Endoplasmic Reticulum Degradation of a Mutated ATP-binding Cassette Transporter Pdr5 Proceeds in a Concerted Action of Sec61 and the Proteasome*

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Degradation of misfolded or tightly regulated proteins in the endoplasmic reticulum (ER) is performed by the cytosolic ubiquitin-proteasome system and therefore requires their prior transport back to the cytosol. Here, we report on the extraction and degradation mechanism of a polytopic membrane protein. Rapid proteasomal degradation of a mutated form of the ATP-binding cassette transporter Pdr5 retained in the ER is initialized at the lumenal face of the ER membrane. Using different antibodies directed against the cytosolic tails or a lumenal loop of the transmembrane protein, it could be demonstrated that the turnover of Pdr5* demands the concerted action of both the Sec61 translocon and the ubiquitin-proteasome system. We observed a stabilization of the entire molecule within the ER membrane in yeast mutants characterized by a reduced translocation capacity or by functionally attenuated proteasomes. Moreover, no degradation intermediates were detected in any of the mutants that impede degradation of Pdr5*. Therefore, initial steps are rate-limiting for cleavage and mutations that impede downstream events prevent initiation of the process.

Our data suggest that ER degradation is a mechanistically highly integrated process, requiring the combined operation of components of the degradation system acting at the lumenal face of the ER membrane, the Sec61 translocon, and the ubiquitin-proteasome system.

The endoplasmic reticulum (ER) is the port of entry of membrane and secretory proteins into the central vacuolar system of all eukaryotic cells (1). It is the site at which these proteins are folded to acquire the biological function at their final destination (2). Moreover, the ER is responsible for delivering only properly folded proteins to their site of action, which requires a very efficient quality control system to recognize aberrant proteins and prevent their further transport (3–5). Improperly folded proteins are subjected to rapid proteasomal degradation after retrograde transport back to the cytosol (6, 7). This process of retrograde transport (8) requires the Sec61 translocon, as demonstrated for a mutated yeast carboxypeptidase yscY (CPY*) (9), a mutated pro-a-factor (10) and the major histocompatibility complex class I heavy chain after infection with human cytomegalovirus (11). The latter demonstrates the implication of the ER degradation system in the development of severe human diseases: in addition to human cytomegalovirus, the human immunodeficiency virus type 1 has been postulated to take advantage of this pathway to suppress the immune response of the host, thereby increasing the virulence of the infection (12, 13). Likewise, the dedifferentiation of malignant melanocytes seems to be affected by the ER degradation system (14). Moreover, in the common genetic disease cystic fibrosis a mutated form of the cystic fibrosis conductance transmembrane regulator, although otherwise functional, is retained in the ER and rapidly degraded via the proteasome (15, 16). However, the molecular basis of cystic fibrosis conductance transmembrane regulator degradation, as well as of degradation of other polytopic membrane proteins known to undergo the ER degradation (as are, for instance, HMG-CoA reductase (17) and the mutant Sec61–2 protein (18), remained a mystery: are entire transmembrane proteins extracted from the ER membrane, or are instead only cytosolic domains shaven off by the proteasome acting like a razor? Is this process generally dependent on components known to be crucial for breakdown of ER-localized proteins (7)?

Cystic fibrosis conductance transmembrane regulator and Pdr5, on which we focus in this study, are both members of the ATP-binding cassette (ABC) transporter superfamily of membrane transport proteins, operating from microorganisms to mammalian cells (19, 20). Pdr5, a protein with 12 putative transmembrane spanning domains, is localized in the plasma membrane of Saccharomyces cerevisiae (21, 22) and is crucial for the export of cytotoxic compounds, such as antifungal azoles, cycloheximide, rhodamines, and steroids, thereby mediating pleiotropic drug resistance (pdr) (23, 24). Recently, the mutant allele pdr5–26 was described, the product of which, Pdr5–C1427Y, or simply Pdr5*, is retained in the ER membrane and found to have a remarkably reduced steady-state level as compared with wild type Pdr5 localized at the plasma membrane. The mutation is predicted to reside in the luminal loop of Pdr5, most likely disturbing proper folding inside the ER (24). If Pdr5* were subjected to the ER degradation process generally dependent on components known to be crucial for breakdown of ER-localized proteins (7)?

