Genetic interactions of Hrd3p and Der3p/Hrd1p with Sec61p suggest a retro-translocation complex mediating protein transport for ER degradation

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SUMMARY

The endoplasmic reticulum contains a quality control system that subjects misfolded or unassembled secretory proteins to rapid degradation via the cytosolic ubiquitin proteasome system. This requires retrograde protein transport from the endoplasmic reticulum back to the cytosol. The Sec61 pore, the central component of the protein import channel into the endoplasmic reticulum, was identified as the core subunit of the retro-translocon as well. As import of mutated proteins into the endoplasmic reticulum lumen is successfully terminated, a new targeting mechanism must exist that mediates re-entering of misfolded proteins into the Sec61 pore from the lumenal side de novo. The previously identified proteins Der3p/Hrd1p and, as we show here, Hrd3p of the yeast *Saccharomyces cerevisiae*, are localised in the endoplasmic reticulum membrane and are essential for the degradation of several substrates of the endoplasmic reticulum

degradation machinery. Based on genetic studies we demonstrate that they functionally interact with each other and with Sec61p, probably establishing the central part of the retro-translocon. In the absence of Hrd3p, the otherwise stable protein Der3p/Hrd1p becomes rapidly degraded. This depends on a functional ubiquitin proteasome system and the presence of substrate molecules of the endoplasmic reticulum degradation system. When overexpressed, Der3p/Hrd1p accelerates CPY* degradation in *Δhrd3* cells. Our data suggest a recycling process of Der3p/Hrd1p through Hrd3p. The retro-translocon seems to be build up at least by the Sec61 pore, Der3p/Hrd1p and Hrd3p and mediates both retrograde transport and ubiquitination of substrate molecules.

Key words: Endoplasmic reticulum, ER degradation, Proteasome, Sec61 translocon, Hrd3p

INTRODUCTION

Secretion of proteins into the extracellular milieu or the lumen of organelles as well as their integration into membranes is essential for viability of all cells. Eukaryotic cells developed the central vacuolar system consisting of endoplasmic reticulum (ER), Golgi apparatus, transport vesicles and the lysosome or, in yeast, the vacuole to fulfill these tasks. The ER provides the site of entry of all soluble and integral membrane proteins into this system (Rapoport et al., 1996). Because newly synthesised polypeptides are transported through the ER membrane in an unfolded state it contains a high amount of molecular chaperones and other enzymes to facilitate folding and assembly of nascent chains into their native conformation (Leitzgen and Haas, 1998). A highly efficient quality control system monitors the folding status and guarantees further transport of only properly folded proteins through the secretory system. Proteins that cannot acquire their native conformation are, in many cases, retained in the ER and rapidly degraded. This process, which is highly conserved in all eukaryotic cells, is known as ER-associated degradation or simply ER

degradation (Brodsky and McCracken, 1998; Plemper and Wolf, 1999; Sommer and Wolf, 1997). Recent studies have revealed that hydrolysis of misfolded or unassembled secretory proteins is mediated by the ubiquitin proteasome system. For example, ubiquitination and/or proteasome dependent degradation could be demonstrated in mammalian cells for a mutated version of the human cystic fibrosis transmembrane conductance regulator CFTR (Jensen et al., 1995; Ward et al., 1995), unassembled T-cell receptor α-chains (Huppa and Ploegh, 1997; Yu et al., 1997) and downregulated HMG-CoA reductase (Moriyama et al., 1998). In yeast, mutant versions of the vacuolar carboxypeptidase Y (Hiller et al., 1996), the plasma membrane ATP-binding cassette transporter Pdr5 (Plemper et al., 1998), the mating-type pheromone α-factor (McCracken and Brodsky, 1996) and downregulated HMG-CoA reductase (Hampton et al., 1996) are subjects of proteasomal degradation.

Substrate proteins of the proteasome are usually marked by the repeated attachment of ubiquitin moieties through isopeptide bonds between the carboxy-terminus of ubiquitin and ε-amino groups of lysine residues within the substrate

protein and ubiquitin itself (Ciechanover, 1998). Binding of ubiquitin to substrate molecules requires an enzyme cascade: after activation through an E1 enzyme, ubiquitin is transferred to a conjugating (E2) enzyme. Due to an E3 enzyme the activated ubiquitin is subsequently attached to substrate proteins. Several multi-subunit E3 enzymes such as APC and SCF were described (Peters, 1998). At least in case of the SCF complex it was suggested that a RING H2 finger protein binding motif could function in bridging the complex or in substrate binding (Tyers and Willems, 1999). Whether RING H2 finger domains are common motifs of E3 enzymes, remains to be demonstrated. Although presently nothing is known about an E3 enzyme involved in ER degradation, the ER membrane localised proteins Ubc6p and Ubc7p were identified as the E2s responsible for degradation of soluble and transmembrane secretory proteins in yeast (Biederer et al., 1996; Hampton and Bhakta, 1997; Hiller et al., 1996; Plemper et al., 1998). While Ubc6p (Sommer and Jentsch, 1993) is an integral membrane protein, Ubc7p is recruited to the ER membrane through the integral ER-membrane protein Cue1p (Biederer et al., 1997). The active sites of both proteins, however, are oriented to the cytosolic face of the ER membrane. As proteasomes are also restricted to the cytosol and nuclear compartments (Enenkel et al., 1998), retrograde transport of soluble secretory proteins from the ER lumen or extraction of transmembrane proteins from the ER membrane has to preceed proteolysis.

The Sec61 pore, known as the central component of the protein import complex into the ER (Rapoport et al., 1996), was identified as the membrane spanning channel for retrograde transport as well: major histocompatibility complex class I heavy chain breakdown intermediates could be co-immunoprecipitated with Sec61β in human cytomegalovirus infected cells (Wiertz et al., 1996). Mutated versions of carboxypeptidase Y, CPY* allele *prc1-1* (Wolf and Fink, 1975) or of mutated Pdr5, Pdr5* allele *pdr5-26* (Egner et al., 1998) are substrates of the ER degradation system in yeast. Strains expressing these proteins and carrying a mutation in Sec61p, allele *sec61-2*, exhibit a considerably slowed down degradation rate of CPY* or Pdr5* (Plemper et al., 1997, 1998). Following the fate of mutated yeast α-factor in vitro, the significance of the Sec61 pore for retrograde transport was further established (Pilon et al., 1997). While several components were identified to interact with the Sec61 pore for protein import giving the process direction, the subunit composition of the postulated retro-translocon remained a mystery.

Remarkably, the entire CPY* chain is imported into the ER lumen prior to its recognition by the ER quality control system (Plemper et al., 1999). This implies the existence of a completely new targeting mechanism of polypeptides to the translocation pore for retrograde transport to occur. Based on these observations, it was speculated that retrograde transport and membrane extraction of proteins must require additional protein components to the Sec61 channel. Mutagenesis of yeast cells and subsequent cloning of the respective genes identified several ER membrane proteins such as Der1p (Knop et al., 1996) and Der3p/Hrd1p (Bordallo et al., 1998; Hampton et al., 1996) to be essential for ER degradation. Remarkably, Der3p/Hrd1p carries a RING H2 finger domain within its lumenal tail that is essential for its function (Bordallo and Wolf, 1999). A truncated version of Der3p/Hrd1p lacking this

domain failed to complement a chromosomal deletion of *DER3/HRD1* (Bordallo et al., 1998). Moreover, expression of this version revealed a dominant negative effect on Pdr5* and CPY* degradation when expressed in a chromosomal wild-type background (Plemper et al., 1998). A similar dominant negative effect was observed with expression of a Der3p/Hrd1p mutant version carrying a single point mutation of a cysteine residue within the RING H2 finger motif (Bordallo and Wolf, 1999). Thus, Der3p/Hrd1p seems to function as a subunit of a larger protein complex rather than as an individual molecule in the ER membrane. Indeed, one could easily imagine that proteins such as Der3p/Hrd1p or Der1p interact with Sec61p resulting in the complex competent for retrograde protein translocation. Alternatively or additionally, they might be involved in targeting substrate molecules to the translocation machinery.

Here we show that Hrd3p, previously identified as a protein necessary for the regulated breakdown of HMG-CoA reductase (Hampton et al., 1996), is a general component of the ER degradation system essential for the degradation of soluble and transmembrane proteins. Hrd3p is a resident protein of the ER membrane carrying five carbohydrate chains. We discovered a genetic interaction of Hrd3p and Der3p/Hrd1p with each other and with Sec61p. In a Δ*hrd3* background Der3p/Hrd1p is subject to rapid degradation by the ubiquitin proteasome system. The instability of Der3p/Hrd1p in a Δ*hrd3* background depends on a functional RING H2 finger domain and the presence of substrate molecules. Ubc7p, Cue1p and functional proteasomes are required for Der3p/Hrd1p degradation. We speculate that the Sec61 pore, Der3p/Hrd1p and Hrd3p together with Cue1p and Ubc7p provide the basis of a complex in the ER membrane mediating retrograde transport and subsequent ubiquitination of substrate molecules.

MATERIALS AND METHODS

Construction and growth conditions of strains

Genetic experiments and methods employing molecular biology were carried out using standard methods (Guthrie and Fink, 1991). Yeast *Saccharomyces cerevisiae* strains used in this study are summarised in Table 1. If not otherwise specified cells were grown in suitable complete synthetic medium containing 2% glucose or 2% galactose, respectively, to an A_{600} of 1.0-1.5.

