Ubiquitin Ligase Hul5 Is Required for Fragment-specific Substrate Degradation in Endoplasmic Reticulum-associated Degradation*

Received for publication, March 3, 2008  Published, JBC Papers in Press, April 24, 2008, DOI 10.1074/jbc.M801702200

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To identify new components of the protein quality control and degradation pathway of the endoplasmic reticulum (ER), we performed a growth-based genome-wide screen of about 5000 viable deletion mutants of the yeast Saccharomyces cerevisiae. As substrates we used two misfolded ER membrane proteins, CTLa and Sec61-2L, chimeric derivatives of the classical ER degradation substrates CPY* and Sec61-2. Both substrates contain a cytosolic Leu2 protein fusion, and stabilization of these substrates enables a restored growth of the transformed LEU2-deficient deletion mutants. We identified the strain deleted for the ubiquitin chain elongating ligase Hul5 among the mutant strains with a strong growth phenotype. Here we show that Hul5 is necessary for the degradation of two misfolded ER membrane substrates. Although the degradation of their N-terminal parts is Hul5-independent, the breakdown of their C-terminal fragments requires the ubiquitin chain elongating ligase activity of Hul5. In the absence of Hul5, a truncated form of CTLa myc remains to a large extent embedded in the ER membrane. Hul5 activity promotes the interaction of this truncated CTLa myc with the AAA-ATPase Cdc48, which is known to pull proteins out of the ER membrane. This study unravels the stepwise elimination of the ER membrane-localized CTLa myc substrate. First, N-terminal, luminal CPY* is transferred to the cytoplasm and degraded by the proteasome. Subsequently, the remaining C-terminal membrane-anchored part requires Hul5 for its effective extraction out of the endoplasmic reticulum and proteasomal degradation.

Ubiquitin-proteasome-triggered protein degradation is an essential process in all eukaryotic cells. Functions of this process include regulation of signal transduction, cell cycle control, and metabolic regulation of protein turnover (1–3). About one-quarter of the proteome of the cell traverses the secretory pathway (4). Most of these proteins are folded in the endoplasmic reticulum (ER) before they continue the journey to their final destination. As a side effect of this huge amount of folding events in the ER, the cell is faced with folding mistakes and failures. Misfolded proteins can disturb cellular functions. Therefore, they have to be eliminated prior to delivery to the site of action. The soluble and membrane-bound misfolded ER proteins are recognized in the ER lumen, retrotranslocated into the cytosol where they are polyubiquitinated, and subsequently degraded by the proteasome (5–8). This cellular disposal pathway is termed ER-associated degradation (ERAD). Degradation of most proteins requires their recognition via a polyubiquitin chain. Catalysts of the polyubiquitination process are E1, E2, and E3 enzymes. Ubiquitin-activating enzyme (E1) transfers ubiquitin to ubiquitin-conjugating enzymes (E2), which in many cases, in cooperation with ubiquitin-protein ligases (E3), assemble a ubiquitin chain on the substrate protein (9, 10). A core component of the recognition and ubiquitination process of proteins harboring a misfolded domain in the ER lumen is the Hrd-ligase protein complex. This complex consists of the single transmembrane-spanning Hrd3 protein and the E3 ubiquitin ligase Der3/Hrd1, which carries six transmembrane spans and a RING finger domain exposed to the cytoplasm (11–13). Recognition of the misfolded glycoproteins includes N-glycan processing (14–16), their binding to the large ER-luminal domain of Hrd3, and the quality check of their carbohydrate via the lectin Yos9 (17–22). Thereafter, the misfolded proteins are retrotranslocated into the cytosol, which requires their polyubiquitination by the Der3/Hrd1 ubiquitin ligase and the action of the hexameric AAA-ATPase Cdc48 and its substrate recruiting factors Ufd1 and Npl4 (5, 23–26). Finally, the function of Dsk2 and Rad23 is necessary for the delivery of the misfolded substrates to the 26S proteasome (27). The 26S proteasome contains the 20S core particle, which is composed of four heptameric rings with an α7β7α7-configuration and harbors the active sites. This core particle is capped at each α-subunit ring by a 19S regulatory particle. As part of the base of the regulatory particle, six different Rpt ATPase subunits dock onto the α-rings. Among other functions, the Rpt subunits regulate the opening of the gate into the 20S core particle. In addition, the three non-ATPase subunits Rpn1, Rpn2, and Rpn13 are...
part of the base, which is linked to the nine subunits containing a lid via the ubiquitin receptor Rpn10 (1, 28–30). Moreover, several proteins with enzymatic activities interact with the proteasome and facilitate the process of binding, unfolding, and ubiquitin chain remodeling of proteasomal substrates (31–33).

