

Sec61p is part of the endoplasmic reticulum-associated degradation machinery

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Endoplasmic reticulum-associated degradation (ERAD) is a cellular pathway for the disposal of misfolded secretory proteins. This process comprises recognition of the misfolded proteins followed by their retro-translocation across the ER membrane into the cytosol in which polyubiquitination and proteasomal degradation occur. A variety of data imply that the protein import channel Sec61p has a function in the ERAD process. Until now, no physical interactions between Sec61p and other essential components of the ERAD pathway could be found. Here, we establish this link by showing that Hrd3p, which is part of the Hrd-Der ubiquitin ligase complex, and other core components of the ERAD machinery physically interact with Sec61p. In addition, we study binding of misfolded CPY* proteins to Sec61p during the process of degradation. We show that interaction with Sec61p is maintained until the misfolded proteins are ubiquitinated on the cytosolic side of the ER. Our observations suggest that Sec61p contacts an ERAD ligase complex for further elimination of ER luminal misfolded proteins.

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Introduction

The occurrence of mistakes is inherent in biological systems. Misfolding of proteins is frequent and has deleterious effects in cells (Aridor and Hannan, 2000; Dobson, 2003). Therefore, protein quality control and elimination pathways have evolved. One such elimination pathway is connected with the endoplasmic reticulum (ER), the organelle responsible for import, folding and distribution of secretory proteins to their site of action. This pathway, termed as ER-associated degradation (ERAD), comprises the retro-translocation of misfolded proteins across the ER membrane into the cytosol, their polyubiquitination and proteasomal degradation (Plemper and Wolf, 1999; Kostova and Wolf, 2003; Meusser *et al.*, 2005; Römisch, 2005; Nakatsukasa and Brodsky, 2008; Schäfer *et al.*, 2008; Vembar and Brodsky, 2008). Depending on the localization of the misfolded domain, proteins are

degraded through different ERAD pathways (Vashist and Ng, 2004; Carvalho *et al.*, 2006; Nakatsukasa and Brodsky, 2008). All these pathways have in common that they require the ubiquitin conjugating enzyme Ubc7p and a complex consisting of the AAA-ATPase Cdc48p, Ufd1p and Npl4p as well as the proteasome for elimination of misfolded proteins. Proteins carrying cytosolic misfolded domains or degradation signals are degraded by the ERAD-C (cytosolic) pathway, which depends in addition on the ER membrane-located ubiquitin ligase Doa10p (Huyer *et al.*, 2004; Vashist and Ng, 2004). The central component of the ERAD-L (lumen) and ERAD-M (membrane) pathway is the Hrd-Der ubiquitin ligase complex of the ER membrane. This complex is necessary for the recognition and ubiquitination process of proteins carrying misfolded domains in the lumen or in the membrane of the ER (Vashist and Ng, 2004; Carvalho *et al.*, 2006). It consists of the ubiquitin ligase Der3p (also known as Hrd1p), which exposes a RING finger domain to the cytoplasm (Bays *et al.*, 2001; Deak and Wolf, 2001) and of Hrd3p carrying a long N-terminal domain in the ER lumen (Hampton *et al.*, 1996; Plemper *et al.*, 1999a). Recognition of misfolded ERAD-L glycoproteins includes binding to Hrd3p assisted by the chaperone Kar2p and the lectin Yos9p in the ER lumen (Kim *et al.*, 2005; Denic *et al.*, 2006; Gauss *et al.*, 2006a). Thereafter, the misfolded proteins are transported into the cytosol, which requires transfer across the ER membrane and their subsequent polyubiquitination by the Der3p ubiquitin ligase (Hiller *et al.*, 1996; Bordallo *et al.*, 1998). In the cytosol, the AAA-ATPase Cdc48p and its substrate recruiting factors, Ufd1p and Npl4p, provide the driving force for final extraction of polyubiquitinated misfolded proteins from the ER membrane (Bays *et al.*, 2001; Ye *et al.*, 2001; Braun *et al.*, 2002; Jarosch *et al.*, 2002; Rabinovich *et al.*, 2002). Thus, the ligase Doa10p and the Hrd-Der ligase complex in conjunction with the Cdc48 complex are central parts of the ER retro-translocation machineries.

The discussion of the retro-translocation channel

The nature of the retro-translocation channel is still under discussion. Several data point at the ER membrane proteins Derlin-1 and Sec61p as possible candidates for the channel-forming protein for retro-translocation. The data concerning Derlin-1 as a candidate were mostly obtained from studies in mammalian cells. In cytomegalovirus-infected mammalian cells, newly synthesized, ER membrane-localized MHC class I molecules are re-directed into the cytosol and thereby interact with p97 and Derlin-1 (Lilley and Ploegh, 2004; Ye *et al.*, 2004), the mammalian homologues of yeast Cdc48 and Der1p, respectively (Knop *et al.*, 1996). This finding led to the proposal that Derlin-1 is a central component of the retro-translocation channel. Furthermore, data obtained by an *in vitro* fluorescence approach have also shown an involvement of Derlin-1 in retro-translocation without the participation of mammalian Sec61 α (Wahlman *et al.*, 2007). These observations are in contrast to an earlier study that favoured

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Sec61p as the retro-translocon (Wiertz *et al*, 1996). Indeed, due to its function as the import channel into the ER, Sec61p was early shortlisted as a possible retro-translocation channel for misfolded proteins. In yeast, indications for a role of Sec61p in ERAD are based on a variety of genetic data. Conditional, temperature-sensitive *sec61* mutant strains showed a disturbed degradation of the misfolded ER-model substrate CPY*, derivatives of CPY* and of mutated pro- α -factor (Pilon *et al*, 1997, 1998; Plemper *et al*, 1997; Willer *et al*, 2008). Genetic interaction studies also point to an involvement of Sec61p in the ERAD process (Plemper *et al*, 1999a). An additional connection with ER-associated proteolysis is indicated by the fact that Sec61p interacts with the 19S sub-complex of the proteasome (Ng *et al*, 2007). All these data reflect a participation of Sec61p in retro-translocation of misfolded proteins in yeast, but so far, no interaction of Sec61p with known core components of the ERAD machinery could be detected.

In this work, we investigated the physical interactions of Sec61p with substrates and components of the ERAD-L pathway. We show the interaction of Sec61p with Hrd3p and other ERAD core components. We show that fully glycosylated and, therefore, completely imported soluble ERAD substrates bind to Sec61p. Interestingly, deletions of Hrd3p or Der3p lead to an enhanced binding of the ERAD substrate CPY* to Sec61p. This indicates that the association of CPY* to Sec61p is retained until ubiquitination of the misfolded proteins on the cytosolic side of the ER takes place. Our data provide strong evidence that Sec61p is a physical part of the ERAD machinery for further transport and subsequent degradation of misfolded ER luminal proteins.

