The EMBO Journal vol.15 no.4 pp.753–763, 1996

Der1, a novel protein specifically required for endoplasmic reticulum degradation in yeast

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The endoplasmic reticulum (ER) of the yeast *Saccharomyces cerevisiae* contains a proteolytic system able to selectively degrade misfolded lumenal secretory proteins. For examination of the components involved in this degradation process, mutants were isolated. They could be divided into four complementation groups. The mutations led to stabilization of two different substrates for this process. The mutant classes were called *'der'* for *'d*egradation in the *ER'*. *DER1* was cloned by complementation of the *der1-2* mutation. The *DER1* gene codes for a novel, hydrophobic protein, that is localized to the ER. Deletion of *DER1* abolished degradation of the substrate proteins. The function of the Der1 protein seems to be specifically required for the degradation process associated with the ER. The depletion of Der1 from cells causes neither detectable growth phenotypes nor a general accumulation of unfolded proteins in the ER. In *DER1*-deleted cells, a substrate protein for ER degradation is retained in the ER by the same mechanism which also retains lumenal ER residents. This suggests that *DER1* acts in a process that directly removes protein from the folding environment of the ER.

Keywords: degradation/endoplasmic reticulum/secretory pathway/yeast

Introduction

The secretory pathway of eukaryotes is organized into distinct membrane-bound structures. Transport of membranes and lumenal contents between these structures results from vesicles that maintain a continuous flow between the endoplasmic reticulum (ER) and the lysosomal system and the plasma membrane. The site of entry into the pathway is the ER, where secretory and lysosomal proteins are translocated in an unfolded state through the membrane into the lumen of this organelle. Parallel to translocation and thereafter, while the proteins are folded, covalent modifications occur in the ER that do not take place in the cytoplasm: *N*- and *O*-linked glycosyl residues are covalently attached and the oxidizing milieu supports the formation of disulphide linkages. The ER has to overcome several problems that go together with the high levels of unfolded proteins that enter the lumen at the same time. These proteins have to be protected from aggregation and they have to be kept in a state in which they are able to fold correctly (reviewed by Pryer *et al.*, 1992; Gaut and Hendershot, 1993).

The lumen of the ER provides high concentrations of chaperones that promote translocation, post-translational folding and oligomerization (reviewed by Rothman, 1989; Gething and Sambrook, 1992; Doms *et al.*, 1993; Hartl *et al.*, 1994). Exposure of hydrophobic, aggregation-promoting regions of unfolded proteins seems to be the key signal that leads to binding of chaperones (Flynn *et al.*, 1989; Blond-Elguindi *et al.*, 1993). Several chaperones may act stepwise on the folding of one protein (Hammond and Helenius, 1994; Melnick *et al.*, 1994).

Unbalanced subunit synthesis, inefficient folding or mutations can cause failure of soluble and membrane-bound secretory proteins to assume their correct shape, as found for mammalian cells (reviewed by Bonifacino and Klausner, 1994) and yeast (Finger *et al.*, 1993; Hill and Stevens, 1994). In most cases this leads to their selective degradation in a pre-Golgi compartment. This so-called ER degradation or pre-Golgi degradation (Lippincott-Schwartz *et al.*, 1988) is independent of the lysosomal/vacuolar proteolytic system (reviewed by Klausner and Sitia, 1990; Bonifacino and Lippincott-Schwartz, 1991; Bonifacino and Klausner, 1994).

The protease(s) that participate in ER degradation are, as yet, unknown. From studies with inhibitors, several types of proteolytic activities have been attributed to this process, i.e. serine proteases, cysteine proteases and metallo proteases (reviewed by Fra and Sitia, 1993), but the difficulties concerning specificities and side effects of the inhibitors used in various studies have not yet been resolved. Moreover, it has to be considered that different substrate proteins for ER degradation may need different proteases for degradation (Inoue and Simoni, 1992), and different proteolytic systems may be involved.

The selectivity of this process has been addressed, showing that the redox potential inside the ER (Young *et al.*, 1993) as well as calcium (Wileman *et al.*, 1991) may play roles in ER degradation, either by regulating the process itself or by providing the optimal conditions. On the other hand, structural features of a protein (either inherent or generated upon exposure to the ER milieu) are suspected to be crucial elements for degradation: sequences within the membrane domain of the unassembled T-cell receptor α chain promote degradation of the entire protein (Bonifacino *et al.*, 1990); the 20 C-terminal amino acid residues with an essential cysteine are necessary and sufficient for ER degradation of the μ heavy chain. Also cathepsin D can be targeted to ER degradation through linkage of these 20 amino acids to its C-terminus (Fra *et al.*, 1993).

Here we report a genetic approach we have undertaken

with the yeast *Saccharomyces cerevisiae* to uncover new molecular components that constitute the ER degradation machinery. We have used two marker substrates for ER degradation to screen for yeast mutants which are no longer able to degrade these proteins. Different '*der*'-mutants (*d*egradation in the *ER*) were isolated. One of the mutants was used to clone a novel gene, *DER1*. We show that the Der1 protein is specifically required for ER degradation and that epitope-tagged Der1 resides in the ER. Subsequent studies were undertaken to characterize more closely the effects that were caused through absence of the Der1 protein.

Results

Screen for mutants defective in ER degradation

To isolate yeast mutants defective in ER degradation we exploited a strain that expresses two substrate proteins for this process, CPY* and PrA*. One protein, PrA*, is a deletion derivative of proteinase yscA (PrA) that lacks the region of the processing site of the propeptide. It is completely inactive *in vivo*, no active proteinase yscB (PrB) can be found; hence, PrA* possesses no activity that is able to initiate the vacuolar activation cascade. The second protein (CPY*) is a mutated version of carboxypeptidase yscY (CPY). It carries a point mutation next to the active site serine. Both proteins were shown to be highly unstable inside the cell due to their degradation in the ER or an ER-related compartment (Finger *et al.*, 1993). In order to isolate mutants with defects in ER degradation, an assay was developed, which is based on the detection of higher antigenic levels of mutated substrate proteins due to their accumulation in mutagenized cells. EMS mutagenized cells were grown on nitrocellulose sheets on solid agar containing complete medium and the sheets were then transferred onto starvation medium to induce synthesis of CPY* and PrA*. Thereafter the cells were incubated on starvation medium containing cycloheximide which prevented new synthesis but not degradation (not shown) of CPY* and PrA* (an example is shown in Figure 1A). Subsequently, the cells were lysed on the nitrocellulose sheets and CPY* and PrA* levels determined immunologically.

From 8000 screened colonies, 307 clones showed an increased immunostaining with the antiPrA and 412 clones with the antiCPY serum as compared with the parental wild-type yeast strain. Among these, 270 clones showed increased staining with both sera. All colonies were picked, streaked out as single colonies and re-analysed. To exclude non-specific mutations (i.e. lysis mutations), each mutant clone was subjected to Western blot analysis (an example is shown in Figure 3A). Cells that did not accumulate CPY* and/or PrA* intracellularly, and by this resembled the parental strain, were discarded. Only six mutants showed significant amounts of CPY* and PrA* antigenic material. None of them accumulated only one of the substrate proteins. Genetic analysis revealed that the mutations were recessive single gene mutations which could be divided into four different complementation groups. Subsequent pulse–chase experiments with the mutants confirmed that the increased amount of CPY* and PrA* antigenic material detected on Western blots resulted indeed from stabilization of these proteins. Figure 1B shows a pulse–chase experiment performed with mutant strains *der1-1* and *der1-2*. Compared with the half-lives for CPY* and PrA* in wild-type cells (20 and 15 min, respectively; Finger *et al.*, 1993) both mutants significantly stabilized both proteins.