In this study, we performed genome-wide screens using a yeast gene deletion library to identify new mutants with defects in ER protein quality control and degradation (ERQD). The screens are based on the stabilization of two misfolded ERAD membrane substrates, CTL* and Sec61-2L, both carrying a cytosolic 3-isopropylmalate dehydrogenase (Leu2 protein). Such stabilization can be observed by a restored growth of the transformed leucine auxotrophic strains of a yeast deletion library (22, 27, 39). Here we report on the discovery of Hul5 as a component of the ERAD process. Hul5 is a HECT domain containing ubiquitin-protein ligase known to interact with the proteasome via its Rpn2 subunit and is involved in ubiquitin chain elongation (E4 activity) of proteasomal substrates (31). We demonstrate that the ability of Hul5 to extend pre-existing ubiquitin chains is necessary for complete degradation of the misfolded ER membrane protein CTL*myc. Interestingly, degradation of CTL*myc occurs stepwise and stops at a defined part of the substrate in the absence of Hul5. The remaining fragment consists of a truncated ER lumenal part of CPY*, a transmembrane region, and the cytoplasmic Leu2myc moiety. Furthermore, this fragment is no longer associated with the Der3-Hrd1 ligase complex. In addition, the absence of Hul5 affects the interaction of Cdc48 with CTL*myc. These results provide further information on the fine-tuning of the extraction and degradation process of misfolded ER membrane proteins.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Growth Conditions**—Media preparation and genetic and molecular biology techniques were carried out using standard methods (34–36). Saccharomyces cerevisiae strains used in this study are summarized in Table 1. Single deletion strains were generated as described in Longtine et al. (37) and Guldener et al. (38). Crossing of single deletion strains and subsequent tetrad analysis resulted in double deletion strains. The genome-wide screens using the EUROSCARF BY4743 diploid yeast library were performed as described in Schäfer and Wolf (39).

| Yeast strains used in this study | Genotype | Source |
|----------------------------------|----------|--------|
| W303prc1-1                      | MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 prc1-1   | Knop et al. (14) |
| Δder3                            | W303 prc1-1 der3-HIS3       | Bordallo et al. (41) |
| Δpdr5                            | W303 prc1-1 pdr5::TRP1     | Plumper et al. (55) |
| Δhul5                            | W303 prc1-1 hul5::HIS3     | This study |
| Δhul5Δubp6                      | W303 prc1-1 ubp6::HIS3   | This study |
| Δubp6                            | W303 prc1-1 ubp6::HIS3 | This study |
| W303Δprc1                       | ade2-1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 prc1-1::LEU2 | Plumper et al. (13) |
| Δpdr5Δder3                      | W303 prc1-1 ubp6::kanMX | This study |
| Δpdr5Δhul5                      | W303 prc1-1 leu2::kanMX | This study |
| Δpdr5Δubp6Δhul5                 | W303 prc1-1 leu2::HIS3 | This study |
| Δpdr5Δubp6Δhul5Δubp6             | W303 prc1-1 leu2::HIS3 | This study |
| UFD1                             | ura3-52 leu2-3,112 his4-19 ade1-100 | Johnson et al. (51) |
| ufd1-1                           | ura3-52 leu2-3,112 his4-19 ade1-100 | Johnson et al. (51) |