Results

Sec61p is associated with core components of the ERAD-L pathway

Any participation of Sec61p in the degradation of misfolded proteins should require an association with the ERAD machinery. Therefore, we wanted to know whether any physical contact between components and substrates of the ERAD pathway and Sec61p exists. To address this question, we isolated the ERAD component Hrd3p, which is part of the Hrd-ubiquitin ligase complex, and analysed it for the presence of Sec61p. We precipitated functional aminoterminally HA-tagged Hrd3p (HA₃Hrd3p) from digitonin-solubilized microsomal extracts using specific α -HA antibodies. As Hrd3p is an ER resident membrane protein, one may assume that it is inserted into the membrane through the Sec61p translocon during import. To exclude that any detectable interaction between Sec61p and ER membrane proteins occurs during the import process, we blocked new protein synthesis for 2 h with cycloheximide (CHX) before immunoprecipitation. This treatment allows complete transfer of already existing polypeptides into the ER. In immunoprecipitates of HA₃Hrd3p without addition of CHX to cells (time zero), we observed an association of Sec61p with HA₃Hrd3p (Figure 1A). The association also exists after 2 h CHX treatment (Figure 1A), even though the amount of Sec61p is somewhat reduced after 2 h (Figure 1B). The continued presence of the Sec61p-Hrd3p interaction even after 2 h of halt of protein synthesis points to a functional association of both proteins independent from Hrd3p import. Besides Sec61p, we detected already known

interaction partners of Hrd3p such as the ubiquitin ligase Der3p as well as Cdc48p (Figure 1A). The amount of Der3p interacting with Hrd3p after 2 h of CHX addition is only marginally reduced, indicating a rather stable interaction over time (Figure 1B). Interestingly, association of Cdc48p with the Hrd complex is decreased after 2 h CHX treatment of the cells. This observation can be explained by the fact that Cdc48p is in a dynamic relation to the Hrd complex and seems to be removed from the ER membrane for delivery of ubiquitinated proteins to the proteasome (Alberts *et al*, 2009). We observed a similar decrease in the Sec61p-Hrd3p contact (Figure 1A and B), indicating that this interaction might also be a dynamic process. As expected, we also found the misfolded ERAD-L substrate CPY* interacting with HA₃Hrd3p in cells without CHX treatment. However, after 2 h of CHX addition to cells, CPY* is completely degraded, whereas Sec61p is still associated with HA₃Hrd3p (Figure 1A). The association might be mediated by further interacting proteins or by endogenous misfolded proteins still present in the ER.

To ensure that the observed interaction of HA₃Hrd3p with Sec61p has not arisen from an unspecific binding of Sec61p to ER membrane proteins, we immunoprecipitated dolichol phosphate mannose synthase (Dpm1p) and analysed for Sec61p interactions. Dpm1p is a stable ER membrane protein with one transmembrane domain similar to Hrd3p. Only insignificant Sec61p background signals were immunoprecipitated with α -Dpm1p (Figure 1A). Similarly, insignificant Sec61p signals were found in α -HA precipitates of wild-type cells confirming the specificity of the applied immunoprecipitation approach. To exclude the possibility of artificial interactions of Sec61p to other proteins during membrane solubilization and precipitation, we performed the following experiment. Cells of two different strains expressing either Sec61p-myc and endogenous Hrd3p or untagged Sec61p and HA₃Hrd3p were mixed. Thereafter, membrane proteins were solubilized and subjected to immunoprecipitation as described before followed by immunoblot analysis. Although endogenous Sec61p was bound to HA₃Hrd3p, no Sec61p-myc was present in the precipitates (Figure 1C). This excludes that the applied experimental procedure results in isolation of Sec61p interactions due to formation of an artefact.

Sec61p loses contact to misfolded proteins during the degradation process

To characterize the contact between Sec61p and a misfolded protein during its degradation at the ER membrane, the ERAD-L substrate CPY*HA₃ was used as a bait. In yeast cells, several pools of CPY* proteins exist, localized at different positions at the ER membrane. CPY* contacts Sec61p during the import step, is quickly recognized as misfolded and thereafter bound to ERAD components on the ER luminal side. An additional pool of CPY* could reside in the retro-translocation channel and in a partly ubiquitinated form on the cytosolic side, but still in contact with the ER membrane. To enrich the endogenous amount of CPY* in the stage of misfolding and retro-translocation, cells were treated for 30 min with CHX. This treatment still allows import of CPY* into the ER, which is completed within 5 min, whereas protein degradation by the ERAD pathway continues (Taxis *et al*, 2002). As the half-life of misfolded CPY* is about 20 min (Hiller *et al*, 1996), all CPY* molecules

