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, Sec61α 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;
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:Sec62ProtA. 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:Sec62ProtA was rapidly degraded with a 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; C15OY) on the ERAD of an unglycosylated form of α-factor (αf) precursor (Pilon et al., 1997).

To explore the role of each of the cysteine residues of Sec61p in the ERAD of Deg1:Sec62ProtA, 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 (t1/2 = 7–8 min; Fig. 1, A and B), whereas Sec61p C121G or C150V had no influence on the degradation rate (t1/2 = 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 (Plumper 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 t1/2 of ~25 min (Fig. 1, C and D). Mutations at position 121 or 150 of Sec61p retained wild-type degradation kinetics, whereas mutation of residue 373 resulted in a slight, but reproducible, stabilization of CPY* (t1/2 = 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 (Plumper et al., 1997). In all experiments, we observed a single electrophoretic species of CPY* unlike the behavior of Deg1:Sec62ProtA.

Deg1:Sec62ProtA is N glycosylated

The effect of Sec61p C373I on the production of one of two electrophoretic species of Deg1:Sec62ProtA 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 during ERAD.

We found that Deg1:Sec62ProtA was sensitive to endoglycosidase H,
which is consistent with a previous report for Deg1:Sec62ProtA (Fig. 2 A; Kim et al., 2006).

The primary sequence of Deg1:Sec62ProtA 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:Sec62ProtA 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 (Deshaias 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:Sec62ProtA 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:Sec62ProtA. 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 αf is synthesized in the cytosol as a prepro precursor form (ppαf). Upon translocation into the ER, ppαf becomes signal cleaved (pαf) and modified by the addition of three N-linked glycans (3gppαf). In Sec61p wild-type and cysteine mutant cells, 3gppαf 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 pαf in the ER (Fig. 3, top, lanes 2, 4, 8, 10, and 12). The translocation mutants sec61-2 and sec61-32 accumulated pppαf, 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:Sec62ProtA 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 αf, 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:Sec62ProtA 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
Deg1 signal localized its degradation determinant to a relatively short peptide segment spanning residues 13–32. We selected mutations that significantly stabilize a Deg1:gal fusion protein (Johnson et al., 1998; half-lives for deg1 mutant hybrids: F18S ≈ 40 min, 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; half-lives for deg1 mutant hybrids: F18S ≈ 40 min, 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

Figure 2. Deg1:Sec62<sup>proSA</sup> is modified by N-linked glycosylation. (A, left) Schematic representation of Deg1:Sec62<sup>proSA</sup> 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<sup>proSA</sup> highlighting the amino acid boundaries of the individual segments of the hybrid protein. (A, right) Steady-state levels of Deg1:Sec62<sup>proSA</sup> 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<sup>proSA</sup> 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<sup>proSA</sup> were pulse labeled for 5 min and chased for the indicated times. Deg1:Sec62<sup>proSA</sup> was immunoprecipitated, resolved on SDS-PAGE, and visualized by autoradiography. (E) Wild-type and mutant Deg1:Sec62<sup>proSA</sup> decay were quantified and plotted as described in Fig. 1 B.
F18S/L29H = 120 min, Δ13–32 = 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 (Δ13–32, 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:Sec62ProtA occurs during degradation.

Glycosylation of Deg1:Sec62ProtA 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:Sec62ProtA 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 (t1/2 = 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:Sec62ProtA 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: Sec62ProtA is also required for glycosylation
We next considered the possibility that cysteine 373 of Sec61p may form a transient disulfide bond to Deg1:Sec62ProtA 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: Sec62ProtA 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, mutagenesis 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
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 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 a 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 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 ~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 ~70-kD species in the nonreducing dimension, which collapsed to 35 kD upon reduction (Fig. 6 B, spot B).

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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 to 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 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 ~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 ~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

Figure 5. Glycosylation of Deg1:Sec62 mutants is dependent on a cysteine residue within the substrate protein. (A) Wild-type and cysteine mutant alleles of Deg1:Sec62 were pulse labeled for 5 min. Deg1:Sec62 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 cysteine mutants in a wild-type Sec61p background. Deg1:Sec62 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 cysteine mutant decay was quantified and plotted as described in Fig. 1 B.

Figure 6. Two dimensional nonreducing/reducing electrophoresis. (A) Membranes from wild-type or Sec61p C373S cells expressing Cys4:Deg1:Sec62 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.
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 Deg1p:Sec62p 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 Deg1p:Sec62p, 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 Doo1p stabilize the hybrid twofold (unpublished data). However, because Doo1p 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 Deg1p:Sec62p. 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 Deg1p:Vma12 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,
by the location of the misfolded domain. Hence, the effect of ways requiring a distinct set of gene products that is dictated Vashist and Ng (2004) supports a view of multiple ERAD path-

faces the ER lumen (G213D; Vashist and Ng, 2004). Work from the Deg-Sec62 hybrid, possesses a mutant residue predicted to
ter defectively in the ERAD of CPY* but not in the ERAD of Walter et al., 2001; Huyer et al., 2004).
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 exten-
sively tested as Der1p, is required for the degradation of Pdr5*

because they require other factors for assembly of the degrada-
tion machinery. Clearly, much remains to be done to understand exactly how Der1p, Doa10p, and Sec61p participate in the retro-

translocation event.

