Membrane Topology and Function of Der3/Hrd1p as a Ubiquitin-Protein Ligase (E3) Involved in Endoplasmic Reticulum Degradation*

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The endoplasmic reticulum (ER) contains a highly effective protein quality control system that discovers malfolded or unassembled secretory proteins and subjects them to degradation in the cytosol. This requires retrograde transport of the respective proteins from the endoplasmic reticulum back to the cytosol via the Sec61 translocon. In addition, a fully competent ubiquitination machinery and the 26 S proteasome are necessary for retrotranslocation and degradation. Ubiquitination of mutated and malfolded proteins of the endoplasmic reticulum is dependent mainly on the ubiquitin-conjugating enzyme Ubc7p. In addition, several new membrane components of the endoplasmic reticulum are required for degradation. Here we present the topology of the previously discovered RING-H2 finger protein Der3/Hrd1p, one of the new components of the endoplasmic reticulum membrane. The protein spans the membrane six times. The amino terminus and the carboxyl terminus containing the RING finger domain face the cytoplasm. Altogether, RING finger-dependent ubiquitination of malfolded carboxypeptidase yscY in vivo, as well as of Der3/Hrd1p itself in vitro and RING finger-dependent binding of Ubc7p, uncovers Der3/Hrd1p as the ubiquitin-protein ligase (E3) of the endoplasmic reticulum-associated protein degradation process.

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* The abbreviations used are: ER, endoplasmic reticulum; CPY, carboxypeptidase yscY, CPY, mutated, malfolded CPY; PCR, polymerase chain reaction; GST, glutathione S-transferase; 5-FOA, 5-fluoroorotic acid; HA, hemagglutinin; Nub and Cub, amino- and carboxyl-halves of ubiquitin.

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completely abolishes degradation of CPY* and Pdr5*, indicating the essentiality of this domain (10, 17, 28). We have furthermore shown that Der3/Hrd1p, Hrd3p, and Sec61p interact genetically, which led us to the proposal that these proteins formed both the retrotranslocon and an E3 complex (19). Here we examine the topology of Der3/Hrd1p in the ER membrane via N-glycosylation scanning and fusion of a topology-sensitive reporter protein domain to carboxyl-terminally truncated versions of the Der3/Hrd1 protein. We show that the carboxy-terminally located RING-H2 domain of Der3/Hrd1p faces the cytoplasm. Altogether, RING finger-dependent ubiquitination of CPY* in vivo as well as self-ubiquitination of Der3/Hrd1p in vitro, and RING finger-dependent binding of Ubc7p identify Der3/Hrd1p as the ubiquitin-protein ligase (E3) of the ER degradation process.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids**

Genetic experiments and molecular biological methods were carried out using standard protocols (29).

**Der3/Hrd1p GST Fusion Constructs**—The plasmid pRS315-DER3 was generated by subcloning a 3,570-base pair BamHI/HindIII fragment from plasmid YEpDER3 (17), containing the DER3 gene under its native promoter, into plasmid pRS315 (30). Fragments of the DER3 gene were PCR amplified from this plasmid using the oligonucleotide 5′-ccggccgctcgagccgttcgtggaacagtg agg-3′ (EagI site within the 3′-untranslated region of DER3) as the upstream primer and one each of the oligonucleotides 5′-ccgccggaattcctgattaacagggggac-3′ (EcoRI) and DER3-GST2 (5′-cggcggcggatccctggtgacgtgccagaaaatagaaggaaac-3′) as the downstream primer (YEp112 (33) is used for yeast and pGEX-4T-1 for E. coli). PCR products were digested with SacI/SacII and inserted into the GST expression vector pGEX-4T-1 (Amersham Pharmacia Biotech), yielding plasmid pGEX-DER3.