For generation of a Δ*hrd3::HIS3* knockout plasmid, plasmid pRH508 (Hampton et al., 1996) was linearised with *Nhe*I, and ends were blunted with Klenow fragment. Subsequently, a 1333 bp fragment containing the *HRD3* gene was removed through digestion with *Nsi*I and replaced with an 1069 bp *Eco*47III *Nsi*I *HIS3* fragment. To delete *HRD3*, the *Cla*I *Xma*I digested plasmid was transformed into yeast. Plasmid 2μ and centromeric plasmids bearing the *HRD3* gene were constructed through ligation of a *Sac*I *Xho*I *HRD3* fragment derived from plasmid pRH508 (Hampton et al., 1996) into *Sac*I *Xho*I linearised vectors pRS426 and pRS314 (Sikorski and Hieter, 1989). An inducible version of *HRD3* under the control of the galactose promoter carrying a HA-tag at its carboxy terminus was constructed through *Cla*I digestion of pRS426-*HRD3*, treatment with Klenow fragment and digestion with *Sma*I. The resulting 2500 bp *HRD3* fragment was ligated with the plasmid pYES2 (Invitrogen), which contained a double HA tag at position 590. The vector was digested with *Xho*I, treated with Klenow fragment and digested with *Pvu*II. *HRD3*-HA versions under the control of the wild-type promoter were generated through ligation of a 1963 bp *Xba*I *Nhe*I *HRD3*-HA

Table 1. Yeast strains used in this study

Strain	Genotype	Reference
W303-1C	*MATα ade2-loc ura3-1 his3-11, 15 leu2-3, 112 trp1-1 can1-100 prc1-1*	Knop et al., 1996
W303-1Ca	*MAT**a** ade2-loc ura3-1 his3-11, 15 leu2-3, 112 trp1-1 can1-100 prc1-1*	This study
YRP181	W303-1C Δ*hrd3::HIS3*	This study
YRP086	W303-1C *sec61-2*	Plemper et al., 1997
YRP250	YRP086 Δ*hrd3::HIS3*	This study
YWO0081	*Matα sec18-1 prc1-1 ade his3-11, 15 cps1-3, prb1-1*	This study
YJB001	YWO0081-C *sec18-1* Δ*hrd3::HIS3*	This study
YJB002	YRP181 Δ*ubc6::LEU2*	This study
YJB003	YRP181 Δ*ubc7::LEU2*	This study
YJB004	YRP181 Δ*ubc6::LEU2* Δ*ubc7::LEU2*	This study
YCT332	W303-1Ca Δ*cue1::LEU2*	This study
YJB005	YCT332 Δ*hrd3::HIS3*	This study
WCGY4a	*MAT**a** his3-11, 15 leu2-3, 112 ura3 prc1-1*	Hiller et al., 1996
YJB006	WCGY4a Δ*hrd3::HIS3*	This study
WCGY4 11/22a	WCGY4a *pre1-1 pre2-2*	Hiller et al., 1996
YJB007	WCGY4 11/22a Δ*hrd3::HIS3*	This study
W303-CD	W303-1C Δ*der1::LEU2*	Knop et al., 1996
YJB008	W303-CD Δ*hrd3::HIS3*	This study
YJB009	W303-1C Δ*der3/hrd1::HIS3*	Bordallo et al., 1998

fragment blunted with Klenow fragment at the *Xba*I site, with *Mlu*NI *Nhe*I fragments derived from plasmid pRS314-*HRD3* and pRS426-*HRD3*. Untagged *HRD3* under the control of the galactose promoter was constructed through ligation of a 2143 bp *Pvu*II *HRD3* fragment derived from plasmid pRS314-*HRD3* with a 6454 bp *Xba*I *Pvu*II fragment of vector pYES2-HAC *HRD3* blunted at the *Xba*I site with Klenow fragment.

Construction of plasmids pRS425-*DER3*, pRS426-*DER3*Δ*R*, YCp*DER3*C399S and pRS426-*DER3*C399S was described previously (Bordallo et al., 1998; Bordallo and Wolf, 1999). Plasmid pTX118 containing the Δ*cue1::LEU2* knockout construct was a gift from T. Sommer (Biederer et al., 1997). For deletion of the *CUE1* gene, pTX118 was linearised through *Apa*I *Sac*I digestion. In all cases, correct integration of the constructs was confirmed through Southern analysis.

Cycloheximide chase analysis

Cells were adjusted to approximately 8 A_{600}/ml. After adding cycloheximide with a final concentration of 0.1 mg/ml, 8 A_{600} of cells were removed at the indicated time points, suspended in sodium azide solution (final concentration, 10 mg/ml) and kept at –80°C. Cell lysis was performed essentially as described before (Bordallo and Wolf, 1999). The material was incubated in sample buffer (8 M urea, 200 mM Tris-HCl, pH 6.8, 5% SDS, 0.1 mM EDTA, 0.03% Bromphenol Blue, 1.5% dithiothreitol) at 40°C for 20 minutes with vigorous agitation and separated on 8.0% SDS-polyacrylamide gels. Proteins on immunoblots were visualised by ECL detection (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Deglycosylation experiments

Cells were lysed essentially as described before (Plemper et al., 1998). Briefly, microsomes were prepared from 5 A_{600} of cells, treated with 0.5 units of endoglycosidase F for 2 hours at 37°C and resuspended in sample buffer. Proteins were analysed by SDS-polyacrylamide gelelectrophoresis and immunoblotting.

Generation of antibodies

The Hrd3p peptide CPSSPLNPNEDTQHS (amino acids 695-708) was coupled to maleimide activated keyhole limpet hemocyanine of *Megathurea crenulata*. The coupled hapten was subsequently used for immunisation of rabbits to generate the polyclonal αHrd3p antiserum. Specificity of the serum was verified by immunoblotting with total cell lysate obtained from wild-type strains expressing *HRD3* from a centromeric or 2μ plasmid and from Δ*hrd3* strains. If not otherwise specified, cells carried wild-type *HRD3* on a 2μ plasmid for detection of Hrd3p in western analysis.

Immunoblotting

For western analysis, 5 A_{600} of cells were taken and subjected to lysis, preparation of membranes, SDS-polyacrylamide gelelectrophoresis and immunoblotting as described before (Plemper et al., 1998).

Microsome extractions

Microsomes were prepared from 25 A_{600} as described before, split into aliquots corresponding to 5 A_{600} and incubated for 30 minutes on ice with the agents indicated, followed by a 20,000 ***g*** centrifugation for 30 minutes and trichloroacetic acid precipitation of the supernatant. Pellets and trichloroacetic acid precipitates were resuspended in sample buffer.

Protease protection experiments

After spheroplasting 50 A_{600} units of cells (Plemper et al., 1997), these cells were gently lysed using a tissue grinder. The lysate was cleared of remaining cells and debris by repeated centrifugation for 5 minutes at 3,000 ***g***. The cleared lysate was split into aliquots corresponding to 12.5 A_{600}. From the time of lysis, all material was kept on ice. Separation of membranes was done by a 30 minute centrifugation at 20,000 ***g*** at 4°C. For protease treatment of the pellet, trypsin was added at a final concentration of 0.5 mg/ml after resuspension of the pellet, and the samples were incubated for 30 minutes on ice. If added, Triton X-100 was present at 1%. All treatments were stopped by adding trichloroacetic acid with a final concentration of 10%. After resuspending the pellet in sample buffer at 40°C for 30 minutes, proteins were analysed by SDS-polyacrylamide gelelectrophoresis and immunoblotting.

Pulse-chase analysis

1 ml of cells corresponding to 2.5 A_{600} were taken for each time point and labelled with 200 μCi of [^{35}S]methionine. The chase period was performed in prewarmed complete synthetic medium supplemented with 0.6% methionine and 0.2% BSA. Cell lysis and immunoprecipitation were performed essentially as described (Plemper et al., 1997). Subsequent to immunoprecipitation, the collected Protein A-Sepharose beads were resuspended in sample buffer and incubated at 40°C for 45 minutes. Proteins were separated on 8% SDS-polyacrylamide gels followed by fluorography.

Subcellular fractionation

Subcellular fractionation was performed essentially as previously described (Knop et al., 1996). Briefly, subsequent to spheroblasting 50 A_{600} units of cells (Plemper et al., 1997), these cells were gently lysed using a tissue grinder as described. The cleared lysate was loaded on a ten step sucrose gradient ranging from 18% to 54% sucrose and subjected to ultracentrifugation at 100,000 ***g*** for 4 hours at 4°C. 20 fractions were collected and examined through trichloroacetic acid precipitation and western analysis, or through enzymatic assays. Guanosine diphosphatase (GDPase) activity was measured as described before (Abeijon et al., 1989). α-Mannosidase activity was determined according to the method of Opheim (1978).

Tunicamycin treatment

Cells harbouring the pGAL-*HRD3* plasmid were grown in suitable complete synthetic medium containing 2% Raffinose to an A_{600} of 1.0. Subsequently, galactose was added to a final concentration of 2%, and cells were incubated in the absence or in the presence of 10 μg/ml, 20 μg/ml and 50 μg/ml tunicamycin followed by cell lysis as

described before. Proteins were analysed through SDS-polyacrylamide gel electrophoresis and immunoblotting.