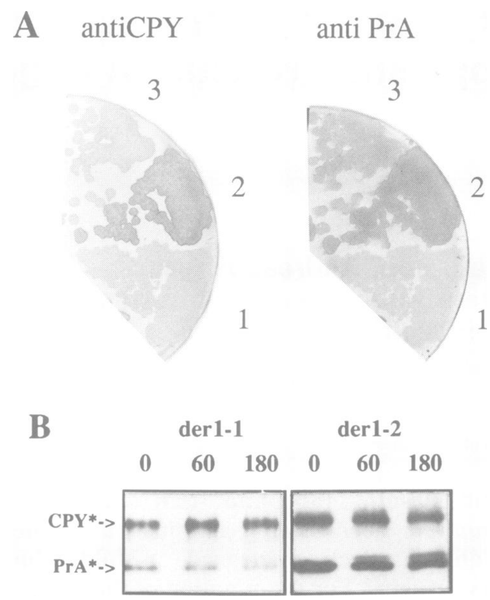

Fig. 1. (**A**) Screen for *der*-mutants and cloning of the *DER1* gene. Patches of strains YAF6 (1) and der1-2 (2 and 3) containing plasmids yEPlac181 (1 and 2) or yEPlac181*DER1* (3) were grown and lysed on nitrocellulose membranes as described in Materials and methods. The nitrocellulose sheets were stained using antibodies specific either for PrA* (anti PrA) or CPY* (antiCPY). (**B**) Pulse–chase of PrA* and CPY* using mutant strains *der1-1* and *der1-2*. The cells were labelled for 30 min with [^{35}S]methionine. After initiation of the chase, aliquots were taken after the indicated times. After cell lysis, CPY* and PrA* were simultaneously immunoprecipitated and the labelled proteins were visualized by SDS–PAGE electrophoresis followed by fluorography.

Cloning and analysis of DER1

DER1 was cloned by complementation of the *der*-phenotype in the mutant strain *der1-2* with a library of yeast genomic DNA in a centromere-containing, *LEU2*-based plasmid (Cvrcková and Nasmyth, 1993). Among 17 000 transformants tested with the immunoassay for the absence of CPY* antigenic material, one single clone contained a plasmid, which after plasmid rescue and retransformation into the *der1-1* and *der1-2* mutant strains, was still able to complement the ER degradation phenotype for both substrate proteins, PrA* and CPY*. Subcloning of the 4.2 kb insert found revealed a complementing 2.2 kb *Hin*dIII DNA fragment. Based on the restriction pattern and on the Olsen clone number determined (Riles *et al.*, 1993), sequence information for an already sequenced 11.7 kb segment of chromosome II (Mallet *et al.*, 1994) was obtained from the data base. The complementing *Hin*dIII fragment contained the open reading frame (ORF) YBR1413. To demonstrate that *DER1* is identical to YBR1413 we constructed subclones by making use of a unique *Pst*I site that is located within the YBR1413 ORF. Neither of these subclones could complement the *der1-2* phenotype. We still had to confirm that the cloned piece of DNA contained the wild-type

DER1 gene and not a suppressor for the *der1-2* phenotype. For this purpose, the cloned DNA was integrated chromosomally at its originating chromosomal locus in the *der1-2* mutant strain. Thereafter, the resulting strain was crossed with a wild-type strain. Subsequent tetrad analysis showed that the cloned *DER1* gene and the *der1-2* mutation were genetically linked, since no spores were found (among 22 tetrads tested) in which the *der1-2* phenotype reappeared (data not shown).

The region of the YBR1413 ORF was re-sequenced. Seven differences to the published sequence were found. Five of the nucleotide differences are conservative exchanges, one leads to a valine instead of an alanine at position 145 of the protein and one causes a frame shift in the N-terminal region that leads to an extended ORF that predicts a 211- instead of 141-amino acid-containing protein. Investigation of the amino acid sequence revealed a hydrophobic protein with only short hydrophilic or charged stretches. Interestingly, sequence comparison found a related ORF from *Hansenula polymorpha* (*Pichia angusta*) in the data base. This ORF predicts a homologous protein which shares ~22% identity with Der1 (see Figure 2A). This 212-amino acid protein exhibits a hydropathy profile which resembles that of Der1 (Figure 2B). The method of Rost and Sander (1994) predicted four *trans*-membrane regions at similar positions within the sequences of both proteins (underlined sequences in Figure 2A).

The Der1 sequence does not contain recognition sites for *N*-glycosylation, nor does it contain a known ER retention signal such as HDEL (Pelham *et al.*, 1988) or KKXX (Nilsson *et al.*, 1989; Townsley and Pelham, 1994). The first potential *trans*-membrane region of Der1 (amino acids 16–32) exhibits some characteristics of a signal sequence: a positive charge at position 17 (arginine) is followed by a hydrophobic stretch and a potential signal peptidase cleavage site at position 31.

Northern blot analysis detected a *DER1*-dependent mRNA of ~0.9 kb. C-terminal prolongation of the *DER1* ORF with 27 nucleotides that code for the HA-tag slightly increased the size of this mRNA. The strong signal observed for the *DER1* mRNA when *DER1* was expressed from a high-copy plasmid (pRS425*DER1*) readily confirms that overexpression of *DER1* is possible at least on this level (Figure 2C).

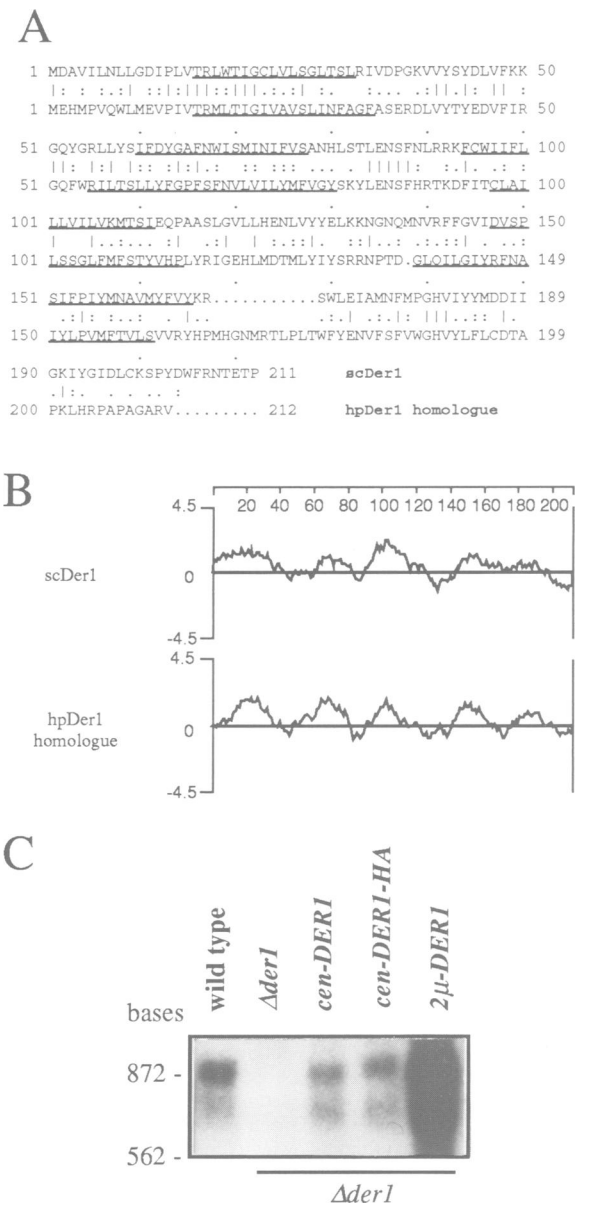

Fig. 2. Properties of the Der1 protein. (**A**) Predicted amino acid sequence of the Der1 protein from *S.cerevisiae* (scDer1) aligned to the sequence of a homologous protein from *Hansenula polymorpha* (hpDer1). The regions where *trans*-membrane regions have been predicted are underlined. (**B**) Kyte–Doolittle hydropathy plots (window size of 19) of scDer1 and the homologous protein from *H.polymorpha*. (**C**) Northern blot and detection of *DER1*-specific mRNA in logarithmically growing cells of strains W303-1C and W303-CD (cen-*DER1* = plasmid pRS315*DER1*; cen-*DER1-HA* = plasmid pRS315 *DER1-HA*; 2μ-*DER1* = plasmid pRS425*DER1*). The numbers indicate the position of RNA standards.

