

Antibodies

Antisera against ppaf (Wuestehube and Schekman, 1993), Kar2p, and CPY/CPY* (Feldheim et al., 1992) have been previously published. α-Flag M2 (Sigma-Aldrich) and α-myc (clone 9B11; Cell Signaling Technology) monoclonal antibodies were obtained from commercial sources and used according to the manufacturer's recommendations.

Pulse chase and immunoprecipitation

Pulse labeling and immunoprecipitation of ppaf, Kar2p, and CPY to evaluate the translocation activities of the Sec61p alleles were performed as previously described (Feldheim et al., 1992; Doering and Schekman, 1996). For CPY* ERAD, strains expressing CPY* (pHIT341; gift from P. Norgaard, Department of Molecular and Cell Biology and Howard Hughes Medical Institute, University of California, Berkeley, Berkeley, CA) were grown overnight at 30°C in SCGlc-Ura medium to OD₆₀₀ = 1.8–2. An aliquot equivalent to 8 ODs was removed and centrifuged and cells were resuspended in 20 ml of fresh SCGlc-Met-Ura. Cells were grown for ~2 h at 30°C to OD₆₀₀ = 0.8–0.9. An aliquot equivalent to 8 ODs was removed and centrifuged and cells were resuspended in 3.6 ml of fresh prewarmed SCGlc-Met-Ura medium. After growth at 30°C for 15 min, cultures were labeled with ³⁵S-Translabel (GE Healthcare) at 50 μCi/OD. Chase was initiated by addition of 0.4 ml of a 10x chase solution (SCGlc-Met-Ura containing 0.6% methionine and 0.4% cysteine). At time zero, and the indicated chase times, 1 ml was removed and immediately quenched by the addition of ice-cold sodium azide to 10 mM. Cell lysis and immunoprecipitation of CPY* was performed by methods described for CPY (beginning of paragraph).

Strains expressing Deg1:Sec62^{ProA} for degradation assays were grown overnight at 30°C in SCRaif-Ura medium. Cells were then subcultured into SCGal-Met-Ura medium at OD₆₀₀ = 0.05 and grown at 30°C to OD₆₀₀ = 0.9–1. An aliquot equivalent to 12 ODs was centrifuged, and cells were resuspended in fresh prewarmed SCGal-Met-Ura medium and allowed to grow at 30°C for 15 min. Labeling was initiated by the addition of ³⁵S-Translabel at 50 μCi/OD. After 5 min, 0.4 ml of a 10x chase solution was added. At time zero and the indicated chase times, 1 ml was removed and immediately quenched by the addition of ice-cold sodium azide to 10 mM. Cell lysis and immunoprecipitation of Deg1:Sec62^{ProA} with IgG Sepharose beads was performed as previously described (Biederer et al., 1996). In brief, cell pellets were thawed and resuspended in 110 μl of ice-cold buffer A (50 mM Tris-Cl, pH 7.4, 10 mM EDTA, and Complete Mini EDTA-free protease inhibitors [Roche]). Cell disruption was performed with 1 vol of glass beads by four cycles of vortex mixing for 30 s, interrupted by 30-s incubation on ice. The lysate was next diluted to 900 μl with ice-cold buffer A and cleared by low-speed centrifugation at 1,000 g. Membranes were isolated from the supernatant by centrifugation at 16,000 g for 10 min, resuspended in 100 μl buffer B (50 mM Tris-Cl, pH 7.5, and 150 mM NaCl), and diluted to a final volume of 900 μl in buffer C (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 11.3 mM EDTA, 1.1% Triton X-100, 0.11% SDS, and complete protease inhibitors). IgG Sepharose (100 μl of a 20% slurry in buffer C) was added and the mixtures were

incubated with rotation at 4°C for 2–4 h. Beads were collected, washed three times with 900 μl of buffer C, and resuspended in 50 μl 2X sample buffer. Samples were heated at 55°C for 10 min and proteins were separated on 9.5% SDS-PAGE. After drying, the gels were exposed to a phosphorimager cassette for detection and quantitation. Degradation kinetics are expressed as the sum of decay for the two substrate species over time. Although the degradation rates for glycosylated and unglycosylated substrate are different, we chose to express decay in this manner because the glycosylation site mutant, N153D, is degraded at a rate identical to the sum of the two wild-type substrate species (Fig. 2, D and E).

Labeling and immunoprecipitation of Deg1:Sec62^{ProA} for pulse-time experiments (Fig. 5) was performed as described for pulse-chase experiments (previous paragraph), with the only difference being the time cells were exposed to radiolabel. Cells were quenched at the indicated pulse time (i.e., 2, 4, 6, or 8 min) by the addition of sodium azide to 10 mM. Data are presented as the ratio of glycosylated to unglycosylated forms of the substrate protein at each labeling time.