Materials and methods

Strains and plasmids

All strains 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\(^{nax}\) to evaluate degra-
dation 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 expression of Deg1:Sec62\(^{nax}\), or pSec61-32 (Pilon et al., 1997) has been previously de-

scribed. A CEN/URA vector for the expression of Deg1:Sec62\(^{nax}\) was constructed by transfer of an EcoRI–HindIII fragment, containing the Deg1:Sec62\(^{nax}\) coding region under control of the Gal1-10 promoter, from a yplac128 (Mayer et al., 1998)-based vector to pRS316. Site-
directed mutants of Sec61p and Deg1:Sec62\(^{nax}\) were constructed using Quick-Change Mutagenesis according to the manufacturer’s recommenda-
tions (Stratagene) and mutations were confirmed by cycle sequencing.
Table I. Strains used in this study

| Strain      | Genotype                          | Source/reference         |
|-------------|-----------------------------------|--------------------------|
| BY4742      | MAAa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0  | Brachmann et al. (1998)  |
| YDS060      | Same as BY4742 but ubc6::His3     | This study               |
| YDS061      | Same as BY4742 but ubc7::leu2     | This study               |
| YDS062      | Same as BY4742 but ubc6::His3 ubc7::leu2 | This study               |
| RSY633      | MAaα con1-100 leu2-3,112 his3-11,15 trp-1-1 ura3-1 ade2-1 s61::His3 [pDF40] | Schekman laboratory      |
| YDS174      | MAaα con1-100 leu2-3,112 his3-11,15 trp-1-1 ura3-1 ade2-1 ubc7::leu2 s61::his3: [loxP-KanMX] [pDF40] | This study               |
| RSY2962     | MAaα con1-100 leu2-3,112 his3-11,15 trp-1-1 ura3-1 ade2-1 plc1::His3 s61::his3: [loxP-KanMX] [pDF40] | Schekman laboratory      |
| RSY524      | MAaα leu2-3,112, ade2 ura3-52 pep4-3 SUC2 s61-12 | Schekman laboratory      |

A CEN/LEU-based vector for the expression of sec61-2 was constructed by PCR amplification of R5524 genomic DNA with the primers 5'TGAAGCCTGGTCAAGGCTTG 3' and 5'AGAAAGTGACCCGGGACCAT 3'. The resulting fragment was digested with SmaI–HindIII and cloned into pRS315. Additional details of strain construction and cloning are available upon request.

Antibodies
Antiserum against pppf (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 pppf, 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* (pHTI341; gift from Wuestehube and Schekman, 1993), Kar2p, and CPY to evaluate the substrate protein at each labeling time.

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:Sec62mmt 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 μl 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:Sec62mmt 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 di-thiothreitol 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 di-thiothreitol). Cells were recollected and resuspended in 1 ml of spheroplast buffer. Cell walls were digested with 140 μg zymolase 100T (Seikagaku Corporation)/1 OD600 equivalent 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 peptatin and 1 mM EDTA, pH 8. Cells were centrifuged again and resuspended in 1 ml of fractionation buffer (200 mM Tris-mannitol, 20 mM sodium phosphate, pH 7.5, and 150 mM NaCl) containing 20 μg/ml peptatin 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 with ice-cold acetone. Pellets were resuspended in 50 μl 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 (micromasol 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 ΔCys4 ΔDeg1 Sec62
two or Δ13-32 ΔCys4 ΔDeg1 Sec62
two (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× SDS-PAGE sample buffer without reducing agent to a concentration approximately corresponding to 0.4 OD600 equivalents/μl (for substrate blots) or 1.5 OD600 equivalents/μl (for Sec61p blots). After 10 min at 55°C, 60 μl 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 bands. 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 two 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 two applied to the two-dimensional gels.

Online supplemental material
Fig. S1 shows the protease susceptibility of Deg1:Sec62 two. Fig. S2 shows the two-dimensional nonreducing/reducing SDS-PAGE analysis of the deg1 determinant deletion mutant Δ13-32 ΔCys4 ΔDeg1:Sec62 two. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200804053/DC1.