**DER3-SUC2-HIS4C Fusion Constructs**—The plasmid pRS426-DER3 was generated by subcloning a 3,570-base pair BamHI/HindIII fragment and transformed into yeast strain W303-1C (17). Immuno-precipitation experiments were carried out according to the manufacturer's instructions. The extracts were incubated with 100 μl (bead volume) of glutathione Sepharose 4B (Amersham Pharmacia Biotech) for 3 h at 4 °C. Sepharose beads were collected (5 min at 3,000 rpm), washed three times with 1 ml of phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄, pH 7.3), and incubated at 4 °C overnight with whole cell extracts prepared from yeast strain W303-1C, carrying the plasmid pTX146 (18) encoding a Myc-tagged version of Ubc7p. Sepharose beads were collected and washed for 5 min with 1 ml of phosphate-buffered saline containing 500 mM NaCl. Proteins from the supernatant and the wash fraction were precipitated with trichloroacetic acid and dissolved in urea buffer. Proteins bound to the Sepharose matrix were then eluted with urea buffer by incubating at 95 °C for 5 min. Samples were subjected to Western blot analysis using monoclonal anti-Myc antibody (Roche).

**In Vitro Ubiquitination Experiments**

Ubiquitination reactions were performed in 25 mM Tris/HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, 10 mM ATP, and 0.5 μg/μl ubiquitin (yeast, Sigma) and contained one more of the following components as indicated in Fig. 6: 0.5 μg of E1 (yeast, Affinity), 1 μg of E2 (human UBC1, Affinity), and approximately 1 μg of GST-Der3 fusion protein (see above). Incubation of the reactions for 60 min at 30 °C was followed by dilution with phosphate-buffered saline and precipitation of GST-Der3 species with Sepharose beads (3 h at 4 °C). The beads were collected, washed three times with phosphate-buffered saline and eluted with urea buffer. Samples were subjected to Western blot analysis using monoclonal anti-ubiquitin antibody (Calbiochem) or polyclonal anti-Der3 antisem (17, respectively).

**Immunoprecipitation of Ubiquitinated CPY**

For in vitro ubiquitination of CPY*, HA-tagged ubiquitin was expressed from plasmid YEp112 (33) by induction with copper as indicated in Fig. 5. The experiment was performed in a DER3 wild type strain (W303-1C) and a der3 deletion strain (YJB009) expressing the C399S mutant of Der3p from plasmid YCpDER3C399S (17). Immunoprecipitation and detection of ubiquitinated CPY* species were done as described previously (9).

**Western Blot Analysis**

For Western blot analysis of complete cellular protein, 3 A₁₅₁ units of cells were subjected to alkaline lysis, and proteins were precipitated with trichloroacetic acid and resuspended in urea buffer. Protein samples were separated on 6–12% SDS-polyacrylamide gels and subjected to immunoblotting as described above (19).

**RESULTS**

**Der3/Hrd1p Spans the ER Membrane Six Times; Its Amino and Carboxyl Terminii Face the Cytoplasm**—Sequence analysis of Der3/Hrd1p had indicated that the protein contains a hydrophobic amino-terminal region with five putative membrane domains and a long hydrophilic carboxyl-terminal tail containing the RING-H2 motif (13, 17). Protease protection and immunolocalization experiments had localized the carboxyl-terminal RING-H2 finger domain to the lumen of the ER. However, because the ubiquitin-conjugating enzyme in charge,
The amino acids Asn-Ser-Thr were introduced between the last two amino acids of Der3/Hrd1p, and the resulting protein (Der3-G) was expressed in a DER3 wild type and a der3 deletion strain and subjected to Western blot analysis. In contrast to a CPY* species modified in the same manner (Pr1-G5) (34), no increase of the molecular mass resulting from N-glycosylation is observed.

Ub7p, as well as the proteasome reside in the cytoplasm, it was hard to reconcile the fact that the RING-H2 domain of the protein resided in the ER lumen with a function for Der3/Hrd1p as a ubiquitin-protein ligase, unless one assumed a process by which the carboxyl terminus with the RING-H2 domain and bound substrate reached back through the translocon into the cytoplasm of the cell (19).