RESULTS

Hrd3p, a glycoprotein of the ER membrane, is an essential component of the ER degradation machinery

In a first attempt to unravel the function of Hrd3p, we wanted to know whether this protein, originally identified as responsible for the regulated turnover of HMG-CoA reductase in yeast (Hampton et al., 1996), also functions as a bona fide component of the ER degradation system of misfolded or unassembled secretory proteins in general. When performing

Fig. 1. Hrd3p is essential for ER degradation of a broad spectrum of misfolded secretory proteins. (A) CPY* degradation is impaired in the Δhrd3 background. Wild-type (W303-1C) and hrd3 deletion cells were pulse labeled with [^{35}S]methionine for 20 minutes and chased for the indicated times. CPY* was immunoprecipitated at each time point. (B) Deletion of HRD3 suppresses the temperature sensitive growth phenotype of sec61-2 mutant cells. Indicated yeast strains were streaked on complete synthetic medium plates and incubated at 30°C or 38°C for 40 hours. Expression of HRD3 from a centromeric plasmid (pCEN HRD3) complements the effect of chromosomal hrd3 deletion in the sec61-2 background. (pCEN: empty plasmid for control).

pulse-chase analyses of CPY* in yeast cells lacking Hrd3p we observed a significant stabilisation of the misfolded protein (Fig. 1A). Unassembled Sec61-2 polypeptide chains had previously been shown to become substrates of the ER degradation machinery at elevated temperature (Biederer et al., 1996; Bordallo et al., 1998). Deletion of HRD3 suppressed the temperature sensitive growth of cells carrying the sec61-2 allele (Stirling et al., 1992) indicating stabilisation of mutated Sec61p (Fig. 1B). Since Hrd3p also abolishes degradation of the polytopic mutant protein Pdr5* (Plemper et al., 1998) we consider Hrd3p as an additional molecule which exhibits a central role in retrograde protein transport across the ER membrane. Recently, Saito et al. (1999) postulated that Hrd3p is rather required for the maintenance of ER membrane functions: this was based on their contradictory finding that a Δhrd3 knockout mutation failed to suppress a mutant sec61 growth phenotype. This difference to our results, however, could be due to the sec61 mutant alleles used by Saito et al. and by us. They analysed loss of function versions of Sec61p for which breakdown through the ER degradation system has not been demonstrated yet.

To further characterise Hrd3p we generated antibodies directed against a peptide within the molecule and, alternatively, added a HA tag to its carboxy-terminus which is predicted to be localised in the cytosol. However, the HA tagged version was biologically inactive; it was unable to complement a chromosomal deletion of HRD3 (data not shown). The newly synthesised antibody directed against Hrd3p recognised a single band with a molecular mass of about 95 kDa that was absent in Δhrd3 cell extracts (Fig. 2A). When cell extracts were subjected to a 20,000 g centrifugation, the antigenic material was exclusively found in the pellet fractions indicating membrane association of Hrd3p (Fig. 2B). Remarkably, Hrd3p could only be detected when expressed from a centromeric (data not shown) or 2μ plasmid (Fig. 2B). This suggests a tight regulation of HRD3 gene expression resulting in a very low steady state level of the protein. However, the plasmid encoded Hrd3p was able to complement a chromosomal deletion of the HRD3 gene indicating complete biological activity (data not shown).

Subcellular fractionation through a sucrose gradient revealed strong co-fractionation of Hrd3p with the ER resident chaperone Kar2p indicating ER localisation (Fig. 2C). We confirmed this finding by immunofluorescence analysis (data not shown). Our findings concerning ER localisation of Hrd3p were consistent with the immunofluorescence data of Saito et al. (1999). To address the question whether Hrd3p is indeed a membrane spanning protein as suggested by its sequence (Hampton et al., 1996), we treated the pellet fractions with different agents such as urea, potassium acetate or sodium carbonate, all known to remove peripheral membrane proteins (Fig. 2D). Solubilisation of Hrd3p, however, was only possible with detergents as Triton X-100 (data not shown) or SDS (Fig. 2D). Because these experiments suggested membrane integration of Hrd3p we determined its topology. Treatment of intact microsome preparations with trypsin did not alter the molecular mass of Hrd3p significantly. As the amino-terminal part of the protein carries the antigenic epitope, the N terminus of Hrd3p must be oriented to the ER lumen (Fig. 2E). The minor shift in molecular mass of the antigenic material corresponding to Hrd3p upon trypsin treatment points to a
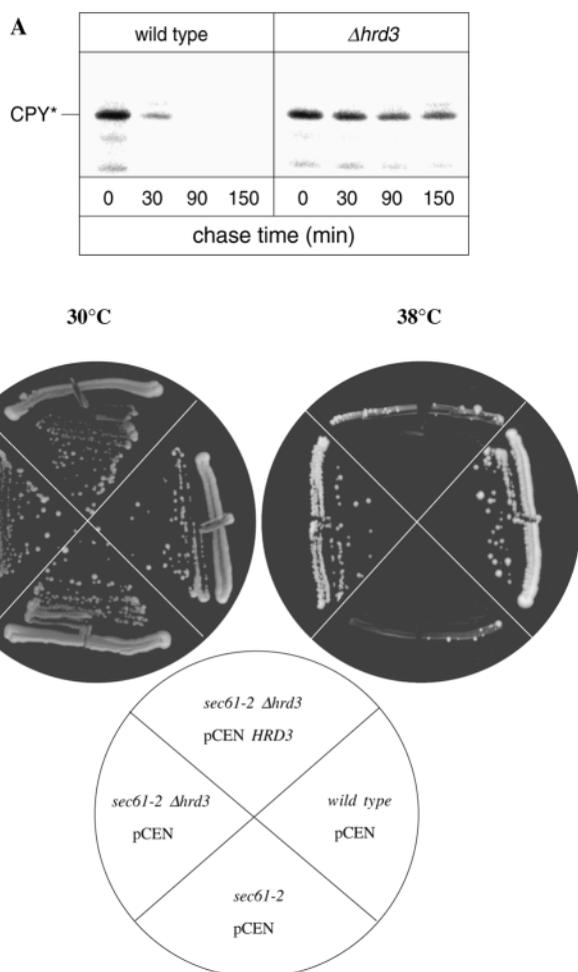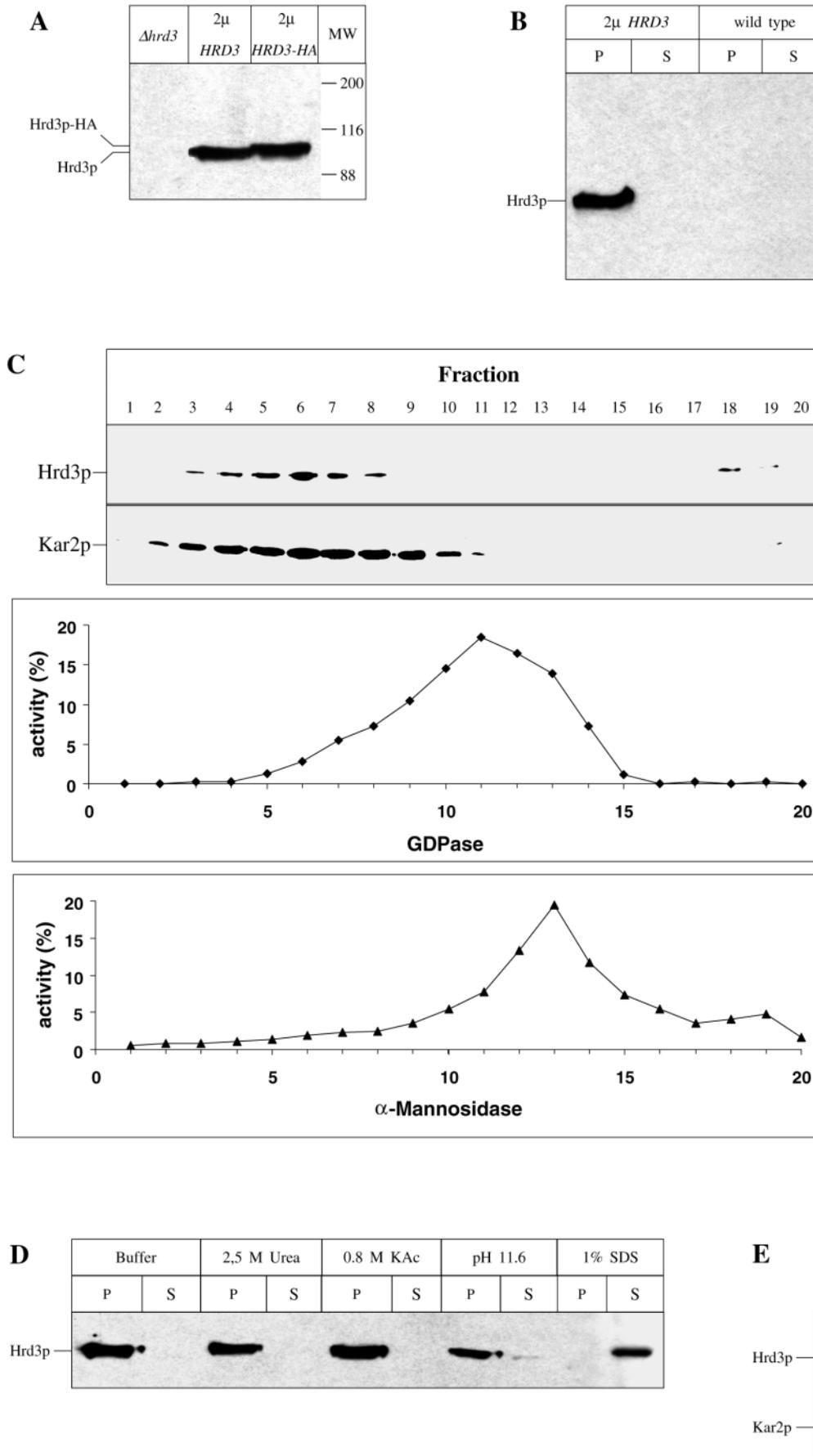
Fig. 2. Hrd3p is an integral glycoprotein of the ER membrane. (A) Western analysis reveals a molecular mass of Hrd3p of about 95 kDa. Yeast cells expressing wild-type or carboxy-terminal HA tagged *HRD3* from a 2μ plasmid or, for comparison, Δ*hrd3* mutants were subjected to alkaline lysis and immunoblotting using polyclonal αHrd3p antiserum. (B) Hrd3p is membrane-associated. Extracts of wild-type cells (W303-1C) and of cells harbouring a 2μ *HRD3* plasmid were subjected to 20,000 *g* centrifugation. Pellet fractions and trichloroacetic acid precipitates were analysed through immunoblotting. The antigenic material was exclusively found in the pellet fraction. In wild-type cells, no antigenic material was detectable due to a very low steady state level of Hrd3p. (C) Subcellular fractionation reveals co-localisation of Hrd3p with the ER resident chaperone Kar2p indicating ER localisation. GDPase activity was analysed as a Golgi marker while α-Mannosidase activity served as vacuolar marker. (D) Hrd3p is an integral membrane protein. Microsomes prepared from wild-type cells were treated with buffer, 2.5 M urea, 0.8 M potassium acetate, 0.1 M Na_2CO_3, pH 11.6, or 1% SDS, and soluble (*S*) and pellet (*P*) fractions were analysed through immunoblotting. (E) The amino-terminal part of Hrd3p carrying the antigenic epitope is oriented to the ER lumen. Western analysis of Hrd3p was performed after preparing spheroplast homogenates, followed by a 20,000 *g* centrifugation. *T*, total cell lysate; *S*, supernatant; *P*, pellet fraction; *P(T)*, pellet treated with trypsin; *P(T+T)*, pellet treated with trypsin and Triton X-100. To confirm the integrity of the prepared vesicles, Kar2p was analysed.
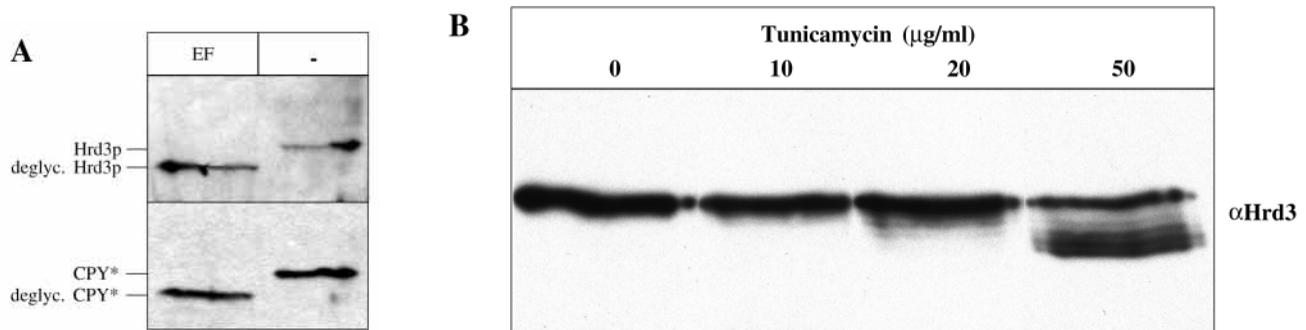
Fig. 3. Hrd3p carries five carbohydrate chains. (A) Western analysis of Hrd3p and, for comparison, fourfold glycosylated CPY* after endoglycosidase F (EF) treatment of crude cell extracts. (B) Immunoblot of Hrd3p expressed under the control of the galactose promoter. Prior to lysis, cells were shifted for 1 hour to medium containing 2% galactose and increasing amounts of tunicamycin as indicated.