Loss of Der1 causes a defect in ER degradation

In order to determine the phenotype of yeast cells that entirely lack the *DER1* gene, we constructed a deletion allele for *DER1* lacking 79% of the coding region and containing the *URA3* gene and used it for chromosomal deletion of the wild-type *DER1* gene in yeast cells.

As found for the *der1-2* mutation, the deletion of the *DER1* gene also led to the accumulation of the mutated proteins PrA* and CPY* as revealed by Western blotting (Figure 3A). Besides the authentic molecular mass protein bands of CPY* and PrA*, a diffuse banding pattern to higher molecular masses was observed, the amount of which varied according to the growth conditions. The stabilization of CPY* in the Δ*der1* background as well as the ability of plasmids with the wild-type *DER1* allele to complement the *DER1* deletion was analysed in pulse–chase experiments. As shown in Figure 3B, deletion of *DER1* led to complete stabilization of CPY*. However, whereas the low-copy *DER1* plasmid restored normal degradation of CPY* in the Δ*der1* cells, the cells with the high-copy *DER1* plasmid exhibited a slight retardation in degradation of CPY* (Figure 3A), suggesting an effect of *DER1* overexpression on CPY* degradation. Alternatively, the same result could also be explained with a heterogeneous population of yeast cells, in which a subpopulation of Δ*der1* cells lost the *DER1* encoding plasmid. If the latter was true, the presence of this high-copy *DER1* plasmid in *DER1*-containing wild-type cells should not

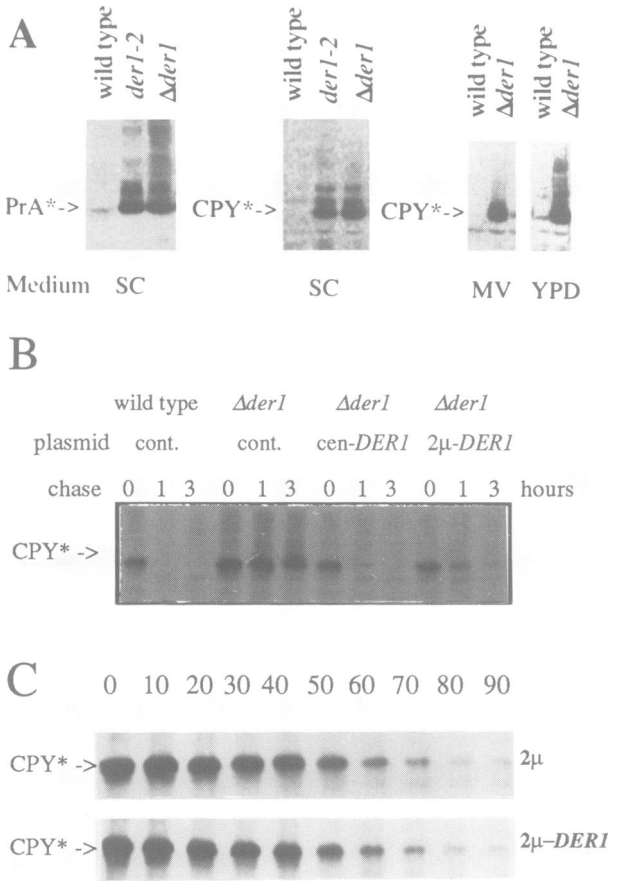

Fig. 3. (**A**) Steady-state levels of PrA* and CPY*. Western blots of crude cell extracts of early stationary phase cells after SDS–PAGE of wild-type strain YAF6, of mutant strain der1-2, of strain FHK7-1 (*Δder1*), that have been grown on SC medium and of strains W303-1C (wild-type) and W303-CD (*Δder1*), grown on MV or on YPD medium. (**B**) Degradation of CPY* followed in a pulse–chase experiment performed with wild-type strain W303-1C and strain W303-CD (*Δder1*). Cells were labelled for 30 min with [^{35}S]methionine and samples were taken after the indicated chase periods. The cells harbour the following plasmids: cont. = plasmid pRS315; cen-*DER1* = plasmid pRS315*DER1*; 2μ-*DER1* = plasmid pRS425*DER1*. (**C**) Overexpression of *DER1*. Pulse–chase experiment of PrA* and CPY* with cells of strains W303-1C with either plasmid pRS425 (cont.) or plasmid pRS425*DER1* (2μ-*DER1*). Cells were labelled for 10 min and aliquots taken after the indicated chase periods.

show the same effect. On the other hand, high copy expression of *DER1* in wild-type cells should also allow to test for an eventual effect of *DER1* overexpression on CPY* degradation. When the respective pulse–chase experiment was performed with *DER1* wild-type cells that contained either the control plasmid or the high copy *DER1* plasmid, no differences that would point to an overexpression phenotype of *DER1* with respect to CPY* degradation were observed (Figure 3C). This also indicated that the inefficient complementation of the *Δder1* phenotype with the high-copy plasmid-encoded *DER1* might be due to plasmid loss. Indeed, when plasmid loss analysis was performed, we found that cultures with the high-copy *DER1* plasmid contained ~55% cells that had lost the plasmid. This value correlated well with the remaining amount of CPY* in such *Δder1* cultures when they were analysed under steady-state conditions by Western blotting (data not shown).

For further characterization of the *DER1* deletion phenotype, we investigated the growth of *Δder1* cells on different media as well as under different temperatures or after heat shock. Also, the influence of various substances inducing misfolded proteins such as canavanine, 2-deoxyglucose, β-mercaptoethanol or tunicamycin, as well as different metal ions, were tested. In no case was a *DER1*-dependent effect on the growth of the cells observed. Also, secretion of wild-type CPY and wild-type PrA was not affected by the absence of the *DER1* gene product (data not shown).