We therefore undertook a new set of experiments to localize the carboxyl-terminal RING finger domain of Der3/Hrd1p and to uncover the membrane topology of the protein. First we undertook an N-glycosylation scanning experiment. Der3/Hrd1p contains two N-glycosylation sites at positions 58 and 137 of its sequence (17). However, these sites are not accessible to the glycosylation machinery because they are predicted to be located in a transmembrane span and on the cytosolic side of the ER membrane, respectively. Therefore, we introduced a new potential N-glycosylation site between the penultimate and last amino acid of Der3/Hrd1p as done with CPY* (34). When expressing this modified Der3/Hrd1 protein either in a DER3 wild type or a der3 deletion background, no shift of the molecular mass to higher molecular mass was visible although a similarly modified CPY* molecule to higher molecular mass was visible although a similarly modified CPY* molecule was shifted to higher molecular mass due to additional glycosylation (Fig. 1). This may indicate that the carboxyl terminus of the ER-localized Der3/Hrd1 protein does not reach the lumen of the organelle where core glycosylation occurs. However, this experiment does not give final proof for cytoplasmic localization of the carboxyl terminus of Der3/Hrd1p.

We set up further experiments to prove this result and to elucidate the exact membrane topology of Der3/Hrd1p. The hydropathy profile of Der3/Hrd1p predicts the existence of five or six transmembrane domains (Fig. 2A). We created fusion constructs of Der3/Hrd1p with a truncated version of the His4 protein (His4C) containing a fragment of invertase (Suc2p) which we used as topology-sensitive reporters. His4C harbors histidinol dehydrogenase activity and is translocated through the ER membrane when fused to a signal sequence (35). Yeast strain STY50 was transformed with the control vector YEp352 or plasmids encoding the different Der3/Hrd1-His4 fusions. Transformants were streaked on selective media supplemented with histidine (left panel) or histidinol (right panel) and incubated for 3–5 days at 30 °C. Analysis of the N-glycosylation state of the Der3/Hrd1-His4C fusion proteins. The proteins were immunoprecipitated from whole cell extracts of STY50 transformed with plasmids pQ37 to pD345 (lanes 1–12) or plasmid YEp352 (lane 13) using polyclonal anti-invertase antibody. Immunoprecipitates were treated with endoglycosidase H (Endo H) as indicated and separated on 6% SDS-polyacrylamide gels. Western blot analysis was performed using anti-invertase antibody.

Our results show that the carboxyl terminus of Der3/Hrd1p resides on the cytoplasmic side of the ER membrane.
first transmembrane region functions as a cleavable signal sequence or that the entire construct fully translocates into the ER lumen. The latter possibility is ruled out on the basis that Der3/Hrd1Q37 is detected in the fraction corresponding to the membrane pellet when anti-invertase antibodies are used. Der3/Hrd1Q37 cannot be solubilized with urea, high salt, or carbonate. Only after treatment with Triton X-100 was Der3/Hrd1Q37 transferred into the soluble fraction (data not shown).

Furthermore, we had shown previously that Der3/Hrd1p genetically interacts with another ER membrane protein necessary for ER degradation, Hrd3p (19). Hrd3p spans the membrane once, its carboxyl terminus being exposed to the cytosol (19). Thus it was feasible that the amino terminus of Der3/Hrd1p was in close proximity to the carboxyl terminus of Hrd3p. To elucidate this possibility and to show that the amino terminus of Der3/Hrd1p indeed faces the cytoplasm, we made use of the split ubiquitin technique (32, 36). This technique monitors interactions between proteins in vivo and is based on the self-reassembly of the amino- and carboxyl-terminal halves (Nub and Cub) of ubiquitin. The reassembled ubiquitin is recognized by ubiquitin-specific proteases that cleave any carboxyl-terminally modified Hrd3p, Hrd3-Cub-RUra3 and the amino-terminally modified ubiquitin (Nub) was attached to the amino terminus of Der3/Hrd1p. Cells coexpressing these two constructs were spotted on uracil-containing minimal medium conditioned with 5-FOA in 1:10 dilution steps. Growth of cells is promoted by reassembly of ubiquitin, leading to cleavage and degradation of the Ura3 protein. To show the specificity of the Der3/Hrd1p-Hrd3p interaction, the Nub fusion of the Golgi protein Sed5p was expressed together with the Cub fusion of Hrd3p.