single membrane spanning domain and a very short cytosolic carboxy terminus. These experimental observations are consistent with predictions based on the Hrd3p sequence.

The Hrd3p sequence contains five potential N-glycosylation sites. To determine the glycosylation status of the molecule we treated the antigenic material with endoglycosidase F. This leads to a considerable loss of molecular mass of the protein. Thus, as previously postulated by Saito et al. (1999), this experiment proved that at least some glycosylation sites are recognised by the ER glycosylation machinery (Fig. 3A). Treatment of cells with increasing amounts of tunicamycin prior to western analysis revealed a pattern of six distinct bands corresponding to Hrd3p with different glycosylation status (Fig. 3B). This indicates complete recognition of all five N-glycosylation sites by the ER glycosylation machinery.

Genetic interaction of Hrd3p, Der3p/Hrd1p and Sec61p

Hrd3p and Der3p/Hrd1p are essential components of the ER degradation machinery (Bordallo et al., 1998; Hampton et al., 1996, and see above). Both proteins are localised in the ER membrane and have apparently an identical substrate spectrum. Thus, we asked the question whether they might act in concert, possibly together with the Sec61 pore, to form the central part of the retrograde transport machinery in the ER membrane. A first hint for a functional interaction of Hrd3p and Der3p/Hrd1p came from our previous finding that overexpression of Hrd3p could partially revert the dominant negative effect of mutant Der3p/Hrd1p defective in its RING H2 finger domain on ER degradation (Bordallo and Wolf, 1999). Strikingly, when we followed the fate of wild-type Der3p/Hrd1p in Δhrd3 deleted cells we observed rapid degradation of the protein; hardly any antigenic material corresponding to Der3p/Hrd1p could be detected (Fig. 4A). While the expression level of Der3p/Hrd1p was unchanged (data not shown), the half life of the normally stable molecule turned out to be greatly reduced (Fig. 4B). Four independent experiments yielded an average value for the half life of Der3p/Hrd1p of 7 minutes. These data strongly support the hypothesis of a direct interaction of Hrd3p and Der3p/Hrd1p in the degradation event of misfolded secretory proteins. The instability of Der3p/Hrd1p in the Δhrd3 background is in a striking contrast to the effect of a *HRD3* gene deletion on degradation of misfolded or downregulated secretory proteins such as CPY*, Pdr5* or HMG-CoA

reductase: under these conditions, CPY* accumulates in the ER lumen (data not shown), Pdr5* and HMG-CoA reductase remain stably integrated in the ER membrane (Hampton et al., 1996; Plemper et al., 1998). Furthermore, 7 minutes half life of Der3p/Hrd1p in the absence of Hrd3p differs substantially from the degradation rate usually reported for misfolded secretory proteins in yeast. Both CPY* and Pdr5* reveal a
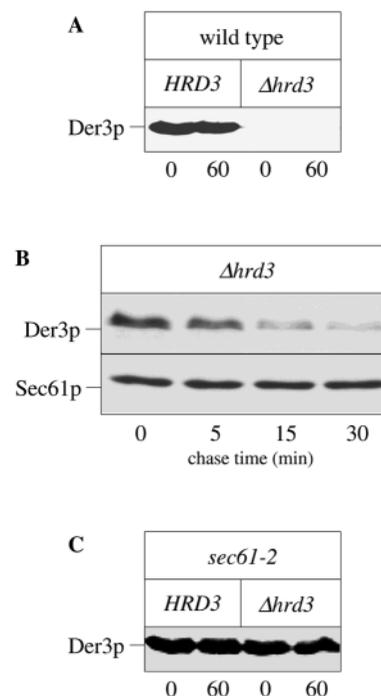

Fig. 4. Genetic interaction of Hrd3p and Der3p/Hrd1p with each other and with Sec61p. (A) Der3p/Hrd1p is rapidly degraded in the absence of Hrd3p. Cycloheximide-chase analysis of Der3p/Hrd1p was performed in wild-type (W303-1C) and Δhrd3 cells at 30°C. After adding cycloheximide, aliquots of cells were subjected to alkaline lysis and western analysis. (B) In a Δhrd3 background, half life of Der3p/Hrd1p is reduced to approximately 7 minutes. Cycloheximide-chase analysis of Der3p/Hrd1p was performed at 30°C. As loading control, the Sec61p level was determined. (C) Degradation of Der3p/Hrd1p is suppressed in Δhrd3 sec61-2 double mutant cells. Cycloheximide-chase analysis of Der3p/Hrd1p was performed as described.

half life of about 20 minutes (Knop et al., 1996; Plemper et al., 1998). Thus, degradation of Der3p/Hrd1p in Δ*hrd3* mutants cannot be explained simply through conversion of Der3p/Hrd1p into an unassembled substrate molecule of the otherwise unaffected ER degradation machinery.

If both Hrd3p and Der3p/Hrd1p were also part of the retrograde translocation machinery, by this functionally interacting with the Sec61 pore, instability of Der3p/Hrd1p in the absence of Hrd3p could be explained by a misregulated activity of the remaining basic retro-translocon lacking Hrd3p. In this case, one would expect that degradation of Der3p is suppressed in Δ*hrd3 sec61-2* double mutants, defective in formation of retro-transloci due to the *sec61-2* mutation (Plemper et al., 1997). Indeed, analysis of Der3p/Hrd1p in Δ*hrd3 sec61-2* double mutants revealed a dramatically restored stability of the protein (Fig. 4C). Remarkably, the complete stabilisation of Der3p/Hrd1p by the *sec61-2* mutation contrasts the 3 fold stabilisation of ER degradation substrates as CPY* (Plemper et al., 1997) or Pdr5* (Plemper et al., 1998). This difference points to a tight connection of Der3p/Hrd1p and Sec61p for retrograde transport of misfolded proteins in wild-type cells. Therefore, our data provide evidence for a retro-translocon build up by functional interaction of at least Hrd3p, Der3p/Hrd1p and Sec61p.