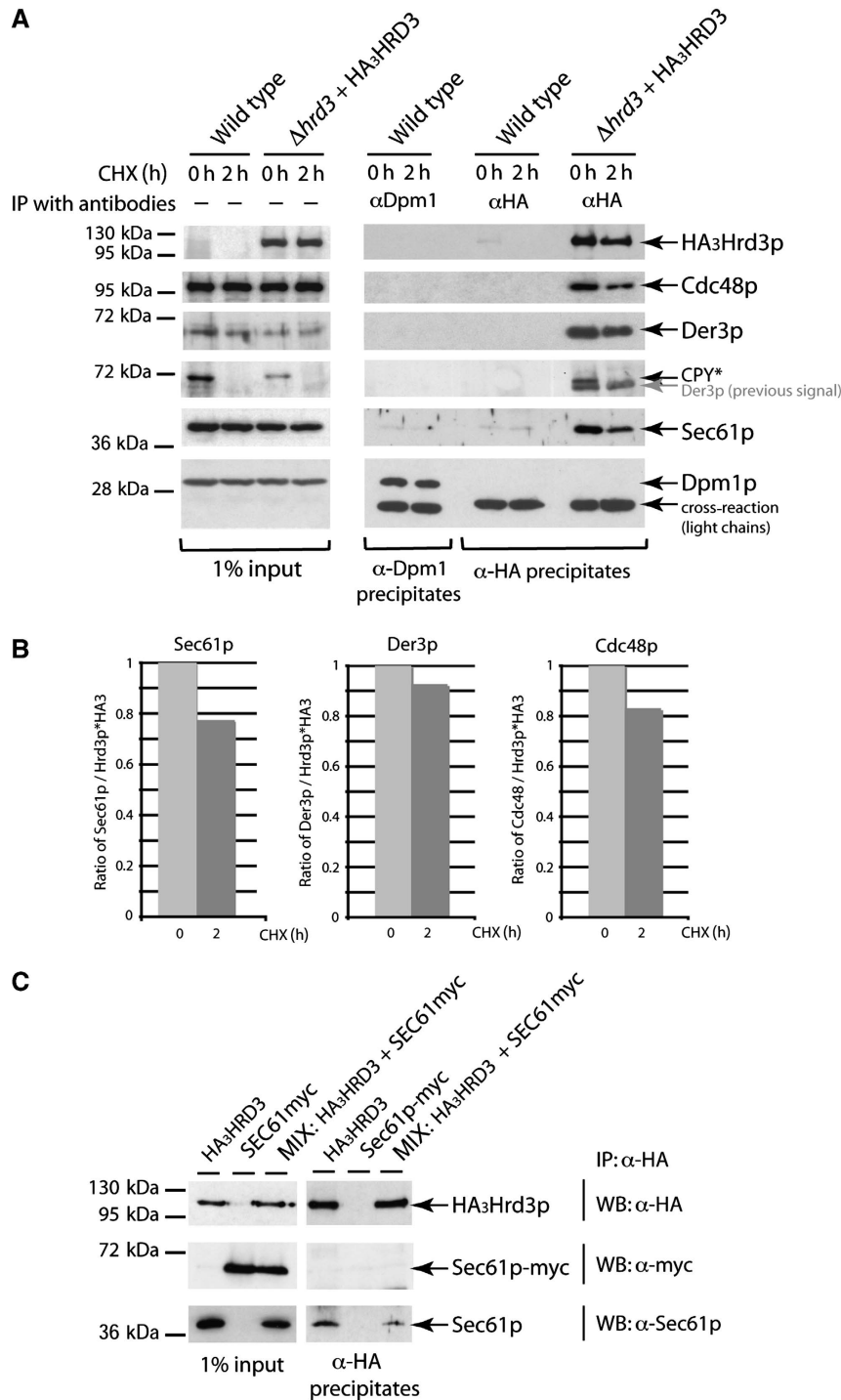


Figure 1 Sec61p is associated with components of the ERAD pathway. (A) Immunoprecipitation experiment using a plasmid encoded, N-terminally HA₃-tagged version of Hrd3p. Digitonin-solubilized microsomal proteins were prepared from *wild type* and Δ hrd3 + HA₃Hrd3p strains (input). Half of the cells were pre-treated with CHX for 2 h, whereas the other half was left untreated. HA₃Hrd3p and its interaction partners were isolated by immunoprecipitation with α -HA-antibodies and ProteinA-Sepharose (α -HA precipitates). The precipitates were analysed by immunoblotting with the indicated antibodies. Dpm1p serves as a negative control as it does not interact with Sec61p and was precipitated using specific α -Dpm1p antibodies. When detecting the CPY* signal, the earlier signal of Der3p could still be seen in α -HA precipitates (indicated in grey letters). (B) Ratios of Der3p, Cdc48 and Sec61p to HA₃Hrd3p obtained by quantification of the corresponding immunoblot signals of the precipitates shown in (A) using the program ImageJ 1.41o. (C) 'MIX' experiment to exclude formation of artificial Sec61p containing protein complexes. Cells from strains expressing either Sec61p-myc and endogenous Hrd3p or untagged Sec61p and HA₃Hrd3p were subjected to immunoprecipitation as described above. In the MIX, sample cells of these two strains were mixed before immunoprecipitation.

present after 30 min of CHX treatment should be on their way to degradation. For analysis, cells deleted for the coding sequence of CPY (Δ prc1) and complemented with a plasmid

encoding CPY*HA₃ were treated with and without CHX. Microsomal CPY*HA₃ extracts were subjected to immunoprecipitation followed by immunoblot analysis. We found an

interaction of CPY*HA₃ with Sec61p even after 30 min of CHX treatment of cells (Figure 2A, lanes 9 and 10). During degradation of CPY*HA₃ over a time period of 30 min, the association of Sec61p with CPY*HA₃ decreased, but still exists. This association points to an import independent contact between Sec61p and CPY*HA₃.

The contact between Sec61p and CPY* is enhanced in ERAD mutant strains

It is known that a deletion of the ubiquitin ligase Der3p leads to a complete block of CPY* degradation. In a Δ *der3* strain, the misfolded protein is not retrograde transported into the cytosol and accumulates in the ER (Bordallo *et al*, 1998). Indeed, we observed an accumulation of CPY*HA₃ in the microsomal membrane extracts of Δ *prc1* Δ *der3* and Δ *prc1* Δ *hrd3* strains after 30 min of CHX addition (Figure 2A, lanes 4–7). Interestingly, after this 30 min treatment, deletions of Der3p and of its complex partner Hrd3p lead to enhanced interactions of Sec61p with CPY*HA₃ at the ER membrane (Figure 2A, lanes 11–14). The ratio of quantified Sec61p and CPY*HA₃ signals revealed an increase of this contact of about 25% in Δ *prc1* Δ *hrd3* and Δ *prc1* Δ *der3* strains

after 30 min of CHX addition in comparison to the wild-type situation in a Δ *prc1* strain (Figure 2B). These data suggest that the contact of Sec61p to misfolded CPY*HA₃ persists until ubiquitination of CPY*HA₃ by the Der3p ligase has occurred, implying an association of CPY*HA₃ with Sec61p during transport out of the ER. In agreement with this, we observed increased relative amounts of polyubiquitinated CPY*HA₃ in the immunoprecipitates of wild-type cells over a time period of 0, 15 and 30 min after CHX addition, whereas the interaction of CPY*HA₃ with Sec61p again decreased with time (Figure 3A, lanes 10–12, and 3B). We also found the known ERAD components Cdc48p and Der3p in the immunoprecipitates (Figure 3A, lanes 10–12), which also show an increased contact with CPY*HA₃ after CHX treatment (Figure 3A and B). Deletions of *DER3* disturb the contact between ER luminal misfolded proteins and the Cdc48 complex in the cytosol (Gauss *et al*, 2006b), resulting in the observed weak Cdc48 signal in CPY*HA₃ immunoprecipitates (Figure 3A, lanes 13–15). The enhanced signals of ubiquitin, Cdc48p and Der3p together with CPY*HA₃ confirm that we isolated in this experiment a pool of CPY*, which is on its way out of the ER after 15 and 30 min of CHX addition to cells.