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regulator, were discovered as to be improperly folded, removed from the ER membrane, and hydrolyzed. Here, we show that Pdr5* is indeed a substrate of the ER degradation process. This led us to uncover the mechanism by which this protein is removed from the ER membrane and degraded.

EXPERIMENTAL PROCEDURES

Construction of Strains and Plasmids—Gene expression and methods employing molecular biology were carried out using standard methods (25). The wild type strains used in this study were W303-1C (MATa ade2-1 ura3-1 his3-1, 15 leu2-3, 112 trp1-1 can1-100 prc1-1) (26) and WCGY4a (MATa his3-11, 15 leu2-3, 112 ura3 prc1-1) (8). Strains W303-1C, YRP154, and YRP175 (MATa, Δpdr5::TRP1 Δhrd3::HIS3 ade2-1 ura3-1 his3-11, 15 leu2-3, 112 trp1-1 can1-100 prc1-1), YRP205 (MATa, Δpdr5::TRP1 Δhrd3::LEU2 Δhrd7::LEU2 ade2-1 ura3-1 his3-11, 15 leu2-3, 112 trp1-1 can1-100 prc1-1), YRP175 (MATa, Δpdr5::TRP1 Δhrd3::HIS3 ade2-1 ura3-1 his3-11, 15 leu2-3, 112 trp1-1 can1-100 prc1-1), YRP193 (MATa, Δpdr5::TRP1 Δhrd3::HIS3 ade2-1 ura3-1 his3-11, 15 leu2-3, 112 trp1-1 can1-100 prc1-1), YRP210 (MATa, Δpdr5::TRP1 s61-2 ade2-1 ura3-1 his3-11, 15 leu2-3, 112 trp1-1 can1-100 prc1-1), and YRP200 (MATa, Δpdr5::TRP1 kar2-159 ade2-1 ura3-1 his3-11, 15 leu2-3, 112 trp1-1 can1-100 prc1-1) were constructed as described before (27). The plasmid MR782 was kindly obtained from Aequorea victoria.

The green fluorescent protein (GFP) of Aequorea victoria was expressed from the ER membrane, and hydrolyzed. Here, we show that Pdr5* is degraded via the ubiquitin-proteasome pathway (21, 22). Furthermore, we performed a pulse-chase analysis of Pdr5* in yeast wild type and Δpdr5 strains.

RESULTS

Pdr5* Is Degraded via the Ubiquitin-Proteasome Pathway—We first addressed the question of whether the lowered steady state level of Pdr5* is due to rapid degradation and, if so, which cellular degradation system is involved in this process. Therefore, we performed a pulse-chase analysis of Pdr5* in wild type yeast cells to determine its turnover rate (Fig. 1A). Pdr5* turned out to be unstable with an estimated halflife of approximately 18 min when cultured in 35 °C. In addition, Pdr5* was partially degraded by trichloroacetic acid precipitation of the supernatant (Fig. 1B). Furthermore, the apparent molecular mass of Pdr5* was slightly reduced from 175 kDa of wild type Pdr5 to approximately 171 kDa. Treatment with endoglycosidase F showed this to result from a different glycosylation pattern of Pdr5* and Pdr5 (Fig. 1B).

Native Pdr5 is removed from the plasma membrane by endocytosis followed by degradation within the vacuole (21, 22).

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Hence, the rapid degradation of Pdr5* suggested either acceleration of the vacuolar proteolytic pathway (32, 33) or utilization of a different degradation pathway, such as the cytosolic ubiquitin-proteasome degradation machinery (34). Cycloheximide chase studies confirmed that the degradation of Pdr5* was unaffected in a proteinase yscA pra1/pep4-deficient yeast strain, ruling out an accelerated vacuolar degradation (35, 36) (Fig. 2A). In contrast, Pdr5* was dramatically stabilized in a pre1–1 pre2–2 double mutant, which has functionally attenuated proteasomes unable to degrade several short-lived proteins (37) (Fig. 2B). In a mutant strain lacking the ubiquitin-conjugating enzymes Ubc6 and Ubc7, almost complete stabilization of Pdr5* was observed (Fig. 2C). These results demonstrate that the mutated ABC transporter is indeed degraded by a process that requires Ubc6, Ubc7, and the cytosolic proteasome. We wanted to address the question of whether polyubiquitination of Pdr5* is necessary for degradation (38). We therefore expressed the mutated ubiquitin species Ub-R48, with Lys48 being replaced by arginine (29). This prevents the formation of polyubiquitin chains and therefore inhibits hydrolysis of proteasomal substrates (38). As shown in Fig. 2D, expression of Ub-R48 led to accumulation of Pdr5*, giving direct proof for the necessity of polyubiquitination for proteolysis to occur.