In Δ*hrd3* cells Der3p/Hrd1p is subject of ubiquitin proteasome dependent degradation

Degradation of Der3p/Hrd1p in strains lacking Hrd3p could imply breakdown of Der3p/Hrd1p through the ubiquitin proteasome system. We first excluded that breakdown of Der3p/Hrd1p in Δ*hrd3* cells required further transport of the protein through the secretory system by analysing Δ*hrd3 sec18-1* double mutants having an impaired vesicular transport at elevated temperature. Indeed, at restrictive temperature of 37°C, no stabilisation of
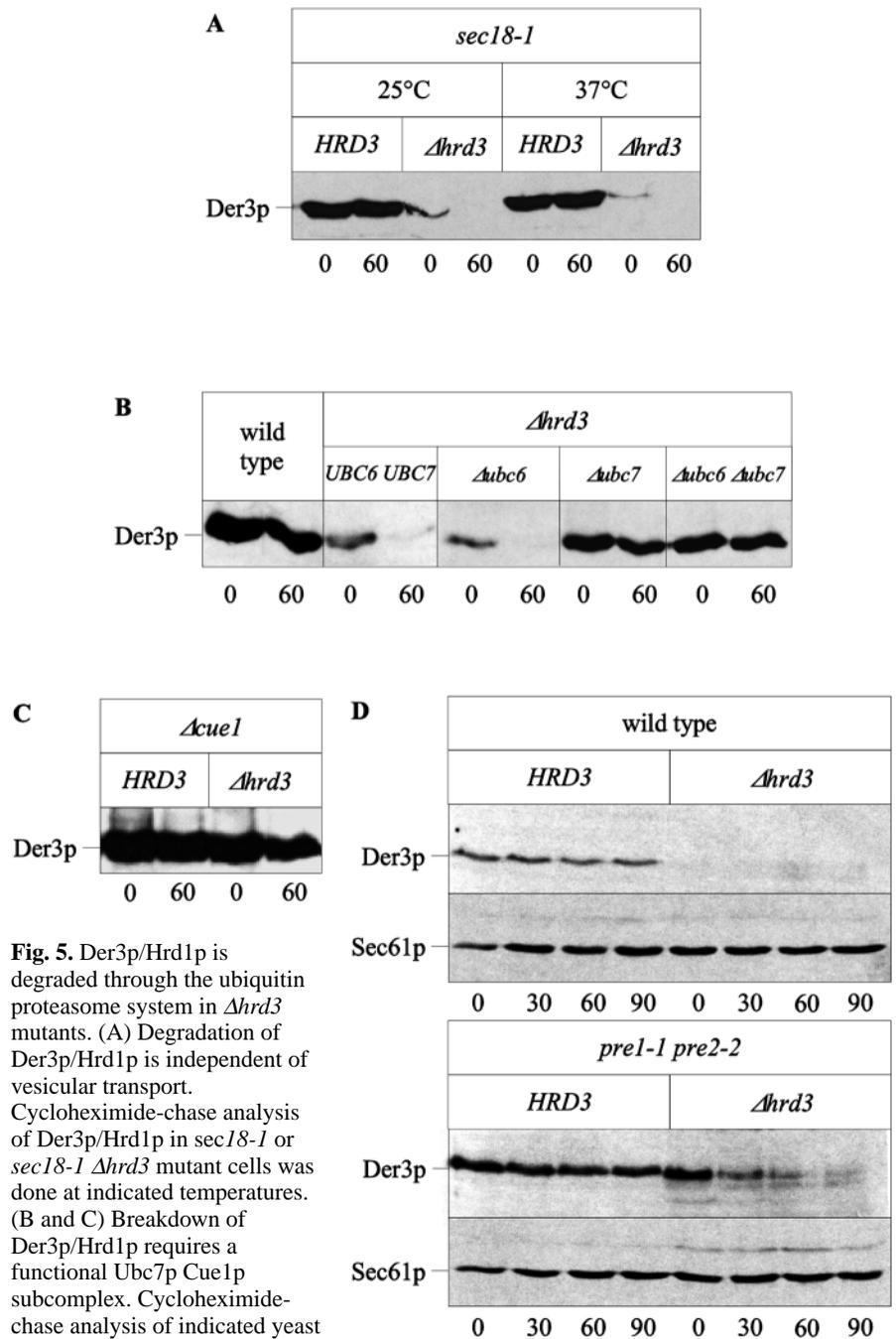

Fig. 5. Der3p/Hrd1p is degraded through the ubiquitin proteasome system in Δ*hrd3* mutants. (A) Degradation of Der3p/Hrd1p is independent of vesicular transport. Cycloheximide-chase analysis of Der3p/Hrd1p in sec*18-1* or *sec18-1* Δ*hrd3* mutant cells was done at indicated temperatures. (B and C) Breakdown of Der3p/Hrd1p requires a functional Ubc7p Cue1p subcomplex. Cycloheximide-chase analysis of indicated yeast strains was performed at 30°C. (D) Degradation of Der3p/Hrd1p is debilitated in proteasomal mutants. Cycloheximide-chase analysis of Der3p/Hrd1p was performed in wild type (WCGY4a)), *hrd3* knockout cells, *pre1-1 pre2-2* proteasomal mutants and Δ*hrd3 pre1-1 pre2-2* triple mutant cells at 30°C.

Der3p/Hrd1p could be observed (Fig. 5A). This suggested ER-associated breakdown of the molecule. Examination of Der3p/Hrd1p degradation in Δ*hrd3* mutant cells lacking the ER membrane associated ubiquitin conjugating enzymes Ubc6p and/or Ubc7p revealed that degradation of Der3p/Hrd1p is dependent on a functional Ubc7p (Fig. 5B). This finding was confirmed by stabilisation of Der3p/Hrd1p in Δ*hrd3* Δ*cue1* double mutants (Fig. 5C). Δ*cue1* cells are unable to recruit active Ubc7p to the ER membrane (Biederer

et al., 1997). If Der3p/Hrd1p were indeed subject to proteasomal proteolysis in Δ*hrd3* cells, the half life of the molecule should be considerably increased in a Δ*hrd3 pre1-1 pre2-2* strain having impaired proteasomal functions (Heinemeyer et al., 1991). Indeed, Δ*hrd3 pre1-1 pre2-2* triple mutants exhibit significant stabilisation of Der3p/Hrd1p (Fig. 5D). From these experiments we conclude that Der3p/Hrd1p is degraded by the ubiquitin proteasome system in cells lacking Hrd3p.

Fig. 6. Rapid degradation of Der3p/Hrd1p requires functional RING-H2 finger domain and presence of its substrate molecules. (A) Any mutation affecting the RING-H2 finger domain of Der3p/Hrd1p leads to stabilisation of the protein in the Δ*hrd3* background. Cycloheximide-chase analysis of wild-type Der3p/Hrd1p and of the mutant versions Der3-ΔRp and Der3_{C399S}p at 30°C. (B) Deletion of *DER1* impeding ER degradation upstream of retrograde protein transport causes impaired breakdown of Der3p/Hrd1p. Cycloheximide-chase analysis of Der3p/Hrd1p was performed in wild-type and Δ*der1* cells carrying *hrd3* deletions as indicated.
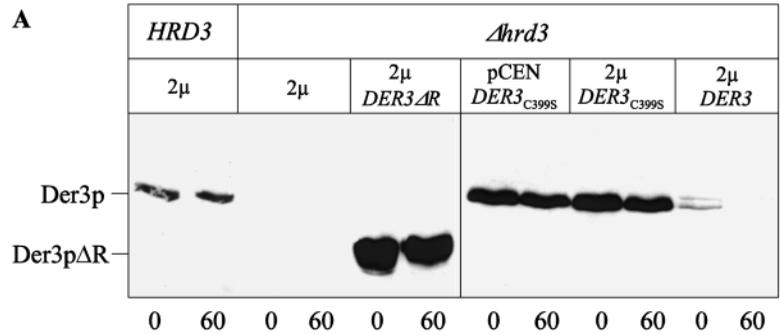
Rapid degradation of Der3p/Hrd1p depends on a functional Ring H2 finger domain and requires its substrate molecules

As import of secretory proteins into the ER lumen is essential for viability, it must have priority over retrograde transport of misfolded proteins, which is not essential under all experimental conditions tested (Plemper et al., 1999). Therefore, we favour the model that only a very limited amount, if any, of pre-existing retro-transloci is present in cells. Formation of retro-transloci should rather be a highly dynamic process taking place only in the presence of substrate molecules. This implies that proteasomal degradation of Der3p/Hrd1p through misguidance of the basic channel lacking Hrd3p has to be preceeded by the formation of the respective complex. If this model were correct one would expect that Der3p/Hrd1p becomes stabilised in Δ*hrd3* mutants by any mutation impeding either the binding of misfolded substrate molecules by Der3p/Hrd1p or its interaction with the Sec61 pore. We had demonstrated that an intact RING H2 finger domain, the highly conserved protein binding motif of Der3p/Hrd1p, is essential for its function (Bordallo et al., 1998; Bordallo and Wolf, 1999). Thus, this domain was a promising target for mutagenesis. Remarkably, when we followed the fate of mutant Der3p/Hrd1p with an impaired RING H2 finger domain we observed stabilisation of the mutated protein in Δ*hrd3* cells. A Der3p/Hrd1p species lacking the entire RING H2 finger domain (Der3-ΔRp) or a species carrying a single point mutation within this motif (Der3_{C399S}p) failed to be degraded (Fig. 6A). So far, we cannot decide whether the RING H2 finger domain of Der3p/Hrd1p, either directly or indirectly, mediates binding to misfolded substrate molecules or to the Sec61 pore. These data strengthen the view of Der3p/Hrd1p being an integral component of the retro-translocon.

Our further aim was to partially block the ER degradation process at a level upstream of retrograde protein transport to analyse the influence of substrate delivery on the degradation rate of Der3p/Hrd1p. In contrast to Der3p/Hrd1p and Hrd3p, another ER membrane located protein, Der1p, was described only to be involved in the degradation of a subset of misfolded secretory proteins such as CPY*, indicating a more specialised function (Knop et al., 1996). From this, it is conceivable that Der1p acts in earlier steps of ER degradation than retrograde translocation. Thus, in Δ*der1* mutants the delivery of this subset of misfolded substrate molecules to the retro-translocon is greatly reduced as could be demonstrated by the strong accumulation of CPY* in the ER lumen in these cells (Bordallo et al., 1998). We found a reduction of the degradation rate of
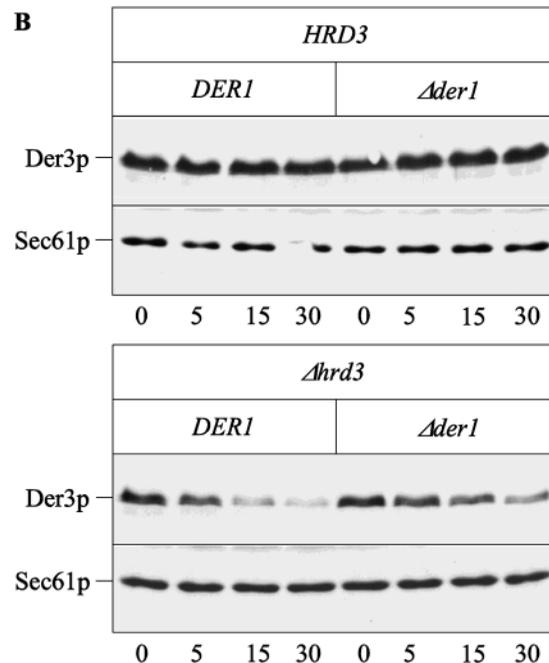
Der3p/Hrd1p in Δ*der1* Δ*hrd3* double mutant cells by a factor of approximately 2 (Fig. 6B). This might be a hint for dependence of Der3p/Hrd1p degradation in Δ*hrd3* mutants on the presence of substrate. However, we cannot determine or even block the overall flow of Der1p independent natural substrate molecules to the retrograde translocation complex due to synthesis errors or failures in protein assembly. These natural substrates might contribute to the remaining degradation of Der3p/Hrd1p in Δ*der1* cells.