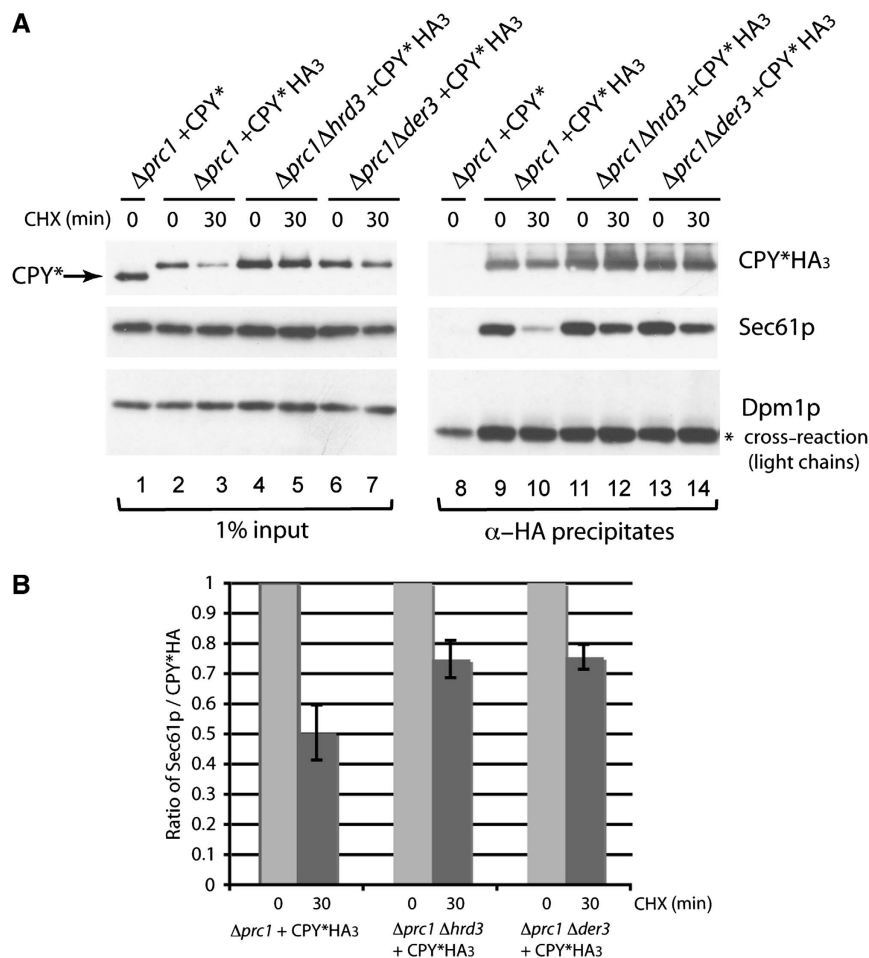


Figure 2 Sec61p loses contact to misfolded proteins during the degradation process. (A) Immunoprecipitation experiment using CPY*HA₃ as a bait. Digitonin-solubilized membrane proteins of isolated microsomes were prepared from *Δprc1*, *Δprc1Δhrd3* and *Δprc1Δder3* strains expressing either CPY* or CPY*HA₃ (input). The cells were pre-treated for 0 and 30 min with CHX. After precipitation using specific α -HA antibodies, the samples (α -HA precipitates) were analysed by immunoblotting with the indicated antibodies. The stars indicate unspecific cross-reactions. (B) Ratio of Sec61p to CPY*HA₃ obtained by quantification of the corresponding immunoblot signals of the immunoprecipitates shown in (A) using the program ImageJ 1.41o. (A). The error bars represent the s.e.m. of three independent experiments.