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References
Biederer, T., C. Volkwein, and T. Sommer. 1996. Degradation of subunits of the Sec61p complex, an integral component of the ER membrane, by the ubiquitin-proteasome pathway. EMBO J. 15:2069–2076.
Brachmann, C.B., A. Davies, G.J. Cost, E. Caputo, J. Li, P. Hieter, and J.D. Boeke. 1998. Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast. 14:115–132.
Brodsky, J.L., and A.A. McCracken. 1999. ER protein quality control and proteasome-mediated degradation. Semin. Cell Dev. Biol. 10:507–513.
Deshaies, R.J., and R. Schekman. 1989. SEC62 encodes a putative membrane protein required for protein translocation into the yeast endoplasmic reticulum. J. Cell Biol. 109:2653–2664.
Doering, T.L., and R. Schekman. 1996. GPI anchor attachment is required for Gas1p transport from the endoplasmic reticulum in COP II vesicles. EMBO J. 15:182–191.
Ellgaard, L., and A. Helenius. 2003. Quality control in the endoplasmic reticulum. Nat. Rev. Mol. Cell Biol. 4:181–191.
Feldhahn, D., J. Rothbatt, and R. Schekman. 1992. Topology and functional domains of Sec61p, an endoplasmic reticulum membrane protein required for secretory protein translocation. Mol. Cell. Biol. 12:3288–3296.
Finger, A., M. Knop, and D.H. Wolf. 1993. Analysis of two mutated vacuolar membrane proteins of yeast. J. Biol. Chem. 268:34774–34782.
Hampton, R.Y. 2002. ER-associated degradation in protein quality control and cellular regulation. Curr. Opin. Cell Biol. 14:476–482.
Hill, K., and A.A. Cooper. 2000. Degradation of unassembled Vph1p reveals novel aspects of the yeast ER quality control system. EMBO J. 19:550–561.
Hitt, R., and D.H. Wolf. 2004. Der1p, a protein required for degradation of malformed soluble proteins of the endoplasmic reticulum: topology and Der1-like proteins. FEBS Yeast Res. 4:721–729.
Ho, C.Y., M. Smith, M.E. Houston, J.G. Adamson, and R.S. Hodgess. 2002. A possible mechanism for partitioning between homo- and hetero- dimerization of the yeast homodimer proteins Mata1 and Mata2p2. J. Pept. Res. 59:34–43.
Huyer, G., W.F. Piltz, A. Fansler, S.G. Kreft, M. Hochstrasser, J.L. Brodsky, and S. Michaelis. 2004. Distinct machinery is required in Saccharomyces cerevisiae for the endoplasmic reticulum-associated degradation of a multipass transmembrane protein and a soluble luminal protein. J. Biol. Chem. 279:38369–38378.
Jackson, D.D., and T.H. Stevens. 1997. VMA21 encodes a yeast endoplasmic reticulum protein required for vacuolar H+-ATPase assembly. J. Biol. Chem. 272:25928–25934.
Kim, L., J. Ahn, C. Liu, K. Tanabe, A. Apodaca, T. Suzuki, and H. Rao. 2006. The Png1–Rad23 complex regulates glycoprotein turnover. J. Cell Biol. 172:211–219.
Kim, W., D.E. Spear, and D.T. Ng. 2005. Yos9p detects and targets glycoproteins for ER-associated degradation. Mol. Cell. 19:753–764.
Knop, M., A. Finger, T. Braun, K. Hellmuth, and D.H. Wolf. 1996. Der1, a novel protein specifically required for endoplasmic reticulum degradation in yeast. EMBO J. 15:753–763.
Kreft, S.G., L. Wang, and M. Hochstrasser. 1998. Degradation signal masking by heterodimerization of MatA2p and MatA1 blocks their mutual destruction by the ubiquitin-proteasome pathway. Cell. 94:217–227.
Lilley, B.N., and H.L. Ploegh. 2004. A membrane protein required for dislocation of misfolded proteins from the ER. Nature. 429:834–840.
Lilley, B.N., and H.L. Ploegh. 2005. Multiprotein complexes that link dislocation, ubiquitination, and extraction of misfolded proteins from the endoplasmic reticulum membrane. Proc. Natl. Acad. Sci. USA. 102:14296–14301.
Liu, Y., P. Choudhury, C.M. Cabral, and R.N. Sifers. 1999. Oligosaccharide modification in the early secretory pathway directs the selection of a misfolded glycoprotein for degradation by the proteasome. J. Biol. Chem. 274:5861–5867.
Mayer, T., T. Braun, and S. Jentsch. 1998. Role of the proteasome in membrane protein extraction of a short-lived ER-transmembrane protein. EMBO J. 17:3251–3257.
Nagasawa, K., T. Higashi, N. Hosokawa, R.J. Kaufman, and K. Nagata. 2007. Simultaneous induction of the four subunits of the TRAP complex by ER stress accelerates ER degradation. EMBO Rep. 8:483–489.
Norgaard, P., W. Vibeke, C. Tachibana, L. Alse, B. Holst, and J.R. Winther. 2001. Functional differences in yeast protein disulfide isomerases. J. Cell Biol. 152:553–562.
Ng, W., T. Sergeyenko, N. Zeng, J.D. Brown, and K. Romisch. 2007. Characterization of the proteasome interaction with the Sec61 channel in the endoplasmic reticulum. J. Cell Sci. 120:682–691.
Oyadomari, S., C. Yun, E. Fisher, N. Kreglinger, G. Kreibich, M. Oyadomari, H.P. Harding, A.G. Goodman, H. Harant, J.L. Garrison, et al. 2006. Cotranslational degradation protects the stressed endoplasmic reticulum from protein overload. Cell. 126:727–739.
Pilon, M., R. Schekman, and K. Romisch. 1997. Sec61p mediates export of a misfolded secretory protein from the endoplasmic reticulum to the cytosol for degradation. EMBO J. 16:4540–4548.
Pilon, M., K. Romisch, D. Quach, and R. Schekman. 1998. Sec61p serves multiple roles in secretory precursor binding and translocation into the endoplasmic reticulum membrane. Mol. Biol. Cell. 9:3455–3473.
Plempér, R.K., S. Bohmler, J. Bordallo, T. Sommer, and D.H. Wolf. 1997. Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation. Nature. 388:891–895.
Plempér, R.K., R. Egner, K. Kuchler, and D.H. Wolf. 1998. Endoplasmic reticulum degradation of a mutated ATP-binding cassette transporter Pdx1 proceeds in a concerted action of Sec61 and the proteasome. J. Biol. Chem. 273:32848–32856.
Ravid, T., S.G. Kreft, and M. Hochstrasser. 2006. Membrane and soluble substrates of the Dna10 ubiquitin ligase are degraded by distinct pathways. EMBO J. 25:533–543.
Schmitz, A., H. Hergen, A. Winkeler, and V. Herzog. 2000. Cholera toxin is exported from microsomes by the Sec61 complex. J. Cell Biol. 148:1203–1212.