**Plasmids**—The plasmids pRS316-PGAL4-prc1-1-PDR5 (bp 4332–4532)-LEU2 and pRS316-PGAL4-Sec61-2-LEU2 encoding CTL* and Sec61-2L, respectively, were described in Medicherla et al. (27) and Buschhorn et al. (22). The plasmid pSK7 (pRS316-PGAL4-prc1-1-PDR5) was obtained by homologous recombination in S. cerevisiae and encodes CTL*myc. The oligonucleotides CTL*L-longtine_fwd (5’CCTCACAATAGGAACAAAGGC-TGGGTACCGGGCCCCCCCTGGTCTCAGAAGGCTGCTCTTAAAC) and CTL*L-longtine_rev (5’CCTCACAATAGGAACAAAGGC-TGGGTACCGGGCCCCCCCTGGTCTCAGAAGGCTGCTCTTAAAC) were used to amplify a fragment using pFA-13Myc-kanMX6 (37) as PCR template. The resulting 2.3-kb PCR fragment and Sall-linearized pRS316-PGAL4-prc1-1-PDR5 (bp 4332–4532)-LEU2 were transformed in a W303 yeast strain. The recombinant plasmid was isolated by plasmid rescue. The plasmid pSK9 (pRS316-PGAL4-Sec61-2-LEU2-MYC13) encodes for Sec61-2L and was created in a similar strategy as described for pSK7. The oligonucleotides CTL*L-longtine_fwd and Sec61-2L-longtine_rev (5’GGCCAGTGAATTGTGACTCTAACGATGTGAATTGTAATACGACTC-ACTAATTATGCAAATATCATAGAGTCGAGATGCTTGTTAAAC) were used to amplify a 2.3-kb fragment, which was transformed in yeast with Sall-linearized pRS316-PGAL4-Sec61-2-LEU2. The plasmids pSK10 (pRS314-PHLUS5-HULS5) and pSK11 (pRS314-PHLUS5-HULS5::C878A) are based on the plasmids pJH84 and pJH85, respectively, in a homologous recombination event in yeast.

**Antibodies**—Precipitations of CTL* and CTL*myc in pulse-chase analyses were done using polyclonal anti-CPY antibodies (Rockland). Specific mouse antibodies generated against c-Myc epitopes (clone 9E10; Sigma) were used in immunoprecipitation experiments to isolate Sec61-2Lmyc and CTL*myc, respectively. For immunodetection, monoclonal
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anti-HA antibodies (clone 16B12, Covance), monoclonal anti-c-Myc antibodies (clone 9E10, Santa Cruz Biotechnology), and monoclonal anti-phosphoglyceraldehyde kinase antibodies (clone 22C5, Molecular Probes) generated in mouse were purchased. For the detection of Der3/Hrd1, specific polyclonal antibodies were used (41). Anti-Cdc48 antibodies were provided by T. Sommer.

Pulse-Chase and Cycloheximide Chase Analyses—Pulse-chase experiments to analyze the degradation of CTL* and CTL*myc proteins were performed as described in Taxis et al. (42) and Medicherla et al. (27). Curves represent data of up to five independent experiments, and the error bars indicate the respective mean ± S.E. For inhibition of the proteasome MG132 (Calbiochem) up to a concentration of 100 μM was added to labeling media and chase media every 20 min. The pulse-chase analysis of Sec61-2L is described in Buschhorn et al. (22). The procedure to perform cycloheximide decay experiments is described in Medicherla et al. (27). Standard methods were used for SDS-PAGE and immunodetection (36).

Subcellular Fractionation and Membrane Extraction Experiments—100 A600 units of yeast cells were harvested in the logarithmic growth phase, washed with water and 10 mM sodium azide. Spheroplasts were obtained at 30 °C using 0.3 mg/ml zymolase, 2.7 μl/ml β-mercaptoethanol, 1.4 mM sorbitol, 50 mM potassium phosphate, pH 7.2, 10 mM sodium azide, 1 mM phenylmethylsulfonyl fluoride, and “complete” inhibitor mix (Roche Applied Science). Spheroplasts were opened in 3 ml of lysis buffer (0.8 mM sorbitol, 10 mM MOPS, pH 7.2, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, complete inhibitor mix (Roche Applied Science) by the use of a douncer. Cell debris was removed by centrifugation for 10 min at 1500 × g to obtain the post-nuclear supernatant (PNS), including intact organelles, microsomes, and cytosolic proteins. For the subcellular fractionation, the PNS was centrifuged for 30 min at 50,000 × g to achieve a pellet fraction, including intact organelles and microsomes, and a supernatant, including cytosolic proteins. To examine the membrane association of CTL*myc, the PNS was separated, and either lysis buffer, 1 M potassium acetate, 2.5 M urea, 0.1 M sodium carbonate, 1% Triton X-100, or 1% SDS was added to the aliquots. The 1% SDS sample was incubated for 30 min at room temperature, whereas the other samples were incubated on ice. The samples were separated into a membrane (pellet) and a soluble protein fraction (supernatant) by centrifugation for 30 min at 50,000 × g. Proteins were analyzed by SDS-PAGE and immunodetection (36).