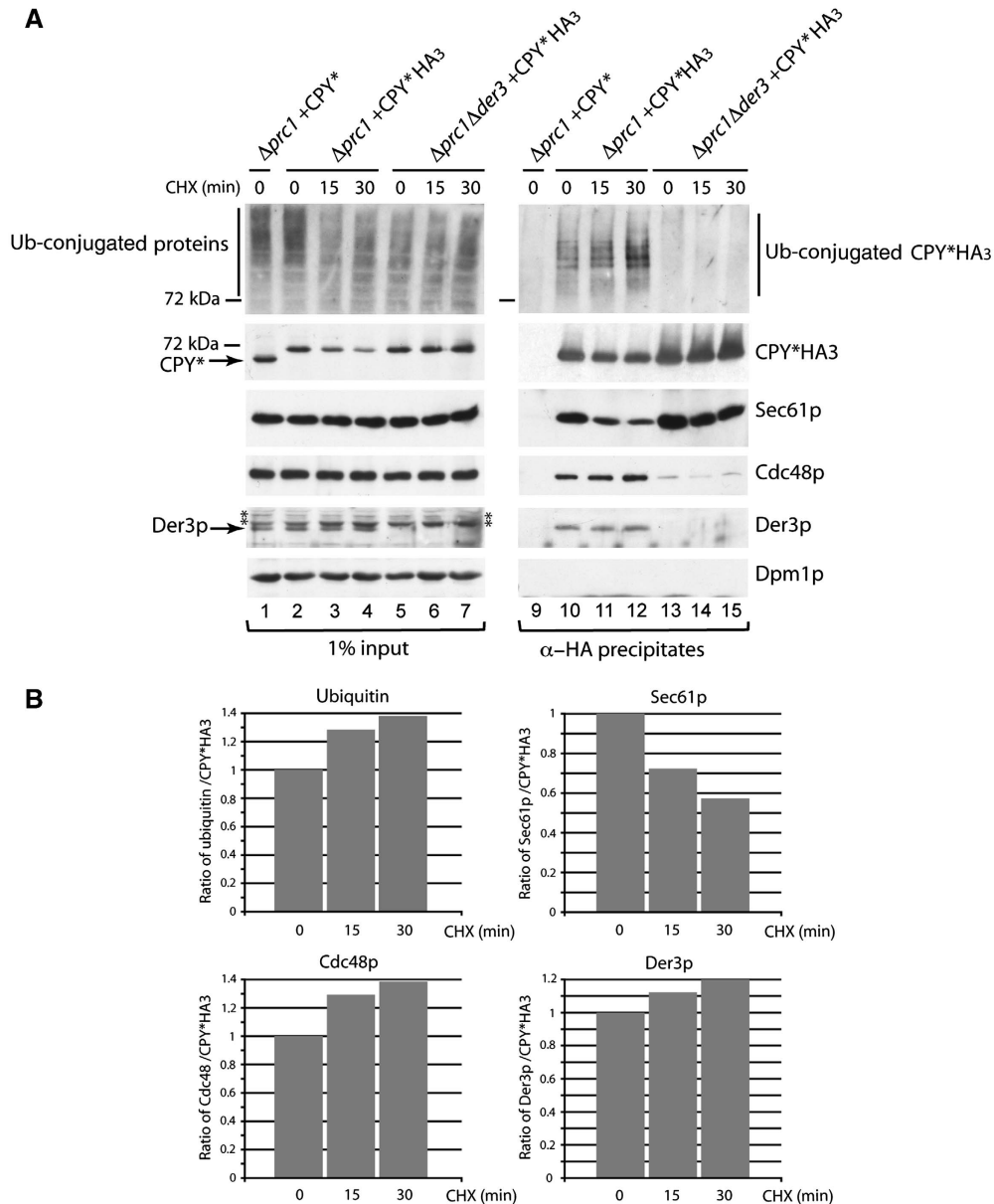


Figure 3 The pool of ubiquitinated CPY* at the ER membrane increases with time, whereas Sec61p loses contact to CPY*. (A) The immunoprecipitation experiment was carried out as described in Figure 2A using $\Delta prc1$ and $\Delta prc1\Delta der3$ strains expressing either CPY* or CPY*HA₃ (input). The cells were pre-treated for 0, 15 and 30 min with CHX. The immunoblot analysis included the detection of ubiquitinated proteins. The stars indicate unspecific cross-reactions. (B) Ratio of the ubiquitin, Sec61p, Cdc48 and Der3p signals to the CPY*HA₃ signals in immunoprecipitates of $\Delta prc1 + CPY*HA_3$ shown in (A) using the program ImageJ 1.41o.

Sec61p is necessary for degradation of the CPY* moiety of the membrane protein CTL*myc

To analyse an interaction between Sec61p and a misfolded membrane protein, we performed additional immunoprecipitation experiments and isolated CTL*myc. CTL*myc consists of an ER luminal CPY* moiety, the last transmembrane segment of Pdr5p and a cytosolically located, myc-tagged 3-isopropylmalate dehydrogenase (Leu2p). Recently, we have shown that CTL*myc is degraded by the ERAD system in a step-wise manner (Figure 4) (Kohlmann *et al*, 2008). Besides the ubiquitin ligase Der3p and the AAA-ATPase Cdc48p, membrane extraction and complete degradation of CTL*myc requires the E4 ligase Hul5p. The absence of Hul5p leads to the appearance of a stable fragment

of CTL*myc, truncCTL*myc (Figure 4). TruncCTL*myc is still membrane embedded and contains the myc-tagged Leu2 protein domain localized in the cytosol, but has lost most of its CPY* moiety (Kohlmann *et al*, 2008). Immunoprecipitation experiments showed that full-length CTL*myc interacts with the ERAD components Der3p and Cdc48p during degradation, whereas the membrane-embedded fragment truncCTL*myc did not exhibit any interaction with the ERAD machinery (Kohlmann *et al*, 2008). Therefore, we tested whether CTL*myc and truncCTL*myc interact with Sec61p. We isolated CTL*myc with α -myc antibodies and observed, besides the interaction with Der3p and Cdc48, also an association of CTL*myc with Sec61p (Figure 4, lanes 11 and 12). Interestingly, the membrane-

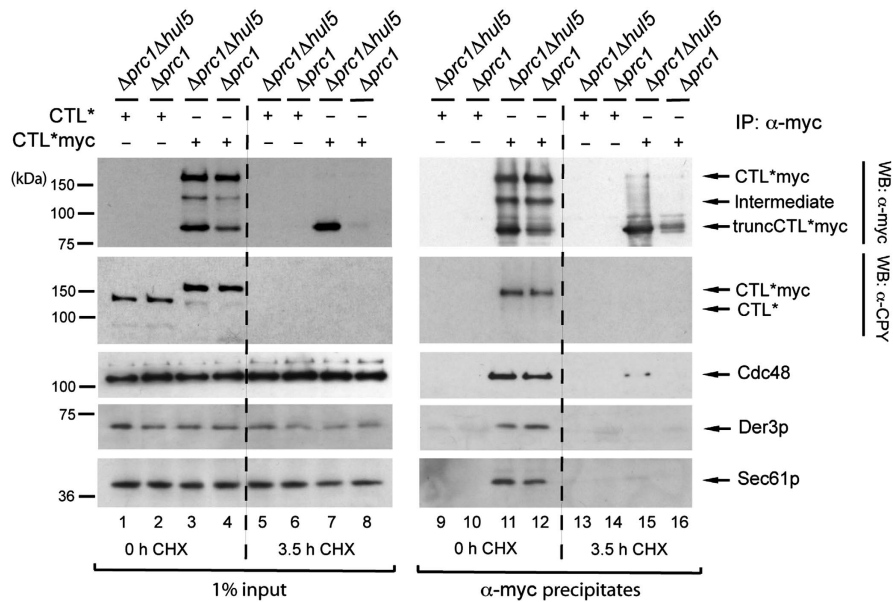


Figure 4 CTL*myc interacts with Sec61p until the CPY* moiety of CTL*myc is degraded. Cells of the yeast strains $\Delta prc1$ and $\Delta prc1\Delta hul5$ expressing either plasmid encoded CTL* or CTL*myc were treated with CHX for 0 and 3.5 h. This enables the accumulation of the fragment truncCTL*myc in a $\Delta prc1\Delta hul5$ strain (lane 7). Solubilized microsomal proteins were subjected to immunoprecipitation using α -myc antibodies (α -myc precipitates) and were analysed by immunoblot analysis with the indicated antibodies. Except for the interaction studies of Sec61p, this figure was earlier published in Kohlmann *et al* (2008). CTL*myc interacts with Sec61p during its degradation (lanes 11 and 12) as well as the known components of the ERAD machinery, Der3p and Cdc48 (Kohlmann *et al*, 2008).

embedded truncCTL*myc, which has lost the contact to Der3p and Cdc48, also does not associate with Sec61p (Figure 4, compare lanes 11 and 12 with lanes 15 and 16). Thus, we conclude that Sec61p is necessary for degradation of the misfolded ER luminal CPY* moiety of CTL*myc. The remaining membrane-embedded part of the protein may traverse a different route.

Fully imported and glycosylated proteins are associated with Sec61p

By isolating CPY*HA₃, we were able to detect an interaction of CPY* with Sec61p, Cdc48 and the ERAD-L ubiquitin ligase Der3p (Figure 3A, lanes 10–12). To address these interactions from the Sec61p side, we performed immunoprecipitation experiments using carboxyterminally HA-tagged Sec61p (Sec61pHA₃) expressed from genomically integrated *SEC61-HA₃*. In addition, we expressed either the plasmid encoded vacuolar carboxypeptidase Y (CPY) or mutated versions in a strain deleted for the coding sequence of endogenous CPY ($\Delta prc1$). The CPY species are all post-translationally imported as pre-pro-forms in an unfolded state into the ER lumen through the Sec61p channel. There, the pre-(signal) sequences are cleaved off and the proteins are glycosylated and folded. Properly folded wild-type CPY is further processed in the ER and the Golgi apparatus and finally delivered to the vacuole in which the enzyme is matured by cleavage of its pro-sequence (Müller and Müller, 1981; Stevens *et al*, 1982). In contrast to wild-type CPY, the mutated versions CPY* and CPY*G5 are unable to fold properly. These proteins are recognized as misfolded, retained in the ER and degraded by the ERAD pathway (Hiller *et al*, 1996; Plemper *et al*, 1999b). Although CPY* is glycosylated at four different sites, the CPY* derivative CPY*G5 contains an additional glycosylation site positioned at three amino acids from the

C-terminus (Figure 5A) (Plemper *et al*, 1999b). During import through the Sec61p channel, the proteins are glycosylated by the oligosaccharyl-transferase (OST) complex. The spatial gap between the active site of the OST complex on imported polypeptides and the Sec61p import channel was identified to be 15 amino-acid residues (Figure 5B) (Whitley *et al*, 1996). As CPY*G5 is glycosylated at all five sites, one can conclude that its import into the ER has been fully completed and contact with Sec61p during import has been lost.