Epitope-tagged Der1 is localized to the ER

For intracellular localization of Der1 we tried to obtain polyclonal antibodies. Due to toxic effects, expression of two-thirds of the Der1 sequence (amino acid positions 70–211) in *Escherichia coli* for production of Der1 antigen was not successful. Specific antisera raised against N-terminal and C-terminal parts of Der1 did not recognize the protein in yeast cells, even when it was overexpressed. Therefore several epitope-tagged versions of Der1 were constructed. While a haemagglutinin (HA) tag at the N-terminus of Der1 led to an inactive protein, C-terminally tagged Der1 containing either one (Der1-HA) or two (Der1-HA2) tags in frame did fully (Der1-HA) or partially (Der1-HA2) complement a DER1 deletion mutant as measured by the ability to mediate CPY* degradation (Figure 4A). Using HA-tag-specific monoclonal antibodies(mAb) (12CA5), overexpressed Der1-HA2, containing two antigenic sites in frame, was easily detected in crude cell extracts. In contrast, chromosomally expressed Der1-HA and Der1-HA2 as well as overexpressed Der1-HA could only be weakly seen in preparations enriched for membranes (Figure 4A) and are not detectable in total cell extracts (not shown). For intracellular localization of Der1-HA2 we performed subcellular fractionation experiments. For this purpose we used cells harbouring the high-copy Der1-HA2 plasmid. As can be seen in Figure 4B, Der1-HA2 co-fractionates with the ER marker proteins Kar2 and NADPH cytochrome C reductase. This localization of Der1-HA2 in the ER was verified in whole cells using immunofluorescence. Thereby the typical staining pattern for the ER, a staining of the perinuclear region and staining of regions along the plasma membrane (Preuss *et al.*, 1991), was observed when Der1-HA2 antigenic material was visualized (Figure 4C). In order to verify this localization we also performed a subcellular fractionation experiment with cells that chromosomally express Der-HA2. For Der-HA2 a similar distribution was detected in the gradient as for CPY* (Figure 4D) and for the ER marker enzymes shown in Figure 4B (not shown). The protein was only visible when almost the entire membrane content of the individual fractions was used for a single Western blot.

ER localization of CPY in Δder1 cells involves chaperone-mediated ER retention*

To obtain clues about the fate of CPY* in *Δder1* cells, we localized CPY*-specific material within the cells. Subcellular fractionation of *Δder1* cells on a sucrose gradient showed that CPY* co-fractionates with ER markers (Figure 5A). The staining pattern obtained when CPY* was visualized in these cells by immunofluorescence (Figure 5B) was typical for ER structures. These experi-

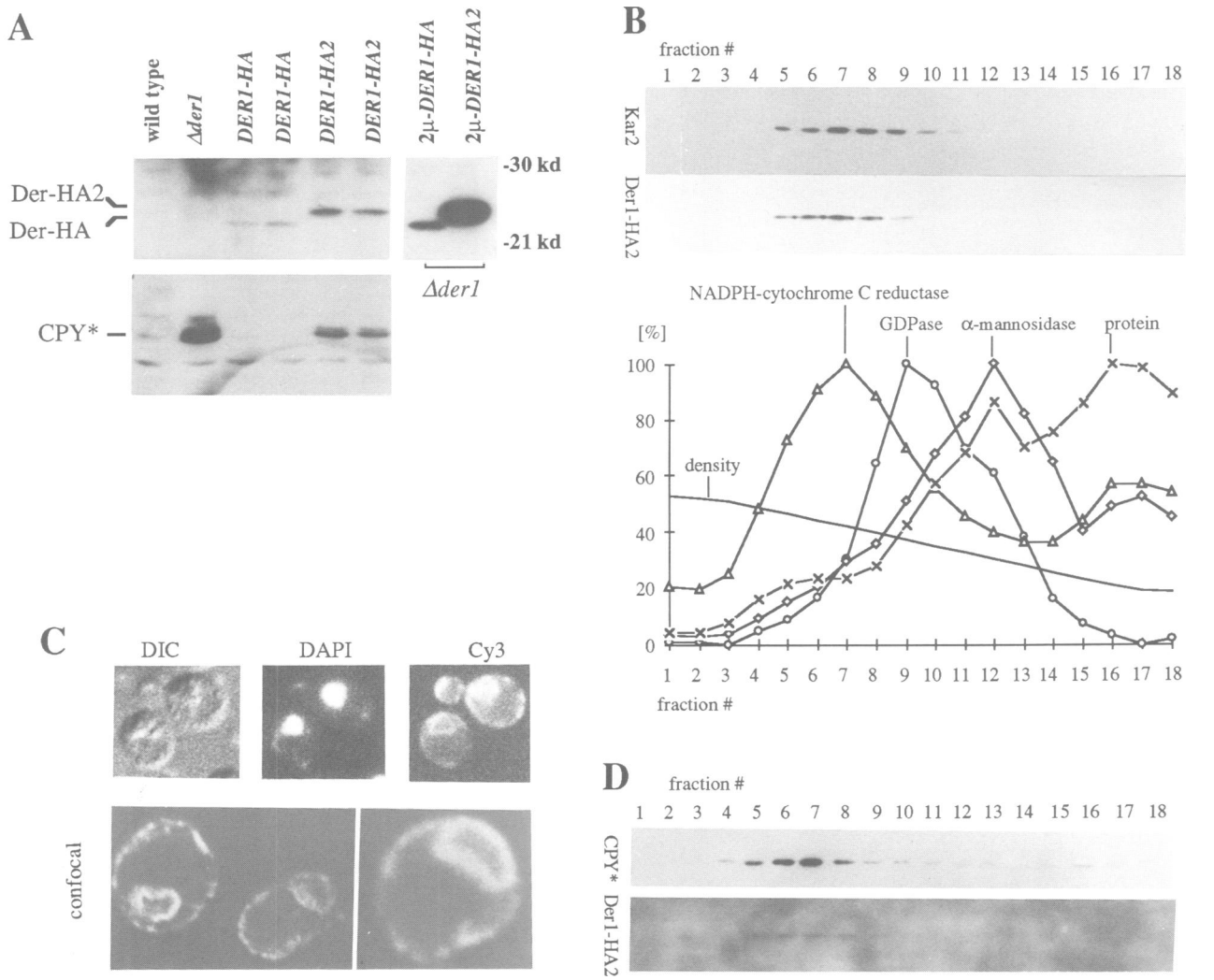

Fig. 4. Detection of epitope-tagged Der1 proteins and their complementation efficiencies. (**A**) The upper panel represents immunoblotting of cell preparations enriched for membranes and detection of epitope-tagged Der1 proteins with 12CA5 mAb. The blot was developed with the ECL™-system (Amersham) and the film was exposed for 2 h. The lower panel shows immunoblotting of total cell extracts followed by detection of CPY* with antiCPY serum. Cells of strains W303-1C (wild-type), W303-CD (*Δder1*) and of strains W303-CtD and Ct^{2}D, that harbour chromosomal copies of epitope-tagged *DER1* alleles (*DER1-HA* and *DER1-HA2*) and of W303-CD cells that overexpress epitope-tagged Der1 from plasmids (2μ-*DER1-HA* = pRS425*DER1-HA* and 2μ-*DER1-HA2* = pRS425*DER1-HA2*). Numbers to the right indicate the positions of the molecular weight standards. (**B**) Epitope-tagged Der1 co-fractionates with the ER markers Kar2 and NADPH–cytochrome C reductase. A spheroplast homogenate of strain W303-CD with plasmid pRS425*DER1-HA2* was fractionated on a 10-step sucrose gradient (54–18%). Aliquots of the collected fractions were subjected to Western blot analysis and used to assay the marker enzymes NADPH–cytochrome C reductase (ER fraction), guanosine diphosphatase (GDPase, Golgi) and α-mannosidase (vacuoles). The activities of NADPH–cytochrome C reductase, GDPase and α-mannosidase are given as % activity of the highest values measured. 100% protein corresponds to 3.2 mg/ml. The density is given as % sucrose (w/w). (**C**) Epitope-tagged Der1 is localized to ER like structures. Indirect immunofluorescence of logarithmically growing W303-CD cells that contain plasmid pRS425*DER1-HA2* were stained with the anti-HA mAb and the goat-anti-mouse Cy3 antibody (DIC, Normarski optics; DAPI, staining of DNA; Cy3, Cy3-fluorescence of the goat-anti-mouse Cy3 antibody; confocal, Cy3-fluorescence monitored with a confocal microscope). (**D**) Chromosomally expressed Der1-HA2 co-fractionates with CPY* in ER fractions. W303-Ct^{2}D cells were fractionated and the fractions analysed as in (B). The marker distribution was similar to (B). For detection of Der1-HA2, 450 μl of each fraction were diluted 8-fold with 10 mM HEPES, pH 7.5, the membranes were collected (100 000 *g*, 1 h) and the entire pellets were used for immunoblotting. At position 18 of the blot performed with the 12CA5 antibody, similarly treated fraction 5 of a fractionation experiment performed with strain W303-CD was applied as a negative control.

ments demonstrate that CPY* mainly resides in the ER, when ER degradation is abolished.