Pdr5* Hydrolysis Requires Components of the ER Degradation System—In previous studies, several components of the ER degradation system were described that are crucial for the efficient degradation of soluble (e.g. CPY* (8)) and transmembrane proteins. In the current study, we focused on the role of the ER degradation system in the degradation of Pdr5*. We found that the degradation of Pdr5* requires components of the ER degradation system, specifically the ubiquitin-proteasome pathway.

Fig. 2. Degradation of Pdr5* occurs via the ubiquitin-proteasome pathway. A and B, degradation of Pdr5* was debilitated in proteasomal mutants but unaffected in cells lacking vacuolar proteolytic activity. Cycloheximide chase was performed in the isogenic strains YRP230–26 (Dpra1/pep4), YRP154–26 (wild type), and WCGY4–11/22a-26 (pre1–1 pre2–2), WCGY4a-26 (wild type). After adding cycloheximide, aliquots of cells were lysed at each time point, and the proteins contained in the membrane fractions were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting using HA epitope-specific antibodies. As a control for the amount of loaded material, the ER-resident protein Sec61 was used. C, degradation of Pdr5* was blocked in a Dubc6 Dubc7 double deletion strain. Pulse-chase analysis of the isogenic strains YRP205–26 (Dubc6 Dubc7) and YRP154–26 (wild type) was performed as described in Fig. 1A. D, expression of mutated ubiquitin R48 (Ub-R48) led to accumulation of Pdr5*. Strains W303–1C-26 and W303–1C-26/R48 expressing Ub-R48 under the control of the CUP1 promoter were incubated in the presence of 0.1 mM copper as indicated. Membrane fractions were subjected to immunoprecipitation using HA epitope-specific antibodies followed by immunodetection with the Pdr5-specific antibody Pdr5-aN1.
brane ER-localized proteins (e.g. Sec61–2p (18) and HMG-CoA reductase (17)). These prerequisites of degradation include, for example Der3/Hrd1 (30) and Hrd3 (17). 2

In order to investigate whether the rapid degradation of Pdr5* requires processes in common with the turnover of CPY*, Sec61–2p or HMG-CoA reductase, we expressed Pdr5* in mutant strains lacking the DER3/HRD1 or HRD3 gene. Pulse-chase analysis performed in a der3/hrd1 deletion strain revealed a 4-fold stabilization of Pdr5* (Fig. 3A), and Western blot with extracts from a Δhrd3 strain demonstrated a dramatic increase in the steady state level of Pdr5* (Fig. 3B). Immunofluorescence analysis of Pdr5* confirmed for both mu-