Immunoprecipitation Experiments—Immunoprecipitation experiments to isolate membrane-embedded CTL*myc and truncTC1R4myc were performed as described in Biederer et al. (43) and Neuber et al. (44) except for the following treatments. The membrane fractions of 150 A600 units of cells expressing CTL*myc were prepared before and after 3.5 h of cycloheximide treatment (150 μg of cycloheximide/A600 unit of cells), respectively. After solubilization of the membrane proteins for 1 h in 1% digitonin, the lysates were cleared by centrifugation at 100,000 × g for 30 min in a TLA110.1 Beckman rotor. Each of the resulting supernatants was incubated overnight with 6 μl of anti-Myc antibodies (mouse clone 9E10; Sigma) and 7 mg of 10% bovine serum albumin blocked protein A-Sepharose (Amersham Biosciences) in buffer (50 mM Tris, pH 7.5, 200 mM NaAc, 10% glycerol). During the washing steps the concentration of digitonin was lowered to 0.5%. For elution of the proteins 50 μl of urea loading buffer (8 M urea, 200 mM Tris/HCl, pH 6.8, 0.1 mM EDTA, 5% (w/v) SDS, 0.03% (w/v) bromphenol blue, 1% β-mercaptoethanol) was added, and the samples were shaken for 15 min at 37 °C. 25 μl of each sample was subjected to immunoblot analysis.

RESULTS

To gain deeper insight into the components of ERQD (39), a genome-wide screen was performed using the EUROSCARF yeast library consisting of about 5000 diploid S. cerevisiae strains with a leucine auxotrophy. In each mutant a single nonessential gene is deleted (22, 27, 39). Use of this genomic library for our screen is possible, because cells defective in ERQD can tolerate defects in this process and survive as long as the unfolded protein response is not impaired (45, 46). To screen for new mutants that influence the ERQD process, we used two ERAD substrates with different misfolded domains. The first substrate, CTL*, includes a fusion of a mutated ER luminal carboxypeptidase yscY (CPY*), the last transmembrane domain of Pdr5, and cytosolic 3-isopropylmalate dehydrogenase (Leu2) (27). The second substrate, Sec61-2L, is based on the nonglycosylated ERAD substrate Sec61-2, including 10 transmembrane domains (43, 47) fused at its C terminus to cytosolic Leu2 (Fig. 1A). In cells intact for ERQD, the degradation of Sec61-2L is induced at a restrictive temperature of 33 °C. Cells with an active ERQD are able to degrade the Leu2 fusion proteins and are leucine auxotrophs (Fig. 1, B and C, left panel). In contrast, strains defective in ERQD like Δter3 mutants stabilize the misfolded, Leu2 containing substrates and therefore complement the Leu2 deficiency (22, 27, 39), which can be easily observed by restored growth (Fig. 1, B and C, left panel). We analyzed the growth phenotype of about 5000 individual deletion mutants of the EUROSCARF yeast library expressing either Sec61-2L or CTL*. Besides previously found ERQD components discovered in screens performed with CTL* (22, 27), we identified in addition the mutant strain deleted for the encoding sequence of Hul5. When expressing either CTL* or Sec61-2L, ∆hul5 cells showed growth on medium lacking leucine (Fig. 1, B and C).

Hul5 Is Required for Degradation of Specific Protein Fragments—Remarkably, when testing the degradation of CTL* in pulse-chase analysis by the use of specific anti-CPY antibodies, the fusion protein disappeared in ∆hul5 mutant cells with similar kinetics as in wild type cells (Fig. 1D). Similarly, a mutated Sec61-2HA protein was degraded with wild type kinetics in the ∆hul5 mutant strain (Fig. 1E). These results were in contrast to the positive growth behavior of ∆hul5 mutant cells expressing either the fusion proteins CTL* or Sec61-2L, which implied substrate stabilization (Fig. 1, B and C). The fusion proteins contain two parts, misfolded CPY* or Sec61-2 and properly folded, active Leu2. The possibility existed that in ∆hul5 mutant cells the mis-
folded CPY* and Sec61-2 moieties were degraded, whereas the part that includes the properly folded, active Leu2 protein was spared from degradation. For selection of plasmid carrying cells uracil was omitted. Degradation of the conditional ERAD substrate Sec61-2L is induced at 33 °C. The transformed wild type strain BY4743 fails to grow on CM medium lacking leucine (left panel). In contrast a strain deleted in the ERAD component Der3/Hrd1 expressing Sec61-2L and CTL, respectively, permits growth and was used as a positive control. The presence of either Sec61-2L or CTL* complements the leucine auxotrophy of the Δhul5 strain indicating substrate stabilization. D, pulse-chase analyses were carried out using W303 wild type and Δhul5 cells expressing CTL*, respectively. The cells are metabolically labeled with [35S]methionine, chased for the indicated times with nonlabeled methionine, and immunoprecipitated using anti-CPY antibodies. The precipitates were further processed by SDS-PAGE, overlaid with a phosphor screen, and quantified in a PhosphorImager. The corresponding phosphor screen signals of the 0-min time point were taken as 100%. Curves represent data of four independent experiments and error bars indicate the respective S.E. E, cycloheximide chase experiments performed with W303 wild type and Δhul5 cells expressing plasmid encoded C-terminally tagged Sec61-2HA. After addition of cycloheximide, cell samples were taken at the indicated time points, and the cell lysates were subjected to immunoblot analyses using anti-HA antibodies. The detection of the cytosolic phosphoglycerate kinase (PGK) serves as a control for equally loaded protein amounts.