On Western blots performed with crude extracts of *der1-2* and *Δder1* cells, we had previously observed CPY* species with higher molecular masses than the ER-modified form of CPY* (Figure 3A). An explanation for this observation could be that fractions of CPY* obtain Golgi-specific mannosyl modifications. This would indicate that some CPY* escapes the ER in *der1-2* and *Δder1* cells. We investigated this hypothesis by following CPY* in a pulse–chase experiment in the *der1-2* mutant cells. The results shown in Figure 6 indicate that, parallel to the duration of the chase period, a smear of more and more CPY* with increased molecular masses appeared. This increase in molecular mass was indeed due to glycosyl modifications, since deglycosylation of the immunoprecipitates with Endo F resulted in a protein species with the molecular mass of deglycosylated CPY*. As could be demonstrated with antibodies specific either for α1,6- or α1,3-mannosylations, these glycosyl modifications of CPY* consisted mainly of α1,6-linked mannose residues (Figure 6). From these results we conclude

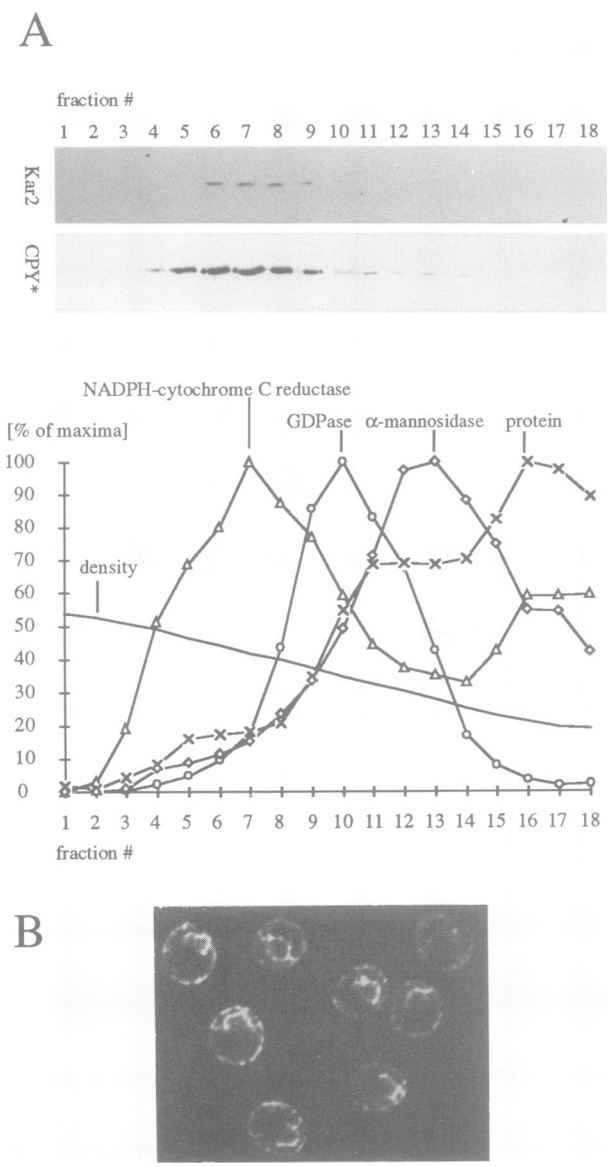

Fig. 5. (**A**) Deletion of *DER1* leads to accumulation of CPY* in the ER. A subcellular fractionation experiment was performed with cells of strain W303-CD as described in the legend to Figure 4B. (**B**) CPY* is detected in ER-like structures. Indirect immunofluorescence with antiCPY and goat-anti-rabbit Cy3 antibodies was performed with W303-CD cells. Fluorescence was visualized with a confocal microscope.

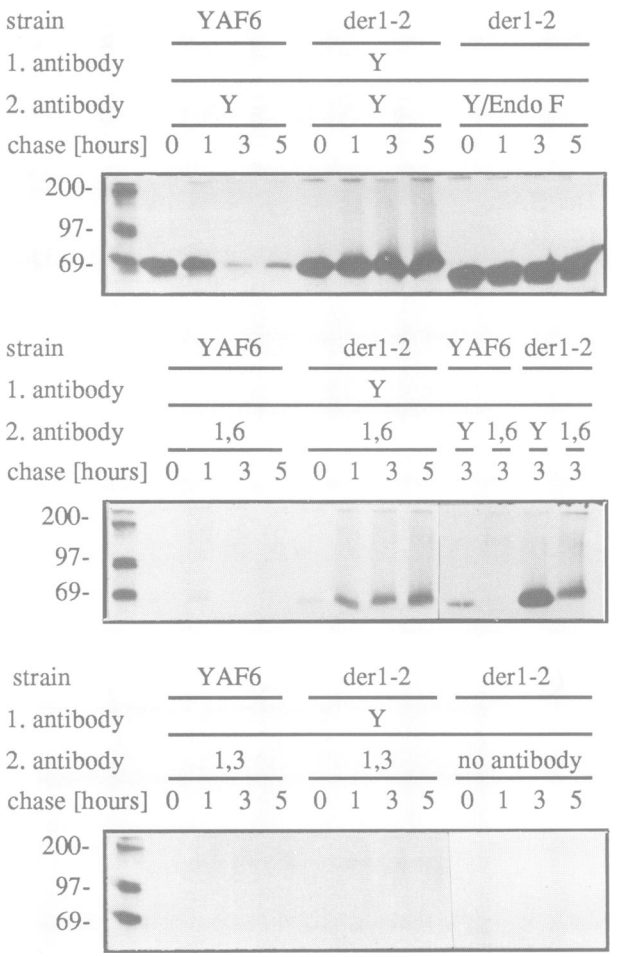

Fig. 6. CPY* becomes early Golgi glycosylated in the *der1-2* strain. Pulse–chase of cells of strains YAF6 and mutant strain *der1-2*. Labelling time was 30 min, chase times as indicated. After immunoprecipitation with the antiCPY antibody (1. antibody) the immunocomplexes were solubilized and aliquots thereof were subjected to a second round of immunoprecipitation with the indicated antibodies [2. antibody; α1,6 and α1,3, antibodies specific for α1,6- and α1,3-mannose linkages (Franzusoff and Schekman, 1989); no antibody, control immunoprecipitation with no antibody]. All samples were treated as required for Endo F digestion, but Endo F was added only where indicated. Numbers to the left indicate the positions of the molecular weight standards shown on the first lanes.

that most of CPY* is retained in the ER, but some CPY* is able to leave the ER. It then becomes early-Golgi mannosylated with characteristics of the outer chain-elongated type of mannosyl modifications (Herscovics and Orlean, 1993).

Similar characteristics were described for glycoproteins which undergo receptor-mediated retrieval from early-Golgi compartments (Dean and Pelham, 1990). We wanted to know, whether the HDEL-triggered ER retrieval system is involved in ER retention of CPY*, in spite of the fact that CPY* contains no C-terminal HDEL-tetrapeptide. For this purpose we examined the effect of an *ERD1* deletion on the glycosylation of CPY*. *ERD1* is a gene required for the retention of lumenal ER proteins. Deletion of *ERD1* leads to secretion of HDEL-containing proteins from the ER. Furthermore, it reduces the extent of Golgi-glycosylation and slightly affects vacuolar protein sorting (Hardwick *et al.*, 1990).