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**Fig. 3**. Proteolysis of Pdr5* requires components of the ER degradation system acting at the lumenal face of the ER membrane. A, pulse-chase analysis of strains YRP175–26 (Δder3) and YRP154–26 (wild type) was performed as described in Fig. 1A. B, functional Hrd3 is crucial for the degradation of Pdr5*. For Western analysis of YRP161–26 (Δhrd3) and YRP184–26 (wild type), proteins contained in the membrane fraction were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting using the Pdr5-specific antibody Pdr5-aN1. C, ER localization of Pdr5* was unaffected in a Δder3 mutant. For immunofluorescence analysis of strain YRP175–26 (Δder3), the Pdr5-specific antibody Pdr5-aN1 and goat anti-rabbit Cy3 antibodies were used. For fluorescence analysis of GFP-tagged Pdr5* strain YRP175–26GFP was examined. D, expression of the truncated protein Der3-ΔR lacking the lumenal RING-H2 finger motif does not complement deletion of DER3. Cycloheximide chase analysis of YRP175–26 harboring the plasmid YCpDER3AR-316 (der3ΔR), YRP175–26 (Δder3), and YRP154–26 (wild type) was performed as described in Fig. 2A using the Pdr5-specific antibodies Pdr5-aN1.
tant strains that the accumulated antigenic Pdr5* material was still ER-localized (Fig. 3C). Moreover, we expressed a carboxyl-terminal GFP-tagged version of Pdr5* in the der3Δ/ 
hrd1 strain. Likewise, this Pdr5* fusion protein exhibited perinuclear staining indicating ER localization (Fig. 3C). Based on the finding that wild type GFP-tagged Pdr5* is fully functional, we assume no further effects of the GFP tag itself on Pdr5*. In accordance with our results for Sec61–2p, the Pdr5* steady state level was unaffected in yeast cells lacking the DER1 gene required for CPY*-degradation (26). Similarly, we observed no participation of Kar2p in the degradation of Pdr5*. Moreover, ER localization of the Pdr5* protein was even further reduced when compared with the DER3/HRD1 strain. In a cycloheximide chase experiment, the mutated protein Der3-AR, lacking this domain, was expressed in a DER3/HRD1 deletion strain. In a cycloheximide chase experiment, the mutated protein Der3-AR was unable to complement the DER3/HRD1 deletion; the degradation rate of Pdr5* was even further reduced when compared with the Δder3/hrd1 strain (Fig. 3D). Because we obtained similar results for the degradation rate of CPY* and Sec61–2p even when expressing Der3-AR in DER3 wild type strains, we consider this strong stabilization of Pdr5* to be due to a dominant negative effect of the der3-ΔR allele. Based on these findings and considering the lumenal mutation of Pdr5*, we conclude that the ER degradation process of Pdr5* as a whole is initiated within the lumen of the ER, most likely at the lumenal face of the ER membrane, followed by ubiquitination and proteasomal degradation at the cytosolic face.

Membrane Extraction of Pdr5* Is Mediated by the Sec61 Channel—The complete degradation of lumenal ER or single membrane-spanning proteins by the cytosolic proteolytic system requires their dislocation into the cytosol, which is mediated by the Sec61 translocon (9–11), otherwise known as the essential channel for protein import into the ER (1).

Degradation of a multimembrane-spanning protein like Pdr5* in a Sec61p-dependent fashion would indicate retrograde transport of all transmembrane and lumenal domains back into the cytosol. To address this question, we performed pulse-chase analysis of Pdr5* in a yeast strain carrying the temperature-sensitive sec61–2 mutation (39) at 25 °C, conditions under which protein import into the ER is unaffected (9). Consistent with the results obtained for CPY* (9), the half-life of Pdr5* increased by 2.3-fold, suggesting a role of Sec61p in the degradation of the polytopic protein (Fig. 4, A and B). Therefore, membrane extraction of at least some transmembrane domains of the protein has to be assumed. Furthermore, our quantification is based on the disappearance of the antigenic material with a molecular mass of 171 kDa, representing the full-length glycosylated Pdr5* molecule. This excludes an initial cut by additional proteases within cytosolic regions of Pdr5* prior to its rapid degradation by the proteasome, because in such a case one would expect the rapid appearance of at least one degradation intermediate eventually followed by its delayed further degradation. However, we observed no such degradation intermediate of Pdr5* in our pulse-chase analysis. Also, removal of any of the cytosolic tails of Pdr5* by the proteasome prior to extraction can be excluded as the entire Pdr5* molecule is stabilized in the sec61–2 background.

To confirm our conclusion that degradation of Pdr5* requires complete membrane extraction, it was important to demonstrate a proper insertion of Pdr5* into the ER membrane in the sec61–2 background. Indeed, ER localization of the Pdr5* protein was unaffected by the sec61–2 mutation. When the GFP-tagged version of Pdr5* was expressed in the sec61–2 strain, perinuclear staining indicating ER localization was observed (Fig. 4C).

Furthermore, we determined the general topology of the molecule by analyzing the trypsin sensitivity of the antigenic material after separating cells into a pellet fraction containing the microsomes and a supernatant fraction. Wild type-like correct cytosolic localization of the amino terminus of Pdr5* in sec61–2 mutant cells could be demonstrated by trypsin treatment of the pellet fraction; the antigenic epitope disappeared (Fig. 4D). As expected, the GFP tag, also, was removed by this treatment of vesicles of sec61–2 cells, indicating proper cytoplasmic localization of the carboxyl terminus of Pdr5*. In both cases, the integrity of the prepared ER vesicles was confirmed with CPY*-specific antibodies (26).

