Sec61p Is Required for ERAD-L

GENETIC DISSECTION OF THE TRANSLLOCATION AND ERAD-L FUNCTIONS OF SEC61P USING NOVEL DERIVATIVES OF CPY*1

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Misfolded proteins in the endoplasmic reticulum (ER) are exported to the cytosol for degradation by the proteasome in a process known as ER-associated degradation (ERAD). CPY* is a well-characterized ERAD substrate whose degradation is dependent upon the Hrd1 complex. However, although the functions of some of the components of this complex are known, the nature of the protein dislocation channel remains obscure. Sec61p has been suggested as an obvious candidate because of its role as a protein-conducting channel through which polypeptides are initially translocated into the ER. However, it has not yet been possible to functionally dissect any role for Sec61p in dislocation from its essential function in translocation. By changing the translocation properties of a series of novel ERAD substrates, we are able to separate these two events and find that functional Sec61p is essential for the ERAD-L pathway.

Perturbations in protein biogenesis in the ER2 can lead to the accumulation of misfolded proteins with potentially catastrophic cytotoxic consequences. The ERAD quality control system identifies aberrant proteins and targets them for destruction. This disposal mechanism involves misfolded proteins being “dislocated” across the ER membrane to the cytosol where they are ubiquitinated before being delivered to the proteasome for degradation (1).

Three distinct ERAD pathways can be distinguished according to the topology of the misfolded lesion. ER luminal proteins are degraded via the ERAD-L pathway, as are integral membrane proteins with lesions in a luminal domain. Membrane proteins with cytosolic lesions are degraded by the ERAD-C pathway (2), whereas those with misfolded transmembrane domains are degraded by the ERAD-M pathway (3, 4). All three pathways require the cytosolic Cdc48p-Ufd1p-Npl4p complex, which delivers ubiquitinated substrates to the proteasome anchor, Cue1p. The ubiquitinated substrate is then passed to the Cdc48p-Ufd1p-Npl4p complex, which is itself anchored to the membrane by the Ubx2p receptor (1).

The identity of the protein dislocation channel has been the subject of considerable debate. Derlin-1, a recently identified human homologue of yeast Der1p has been suggested to form a pore in the ER membrane through which unfolded ERAD substrates are exported for degradation (5, 6). Yeast Der1p is an integral membrane protein required for the degradation of ERAD-L substrates including CPY*, KHN, and KWW (2, 7), but the molecular function of Der1p/Derlin-1 is not yet known. In yeast, Der1p is not essential for viability, but its widespread conservation does suggest an important role in eukaryotes.

Another candidate for the dislocation channel is Sec61p, which is a core component of the translocation channel through which proteins are imported into the ER (8). A number of observations support a role for Sec61p in ERAD including its association with a variety of ERAD substrates (9–11) plus the intriguing observation that proteasomes interact with the Sec61 complex both in vivo and in vitro (12). Studies in yeast have shown that the degradation of an unfolded mutant form of alpha factor is reduced in a cell free assay using microsomes from various sec61 mutant strains (13), whereas the degradation of CPY* is delayed in sec61-2 mutant cells (14). However, the interpretation of these data is complicated by the fact that the sec61 mutant alleles examined were also defective in the initial translocation of both alpha factor and CPY*.

In this paper we employ the sec61-3 mutant, which has a cold-sensitive defect in the signal recognition particle (SRP)-dependent co-translational translocation pathway. We therefore engineered two novel SRP-dependent derivatives of CPY*, one integral membrane form and one soluble, and examined their translocation and ERAD properties. We demonstrate that Sec61p is required for ERAD of these novel substrates in a manner that is independent of any effect on translocation.

EXPERIMENTAL PROCEDURES

Yeast Strains—The strains used in this study are listed in Table 1. Yeast strains were grown in either YPD medium (2% peptone, 1% yeast extract, 2% glucose) or minimal medium.
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(0.67% yeast nitrogen base, 2% glucose, appropriate supplements) at temperatures required for individual experiments (17, 24, 30, or 37 °C). Analysis of plasmid-borne forms of CPY*, DPY*, and OPY* were performed in strains lacking any endogenous CPY (prc1::KANMX). To make strains of the required genotypes, we re-engineered the sec61-3 allele in target strains in the following way. The sec61-3 mutation was introduced into pBW11 (15) by site-directed mutagenesis with Primers 1 and 2 generating plasmid pCW11 Table 2. A 2.4-kb KpnI-PstI fragment was cloned from pMW339 into pMW319 to generate pMW342. To be able to make strains of the required genotypes, we decided to regener-
membrane protein with an amino-terminal signal anchor domain whose targeting is SRP-dependent (23). Ost1p is a type I integral membrane protein with a cleavable signal sequence (24) whose hydrophobicity led us to predict a likely dependence upon SRP.