The appearance of high molecular weight species of CPY* was taken as a criterion for CPY* that had left the ER. Deletion of *ERD1* caused the escape of some CPY* from ER degradation in cells wild-type for *DER1* (Figure 7A, upper panel, compare lanes 1 and 3). In a strain deleted in *DER1*, the additional deletion of *ERD1* led to an increase in the appearance of higher molecular mass CPY* species, indicating that more molecules reached the compartments where visible mannosylation takes place (Figure 7A, upper panel, compare lanes 2 and 4). This interpretation is also supported by the fact that short-term exposure of the immunoblot (Figure 7A, lower panel) revealed a reduced amount of ER glycosylated CPY* in Δ*der1* Δ*erd1* cells as compared with Δ*der1* cells (Figure 7A, lower panel, compare lanes 2 and 4). Even more of

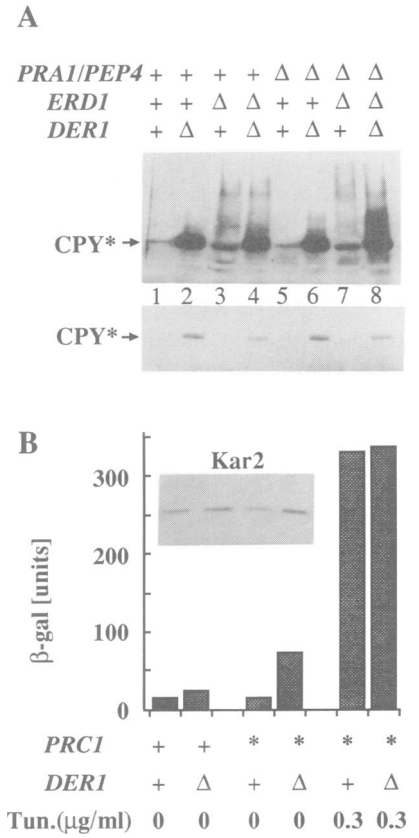

Fig. 7. Chaperone-mediated retrieval contributes to the localization of CPY* in the ER. (**A**) Immunoblot of crude cell extracts of a set of congenic strains to strain W303-1C (*PEP4 ERD1 DER1 prc1-1*) that are wild-type (+) or deleted (Δ) in the indicated alleles. Two different exposure times of the same blot are shown (upper picture, 30 min; lower picture, ~30 s). The arrow indicates the 67 kDa form of CPY*. (**B**) The unfolded protein response depends on *DER1* and CPY*. β-galactosidase activities in cells of strains W303-1B, BΔD, 1C and CΔD containing plasmid pSZ1 expressing β-galactosidase under the control of an UPR element-regulated CYC1 promoter. Cells were grown on MV medium to mid log growth phase (corresponding to 1.8–2.2 OD_{600} of cells). For each strain, two independent measurements were performed using each time three independent transformants. Values were corrected for the activity in cells with plasmid pLGΔ-178 (without the UPR element). The range of error is <10%. Tunicamycin (Tun.) was added 4 h before the measurement. For the immunoblot with anti-Kar2 serum, crude extracts of cells of the same strains (but without plasmids) were used.

the Golgi-modified species of CPY* were visible when, in addition to the Δ*der1* and the Δ*erd1* deletion, vacuolar proteolysis was abolished through deletion of the gene for PrA. This indicates that in this strain additional material accumulated in the vacuole (Figure 7A, upper panel, compare lanes 4 and 8). As expected, vacuolar PrA deficiency has no effect on CPY* amounts, when its retention in the ER is possible due to presence of Erd1 (Figure 7A, compare lanes 2 and 6). However, it is not clear why the absence of PrA does not lead to an increase in the amounts of CPY* in *DER1* wild-type cells when *ERD1* is deleted (Figure 7A, upper panel, compare lanes 3 and 7). This might be due to the rather low steady-state level of CPY* in ER degradation-competent cells.

We draw the following conclusions from this experiment: the *ERD1*-dependent ER retention mechanism retrieving HDEL-containing proteins is involved in ER retention of CPY* in *DER1*-deleted cells, despite the fact that CPY* does not contain this retention motif. Interestingly, the lack of the Golgi-localized Erd1 protein (Hardwick *et al.*, 1990) also has an effect on the degradation of CPY* which takes place in the ER of *DER1* wild-type cells (Finger *et al.*, 1993).

Soluble ER chaperones usually contain the HDEL retrieval signal. Some of these proteins are up-regulated in response to the accumulation of incompletely or malfolded proteins in the ER (Kozutsumi *et al.*, 1988; Mori *et al.*, 1992; Ng *et al.*, 1992; Tachibana and Stevens, 1992). We were interested in the question of whether CPY* is recognized by molecular chaperones as an unfolded protein in Δ*der1* cells. In order to investigate the ability of CPY* to induce such a response we made use of a reporter construct containing the β-galactosidase gene under the control of the so-called UPR-element known to be responsible for the regulation of the unfolded protein response (Mori *et al.*, 1992; Kohno *et al.*, 1993).

We compared β-galactosidase activities in strains that either express wild-type CPY or CPY* from the chromosome, each in the *DER1* wild-type and the *DER1* deletion background. Deletion of *DER1* in a strain that expresses wild-type CPY led to an increase of β-galactosidase activity of ~1.6-fold. In contrast, the *DER1* deletion in a strain expressing CPY* increased β-galactosidase activity ~4-fold (Figure 7B). These results could be confirmed qualitatively when levels of Kar2 (the yeast homologue to BiP) were visualized in these cells on a Western blot (Figure 7B). These results demonstrate that accumulated CPY* in Δ*der1* cells is readily recognized as an unfolded protein. If one assumes that this recognition results from binding of CPY* to chaperones, this then could explain the observed *ERD1*-dependent retention of CPY* in the ER.

Tunicamycin is known to inhibit *N*-glycosylation and by this to increase the amount of unfolded proteins in the ER (Kozutsumi *et al.*, 1988). We analysed whether *DER1* has an effect on the intensity of the unfolded protein response in the presence of tunicamycin. No considerable differences in the levels of β-galactosidase activity were measured (Figure 7B). This demonstrates that under these conditions, the presence or absence of *DER1* exhibits no effects which considerably influence the amount of unfolded proteins in the ER (Figure 7B). Similar results were also obtained when 2-deoxyglucose or β-mercaptoethanol were used (not shown) instead of tunicamycin to induce the unfolded protein response.

Discussion

We developed a screening procedure to isolate mutant strains that are selectively defective in genes required for ER degradation. From 8000 colonies screened, six clones were found that significantly stabilize the two mutated proteins, PrA* and CPY*. All mutant colonies were able to stabilize both mutant proteins, pointing to the fact that the same proteolytic system is responsible for degradation of both proteins. The mutants fell into four different complementation groups. Since the mutant screen was not saturating, one should expect even more complementation groups that constitute this pathway. From one of the mutants we cloned a novel gene, *DER1*, that is required

for ER degradation. The other *DER* genes are presently under investigation and will be subject of further work.