Membrane Extraction Governs the Degradation Rate of the Entire Pdr5* Molecule—Membrane extraction seems to be rate-limiting for the degradation of Pdr5* because we could not observe any degradation intermediate in our pulse-chase and immunoblot analysis using domain-specific antibodies against the cytosolic amino terminus of Pdr5* or against the amino-terminally inserted HA tag, respectively. However, despite our findings using sec61–2 mutant cells, it remained an open question whether Pdr5* is indeed completely degraded or whether the amino terminus carrying the antigenic regions is simply cleaved off, resulting in a truncated but otherwise stable molecule. If degradation starts at the amino terminus of the molecule, one could envision that about 90% of the polypeptide remains integrated in the ER membrane, whereas only the antigenic epitope is removed by the proteasome.

If such a hypothetical degradation intermediate exits, it would perhaps be more readily detectable in cells carrying functionally impaired proteasomes, thus resulting in slower downstream processing. We therefore analyzed the GFP-tagged Pdr5* expressed in wild type and proteasomal mutant cells by immunoblotting, using specific antibodies against either GFP or the amino terminus of Pdr5*. Strikingly, both antibodies failed to detect antigenic material that was absent in mutant cells completely lacking Pdr5, suggesting that membrane extraction and subsequent degradation of Pdr5* occurs as a smooth, continuous process (Fig. 6A). Moreover, we could not observe any soluble already extracted fraction of Pdr5* even in the cytosol of pre1–1 pre2–2 mutant cells (Fig. 6A) characterized by a reduced proteasomal efficiency in the presence of fully translocation competent transloci. If Pdr5* would be extracted from the membrane but not simultaneously degraded by the proteasome, one could expect the formation of aggregates attached to the cytosolic face of the ER membrane or in the cytosol. We therefore treated the pellet fraction of pre1–1 pre2–2 mutant cells containing the antigenic material with agents...
known to dissolve protein aggregates and peripheral membrane proteins (Fig. 6B). In no case could any Pdr5* protein be found in the soluble fraction, indicating that Pdr5* remained integrated in the membrane. Only with the detergent CHAPS, which destroys membranes, could solubilization of Pdr5* be observed. These observations support a direct connection between the process of membrane extraction and the proceeding of proteolysis.
FIG. 5. Hydrolysis of Pdr5\(^*\) does not require functional Kar2p, and degradation of Pdr5\(^*\) is unaffected in a kar2-159 mutant strain. Cycloheximide chase of YRP200–26 (kar2-159) and YRP154–26 (wild type) was performed as described in Fig. 2A. After incubation at the permissive temperature of 19.5 °C, equal amounts of cells were treated with cycloheximide followed by an immediate shift to the restrictive temperature of 32 °C for 90 min. Aliquots of cells were taken at the indicated time points. For comparing soluble and transmembrane proteins subjected to ER degradation, the turnover of CPY\(^*\) was analyzed.

FIG. 6. Membrane extraction and degradation of Pdr5\(^*\) are inseparable. A, Western analysis of the amino and carboxyl termini of Pdr5\(^*\) revealed no degradation intermediate in proteasomal mutant WCGY4–11/22a-26GFP (pre1–1 pre2–2) or in YRP154–26GFP (wild type). Likewise, no soluble fraction of Pdr5\(^*\) could be detected in the cytosol. Immunoblotting was performed using GFP-specific antibodies. B, Pdr5\(^*\) accumulates integrated in the membrane. Microsomes prepared from pre1–1 pre2–2 mutant cells were treated with buffer, 2.5 M urea, 0.8 M potassium acetate, 0.1 M Na\(_2\)CO\(_3\) pH 11.6, or 1% CHAPS, and degradation of Pdr5\(^*\) is unaffected in a kar2–159 mutant cells. Microsomes treated with buffer, 2.5 M urea, 0.8 M potassium acetate, 0.1 M Na\(_2\)CO\(_3\) pH 11.6, or 1% CHAPS, and soluble (S) and pellet (P) fractions were analyzed with Pdr5-specific antibody Pdr5\(-\alpha\)N1. Only detergent is able to solubilize Pdr5\(^*\). Other protein-solubilizing treatments had no effect. C, following the fate of the carboxyl terminus of Pdr5\(^*\), no breakdown product could be detected. Pulse-chase analysis of strain YRP154–26GFP was performed as described in Fig. 1A using GFP-specific antibodies.