**ER Translocation of DPY* and OPY** — When expressed in wild type cells, both DPY* and OPY* were efficiently translocated and glycosylated, indicating that their respective ER targeting signals are functional. Our hypothesis predicted that DPY* and OPY* would translocate efficiently in sec61-3 cells at 30 °C, and this proved to be the case (Fig. 1C). Interestingly, these proteins also translocate well at 17 °C, suggesting that the SRP-dependent targeting of a polypeptide that is competent for post-translational import can overcome the sec61-3 translocation defect. This finding suggested that the translocation defect in sec61-3 cells might be kinetic in nature, and this has been confirmed by pulse-chase analysis (see supplemental Fig. 1). To confirm that DPY* and OPY* were being targeted via the SRP-dependent pathway, we also examined their translocation in sec65-1 cells, which express a temperature-sensitive form of SRP (23). As expected, preproCPY* translocation is unaffected in sec65-1 cells with only the ER glycosylated form of proCPY* being evident at either 24 or 37 °C. In contrast, DPY* and OPY* translocate efficiently at 24 °C but accumulate as precursor forms at 37 °C (Fig. 1D). These results demonstrate that DPY* and OPY* require functional SRP for their ER targeting. A further characteristic of a genuinely SRP-dependent precursor is that it does not depend on Sec62p for translocation. sec62-1 cells have defects in post-translational translocation at their permissive temperature (24 °C) and are temperature-sensitive for growth at 37 °C (25). We therefore examined translocation of DPY* and OPY* in sec62-1 cells. A profound defect in translocation of CPY* was observed at both 24 and 37 °C, but DPY* and OPY* translocation were unaffected (Fig. 1D). We therefore conclude that unlike CPY*, both DPY* and OPY* translocate via the co-translational SRP-dependent pathway. Most importantly, the sec61-3 mutation has no detectable effect on translocation of these new precursors.

**Membrane Association of DPY* and OPY** — The signal peptide of preproCPY* is cleaved during translocation by signal peptidase. OPY* was predicted to be similarly cleaved, whereas DPY* was predicted to insert into the bilayer as an integral membrane protein. To test these predictions we used carbonate extraction of microsomes to examine the membrane association of the various proteins. We found that DPY* behaved as an integral membrane protein, whereas both CPY* and OPY* were readily extracted by carbonate (Fig. 2). We therefore conclude that OPY* is soluble, whereas DPY* is membrane associated. DPY* and OPY* thus behave entirely differently from CPY* with

![FIGURE 2. Membrane association of novel derivatives of CPY*. Microsomes from wild type cells expressing CPY*, DPY*, or OPY* were treated with Na2CO3 as described under "Experimental Procedures." Total (T), pellet (P), and supernatant (S) fractions were analyzed by Western blots using antibodies against CPY, Kar2p, or Sec61p as indicated.](image-url)
regards to their mode of translocation and differently from one another with regards to their solubility/membrane association properties.

**DPY** and **OPY** Are Subject to Der1p-dependent ERAD-L—
Next we examined whether the translocated forms of DPY* and OPY* were subject to ERAD. Wild type cells expressing CPY*, DPY*, or OPY* were subjected to pulse-chase studies demonstrating that all three substrates degraded with similar kinetics (Fig. 3). Signal cleaved OPY* behaved as a lumenal protein in our carbonate extraction studies, and so one might expect that it would be a substrate for the Der1p-dependent ERAD-L pathway (7). This was confirmed by pulse-chase studies in \( /H9004 \) der1 cells in which the rate of OPY* degradation was indistinguishable from that of CPY* (Fig. 4). DPY* shares the same lumenal lesion as CPY*/OPY* but in a membrane-tethered form. Recent studies suggest that ERAD-M can supersede the ERAD-L pathway for substrates that have lesions in both a membrane anchor and a lumenal domain (26, 27); thus any lesion in the DPY* membrane anchor might have led to degradation via the Der1p-independent ERAD-M pathway. However, our data demonstrate that DPY* degradation requires Der1p (Fig. 4) and so conclude that this degradation occurs as a result of the lumenal lesion in this protein. Finally we found that the rates of degradation of DPY* and OPY* were unaffected in \( /doa10 \) cells (data not shown), confirming that neither was dependent on the

**FIGURE 3.** **DPY** and **OPY** are substrates for ERAD in wild type cells. A, wild type cells expressing CPY*, DPY*, or OPY* were pulse-labeled with \([35S]\)methionine as described under “Experimental Procedures.” After the addition of cold methionine (time 0), the samples were taken at 30 min intervals, whole cell extracts were subjected to immunoprecipitation with anti-CPY antibodies, and labeled proteins were analyzed by SDS-PAGE. B, quantification of data shown in A.

**FIGURE 4.** ERAD of DPY* and OPY* is Der1p-dependent. A, wild type and \( \Delta \)der1 cells expressing CPY*, DPY*, or OPY* were analyzed as in Fig. 3A. B, quantification of data shown in A.