FIGURE 1. HUL5 was identified in a screen for new ERAD components. A, schematic representation of the ERAD substrates Sec61-2L and CTL*. B and C, growth tests of isogenic yeast strains expressing plasmid-encoded Sec61-2L (B) and CTL* (C). The cells were plated in serial dilutions on CM medium with or without leucine. For selection of plasmid carrying cells uracil was omitted. Degradation of the conditional ERAD substrate Sec61-2L is induced at 33 °C. The transformed wild type strain BY4743 fails to grow on CM medium lacking leucine (left panel). In contrast a strain deleted in the ERAD component Der3/Hrd1 expressing Sec61-2L and CTL, respectively, permits growth and was used as a positive control. The presence of either Sec61-2L or CTL* complements the leucine auxotrophy of the Δhul5 strain indicating substrate stabilization. D, pulse-chase analyses were carried out using W303 wild type and Δhul5 cells expressing CTL*, respectively. The cells are metabolically labeled with [35S]methionine, chased for the indicated times with nonlabeled methionine, and immunoprecipitated using anti-CPY antibodies. The precipitates were further processed by SDS-PAGE, overlaid with a phosphor screen, and quantified in a PhosphorImager. The corresponding phosphor screen signals of the 0-min time point were taken as 100%. Curves represent data of four independent experiments and error bars indicate the respective S.E. E, cycloheximide chase experiments performed with W303 wild type and Δhul5 cells expressing plasmid encoded C-terminally tagged Sec61-2HA. After addition of cycloheximide, cell samples were taken at the indicated time points, and the cell lysates were subjected to immunoblot analyses using anti-HA antibodies. The detection of the cytosolic phosphoglycerate kinase (PGK) serves as a control for equally loaded protein amounts.

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Hul5 (not shown). For CTG*, this fact is rather surprising as this substrate has a similar modular assembly as CTL*myc with the exception that the green fluorescent protein is replaced by Myc-tagged Leu2 (48). The different degradation behavior of these two substrates in HUL5-deleted cells might be due to the fact that in addition to the known ERAD...
components CTG* degradation requires the cytosolic Ssa chaperone machinery (48), whereas CTL*myc does not.6

The deubiquitinating enzyme Ubp6 processes polyubiquitin chains on proteasomal substrates and is described as an antagonist of Hul5. The binding of substrates on the proteasome is thought to be regulated through the balanced action between Hul5 and Ubp6 (31, 49). However, deletion of Ubp6 had no influence on the degradation behavior of CTL*myc. Moreover, a double deletion of UBp6 and HUL5 changed neither the degradation rate of full-length CTL*myc nor the accumulation of truncCTL*myc as compared with the Hul5 single mutant (Fig. 2, D and E). It has also been suggested that the ubiquitin-conjugating enzyme Ubc4 could be a physiological partner of Hul5 (31). As can be seen in Fig. 2F, deletion of UBC4 did not alter CTL*myc degradation as compared with wild type. Therefore, neither Ubp6 nor Ubc4 are required for elimination of the fragment.