We further examined the degradation of GFP-tagged Pdr5\(^*\) in wild type cells by pulse-chase analysis performed at 25 °C. Using antibodies directed against GFP for immunoprecipitation of the antigenic material, again, no degradation intermediates were detected (Fig. 6C). Moreover, the rate of degradation was similar to that seen when following the fate of the Pdr5\(^*\) amino terminus (Fig. 4B, wild type). Consistent with the pulse-chase data, we also noticed a complete disappearance of the green fluorescence within a period of 90 min after adding cycloheximide. Therefore, the chromophore itself must have been degraded, excluding a simple masking of the antigenic epitopes in our pulse-chase and immunoblot experiments.

Even considering our findings about the similar turnover of both cytosolic tails of Pdr5\(^*\), it remained to be shown that the luminal and transmembrane domains of Pdr5\(^*\) are degraded via the same pathway. To address this question, we generated an antibody (Pdr5-\(\alpha\)L6) directed against a peptide localized within the last luminal loop of Pdr5\(^*\). We then compared the degradation of Pdr5\(^*\) in a cycloheximide chase analysis in a wild type and a pra1/pep4-deficient strain by reprobing the immunoblots either with the Pdr5-specific antibodies Pdr5-\(\alpha\)N1 (directed against the amino terminus) or Pdr5-L6 (directed against the sixth luminal loop) (Fig. 7). With each antibody, even at time zero, representing the steady state level, no degradation intermediates could be observed, indicating the entire breakdown of the whole Pdr5\(^*\) molecule by the ubiquitin-proteasome system. Moreover, in a \(\Delta\)pra1/pep4 strain lacking all vacuolar proteolytic activity, no antigenic material representing a truncated version of Pdr5\(^*\) without cytosolic tails could be detected. However, one would expect the appearance of such an intermediate with a molecular mass of approximately 120 kDa in a vacuolar mutant if the luminal and transmembrane domains would be transported into the vacuole after removal of the cytosolic tails.

FIG. 7. Breakdown of the entire Pdr5\(^*\) molecule occurs without initial cuts. Cycloheximide chase analysis of YRP154–26 (wild type) and for control YRP230–26(\(\Delta\)pra1/pep4) was performed as described in Fig. 2A. As indicated, the immunoblots were consecutively reprobed with the Pdr5-specific antibodies Pdr5-\(\alpha\)N1 and Pdr5-\(\alpha\)L6 directed against a peptide located in the last luminal loop of Pdr5.

Pdr5\(^*\) amino terminus (Fig. 4B, wild type). Consistent with the pulse-chase data, we also noticed a complete disappearance of the green fluorescence within a period of 90 min after adding cycloheximide. Therefore, the chromophore itself must have been degraded, excluding a simple masking of the antigenic epitopes in our pulse-chase and immunoblot experiments.

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**DISCUSSION**

Here, we report that the plasma membrane localized ABC transporter Pdr5, when mutated (Pdr5-C1427Y), is retained in the ER membrane and degraded via the ubiquitin-proteasome system. Our studies on Pdr5\(^*\) give indication on the mechanism of how a polytopic membrane protein is removed from the ER membrane and delivered to proteasomal proteolysis. The fact that under no conditions any Pdr5\(^*\) intermediate can be detected leads us to conclude that membrane extraction and complete degradation of Pdr5\(^*\) are inseparable, taking place in a concerted action of the Sec61-translocon and the ubiquitin-proteasome system. The stabilization of Pdr5\(^*\) by the sec61-2 mutation is in the same magnitude as found for CPY\(^*\) (9).
Because the entire molecule is stabilized in a sec61–2 background, its degradation must be tightly linked to the progress of membrane extraction. We consider it unlikely that the retarded degradation of Pdr5* in sec61–2 mutants is due to import defects resulting from the mutated translocon. (i) Testing the import of a variety of proteins in the sec61–2 mutant under conditions used in this study (25 °C) did not exhibit any precursor accumulation, which would be expected for a defective import system (9). (ii) The sec61–2 protein is itself a substrate of the ER degradation machinery (18). Components necessary for ER degradation as for instance Der3p are synthesized at wild type levels in a sec61–2 mutant (data not shown). (iii) Most importantly, deletion of Der3p results in suppression of the temperature-sensitive phenotype of sec61–2 cells; sec61–2 Δder3 mutant cells are able to grow at 38 °C (30). This proves that the ER degradation system is fully functional in a sec61–2 mutant, even at the restrictive temperature of 38 °C.