The ORF of *DER1* predicts a short hydrophobic protein of 211 amino acids with a predicted molecular mass of 24.4 kDa. By using an epitope-tagged *DER1* allele, a protein with an appropriate molecular mass of 22–23 kDa could be detected (Figure 4A). One HA epitope attached on the C-terminus of Der1 did not interfere with its function in respect to degradation of CPY*. When a second HA tag was introduced at the C-terminus the resulting Der1-HA2 protein showed reduced *in vivo* function, but it was still able to mediate degradation of most of CPY*. Due to the low levels of epitope-tagged Der1 proteins we had to use the double-tagged *DER1* allele for subcellular localization of the protein. Thereby we found that Der1-HA2 localizes to the ER. Expression levels seem not to affect subcellular localization of the Der1-HA2 protein, since chromosomally and high-copy plasmid expressed *DER1-HA2* alleles both led to a similar distribution of Der1-HA2 antigenic material within the gradient. This ER localization of Der1-HA2 was not surprising, since the degradation of CPY* and PrA* in which Der1 (and also Der1-HA2) acts is associated with this organelle.

For *DER1*, a specific mRNA with an appropriate size was detected. The gene seems to be constitutively expressed under logarithmic (Figure 2C) and also under early stationary phase growth conditions (not shown). Expression of CPY* from a high copy number plasmid resulted in significant accumulation of CPY* in the ER (Finger *et al.*, 1993) but did not increase *DER1* mRNA levels (not shown).

Deletion of *DER1* blocked degradation of PrA* and CPY* (Figure 3A and B), but did not affect vacuolar delivery of wild-type PrA and CPY. *DER1*-deleted strains exhibited no growth defects under any of the tested conditions. The *DER1* deletion caused only a marginal increase of the unfolded protein response of ~1.6-fold (Figure 7B). This may mean that under normal growth conditions yeast cells do not produce many aberrant proteins that are needed to be removed from the ER, at least through the *DER1*-dependent ER degradation pathway. This also indicates that Der1 is not generally required for folding of proteins in the ER. Under conditions where the formation of unfolded proteins is stimulated, no visible *DER1* dependency for the amounts of unfolded proteins present in the ER is measured, either because under such conditions this degradation pathway is oversaturated, or because the cells do react to this situation in a way that ER degradation is abolished (Figure 7B). Taken together, these findings indicate that the Der1 protein is not needed for the overall integrity of ER-functions, but seems to be involved in the specific functions that are required for the degradation of CPY* and PrA*. These functions seem to be redundant for cell survival.

The fate of CPY* was followed in more detail when its degradation was blocked through a deletion or a mutation in the *DER1* gene. The CPY* protein was mainly detectable in the ER (Figure 5). The unfolded protein response observed in Δ*der1* cells that expressed CPY* was significantly increased compared with Δ*der1* cells that expressed wild-type CPY (Figure 7B). This demonstrates that accumulated CPY* is recognized as an unfolded or malfolded protein, which implies interaction of CPY* with components of the ER and hence confirms not only the observed ER localization of accumulated CPY* but also the aberrantly folded nature of CPY* (Finger *et al.*, 1993). However, this result gives no information on whether all CPY* is recognized as an unfolded protein and whether there are different pools of CPY* present in the ER that behave differently.

Evidence has accumulated that the unfolded protein response is stimulated through reduced levels of free Kar2 (Hardwick *et al.*, 1990; Dorner *et al.*, 1992; Ng *et al.*, 1992; Kohno *et al.*, 1993). With respect to these findings, CPY* would induce the unfolded protein response through binding to Kar2. The observed attachment of mainly early-Golgi α1,6-linked mannose residues to CPY* in the *der1-2* background can then be interpreted as recycling of CPY* through compartments of the early-Golgi apparatus back into the ER which may be mediated by Kar2 and other chaperones that undergo ER-retrieval. Deletion of *ERD1* encoding a protein required for retrieval of HDEL-containing proteins from the Golgi to the ER (Hardwick *et al.*, 1990) leads to release of some CPY* from the ER (Figure 7A). This may also be taken as a hint that ER-retrieval of CPY* via Kar2 or other HDEL-containing chaperones takes place. These results again raise the question about the involvement of the known chaperones, especially BiP, in the ER degradation process. Recent findings demonstrated that the half-life of IgG light chains corresponds to the dissociation of the IgG light chain–BiP complex and that all remaining IgG light chain is associated with BiP (Knittler and Haas, 1992; Knittler *et al.*, 1995). Does BiP therefore target a protein for its degradation in the ER (no BiP binding, no ER degradation) or is release from BiP a preliminary step before degradation can occur (BiP binding protects from ER degradation)? The same question also arose from recent findings which showed that BiP binding sites co-localized with signals required for degradation of misfolded proinsulin in the ER (Schmitz *et al.*, 1995).

It remains to be explained why the glycosylation pattern of some CPY* that has escaped the ER contains outer chain elongated species that resemble the glycosylation of invertase or proalpha-factor and why it is different to the distinct core-matured glycosylation type of wild-type CPY (reviewed by Herscovics and Orlean, 1993). Our favourite explanation is that only correct folding of CPY forms a signal that leads to the distinct mannosylation pattern found in wild-type CPY. This is supported by the finding that wild-type CPY carries a dominant signal which is able to convert the mannosylation pattern of C-terminally attached invertase to the type observed for wild-type CPY (Johnson *et al.*, 1987). This signal may be disturbed in the improperly folded CPY* molecule. This mechanism could be similar to the one that leads to the synthesis of mannose-6-phosphate residues in the Golgi on cathepsin D and which is dependent on a surface patch present only on correctly folded cathepsin D (Baranski *et al.*, 1991; Metcalf and Fusek, 1993). An alternative explanation for the non-homogenous mannosylation of CPY* were an altered duration of stay of the molecule in the mannosylating Golgi compartments as compared with wild-type CPY.

So far, very little is known about the organization of the proteolytic system of the ER, nor is it known how the

substrate proteins are recognized or in which way they are delivered to the protease(s). What then could be the function of Der1?

One model for ER degradation assumes a system that specifically recognizes substrate proteins for ER degradation. In this model, the protease(s) are only able to degrade substrate proteins when they are delivered to them. This could either be achieved when the proteases were separated from the ER lumen, e.g. in a specific subcompartment (Sitia and Meldolesi, 1992), or when the protease(s) cooperates with the system that recognizes the substrate proteins. In such a model, Der1 could act as a component of the substrate-recognizing system, but it could also act in mechanisms that are involved in correct localization or function of the protease(s) or the associated specific components.

Another model for ER degradation proposes the presence of highly active and rather non-specific proteases in the ER. Hereby every lumenal ER protein is endangered from the proteases. To protect a 'normal' protein from being degraded one could propose two mechanisms: (i) correct and therefore protease-resistant folding; and (ii) binding of yet-to-be-folded proteins to chaperones. In this case Der1 could be the protease or part of the protease complex.

Whether Der1 functions not only in the degradation of soluble lumenal substrate proteins but also in that of membrane proteins that become (for one reason or another) substrate for such a degradation pathway, remains to be established.

Materials and methods

The screening procedure for 'der' mutants

Parental strain YAF6 was used for EMS (ethylmethanesulfonate) mutagenesis (30% surviving cells) (Guthrie and Fink, 1991). Cells were plated on YPD plates at a density of 250 clones per plate. Cells were grown for 2 days and thereafter replica-plated onto a fresh YPD or SC plate covered with a nitrocellulose membrane. After growth for 2 days, the membrane was removed and placed overnight on SPO plates (1% potassium acetate, 2% bacto-agar, supplemented as required) following incubation for 10 h on SPO plates containing cycloheximide (4 μg/ml). The membranes were then placed on a round filter paper soaked with lysis solution (~2.8 ml of 0.1% SDS, 0.2 M NaOH, 0.5% β-mercaptoethanol) and incubated for 1 h. Cells were then washed away with a sharp water jet and the filters stained with polyclonal antiCPY or antiPrA sera.