We studied the degradation of CTL*myc in other strains impaired in ERAD. In cells defective in the ubiquitin ligase Der3/Hrd1, full-length CTL*myc was stabilized (Fig. 3A), and no intermediate of 92 kDa accumulated (not shown). The absence of Der3/Hrd1 completely suppresses the formation of truncCTL*myc in a Δder3Δhul5 double deletion strain (Fig. 3A), indicating that Hul5 acts downstream of Der3/Hrd1-triggered polyubiquitination. This is in accordance with the findings of Crosas et al. (31), who assign a ubiquitin chain elongation function to Hul5 as described for E4 enzymes (50). In the mutant strain ufd1-1, the function of the Cdc48-Ufd1-Npl4 complex in ERAD is impaired (51) and the degradation of CTL*myc is completely blocked without generation of trunc-CTL*myc (Fig. 3B). Thus, a disturbed ERAD does not generally lead to an accumulation of the intermediate. Obviously, the accumulation of the CTL*myc intermediate is a specific feature of HUL5-deleted cells.

The E4 Activity of Hul5 Is Necessary for Degradation of truncCTL*myc—To examine whether the elongation function of Hul5 at ubiquitin chains is required for degradation of truncCTL*myc, experiments were performed using the catalytic inactive form Hul5C878A (Fig. 3C). As breakdown of soluble misfolded CPY* is independent of Hul5, most of the CPY* portion of CTL*myc is degraded. As a result of the defective enzymatic

FIGURE 3. The E4 ligase activity of Hul5 is required for degradation of truncCTL*myc. A, pulse-chase analyses of wild type, Δhul5, and Δder3Δhul5 strains expressing CTL*myc are performed as described in Fig. 1D using anti-Myc antibodies. B, pulse-chase analyses were done as described in A using wild type and ufd1-1 cells expressing CTL*myc. Curves represent data of three independent experiments, and error bars indicate the respective S.E. C, cycloheximide chase experiments using wild type and Δhul5 cells, each expressing CTL*myc and in addition prS314 or plasmids encoding Hul5 and its catalytically inactive form Hul5C878A, respectively. PGK, phosphoglycerate kinase.

6 S. Besser and D. H. Wolf, unpublished data.
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ER membrane and remained in a loose membrane contact. Therefore, the microsomal membranes were treated with low and high concentrated salts or detergents. Only Triton X-100 and SDS led to solubilization of truncCTL*myc (Fig. 4C) indicating that truncCTL*myc is still firmly embedded in the membrane like full-length CTL*myc. Obviously, a large fraction of 92-kDa truncCTL*myc cannot be liberated from the ER membrane in the absence of Hul5 in vivo. As truncCTL*myc is not detectable with specific anti-CPY antibodies (Fig. 5, lanes 3 and 4), the major ER-lumenal, N-terminal CPY* part of CTL*myc must be degraded, whereas the cytosolic C-terminal Leu2myc fused with the transmembrane domain remains in the ER membrane (Fig. 4, B and C).

**FIGURE 4.** The fragment of CTL*myc is not generated when proteasomal activity is blocked. A, proteasomal activity was inhibited using MG132 in Δpdr5 and Δpdr5Δhul5 strains expressing CTL*myc. Strains are deleted for the gene encoding for Pdr5 to enhance the concentration of MG132 in the cells. The cells were subjected to pulse-chase analyses as described in Fig. 1A using anti-Myc antibodies. Curves represent data of three independent experiments, and the error bars indicate the respective S.E. B, PNS of Δhul5 + CTL*myc cells was prepared, and the supernatant fraction, including cytosolic proteins (supernatant, S), and pellet fraction, including organelles and microsomes (pellet, P) were separated. C, membrane extraction experiment. The postnuclear supernatant of Δhul5 + CTL*myc cells was subdivided into parts and treated either with 1 M KAc, 2.5 M urea, 0.1 M Na2CO3 pH 11.2, 1% Triton X-100, or 1% SDS. The samples were separated into a supernatant (S) and pellet fraction (P) by high speed centrifugation and analyzed by immunoblotting with anti-Myc antibodies. Detection of the cytosolic phosphoglycerate kinase (PGK) and the ER membrane protein Sec61 serve as controls for the agent treatments.