No extracted cytosolic or membrane attached fraction of Pdr5* could be detected in pre1–1 pre2–2 mutant or wild type cells. This indicates a feedback regulatory loop, inhibiting membrane extraction in case of an impaired proteolysis and thereby inhibiting accumulation of secretory proteins within the cytosol prior to their degradation. With this mechanism, the cell would avoid the accumulation of possibly toxic protein aggregates or breakdown intermediates. Furthermore, we observed that deletion of the ER luminal RING-H2 finger domain of Der3/Hrd1 abolishes Pdr5* hydrolysis. This demonstrates that ER degradation of the polytopic protein is indeed an ER-dependent process. Initial steps, such as recognition of the malfolded protein and its retention and targeting to the translocon, seem to occur within the ER lumen. Consequently, degradation of both soluble proteins (such as CPY*) and transmembrane proteins (such as Pdr5*, Sec61–2p, and HMG-CoA reductase) is dependent on the same core components of the ER degradation machinery acting at the luminal face of the ER membrane. e.g. Der3/Hrd1 and Hrd3. Because the sec61–2 mutation leads to a reduced degradation rate of CPY* (9) and Pdr5*, the Sec61 channel seems to be crucial for both retrograde transport and membrane extraction. The mechanism of reentering the translocon may, however, differ between soluble and transmembrane proteins, as only the former requires the permeability barrier of the ER membrane (40). Therefore retrograde transport of ER lumenal, soluble proteins should require some action of Kar2p to open the channel at the luminal side. One could imagine that this does not require active participation of Kar2p. This model would also imply that Kar2p does not participate in the unfolding of proteins prior to their retrograde transport.

Once initiated, reverse transport seems to be a continuous process clearly dependent upon the action of the ubiquitin-conjugating enzymes Ube6 and Ubc7 and the proteasome. The driving force for the actual dislocation event could be provided by the ATPases of the 19S cap of the 26S proteasome itself (34, 38). Alternatively, it is possible that additional cytosolic chaperones may act in concert with the ubiquitin-conjugating enzymes and the proteasome in membrane extraction. In such a case, the activity of these chaperones, however, should be linked to the progress of proteolysis.

After our work had been completed, Mayer et al. (41) published membrane extraction of the ER-resident membrane protein Sec62 onto which the authors had fused the Deg1 domain of the MATa2 repressor. This domain targets proteins for degradation by the ubiquitin-conjugating enzymes Ube6 and Ubc7 and the proteasome (42). In case of Mayer et al. (41), mutants with impaired proteasomes did not stabilize the full-length Sec62 fusion protein but accumulated degradation intermediates. The authors did not show whether the translocon or known ER components of the ER degradation process are involved in extraction of their hybrid protein. Moreover, the authors only followed the fate of carboxyl-terminal cytosolic tags attached to their Sec62 fusion protein. Therefore, the possibility cannot be excluded that only the cytosolic portions of the fusion protein are shved off by the proteasome, leaving behind the luminal loop and the two transmembrane domains, which might remain in the ER. From their data, Mayer et al. (41) conclude that the proteasome initiates membrane extraction. We did not find any breakdown intermediates of Pdr5* in proteasome mutants when following the N- or carboxyl-terminal tail of our protein or even a luminal loop (Figs. 2B, 6, and 7). We show that full-length Pdr5* remained stabilized in proteasomal mutants and in mutants carrying a defective translocon (Figs. 2B and 4A). These differences may be due to the fact that Pdr5*, due to its luminal mutation, is actively retained in the ER and selected for degradation by the quality control system of the ER, whereas the nonmutated Sec62 protein studied by Mayer et al. (41) is targeted for degradation by the cytosolic Deg1 domain independently of the ER quality control. The Deg1 domain initiates proteasome directed proteolysis of every protein studied so far.

We propose that the ER degradation process of soluble luminal and polytopic membrane proteins is a vectorial event, which is initiated and directed from the ER lumen via the Sec61 channel to the cytosolic proteasome. Considering the close evolutionary relationship of all members of the ABC transporter superfamily, we speculate that further details about the mechanisms of the retention, membrane extraction, and degradation of Pdr5* will greatly improve our knowledge about the molecular cause of a severe human disease, cystic fibrosis, linked to the ER degradation process.

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