**FIGURE 5.** ERAD of DPY* and OPY* is unaffected in sec61-3 cells at 30 °C. A, pulse-chase experiment using wild type and sec61-3 cells at 30 °C as described in Fig. 3A. B, quantification of data shown in A.
ERAD-C pathway (28). We therefore conclude that DPY* and OPY* are efficiently degraded via the ERAD-L pathway.

**ERAD of DPY* and OPY* Requires Functional Sec61p**—Next we sought to examine any requirement for Sec61p. We have earlier shown that DPY* and OPY* can be efficiently translocated into the ER in sec61-3 mutant cells at 30 °C. We therefore tested whether these translocated substrates were competent for ERAD under these same conditions. Wild type or sec61-3 mutant cells expressing either DPY* or OPY* were labeled at 30 °C, and samples taken at various time points during a chase performed at the same temperature. We found the half-life of both DPY* and OPY* in sec61-3 cells to be indistinguishable from that in wild type cells under these conditions (Fig. 5). These results demonstrate that DPY* and OPY* are available for ERAD after translocation at 30 °C in sec61-3 cells and that their degradation occurs with essentially wild type kinetics.

Next we tested for any effect on ERAD following inactivation of the Sec61p-dependent translocase at 17 °C. The ER was preloaded with ERAD-competent substrate by pulse labeling at 30 °C in either wild type or sec61-3 mutant cells. The cells were then shifted to 17 °C and chased in the presence of unlabeled methionine for the times indicated (Fig. 6, A and B). We observed a dramatic increase in the stability of both substrates in sec61-3 cells when compared with wild type controls. Thus functional Sec61p is required for the ER-associated degradation of both DPY* and OPY*.

It remains formally possible that the severe translocation phenotype associated with sec61-3 at 17 °C might lead to an indirect effect on ERAD, perhaps by blocking the import of some essential factor. To rule this out we first examined ERAD in sec62-1 mutant cells in which post-translational translocation is specifically blocked but found no significant difference in the rate of degradation of either substrate when compared with wild type controls (Fig. 6C). Thus ERAD of these novel substrates does not require ongoing post-translational translocation. Of course sec61-3 cells are also deficient in co-translational translocation at 17 °C. We therefore tested the effects on our ERAD substrates of a complete block in all protein translocation by treating wild type cells with cycloheximide to inhibit protein synthesis. The cells were pulse-labeled at 30 °C and then chased at either 30 or 17 °C in the presence or absence of cycloheximide (Fig. 6D). We found no delay in the degradation of DPY* in the presence of drug. Because protein synthesis is not required for ERAD, it naturally follows that ongoing co-translational import of factors into the ER cannot be required. This is consistent with numerous studies in which ERAD has been observed in cells treated with cycloheximide (29).

**DISCUSSION**

A variety of studies implicate Der1p in ERAD, but its precise function remains unknown. It is clearly required for the degradation of a range of misfolded luminal proteins including CPY* (7). In contrast, the majority of membrane proteins tested appear to be degraded independently of Der1p (2, 26, 30, 31). One notable exception is the type I integral membrane protein, KWW, whose degradation is defective in der1 mutant cells (2). The misfolded lesion in KWW is located within its luminal domain leading to the suggestion that Der1p is required for the degradation of substrates with misfolded luminal domains via a pathway now known as ERAD-L (2).

A number of studies have similarly implicated Sec61p in ERAD, but the role of Sec61p in the initial translocation of such substrates into the ER complicates the analysis of degradation kinetics. We have sought to temporally separate the known role of Sec61p in translocation from any subsequent role in ERAD by exploiting the properties of the sec61-3 mutant. We have created two new ERAD substrates, both of which are imported into the ER in an SRP-dependent manner. The first, DPY*, inserts into the ER membrane as a type II integral membrane protein, whereas the second, OPY*, is subject to signal peptide cleavage and is released into the ER lumen. Both DPY* and OPY* are substrates for Der1p-dependent ERAD and so, like...
CPY*, must be substrates for the ERAD-L pathway. As expected, both DPY* and OPY* were efficiently translocated in sec61-3 cells at the permissive temperature of 30 °C. These translocated forms of DPY* and OPY* were evidently available to the ERAD machinery because both were degraded with kinetics that were indistinguishable from those observed in wild type cells. This allowed us to load the ER in sec61-3 cells with ERAD-competent substrate and to then inactivate Sec61p by shifting cells to 17 °C. Our data demonstrate that the inactivation of sec61-3p at 17 °C results in a rapid and substantial block in the ERAD of both DPY* and OPY*. This cannot be explained by some indirect effect of a block in sec61-3-dependent protein import because neither sec62-1 nor cycloheximide treatment had any similar effect on ERAD.

Our results demonstrate that functional Sec61p is essential for degradation of ERAD-L substrates. This finding supports a model in which Sec61p forms a bi-directional protein-conducting channel for the transport of polypeptide chains both into and out of the lumen of the endoplasmic reticulum. It will be interesting to determine whether or not different accessory factors might engage with Sec61p to determine the directionality of transport.

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