Overexpression of Hrd3p reduces the rate of ER degradation

The question must be posed why the presence of Hrd3p prevents degradation of Der3p/Hrd1p. One possible explanation were that in the absence of Hrd3p, Der3p/Hrd1p unfolds and becomes a regular substrate of the ER degradation machinery. This is, however, hard to reconcile with the fact that its degradation features are completely different from those of all other tested ER substrates (Knop et al., 1996; Plemper et al., 1997, 1998): (i) Der3p/Hrd1p is degraded in the Δ*hrd3* background, while all other ER-substrates are stabilised; (ii) its
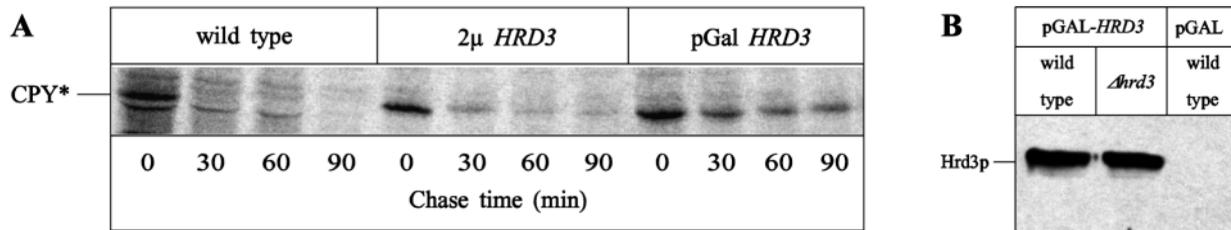
Fig. 7. Strong overexpression of Hrd3p leads to impaired ER degradation. (A) Pulse-chase analysis of CPY* in cells harbouring empty plasmids, 2μ *HRD3* plasmids and plasmids carrying *HRD3* under the control of the galactose promoter was performed at 30°C. Pulse labelling was preceded by incubation of cells in suitable complete synthetic medium containing 2% galactose for 2 hours. (B) Overexpression of *HRD3* under the control of the galactose promoter. Western analysis of Hrd3p and, for comparison, CPY* in wild-type and Δh*rd3* cells harbouring *HRD3* under the control of the galactose promoter on plasmid. For control, wild-type cells carrying empty plasmid were analysed. Cells were shifted to complete synthetic medium for 2 hours and subjected to alkaline lysis.

half life is very different from that of all other ER-substrates and (iii) it is completely stabilised in *sec61-2* mutant cells while all other ER-substrates tested undergo only an about 3-fold stabilisation.

The fact that mutations in the RING H2 finger domain lead to full stabilisation of Der3p/Hrd1p in a Δ*hrd3* background argue for a role of this domain in a process which leads to Der3p/Hrd1p degradation in the absence of Hrd3p. Considering the data above (Fig. 6B), that also the ER substrate flow influences degradation of Der3p/Hrd1p, one could hypothesize that the RING H2 finger domain together with binding of the substrate leads to degradation of Der3p/Hrd1p in the Δ*hrd3* background. One speculation were that the RING H2 finger domain of Der3p/Hrd1p, either directly or together with ancillary proteins, binds the degradation substrates in the ER and delivers them to the cytoplasmic ubiquitin proteasome system. Hrd3p could have the function of separating the RING H2 finger domain of Der3p/Hrd1p from the substrate and the degradation machinery to prevent its degradation. This speculation presupposes that the complex for retrograde transport build up by Der3p/Hrd1p and the Sec61 pore transiently interacts with Hrd3p. Genetic interaction of these proteins has been shown above. If this model were correct, it should be possible to reduce the rate of ER-degradation in wild-type cells by strong overexpression of Hrd3p due to premature release of substrate molecules from Der3p/Hrd1p and thus preventing their degradation. When we performed pulse-chase analyses of CPY* in wild-type cells expressing Hrd3p from a centromeric plasmid (data not shown) or from a 2μ plasmid under the control of its own promoter we detected, as expected, hardly any CPY* stabilisation (Fig. 7A). However, substantial overexpression of the *HRD3* gene under the control of the galactose promoter (Fig. 7B) led to significant stabilisation of CPY* (Fig. 7A). These data are consistant with the proposed hypothesis. We would like to stress, however, that alternate explanations are possible.

In Δ*hrd3* mutants overexpression of Der3p/Hrd1p increases degradation of CPY*

Transport of substrate molecules into the retro-translocon through the lumenal tail of Der3p/Hrd1p and subsequent rescue of Der3p/Hrd1p through Hrd3p would suggest that only in the presence of Hrd3p, Der3p/Hrd1p can function catalytically in

ER degradation. In the Δ*hrd3* background, it can be assumed that at least the lumenal tail of Der3p/Hrd1p is subjected to proteasomal degradation, together with a single misfolded substrate molecule. To examine this possibility, we analysed the effect of overexpression of Der3p/Hrd1p on CPY* degradation in Δ*hrd3* cells. Indeed, when *DER3/HRD1* was expressed from a 2μ plasmid in Δ*hrd3* cells, CPY* degradation was substantially increased as compared to control cells carrying empty plasmids (Fig. 8A). For all time points, we confirmed the expression level of Der3p/Hrd1p through western analysis (Fig. 8B). The increased degradation of CPY* could point to the fact that in the absence of Hrd3p, Der3p/Hrd1p acts stoichiometrically in ER degradation. In the wild-type situation, however, Der3p/Hrd1p seems to act catalytically in the degradation event.

DISCUSSION

The cytosolic ubiquitin proteasome system was identified as the major proteolytic system involved in ER degradation of misfolded, unassembled or downregulated secretory proteins (Kopito, 1997; Plemper and Wolf, 1999; Sommer and Wolf, 1997). The location of substrate molecules in the ER and the protease in the cytoplasm requires retrograde transport of the substrate proteins from the ER back into the cytosol for proteolysis. The Sec61 pore of the ER membrane, which is crucial for protein import (Rapoport et al., 1996), was discovered as the core component mediating reverse membrane transport as well (Pilon et al., 1997; Plemper et al., 1997; Wiertz et al., 1996). Three novel proteins, Der1p, Der3p/Hrd1p and Hrd3p, have been found, which are specifically involved in ER degradation (Bordallo et al., 1998; Hampton et al., 1996; Knop et al., 1996). Der1p (Knop et al., 1996) and Der3p/Hrd1p (Bordallo et al., 1998) had been localised to the ER membrane.

In this study we aimed to identify interactions between components of the ER degradation system and the subunit composition of the retro-translocon. Our results show that also Hrd3p is a component of the ER membrane. Our genetic studies favour a model in which Hrd3p and Der3p/Hrd1p are constituents of a highly dynamic complex organised around the Sec61 pore. It might be this complex which could be capable of delivering misfolded substrate molecules into the
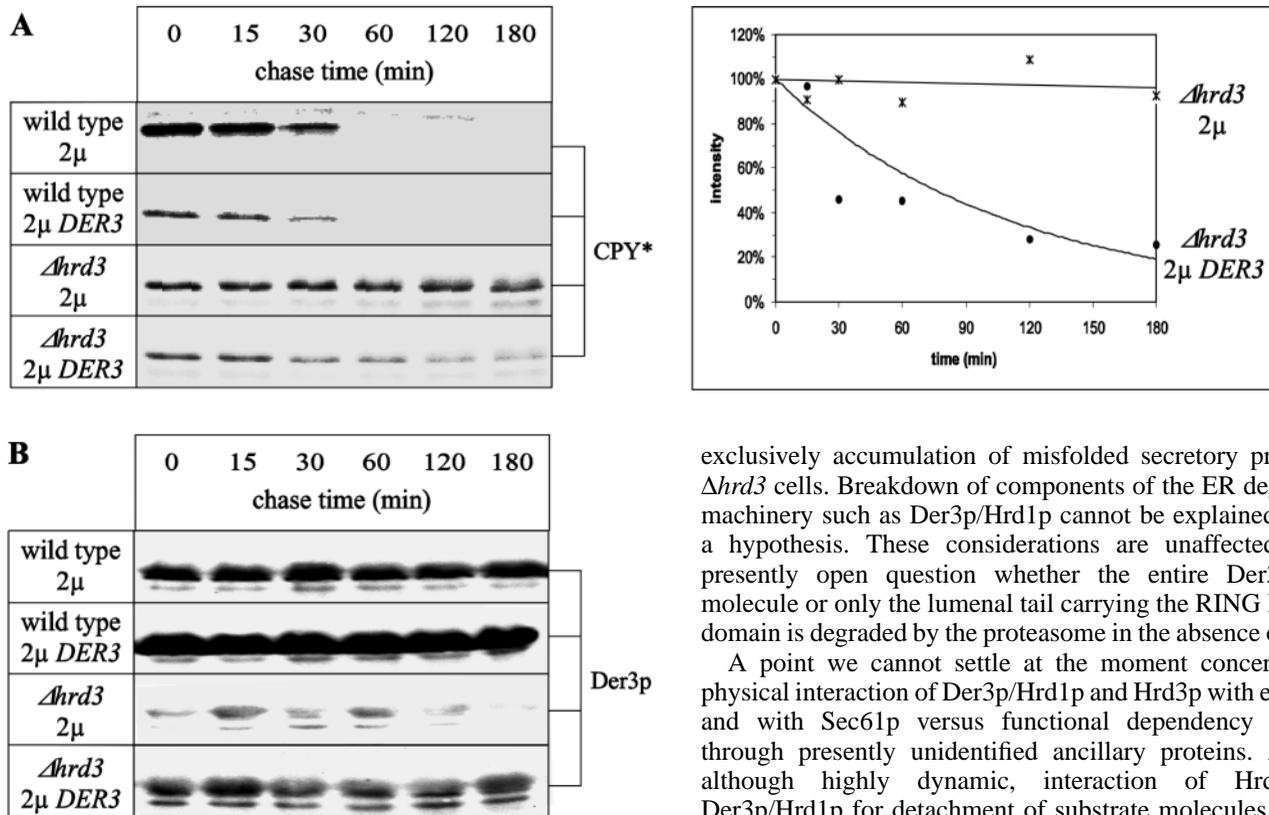
Fig. 8. Der3p/Hrd1p acts stoichiometrically on degradation of misfolded secretory proteins in the absence of Hrd3p. (A) Pulse-chase analysis of CPY* was performed with wild-type and Δ*hrd3* strains harbouring 2μ plasmids carrying *DER3/HRD1* or empty plasmids at 30°C. After pulse labelling with [³⁵S]methionine for 20 minutes, cells were chased for the indicated times. (B) For comparison, the Der3p/Hrd1p levels were determined at each time point through western analysis. Note, that double amounts of extracts from Δ*hrd3* cells were loaded compared to wild type.