For immunoprecipitation experiments, functional microsomal Sec61pHA₃ was isolated from a $\Delta prc1$ Sec61pHA₃ strain expressing either CPY, CPY* or CPY*G5 (Figure 5C, lanes 2–4). CPY* and CPY*G5 can be clearly immunoprecipitated with Sec61pHA₃ (Figure 5C, lanes 7 and 8). In contrast, no interaction was observed with wild-type CPY or with its pro-forms (Figure 5C, lane 6), indicating a normal import of CPY in a strain carrying the HA-tagged version of Sec61p. In the microsomal extracts (input), we detected an additional CPY* and CPY*G5 signal in the $\Delta prc1$ Sec61pHA₃ strain (Figure 5C, lanes 3 and 4, indicated by a star) compared with the CPY* signal in $\Delta prc1$ (Figure 5C, lane 1). Most likely, these additional signals represent unglycosylated pre-pro-forms of CPY* and CPY*G5. Such forms can sometimes be detected and disappear when import is completed, as it is for instance after the addition of CHX to the cells for 30 min (Figure 5D, lanes 3 and 4). Interestingly, neither the pre-pro-forms of mutated CPY*, CPY*G5 nor of wild-type CPY were found in the Sec61pHA₃ immunoprecipitates (Figure 5C, lanes 6–8). Obviously, these forms are rapidly imported and have, therefore, only a short and non-detectable contact with Sec61pHA₃. The observation that only fully glycosylated, misfolded CPY* species are bound to Sec61pHA₃ indicates that this binding occurs after their complete import and glycosylation in the ER.

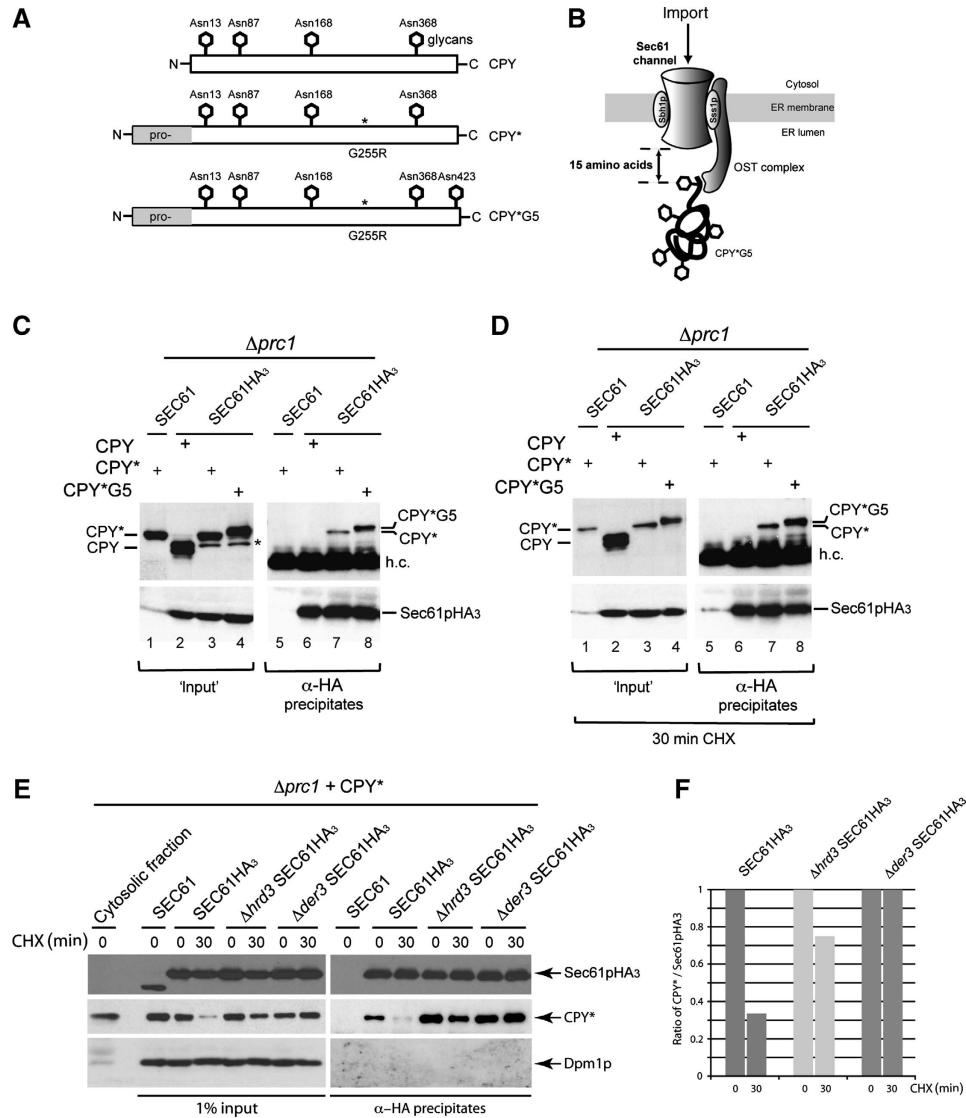


Figure 5 Fully glycosylated proteins are associated with Sec61p. (A) Schematic representation of carboxypeptidase Y (CPY), point mutated CPY G255R (CPY*) and CPY* including an additional glycan at the amino-acid position 423 (CPY*G5). (B) Import model of CPY*G5. The active site of the OST complex is located in a distance of ~15 residues from the end of the hydrophobic transmembrane segments of Sec61p. (C) Immunoprecipitation experiment using a C-terminally HA₃-tagged version of Sec61p. Digitonin-solubilized microsomal proteins (input) were prepared from strains deleted for the coding sequence of CPY ($\Delta prc1$) and expressing either plasmid encoded CPY, CPY* or CPY*G5. Sec61pHA₃ was precipitated with α -HA antibodies and ProteinA-Sepharose (α -HA precipitates) and analysed by immunoblotting using specific α -CPY and α -Sec61p antibodies. The star indicates pre-pro-forms of CPY* and CPY*G5; h.c. indicates a cross-reaction with the heavy chains of the antibodies used for precipitation (D) Immunoprecipitation experiment carried out as described above. The cells were pre-treated with CHX for 30 min at 30°C. (E) Immunoprecipitation experiment using the strains SEC61HA₃, $\Delta hrd3$ SEC61HA₃ and $\Delta der3$ SEC61HA₃. All strains carry in addition a $\Delta prc1$ deletion and plasmid encoded CPY*. The deletion of the core components of the Hrd-ubiquitin ligase complex, Der3p and Hrd3p, leads to an enhanced interaction of CPY* with Sec61p. (F) Ratio of immunoblot signals of CPY* to Sec61p signals of immunoprecipitates shown in (E).