Spear, E.D., and D.T. Ng. 2005. Single, context-specific glycans can target misfolded glycoproteins for ER-associated degradation. J. Cell Biol. 169:73–82.

Swanson, R., M. Locher, and M. Hochstrasser. 2001. A conserved ubiquitin ligase of the nuclear envelope/endoplasmic reticulum that functions in both ER-associated and Matalpha2 repressor degradation. Genes Dev. 15:2660–2674.

Taxis, C., R. Hitt, S. Park, P.M. Deak, Z. Kostova, and D.H. Wolf. 2003. Use of modular substrates demonstrates mechanistic diversity and reveals differences in chaperone requirement of ERAD. J. Biol. Chem. 278:35903–35913.

Tirosh, B., M.H. Furman, D. Tortorella, and H.L. Ploegh. 2003. Protein unfolding is not a prerequisite for endoplasmic reticulum-to-cytosol dislocation. J. Biol. Chem. 278:6664–6672.

Van den Berg, B., W.M. Clemens, I. Collinson, Y. Modis, E. Hartmann, S.C. Harrison, and T.A. Rapoport. 2004. X-ray structure of a protein-conducting channel. Nature. 427:36–44.

Vashist, S., and D.T. Ng. 2004. Misfolded proteins are sorted by a sequential checkpoint mechanism of ER quality control. J. Cell Biol. 165:41–52.

Wahlman, J., G.N. DeMartino, W.R. Skach, N.J. Bulleid, J.L. Brodsky, and A.E. Johnson. 2007. Real-time fluorescence detection of ERAD substrate retro-translocation in a mammalian in vitro system. Cell. 129:943–955.

Walter, J., J. Urban, C. Volkwein, and T. Sommer. 2001. Sec61p-independent degradation of the tail-anchored membrane protein Ubc6p. EMBO J. 20:3124–3131.

Ward, C.L., S. Omura, and R.R. Kopito. 1995. Degradation of CFTR by the ubiquitin-proteasome pathway. Cell. 83:121–127.

Wiertz, E.J., D. Tortorella, M. Bogyo, J. Yu, W. Mothes, T.R. Jones, T.A. Rapoport, and H.L. Ploegh. 1996. Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. Nature. 384:432–438.

Wuestehube, L.J., and R. Schekman. 1993. Selection and screening for yeast secretory mutants. Methods in Molecular Genetics. 1:88–106.

Ye, Y., Y. Shibata, C. Yun, D. Ron, and T.A. Rapoport. 2004. A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. Nature. 429:841–847.

Ye, Y., Y. Shibata, M. Kikkert, S. van Voorden, E. Wiertz, and T.A. Rapoport. 2005. Recruitment of the p97 ATPase and ubiquitin ligases to the site of retrotranslocation at the endoplasmic reticulum membrane. Proc. Natl. Acad. Sci. USA. 102:14132–14138.