translocation pore from the lumenal side of the ER. Although we cannot exclude the possibility that Hrd3p and Der3p/Hrd1p perform as subunits of a different protein complex acting upstream of the Sec61 pore itself, we consider direct assembly of these three components into a larger structure to be more likely for several reasons: (i) in Δ*hrd3* cells, Der3p/Hrd1p cannot be regarded simply as another unassembled substrate molecule of the ER degradation machinery, because in the absence of Hrd3p, misfolded or downregulated secretory proteins, in contrast to Der3p/Hrd1p, are not degraded but accumulate instead in the ER lumen or in the ER membrane. Furthermore, under these conditions half life of Der3p/Hrd1p is about 3 times shorter than reported for the degradation of typical misfolded secretory proteins. (ii) Sec61p dependent degradation of wild-type Der3p/Hrd1p in the absence of Hrd3p suggests a close interaction of Der3p/Hrd1p and the channel during retrograde transport of misfolded proteins. According to this assumption, turnover of Der3p/Hrd1p can be explained by an uncontrolled activity of the retro-translocation complex lacking Hrd3p. (iii) If Hrd3p would be required for recognition or transfer of substrate molecules to the channel rather than for proper function of the retro-translocon itself, one would expect

exclusively accumulation of misfolded secretory proteins in Δ*hrd3* cells. Breakdown of components of the ER degradation machinery such as Der3p/Hrd1p cannot be explained by such a hypothesis. These considerations are unaffected by the presently open question whether the entire Der3p/Hrd1p molecule or only the lumenal tail carrying the RING H2 finger domain is degraded by the proteasome in the absence of Hrd3p.

A point we cannot settle at the moment concerns direct physical interaction of Der3p/Hrd1p and Hrd3p with each other and with Sec61p versus functional dependency mediated through presently unidentified ancillary proteins. A direct, although highly dynamic, interaction of Hrd3p and Der3p/Hrd1p for detachment of substrate molecules from the lumenal tail of Der3p/Hrd1p seems to be a possibility. The assembly of the whole complex could be based on hydrophobic interactions within or close to the ER membrane. This view is corroborated by our finding that attachment of a hydrophilic HA tag to the very short cytosolic carboxy terminus of Hrd3p leads to inactivity of the molecule: cells expressing this Hrd3p version were characterised by accumulation of misfolded secretory proteins in the ER and rapid degradation of Der3p/Hrd1p. The tag might disturb hydrophobic contacts.

Binding of misfolded substrates to Der3p/Hrd1p, probably through its RING H2 finger domain, could easily be assisted by a subset of additional linker proteins. A conceivable candidate for a member of such protein species functioning upstream in the ER degradation process seems to be Der1p, responsible for breakdown of a limited substrate spectrum including CPY*. Furthermore, participation of ER lumenal chaperones such as Kar2p and calnexin was reported for degradation of several substrate molecules (Brodsky et al., 1999; McCracken and Brodsky, 1996; Plemper et al., 1997). They could be involved in partial unfolding of polypeptide chains prior to their transport to the retro-translocon or their delivering into the channel.

Presently, we favour the simplified model of direct contact of Der3p/Hrd1p and the Sec61 pore in the presence of misfolded secretory proteins. Substrate molecules could be imported into the membrane spanning channel upon its opening from the lumenal side. The latter function could be due to the action of Der3p/Hrd1p. The core complex constituted by the Sec61 pore and Der3p/Hrd1p could provide a high affinity binding site for Hrd3p. Once the mutated polypeptide chains are inserted into the pore, Hrd3p could initiate, either by direct interaction or via further helper proteins, recycling of the Der3p/Hrd1p lumenal tail through

detachment of substrate molecules. If the substrate is not detached from Der3p/Hrd1p, ubiquitination of the substrate initiates not only its own degradation but also breakdown of the binding protein Der3p/Hrd1p. Indeed, such a degradation *in trans* has been reported previously (Johnson et al., 1990). These considerations are also based on the striking finding that in Δ*hrd3* knockout cells Der3p/Hrd1p is rapidly degraded, leading to strong stabilisation of ER degradation substrates. In Δ*hrd3* cells, overexpression of Der3p/Hrd1p increases the ER degradation rate again to some degree. Under these conditions, Der3p/Hrd1p seems to function stoichiometrically in ER degradation, while in *HRD3* wild-type cells Der3p/Hrd1p acts catalytically.

Der3p/Hrd1p seems to be in surplus of Hrd3p. Our studies show that this is a necessary prerequisite, as overexpression of Hrd3p slows down ER degradation. This may be due to the fact that under these conditions Hrd3p might permanently liberate Der3p/Hrd1p-bound substrates and by this prevent their degradation. To allow undisturbed protein import into the ER, which is essential for cellular life, only rather few Sec61 channels might be assembled in a complex for retrograde transport. These active retro-transloci might depend on the amount of Hrd3p.

Substrate dependence of Der3p/Hrd1p degradation could also be inferred from an additional Δ*der1* deletion in a Δ*hrd3* background: a block in CPY* degradation due to absence of Der1p lowers the degradation rate of Der3p/Hrd1p. Thus, a basic retro-translocon lacking Hrd3p seems to be capable of turnover of only one misfolded polypeptide chain. Functional Hrd3p is required, however, to prevent breakdown of Der3p/Hrd1p by the proteasome and recycle the complex for another round of retrograde transport. This idea is underlined by the fact that a mutation in the RING H2 finger motif of Der3p/Hrd1p, which prevents degradation of substrates, also prevents its own degradation in Δ*hrd3* cells. Subsequently to delivery of substrate molecules into the membrane spanning channel, a driving force has to be generated mediating further movement of the polypeptide chain to the cytosol. Recently, three different hypotheses concerning driving force were intensively discussed: according to a Brownian ratchet, retrograde transport could become irreversible upon co-ubiquitination of substrates during export (Bordallo et al., 1998). Alternatively, AAA ATPases of the 26S proteasome (Baumeister et al., 1998) could directly pull substrate molecules out of the channel (Mayer et al., 1998; Yu et al., 1997). Finally, cytosolic chaperones could mediate extraction of polypeptide chains from the pore. However, members of the cytosolic HSP70 chaperone family (Hartl et al., 1994) were shown not to be involved in ER degradation (Brodsky et al., 1999) (our unpublished observations). Our data suggest that the postulated Cue1p Ubc7p subcomplex of the ER membrane (Biederer et al., 1997) could be another integral component of the retro-translocon. Deletion of *UBC7* or *CUE1*, respectively, led to stabilisation of both misfolded secretory proteins (Biederer et al., 1996; Hampton and Bhakta, 1997; Hiller et al., 1996) and Der3p/Hrd1p in the Δ*hrd3* background. In contrast, knockout of *UBC6*, another ER membrane resident E2 enzyme, had no measurable effect on Der3p/Hrd1p stability in Δ*hrd3* cells. Recent discussions focus on RING H2 finger domains as conserved motifs of E3-like protein complexes involved in the final transfer of ubiquitin to substrate molecules (Tyers and

Willems, 1999). Thus, it is intriguing to speculate that the Sec61 pore, Der3p/Hrd1p, Hrd3p, Cue1p and Ubc7p might be part of an ER membrane localised E3-like complex required for ER degradation. This view would imply that translocation of substrates into the channel, retrograde transport and ubiquitination are tightly coupled events. Indeed, such a close link between retrograde transport and the proceeding of proteolysis was described for several substrates of the ER degradation system (Bordallo et al., 1998; de Virgilio et al., 1998; Mayer et al., 1998; Plemper et al., 1997; Yu et al., 1997).

In conclusion, our data might suggest Der3p/Hrd1p, Hrd3p and the Sec61 pore as core components of the retro-translocon mediating reverse protein translocation for ER degradation. Further binding of the Cue1p Ubc7p subcomplex seems also to be required for retrograde protein transport. It is likely that other, yet unidentified soluble and membrane components are required to constitute the complete retro-translocon. Final proof for the validity of this concept must come from experiments determining the direct protein interactions between the partners of the retro-translocation complex.