The OST complex interacts directly with the protein Sss1p, which is part of the trimeric Sec61p complex (Scheper *et al.*, 2003). One might argue that the observed association of mutated CPY* and CPY*G5 with Sec61pHA₃ may be the result of an indirect interaction through the OST complex. To exclude this possibility, cells were treated with CHX for 30 min before immunoprecipitation as described before, allowing complete import of soluble CPY* and CPY*G5 into the ER lumen. Under these conditions, we still observe an association of misfolded CPY* and CPY*G5 with Sec61pHA₃ (Figure 5D, lanes 7 and 8). For quantitative analysis of this association, we analysed the samples side by side on one SDS

gel (Figure 5E) and calculated the ratio between the Sec61p and CPY* immunoblot signals (Figure 5F). We observed a decrease of this binding, which we also found earlier when isolating Sec61p through CPY*HA₃ (Figure 2A). Deletion of the ubiquitin ligase Der3p and its complex partner Hrd3p leads to enhanced interaction between Sec61pHA₃ and CPY* at the ER membrane (Figure 5E), which we also observed in the earlier analysis of CPY*HA₃ immunoprecipitates (Figure 2A). This association is not mediated through the pre-sequence of CPY*, which may have contact with Sec61p until cleavage by the signal peptidase occurs (Johnson *et al.*, 1987; Ng *et al.*, 1996). The molecular mass of soluble, pre-

sequence cleaved CPY* (Figure 5E, cytosolic fraction) is identical with the molecular mass of microsomal, ER membrane-associated CPY* found in the input fractions and in the immunoprecipitates (Figure 5E). Thus, Sec61pHA₃ must be considered to be associated with pre-sequence cleaved CPY*. We were unable to detect Cdc48 or Der3p in the Sec61pHA₃ immunoprecipitates (data not shown). Interestingly, this import independent Sec61p–CPY* contact at the ER membrane seems to exist even without the presence of Hrd3p (Figures 2A and 5E), which was proposed to be necessary for the recruitment of misfolded proteins in the ERAD process (Denic *et al*, 2006; Gauss *et al*, 2006b). The absence of the Hrd-Der ligase complex as well as Cdc48 in Sec61pHA₃ immunoprecipitates may point to the existence of a protein complex composed of Sec61p and misfolded proteins without known ERAD components. This complex seems to exist in addition to the described Sec61p containing Hrd complex (Figure 1A).

Discussion

Sec61p is part of the ERAD machinery

Several genetic studies implicated a function of Sec61p in the ERAD process for elimination of misfolded proteins (Pilon *et al*, 1997; Plemper *et al*, 1997, 1999a). Recent data using a novel *sec61-3* mutant strain and co-translationally imported CPY* derivatives corroborated that a functional Sec61p is essential for the elimination process of ERAD-L substrates (Willer *et al*, 2008). Up to now, no interaction between Sec61p and known ERAD components could be determined on a biochemical basis. Therefore, we investigated the physical contact between components and substrates of the ERAD pathway with Sec61p. The essential role of Sec61p in the import process of proteins into the ER complicates the analysis of an additional function of Sec61p in the ERAD process. As most of the ERAD components have to be imported into the ER, detection of an interaction between Sec61p and ERAD substrates or ERAD components such as Hrd3p might represent only contact during the import step. To analyse completely imported proteins, we pre-treated the cells with CHX before immunoprecipitation experiments. This treatment prevents new protein synthesis, whereas the import of already existing polypeptides and the ERAD process continues. Even after the addition of CHX, we observed an association of Sec61p with the Hrd-Der ligase complex and Cdc48p using HA₃Hrd3p as a bait (Figure 1A). Furthermore, we confirmed this interaction by isolating the misfolded ERAD-L substrate CPY*HA₃ together with Sec61p, Cdc48 and the ubiquitin ligase Der3p (Figure 3A). As Der3p is always connected to its complex partner Hrd3p (Plemper *et al*, 1999a; Gardner *et al*, 2000; Denic *et al*, 2006), Hrd3p can also be assumed to be present in the immunoprecipitates. Thus, we propose that Sec61p is a dynamic part of the Hrd-Der ligase complex. In an additional experiment using Sec61pHA₃ as a bait, the interaction between Sec61p and misfolded CPY* and CPY*G5 was proven (Figure 5C and D). However, in the same experiment, we did not observe any Cdc48 or Der3p signals (data not shown). One explanation for this observation might reside in the existence of a protein complex, including Sec61p and misfolded proteins, but excluding the Hrd-Der ligase complex. Interestingly, the association between CPY* and Sec61p is not disrupted in a $\Delta hrd3$ strain (Figures 2A and 5E). This observation is somewhat

surprising because Hrd3p is suggested to be the central binding partner for misfolded proteins in the ER lumen, acting before the retro-translocation event (Kim *et al*, 2005; Gauss *et al*, 2006a). Therefore, we expected disruption of the Sec61p–CPY* interaction in a $\Delta hrd3$ strain. Instead, the amount of Sec61p associated with CPY* is increased in $\Delta hrd3$ and $\Delta der3$ strains (Figures 2A, 3A and 5E). Obviously, binding of misfolded CPY* to Sec61p can be independent of the presence of the Hrd-Der ligase complex. This observation may underline the possibility of the existence of a second Sec61p–CPY*-containing complex. One may assume that binding between Sec61p and CPY* continues until ubiquitination by the Der3p ligase takes place. Indeed, in the presence of Der3p, Sec61p loses contact with CPY* during the degradation process when it is ubiquitinated and retro-translocated to the cytosolic side of the ER (Figure 3A). Thus, one function of Sec61p in the ERAD-L pathway might reside in a process upstream of the function of the Hrd-Der ligase complex. We observed that solely the ER luminal CPY* moiety of the misfolded membrane protein CTL*myc contacts Sec61p during degradation, whereas the part of CTL*myc containing the transmembrane region fused with a cytosolic Leu2p-myc loses contact to Sec61p (Figure 4). Due to this finding, one might conclude that the functional capacity of Sec61p might be restricted to misfolded proteins of the ER lumen, whereas the handling of certain membrane spanning protein stretches for degradation might be executed by other devices.