### Table I

| Der3/Hrd1-His4C fusion | Molecular mass | Growth on histidinol | Glycosylation |
|------------------------|---------------|---------------------|--------------|
| Der3/Hrd1Q37           | 126,398       | -                   | +            |
| Der3/Hrd1E78           | 131,138       | +                   | -            |
| Der3/Hrd1R103          | 134,233       | +                   | -            |
| Der3/Hrd1Q134          | 138,031       | +                   | -            |
| Der3/Hrd1Q176          | 142,828       | -                   | -            |
| Der3/Hrd1D345          | 162,548       | +                   | -            |

FIG. 3. Split ubiquitin assay confirms the cytoplasmic orientation of the Der3/Hrd1p amino terminus. The carboxyl-terminal half of ubiquitin (Cub), followed by an arginine residue and the Ura3 protein (RUra3), was fused to the cytosolic carboxyl terminus of Hrd3p. The amino-terminal half of ubiquitin (Nub) was attached to the amino terminus of Der3/Hrd1p. Cells coexpressing these two constructs were spotted on uracil-containing minimal medium conditioned with 5-FOA in 1:10 dilution steps. Growth of cells is promoted by reassembly of ubiquitin, leading to cleavage and degradation of the Ura3 protein. To show the specificity of the Der3/Hrd1p-Hrd3p interaction, the Nub fusion of the Golgi protein Sed5p was expressed together with the Cub fusion of Hrd3p.

FIG. 4. Membrane topology of Der3/Hrd1p.
enzymes and Mg$^{2+}$ main of Der3/Hrd1p (amino acids 208–551) was expressed as a
soluble RING-H2 finger-containing carboxyl-terminal domain in the absence of other substrate
proteins (20, 23, 27). We tested Der3/Hrd1p for this property.

It has been demonstrated that the RING domain mediates the recruitment of the respective ubiquitin-conjugating enzyme $E2$ involved in the degradation reaction (25, 37). In the case of the ubiquitin-protein ligase c-Cbl, the RING finger motif has been shown to be essential for binding of the $E2$ enzyme (37, 38). We tested the ability of Der3/Hrd1p to bind the ubiquitin-conjugating enzyme Ubc7p, which is known to be necessary for all ubiquitin conjugation reactions dependent also on Der3/Hrd1p (10, 13, 14, 17). The soluble GST-fused RING-H2 finger-containing domain of Der3/Hrd1p was bound to glutathione-Sepharose. Application of extracts of cells expressing a functional Myc-tagged Ubc7p (18) to the GST-Der3/Hrd1 Sepharose beads resulted in specific binding of Ubc7p to the protein (Fig. 7A). Interestingly, the inactive C399S mutant of Der3/Hrd1p is also defective in binding of Ubc7p (Fig. 7B). Taken together, the lack of CPY* ubiquitination in cells carrying a mutated Der3/Hrd1 C399S protein, the ability to ubiquitinate itself, and its interaction with Ubc7p clearly identify Der3/Hrd1p as the ubiquitin-protein ligase (E3) of the ER degradation process. The results also show that the RING-H2 domain of the ligase is crucial for recruitment of the ubiquitin-conjugating enzyme Ubc7p.

**DISCUSSION**

This study presents a first analysis of the complete membrane topology of Der3/Hrd1p, an essential component of the ER degradation machinery. We furthermore provide evidence that Der3/Hrd1p is the ubiquitin-protein ligase (E3) of the ER degradation process. Using a topology-sensitive reporter protein domain, we show that Der3/Hrd1p contains six transmembrane domains. The amino terminus and the carboxyl terminus of the protein face the cytoplasmic side of the ER membrane. The newly established cytoplasmic localization of the carboxyl-
same experiment was performed using the mutated C399S species of control, GST alone was attached to the Sepharose beads. By Western blot analysis using monoclonal anti-Myc antibodies. As proteins were eluted with urea buffer (E), and samples were analyzed washed with incubation buffer containing 500 mM NaCl (S) was removed and the gel beads prepared from a strain expressing a functional Myc-tagged version of Ubc7p. The nonbound supernatant (W) was analyzed by Western blot analysis using monoclonal anti-Myc antibodies. As a control, GST alone was attached to the Sepharose beads. When the same experiment was performed using the mutated C399S species of Der3/Hrd1p, specific binding of Myc-Ubc7p to the GST fusion protein was completely abolished.