**FIGURE 5.** Substrate-Der3/Hrd1-Cdc48 interaction is disturbed in a Δhul5 strain. Cells of the yeast strains Δprc1 and Δprc1Δhul5 both deleted for the coding sequence of CPY (PRC1) and expressing plasmid encoded CTL* or CTL*myc, respectively, were treated with cycloheximide (CHX) for 0 and 3.5 h. This allows the accumulation of truncCTL*myc fragment in the Δprc1Δhul5 strain. Microsomal extracts of all cells were prepared, and the membrane proteins were solubilized using 1% digitonin. The 100,000 × g supernatant (input) was subjected to immunoprecipitation using anti-Myc antibodies (α-myc precipitates) and analyzed by immunoblotting with the indicated antibodies. Asterisk indicates an unspecific reaction of anti-Der3 antibodies.

Npl4 complex is implicated in release of misfolded substrates from the ER membrane prior to proteasomal degradation (25, 26, 52). In agreement with this model, a mutation in Ufd1 pre-
vents degradation of CTL*myc (Fig. 3B). Therefore, we studied the direct interaction of CTL*myc with the Cdc48 complex. Immunoprecipitation experiments using digitonin-solubilized membrane proteins demonstrate an interaction of full-length CTL*myc with Cdc48 (Fig. 5, lanes 11 and 12). Only a very weak interaction between Cdc48 and truncCTL*myc was observed in a /H9004 hul5 mutant strain after 3.5 h of cycloheximide treatment (Fig. 5, lanes 15 and 16). Therefore, the action of Hul5 on truncCTL*myc allows complete extraction of the truncated fragment out of the ER membrane in wild type cells. After partial degradation of the CPY* moiety of CTL*myc in Δhul5 mutant cells, truncCTL*myc loses its interaction with Cdc48 (Fig. 5, lane 15). Even the association with the ER membrane-localized E3 ligase Der3/Hrd1 was abandoned (Fig. 5, lanes 15 and 16). Obviously, in the absence of a ubiquitin chain elongation by Hul5, truncCTL*myc does not have an affinity for the Cdc48 complex. This prevents complete extraction of truncCTL*myc from the ER membrane, its ubiquitination by Der3/Hrd1, and subsequent degradation.

**DISCUSSION**

The use of two substrates CTL* and Sec61-2L, each carrying a misfolded protein moiety in the ER, allowed for a genome-wide screen of mutant strains defective in ERQD. Besides other ERQD components (22, 27), Hul5 was identified to be essential for complete degradation of both substrates (Fig. 1A). Finley and co-workers (31) discovered Hul5 as an E4-HECT domain ubiquitin ligase involved in ubiquitin chain elongation. When Hul5 is bound to the proteasome its catalytic function is stimulated, and the enzyme extends pre-existing polyubiquitin chains on proteasomal substrates. The ubiquitin chain elongation may stabilize the interaction between proteasome and its substrates and thus lead to efficient substrate degradation. The E4 ligase activity of Hul5 has been found to act on a variety of soluble proteasomal substrates in vitro and in vivo. Examples are the transcriptional activator Gcn4, N-end rule substrates, and cyclin B (31). Here we show that the activity of Hul5 is also required for ERAD of certain misfolded ER membrane proteins.

Interestingly, we observed a stepwise degradation of the misfolded ER membrane proteins CTL*myc and Sec61-22Lmyc, which proceeded via two intermediates, respectively (Fig. 2A). The CTL*myc intermediate of about 92 kDa (truncCTL*myc) accumulates in HUL5-deleted cells, whereas it is degraded in wild type cells (Fig. 2, A and B). Studying elimination of CTL*myc in more detail, we could demonstrate that truncCTL*myc remains to a large extent embedded in the membrane in the absence of Hul5 (Fig. 4, B and C). Considering the topology of CTL*myc (Fig. 1A), we conclude that the ER-lumenal CPY* moiety must have reached the cytoplasm via its N terminus to become a target of the proteasome. Most likely, this occurs by looping of the N terminus across the ER membrane. In contrast, the truncated form of CTL*myc remains in the ER membrane and resists degradation (Fig. 4, B and C). Obviously, the ubiquitin chain elongating activity of Hul5 is absolutely necessary for complete removal of this C-terminal fragment from the ER membrane, but it is not required for degradation of the misfolded CPY* moiety of CTL*myc (Fig. 3C). The AAA-ATPase Cdc48 was shown to remove ERAD substrates from the ER membrane in conjunction with Ufd1 and Npl4 (25, 52). As compared with full-length CTL*myc, truncated CTL*myc is associated only in minor amounts with Cdc48 (Fig. 5, lanes 11 and 15). It is assumed that the affinity of truncCTL*myc to Cdc48-Ufd1-Npl4 is too weak for its efficient extraction out of the ER membrane without a modification by Hul5. The molecular mass of membrane-localized truncCTL*myc of 92 kDa hints to its composition of a cytosolic Leu2myc domain.