Different Sec61p functions in translocation

Any translocation mode of the protein-conducting channel Sec61p is defined through its interaction with additional proteins. Main partners for co-translational import of soluble and membrane proteins are ribosomes. Binding of ribosomes to Sec61p enables the transfer of nascent polypeptide chains into the ER lumen as well as the lateral insertion of proteins into the ER membrane during their synthesis (Osborne *et al*, 2005). Post-translational import of already synthesized polypeptides requires the assembly of a hetero-heptameric Sec61p complex in the ER membrane and the assistance of the chaperone Kar2p in the ER lumen (Deshaies *et al*, 1991; Panzner *et al*, 1995; Matlack *et al*, 1999; Rapoport, 2008). Interestingly, the mode of action can be altered under ER stress. Through the association of cytosolic chaperones, the translocation channel is reprogrammed from import to co-translational proteasomal degradation of apolipoprotein B (Pariyath *et al*, 2001; Fisher and Ginsberg, 2002; Oyadomari *et al*, 2006). This implies that Sec61p can change its transport directionality. Such a switch in the directionality of Sec61p was also suggested for the retro-translocation step of misfolded ERAD substrates (Plemper *et al*, 1997, 1998, 1999a; Pilon *et al*, 1998; Römisch, 1999; Meusser *et al*, 2005). One may expect that docking of ERAD core components to Sec61p leads to reprogramming of the channel and to transport of misfolded proteins to the cytosol. Indeed, we could show that Sec61p is associated with Hrd3p, Der3p and Cdc48p, which are all components required for the degradation of misfolded luminal ER proteins similar to CPY*. Thus, our results support the idea that the mode of Sec61p can be switched into one or the other direction by the assistance of additional recruited partners. Nevertheless, our data also allow different interpretations about the nature of the retro-

translocation channel. When Der3p is missing, ubiquitination and retro-translocation of misfolded proteins is disturbed, whereas their interaction with Sec61p still persists (Figures 2A, 3A and 5E). Therefore, one might speculate that the Hrd-Der ligase complex forms a channel whereby the function of Sec61p resides in the delivery of misfolded proteins to the Hrd complex. This delivery function could also include the formation of a hybrid channel composed of Sec61p and the Hrd-Der ligase complex. The retro-translocation process might also require the assistance of additional ER membrane spanning proteins. Sec61p seems to be highly dynamic having a multitude of options to deal with the needs of the cell to guarantee a functional ER. From our biochemical and the existing genetic data, we conclude that one of such options resides in its participation in the ERAD-L process for elimination of misfolded proteins.

Materials and methods

Yeast and plasmids

Media preparation, genetic and molecular biology techniques were carried out using standard methods (Guthrie and Fink, 1991; Ausubel *et al.*, 1992). The *Saccharomyces cerevisiae* strains W303 Δ prc1 and W303 Δ prc1 Δ hul5 were described before (Plempner *et al.*, 1998, 1999b; Kohlmann *et al.*, 2008). The double deletion strains W303 Δ prc1 Δ hrd3 and W303 Δ prc1 Δ der3 were generated by tetrad analysis after crossing W303 Δ prc1::LEU2 with W303prc1-1 Δ der3::HIS3 Δ hrd3::HIS3 (Bordallo *et al.*, 1998). Epitope tagging of Sec61pHA₃ and Sec61pmyc were achieved by homologous recombination as described in Knop *et al.* (1999) generating the strains W303 Δ prc1::LEU2, SEC61-HA₃-HIS3MX and W303prc1-1, SEC61-MYC9-TRPMX. W303 Δ prc1::LEU2 Δ der3::HIS3, SEC61-HA₃-HIS3MX and W303 Δ prc1::LEU2 Δ hrd3::HIS3, SEC61-HA₃-HIS3MX were generated by crossing W303 Δ prc1::LEU2, SEC61-HA₃-HIS3MX with W303 Δ prc1::LEU2 Δ der3::HIS3 and W303 Δ prc1::LEU2 Δ hrd3::HIS3, respectively, and subsequent tetrad dissection. Plasmids encoding for CPY*G5, CPY*HA₃, CTL* and CTL*myc were described earlier (Plempner *et al.*, 1999b; Taxis *et al.*, 2003; Medicherla *et al.*, 2004; Kohlmann *et al.*, 2008). HA₃Hrd3p was

subcloned as a 3.3-kb *KpnI/SacI* fragment from pYS14 (Saito *et al.*, 1999) into pRS314 (Sikorski and Hieter, 1989). For generation of pRS316-CPY, a 2.6-kb PCR fragment was generated using genomic W303-DNA as template and the oligonucleotides fwdCPY (5'AACTGCAGGAATTCTACGGTATGTGTGGCGGTTA3') and revCPY (5'ACGC TCG AGGTCGACCGTGCCCAAAGCTTGATTAC3'). This fragment was *EcoRI/SalI* cloned into pRS316.

Antibodies

Monoclonal antibodies specific for detection of Dpm1p (Molecular Probes through Invitrogen), c-myc (clone 9E10, Sigma-Aldrich) and HA epitope (clone 16B12, Covance, CA) were used in immunoprecipitation experiments and for immunodetection. For the detection of CPY (Rockland, PA) and Der3p, specific polyclonal antibodies were used (Bordallo *et al.*, 1998). Monoclonal ubiquitin antibodies (P4G7) were purchased from Covance (CA). α Cdc48 and α Sec61p antibodies were provided by T Sommer.

Immunoprecipitation and immunoblotting

Immunoprecipitation experiments were performed as described in Kohlmann *et al.* (2008). The cells were pre-treated with CHX (150 μ g/l OD₆₀₀ of cells) at 30°C for 15, 30 min, 2 or 3.5 h, as indicated in the results and figures. Washed microsomal membranes of 150–250 OD₆₀₀ of cells were used for the solubilization step with 1% digitonin. Standard methods were used for SDS-PAGE and immunoblot analysis (Ausubel *et al.*, 1992).

Quantification of immunoblot signals

The corresponding signals of the immunoprecipitates were quantified using the program ImageJ 1.41o. The immunoblot signals were normalized to the signals of proteins used as baits similar to HA₃Hrd3p (Figure 1A), CPY*HA₃ (Figures 2A and 3A) or Sec61pHA₃ (Figure 5E) respectively, resulting in the presented ratios in Figures 1B, 2B, 3B and 5F.

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