REFERENCES

Abeijon, C., Orlean, P., Robbins, P. W. and Hirschberg, C. B. (1989). Topography of glycosylation in yeast: characterization of GDPmannose transport and lumenal guanosine diphosphatase activities in Golgi-like vesicles. *Proc. Nat. Acad. Sci. USA* **86**, 6935-6939.

Baumeister, W., Walz, J., Zuhl, F. and Seemuller, E. (1998). The proteasome: paradigm of a self-compartmentalizing protease. *Cell* **92**, 367-380.

Biederer, T., Volkwein, C. and Sommer, T. (1996). Degradation of subunits of the Sec61p complex, an integral component of the ER membrane, by the ubiquitin-proteasome pathway. *EMBO J.* **15**, 2069-2076.

Biederer, T., Volkwein, C. and Sommer, T. (1997). Role of Cue1p in ubiquitination and degradation at the ER surface. *Science* **278**, 1806-1809.

Bordallo, J., Plemper, R. K., Finger, A. and Wolf, D. H. (1998). Der3p/Hrd1p is required for endoplasmic reticulum-associated degradation of misfolded lumenal and integral membrane proteins. *Mol. Biol. Cell* **9**, 209-222.

Bordallo, J. and Wolf, D. H. (1999). A RING-H2 finger motif is essential for the function of Der3/Hrd1 in endoplasmic reticulum associated protein degradation in the yeast Saccharomyces cerevisiae. *FEBS Lett.* **448**, 244-248.

Brodsky, J. and McCracken, A. A. (1998). ER-associated degradation: An unconventional route to a familiar fate. *Advan. Mol. Cell Biol.* **27**, 165-200.

Brodsky, J. L., Werner, E. D., Dubas, M. E., Goeckeler, J. L., Kruse, K. B. and McCracken, A. A. (1999). The requirement for molecular chaperones during endoplasmic reticulum-associated protein degradation demonstrates that protein export and import are mechanistically distinct. *J. Biol. Chem.* **274**, 3453-3460.

Ciechanover, A. (1998). The ubiquitin-proteasome pathway: on protein death and cell life. *EMBO J.* **17**, 7151-7160.

de Virgilio, M., Weninger, H. and Ivessa, N. E. (1998). Ubiquitination is required for the retro-translocation of a short-lived luminal endoplasmic reticulum glycoprotein to the cytosol for degradation by the proteasome. *J. Biol. Chem.* **273**, 9734-9743.

Egner, R., Rosenthal, F. E., Kralli, A., Sanglard, D. and Kuchler, K. (1998). Genetic separation of FK506 susceptibility and drug transport in the yeast Pdr5 ATP-binding cassette multidrug resistance transporter. *Mol. Biol. Cell* **9**, 523-543.

Enenkel, C., Lehmann, A. and Kloetzel, P. M. (1998). Subcellular distribution of proteasomes implicates a major location of protein

degradation in the nuclear envelope-ER network in yeast. *EMBO J.* **17**, 6144-6154.

Guthrie, C. and Fink, G., R. (1991). Guide to Yeast Genetics and Molecular Biology. *Acad. Press* **194**, 1-933.

Hampton, R. Y., Gardner, R. G. and Rine, J. (1996). Role of 26S proteasome and HRD genes in the degradation of 3-hydroxy-3-methylglutaryl-CoA reductase, an integral endoplasmic reticulum membrane protein. *Mol. Biol. Cell* **7**, 2029-2044.

Hampton, R. Y. and Bhakta, H. (1997). Ubiquitin-mediated regulation of 3-hydroxy-3-methylglutaryl-CoA reductase. *Proc. Nat. Acad. Sci. USA* **94**, 12944-12948.

Hartl, F. U., Hlodan, R. and Langer, T. (1994). Molecular chaperones in protein folding: the art of avoiding sticky situations. *Trends Biochem. Sci.* **19**, 20-25.

Heinemeyer, W., Kleinschmidt, J. A., Saidowsky, J., Escher, C. and Wolf, D. H. (1991). Proteinase yscE, the yeast proteasome/multicatalytic-multifunctional proteinase: mutants unravel its function in stress induced proteolysis and uncover its necessity for cell survival. *EMBO J.* **10**, 555-562.

Hiller, M. M., Finger, A., Schweiger, M. and Wolf, D. H. (1996). ER degradation of a misfolded luminal protein by the cytosolic ubiquitin-proteasome pathway. *Science* **273**, 1725-1728.

Huppa, J. B. and Ploegh, H. L. (1997). The alpha chain of the T cell antigen receptor is degraded in the cytosol. *Immunity* **7**, 113-122.

Jensen, T. J., Loo, M. A., Pind, S., Williams, D. B., Goldberg, A. L. and Riordan, J. R. (1995). Multiple proteolytic systems, including the proteasome, contribute to CFTR processing. *Cell* **83**, 129-135.

Johnson, E. S., Gonda, D. K. and Varshavsky, A. (1990). Cis-trans recognition and subunit-specific degradation of short-lived proteins. *Nature* **346**, 287-291.

Knop, M., Finger, A., Braun, T., Hellmuth, K. and Wolf, D. H. (1996). Der1, a novel protein specifically required for endoplasmic reticulum degradation in yeast. *EMBO J.* **15**, 753-763.

Kopito, R. R. (1997). ER quality control: the cytoplasmic connection. *Cell* **88**, 427-430.

Leitzgen, K. and Haas, I. G. (1998). Protein maturation in the endoplasmic reticulum. *CHEMTRACTS-Biochem. Mol. Biol.* **11**, 423-445.

Mayer, T. U., Braun, T. and Jentsch, S. (1998). Role of the proteasome in membrane extraction of a short-lived ER-transmembrane protein. *EMBO J.* **17**, 3251-3257.

McCracken, A. A. and Brodsky, J. L. (1996). Assembly of ER-associated protein degradation in vitro: dependence on cytosol, calnexin and ATP. *J. Cell Biol.* **132**, 291-298.

Moriyama, T., Sather, S. K., McGee, T. P. and Simoni, R. D. (1998). Degradation of HMG-CoA reductase in vitro. Cleavage in the membrane domain by a membrane-bound cysteine protease. *J. Biol. Chem.* **273**, 22037-22043.

Opheim, D. J. (1978). alpha-D-Mannosidase of Saccharomyces cerevisiae. Characterization and modulation of activity. *Biochim. Biophys. Acta* **524**, 121-130.

Peters, J. M. (1998). SCF and APC: the Yin and Yang of cell cycle regulated proteolysis. *Curr. Opin. Cell Biol.* **10**, 759-768.

Pilon, M., Schekman, R. and Römisch, K. (1997). Sec61p mediates export of a misfolded secretory protein from the endoplasmic reticulum to the cytosol for degradation. *EMBO J.* **16**, 4540-4548.

Plemper, R. K., Böhmler, S., Bordallo, J., Sommer, T. and Wolf, D. H. (1997). Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation. *Nature* **388**, 891-895.

Plemper, R. K., Egner, R., Kuchler, K. and Wolf, D. H. (1998). Endoplasmic reticulum degradation of a mutated ATP-binding cassette transporter Pdr5 proceeds in a concerted action of Sec61 and the proteasome. *J. Biol. Chem.* **273**, 32848-32856.

Plemper, R. K., Deak, P. M., Otto, R. T. and Wolf, D. H. (1999). Re-entering the translocon from the lumenal side of the endoplasmic reticulum. Studies on mutated carboxypeptidase yscY species. *FEBS Lett.* **443**, 241-245.

Plemper, R. K. and Wolf, D. H. (1999). Retrograde protein translocation: ERADication of secretory proteins in health and disease. *Trends Biochem. Sci.* **24**, 266-270.

Rapoport, T. A., Jungnickel, B. and Kutay, U. (1996). Protein transport across the eukaryotic endoplasmic reticulum and bacterial inner membranes. *Annu. Rev. Biochem.* **65**, 271-303.

Saito, Y., Yamanushi, T., Oka, T. and Nakano, A. (1999). Identification of SEC12, SED4, truncated SEC16, and EKS1/HRD3 as multicopy suppressors of ts mutants of Sar1 GTPase. *J. Biochem. (Tokyo)* **125**, 130-137.

Sikorski, R. S. and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. *Genetics* **122**, 19-27.

Sommer, T. and Jentsch, S. (1993). A protein translocation defect linked to ubiquitin conjugation at the endoplasmic reticulum. *Nature* **365**, 176-179.

Sommer, T. and Wolf, D. H. (1997). Endoplasmic reticulum degradation: reverse protein flow of no return. *FASEB J.* **11**, 1227-1233.

Stirling, C. J., Rothblatt, J., Hosobuchi, M., Deshaies, R. and Schekman, R. (1992). Protein translocation mutants defective in the insertion of integral membrane proteins into the endoplasmic reticulum. *Mol. Biol. Cell* **3**, 129-142.

Tyers, M. and Willems, A. R. (1999). One ring to rule a superfamily of E3 ubiquitin ligases. *Science* **284**, 601-604.

Ward, C. L., Omura, S. and Kopito, R. R. (1995). Degradation of CFTR by the ubiquitin-proteasome pathway. *Cell* **83**, 121-127.

Wiertz, E. J., Tortorella, D., Bogyo, M., Yu, J., Mothes, W., Jones, T. R., Rapoport, T. A. and Ploegh, H. L. (1996). Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. *Nature* **384**, 432-438.

Wolf, D. H. and Fink, G. R. (1975). Proteinase C (carboxypeptidase Y) mutant of yeast. *J. Bacteriol.* **123**, 1150-1156.

Yu, H., Kaung, G., Kobayashi, S. and Kopito, R. R. (1997). Cytosolic degradation of T-cell receptor alpha chains by the proteasome. *J. Biol. Chem.* **272**, 20800-20804.