Endoglycosidase H treatment

Steady-state levels of Deg1:Sec62^{ProA} were immunoprecipitated from wild-type cells as described for the pulse-chase experiments (Pulse chase and immunoprecipitation). Bound protein was eluted from the IgG Sepharose beads by heating in 1% SDS/50 mM Tris, pH 7.4. The eluted sample was diluted 20-fold into a final buffer concentration of 50 mM sodium citrate, pH 5.5. Samples were aliquoted into separate tubes and mock treated or treated with 1 mU of endoglycosidase-H (Boehringer Ingelheim). Mixtures were incubated for 3 h at 37°C and stopped by the addition of 2x sample buffer. Proteins were resolved by electrophoresis on 9.5% SDS-PAGE gels, transferred to nitrocellulose membranes, and developed with anti-Flag M2 monoclonal antibodies.

Microsomal membrane isolation and protease protection

Yeast cells expressing Deg1:Sec62^{ProA} were grown in SD-Ura 2% raffinose, 0.1% glucose overnight at 30°C. Cells were diluted to 0.05 OD₆₀₀/ml in SD-Ura 2% galactose and grown at 30°C for 8 h to induce expression of the hybrid. Cells (OD₆₀₀ = 15) were harvested and resuspended in 1 ml of 10-mM Tris-HCl, pH 9.4, containing 10 mM dithiothreitol and incubated at room temperature for 10 min. Cells were collected by centrifugation and washed with spheroplast buffer (1 M sorbitol, 20 mM sodium phosphate, pH 7.5, 150 mM NaCl, and 2 mM dithiothreitol). Cells were recollected and resuspended in 1 ml of spheroplast buffer. Cell walls were digested with 140 μg zymolase 100T (Seikagru Corporation)/10 OD₆₀₀ equivalents for 20 min at 30°C. Spheroplasts were centrifuged (5 min at 600 g at 4°C) and washed in prechilled spheroplast buffer containing 20 μg/ml pepstatin and 1 mM EDTA, pH 8. Cells were centrifuged again and resuspended in 1 ml of fractionation buffer (200 mM D-mannitol, 20 mM sodium phosphate, pH 7.5, and 150 mM NaCl) containing 20 μg/ml pepstatin and 1 mM EDTA, pH 8. Lysis was achieved by the addition of 300 μl of acid-washed glass beads (Sigma-Aldrich) and 1 min of agitation with a vortex mixer at 4°C. Unlysed cells and cell debris were centrifuged for 5 min at 600 g at 4°C. Crude microsomal fractions were collected by centrifugation and resuspended in buffer (50 mM Tris, pH 7.4, and 200 mM NaCl) at ~0.5 OD₆₀₀ equivalents/ml. The microsome fraction was split into three aliquots (~4 OD₆₀₀ equivalents each). One was mock treated with buffer and the others were digested with 5 μg/ml trypsin in the absence or presence of 1% Triton X-100 for 30 min on ice. Digestions were stopped by the addition of trichloroacetic acid to 15%. After a 30-min incubation on ice,

precipitated material was collected by centrifugation and washed once with ice-cold acetone. Pellets were resuspended in 50 μ l SDS-PAGE sample buffer, heated at 55°C for 15 min, and subjected to electrophoresis. Proteins were transferred to nitrocellulose and subjected to immunoblotting with anti-Kar2 (microsomal integrity control), anti-Protein A (C terminus of hybrid), or anti-Flag (N terminus of hybrid) antibodies.

Two-dimensional nonreducing/reducing SDS-PAGE

Cells expressing Δ Cys4:Deg1:Sec62^{ProtA} or Δ 13-32: Δ Cys4:Deg1:Sec62^{ProtA} (Fig. S2) were grown and membranes were isolated from the cell pellets as described for the pulse-chase experiments (Pulse chase and immunoprecipitation). Membrane pellets were then resuspended in 4 \times SDS-PAGE sample buffer without reducing agent to a concentration approximately corresponding to 0.4 OD₆₀₀ equivalents/ μ l (for substrate blots) or 1.5 OD₆₀₀ equivalents/ μ l (for Sec61p blots). After 10 min at 55°C, 60 μ l of the sample was electrophoresed on 9.5% SDS-PAGE gels. After electrophoresis in the first dimension, the lane was excised with a razorblade and soaked in 5 ml of sample buffer containing 10% β -mercaptoethanol at 90°C for 5 min. The gel sliver was then placed on top of a second 9.5% SDS-PAGE trough gel and electrophoresed in the second dimension. After transfer to nitrocellulose, but before detection with anti-flag or anti-myc antibodies, the membrane was stained with Ponceau S to demarcate the protein diagonal band. Blots were developed with ECL Plus (GE Healthcare) and scanned with a typhoon imager for quantitation. To compare the relative amounts of Sec61p and Deg1:Sec62^{ProtA} between the strain backgrounds, we sampled serial dilutions of the membrane suspension under reducing conditions and quantified the resulting protein species. In all experiments, there was a <5–10% difference in the total amount of Sec61p or Deg1:Sec62^{ProtA} applied to the two-dimensional gels.

Online supplemental material

Fig. S1 shows the protease susceptibility of Deg1:Sec62^{ProtA}. Fig. S2 shows the two-dimensional nonreducing/reducing SDS-PAGE analysis of the deg1 determinant deletion mutant Δ 13–32: Δ Cys4:Deg1:Sec62^{ProtA}. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200804053/DC1>.

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