The carboxyl-terminal domain of Der3/Hrd1p corrects our previous preliminary finding, in which upon protease protection experiments and subsequent immunolocalization studies with an antibody directed against the carboxyl terminus of Der3/Hrd1p, an ER luminal localization of the carboxyl-terminal domain of the protein had been suggested (17). This may have been the result of conditions leading to an artifactual burying of the carboxyl terminus of Der3/Hrd1p because of membrane aggregation and occlusion of membrane surfaces (39) or a carboxyl terminus resistant to protease attack. While our work was nearly completed, a paper appeared which also reported a cytoplasmic localization for the carboxyl-terminal domain of Der3/Hrd1p (40).

The carboxyl-terminal domain of Der3/Hrd1p contains a RING-H2 finger motif that is crucial for its function: deletion of the motif or exchange of one of the cysteine residues (Cys-399) of the RING-H2 domain to serine completely abolishes degragation of mutated and malfolded ER proteins (17, 28). RING finger-containing proteins have been identified as a new class of ubiquitin-protein ligases (3 enzymes) necessary for ubiquitination of substrates destined for degradation via the proteasome (20–27). We show here that cells carrying a mutated Der3/Hrd1p C399S version are unable to ubiquitinate CPY* in vivo (Fig. 5). A common feature of RING finger-containing ubiquitin-protein ligases is their ability to ubiquitinate themselves in vitro in the absence of substrates (20, 23, 27). We found that this also holds true for Der3/Hrd1p (Fig. 6). Function of a protein as an E3 requires recruitment of the respective ubiquitin-conjugating enzyme (E2). Protein-protein interaction studies using a GST-bound carboxyl-terminal fragment of Der3/Hrd1p containing the RING-H2 domain demonstrated that Ubc7p, the major ubiquitin-conjugating enzyme of the ER degradation process, selectively bound to the protein. Altogether, these properties identify Der3/Hrd1p as the ubiquitin-protein ligase (E3) of ER degradation as suggested previously (19). Mutation of a crucial cysteine (C399S) in the RING-H2 finger domain completely abolished binding of Ubc7p, suggesting that the RING-H2 domain is directly involved in the binding of E2. Cue1p, another ER membrane protein, had been shown to bind Ubc7p and to be necessary for Ubc7p activity (18). Our pull-down experiment clearly demonstrates that Der3/Hrd1p carries an intrinsic binding activity toward Ubc7p. The additional binding of Ubc7p by Cue1p might result in a tightening of Ubc7p binding to the membrane and by this inducing the formation of the “active” structure of Ubc7p.

We had shown previously that Der3/Hrd1p and Hrd3p, another essential ER membrane protein involved in ER degradation, interact genetically, indicating complex formation between these two proteins (19). Using the split ubiquitin system, we could show that the cytoplasmically localized amino terminus of Der3/Hrd1p and the carboxyl terminus of Hrd3p are in close proximity, which might indicate that they physically interact (Fig. 3). An interaction between both proteins has also been presented recently by others (40). The fact that the carboxyl terminus of Der3/Hrd1p with its RING-H2 domain is localized to the cytoplasm and never sees the ER lumen makes the previous proposal of Der3/Hrd1p as being a binding partner of the substrate in the ER lumen, which delivers it to the cytoplasmic degradation machinery, unlikely. It also makes a function of Hrd3p as a recycling molecule for the carboxyl terminus of Der3/Hrd1p from the cytoplasm back into the ER lumen improbable (19). A complex formation of Der3/Hrd1p and Hrd3p must have different functions. As induction of Ubc7p-dependent Der3/Hrd1p degradation upon Hrd3p deletion indicates the presence of a fully competent ubiquitination and degradation machinery in Δhrd3 cells, the defective degrad-geration of ER substrates in the absence of Hrd3p must have other reasons. It would be plausible if Hrd3p had substrate recognition and signaling functions, which lead to integration of processes such as substrate delivery via the Sec61 translocon, ubiquitination via Der3/Hrd1p and Ubc7p, and degradation via the proteasome in a fashion that makes the overall process highly regulated and processive. Similar functions for the Der3/ Hrd1p-Hrd3p interaction are also discussed by Gardner et al. (40). Future experiments will uncover the functional relationship of both proteins.

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Membrane Topology and E3 Function of Der3/Hrd1p

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