**FIGURE 6. Model of the degradation process of CTL*myc in wild type cells (A–C) and Δhul5 (D).** The CPY* moiety of CTL*myc (A) comes out of the ER lumen and reaches the cytoplasm. There it is ubiquitinated by Der3/Hrd1, extracted by Cdc48, and degraded by the proteasome (B). The membrane-embedded part of CTL*myc needs the E4 ligase activity of Hul5 for complete extraction and degradation (C). In the absence of Hul5 most of the membrane-embedded part of truncCTL*myc remains stable in the ER membrane and loses contact with Der3/Hrd1 and Cdc48 (D).
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(74 kDa; Fig. 2C), a 7–10-kDa transmembrane domain and probably a residual peptide of misfolded CPY* of about 10 kDa in the ER lumen (Fig. 2C). We assume that this part of the CPY* moiety is extended through the retrotranslocation channel and thereby protected from degradation in the absence of Hul5. This residual part of truncCTL*myc seems to be unable to strongly bind to Cdc48, because it might slip back into the ER lumen. This view is further supported by the fact that no interaction was found between the E3 ligase Der3/Hrd1 of the ERAD pathway and the CTL*myc fragment (Fig. 5, lanes 11 and 15). Der3/Hrd1 is usually associated with Hrd3, which is known to act on the ER lumenal site in recognition and binding of ERAD substrates (13, 20, 21, 53, 54). It is most likely that the remaining truncCTL*myc also loses contact with Hrd3 and, by doing so, cannot be recognized by the ERAD machinery anymore. So far, we cannot exclude that the truncCTL*myc undergoes proteasomal degradation independently of the known ERAD components.

It is a long standing discussion whether membrane proteins are completely extracted prior to proteasomal degradation or whether proteolysis starts while they are still embedded in the ER membrane. Here we show that CTL*myc is not pulled out of the membrane in its complete entity prior to degradation. The pulling and elimination of full-length CTL*myc are stepwise processes even in wild type cells. Combining all results, the following scheme for degradation of the substrate CTL*myc emerges (Fig. 6). First, a limited degradation of the ER-lumenal N-terminal part of CTL*myc takes place, whereas a part of CTL*myc is still embedded in the membrane. Most likely, this mechanism starts with a loop of the soluble CPY* part of CTL*myc through the ER membrane into the cytoplasm (Fig. 6B). In a second step, the remaining truncCTL*myc is pulled out of the membrane by Cdc48 and degraded by the proteasome. For this purpose the E4 activity of Hul5 is essentially required (Fig. 6C). In the absence of Hul5 most of the remaining truncCTL*myc slips back and has less or no contact to the Hrd-ligase complex and to Cdc48 (Fig. 6D). Nevertheless, a portion of truncCTL*myc reaches the cytoplasm where it remains stable (Fig. 4B). Despite the disturbed association to CTL*myc, Cdc48 could partially release truncCTL*myc. If Hul5 is present, its E4 ligase activity ensures degradation of the remaining fragment truncCTL*myc.

Full-length CTL*myc cannot be degraded as long as proteasomal degradation is blocked (Fig. 4A). Most likely, this full-length CTL*myc remains in the ER membrane as well as all CTL*myc intermediates generated during the degradation process (Fig. 4C). The extraction of the membrane-embedded CTL*myc is not achieved without the action of the proteasome. This may indicate that the proteasome has an active role in the extraction process of CTL*myc by modulating the strength of the ubiquitin targeting signal through the E4 function of Hul5. This suggests that the process of ER membrane extraction and the degradation by the proteasome might be a concerted action whereby ER membrane and proteasome are in close contact (Fig. 6).

Acknowledgments—We thank Thomas Sommer for the generous gift of anti-Cdc48 antibodies on regular basis and Daniel Finley for Hul5 encoding plasmids. We appreciate the valuable comments by Wolfgang Hilt, Shahri Raasi, and the Wolf group members (especially Alexandra Stolz) on this manuscript. The help of Elisabeth Tosta on the preparation of the manuscript is acknowledged. We are grateful to Jeannette Juretschke providing the Leu2myc encoding plasmid and to Stefanie Besser for experiments performed in ssa1-45.

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