Cloning of DER1

DER1 was isolated by transforming strain der1-2 with a genomic library in plasmid yCPlac111 (Cvrckova and Nasmyth, 1993). Colonies that no longer stained with antiCPY and antiPrA antibodies on the nitrocellulose membranes were picked, re-tested on Western blots and the plasmids were rescued. The gene was located by testing the ability of fragments of the genomic insert, subcloned into plasmid pRS315 (Sikorski and Hieter, 1989), to complement the *der1-2* phenotype.

Plasmid construction and Northern blot hybridization

A 2.2 kb *Hin*dIII fragment containing *DER1* was cut out from the originally isolated gene bank plasmid yCPlac111*DER1* and cloned into the *Hin*dIII sites of plasmids pRS315 and pRS425 (Christianson *et al.*, 1992) to obtain plasmids pRS315*DER1* and pRS425*DER1*. HA epitope-tagged *DER1* (*DER1-HA*) was obtained by using recombinant PCR (Mullis *et al.*, 1986). The primers were designed to introduce the HA epitope after the last coding codon of the ORF. The amplification product was used to replace the corresponding part in plasmid pRS315*DER1* (to yield plasmid pRS315*DER1-HA*) and sequenced. Plasmid pRS425*DER1-HA* was obtained by cloning the 2.2 kb *Hin*dIII fragment of plasmid pRS315*DER1-HA* containing the *DER1-HA* allele into plasmid pRS425. Plasmid pRS425*DER1-HA2* was obtained by ligating a second repeat of the HA tag into the unique *Aat*II site within the HA tag in plasmid pRS425*DER1-HA*. The *DER1* disruption alleles were constructed as follows: a 4.0 kb *Xba*I–*Sal*I fragment of the isolated gene bank plasmid was cloned into plasmid pBluescript KS+ and a *Bam*HI–*Cla*I fragment within the ORF of *DER1* (479 bp, 79%) was replaced with the *URA3* gene (p*der1::URA3*) or just deleted (p*der1ΔBC*). To construct plasmid pRS306*prc1-1*, a 2.68 kb fragment (*Cla*I–*Hin*dIII) with the entire *prc1-1* allele was cloned into plasmid pRS306. Plasmids pSZ1 and pLGΔ-178 were described elsewhere (Mori *et al.*, 1992).

For Northern blot hybridization, total yeast mRNA was prepared through extracting yeast cells with hot phenol (Ausubel *et al.*, 1988). Preparation of the Northern blots and detection of *DER1*-specific mRNA with the ^{32}P-labelled 479 bp *Bam*HI–*Cla*I fragment of *DER1* were essentially carried out using a method described by Thomas (1980).

Yeast strains and strain construction

The wild-type strains used for all experiments were YAF6 (MAT**a** *pra1ΔSS prc1-1 leu2-3,112*) (Finger *et al.*, 1993), YAF6ura (MAT**a** *pra1ΔSS prc1-1 leu2-3,112 ura3::LEU2*) (this study) and W303-1B (*MATα ade2-1^{oc} ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100*) respective W303-BF (*MATα ade2-1^{oc} ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 pep4::TRP1*) (Chiang and Schekman, 1991). Strain der1-2 was derived from EMS mutagenesis of cells of strain YAF6. Strains W303-1C and -CF were made from strains W303-1B and -BF by replacing the chromosomal *PRC1* allele with the *prc1-1* allele using the two-step gene replacement method (Scherer and Davis, 1979) with *Bgl*II-digested DNA of plasmid pRS306*prc1-1*. Strains FHK7-1, W303-BD, -CD and -CDF were derived from strains YAF6ura, W303-1B, -1C and -CF by transforming cells with *Hin*dIII-digested DNA of plasmid p*der1::URA3* followed by selection for Ura$^+$ transformants. Transformation of the strains W303-BD and -CD with *Hin*dIII digested DNA of plasmid p*der1*ΔBC followed by selection for Ura$^-$ transformants on 5′-fluoro-orotic acid (5′-FOA) resulted in the strains W303-BΔD and -CΔD. The strains W303-CR, -CFR, -CDR and -CDFR were constructed from strains W303-1C, -CF, -CD and -CDF by replacing the *ERD1* gene with the *LEU2* gene (Hardwick *et al.*, 1990). In all cases the correctness of the selected homologous recombination events was checked by Southern blotting or by chromosomal PCR. To obtain strains W303-CtD and -Ct^{2}D that express Der1-HA or Der1-HA2 respectively from the chromosomal *DER1* locus, cells of strain W303-CD that harbour the *LEU2*-based plasmids pRS425*DER1-HA* or pRS425*DER1-HA2* were plated on 5′-FOA. Thereafter, Ura$^-$ cells were streaked twice on non-selective medium. Cells that have lost the plasmids were investigated by chromosomal PCR followed by digestion of the PCR products with *Aat*II, indicating the presence of the tagged *DER1* alleles (*DER1-HA* and *DER1-HA2*) on the chromosome. For all strain constructions standard yeast genetic techniques were used (Sambrook *et al.*, 1989; Guthrie and Fink, 1991).

Subcellular fractionation and enzymatic assays

Subcellular fractionation and enzymatic measurements were carried out as described in Antebi and Fink (1992), with the following alterations: cells were grown to an OD_{600} of 2.0–2.5. A five-times concentrated cell suspension was used for homogenization and the amount of homogenate that corresponds to 300 OD_{600} of cells in 1 ml was fractionated on the sucrose gradient.

β-Galactosidase assays of permeabilized cells and calculation of the specific activities were performed as described previously (Ausubel *et al.*, 1988).

Western blotting and metabolic labelling

For Western blotting and immunodetection of CPY*- and PrA*-specific material, a stationary phase preculture of cells on MV medium (synthetic minimal medium; Guthrie and Fink, 1991) was diluted 1:10 with fresh MV, SC (Guthrie and Fink, 1991) or YPD medium (contained additional 75 mg/l of adenine for *ade2-1^{oc}* strains) and grown for 15–17 h at 30°C into stationary phase. Aliquots of medium containing 3 OD_{600} (0.8–1.0×10^8) of cells were taken, washed once with ice-cold water, resuspended in 1 ml cold water and cells were lysed in analogy to the method of Yaffe and Schatz (1984). Briefly, 150 μl of a solution of 1.85 M NaOH/7.5% β-mercaptoethanol was added to the cell suspension followed by incubation on ice (10 min) and TCA-precipitation of the proteins. The TCA precipitates were resuspended in 100 μl of UREA-sample buffer (8 M urea, 5% SDS, 200 mM Tris–HCl, pH 6.8, 0.1 mM EDTA, bromphenol blue; stored in frozen aliquots and adjusted to 1.5% DL-dithiothreitol before use) by vigorously shaking for 10 min at 60°C.

Thereafter the insoluble material was removed by centrifugation and 15–20 µl of the supernatants were used for SDS–PAGE on 8% acrylamide gels (Laemmli *et al.*, 1970) and Western blotting. For Western blotting and immunodetection of Kar2, logarithmically growing cultures on MV medium were used and treated as described for stationary phase cultures.

Cell lysate preparations that were enriched for membranes, were prepared after the protocol of Serrano (1988). Membrane pellets were solubilized in UREA-sample buffer (membranes of 80 OD_{600} of cells per 100 µl) and incubated at 65°C for 15 min.

For metabolic labelling in pulse–chase experiments, 2 OD_{600} (3.3 OD_{600} for the experiment shown in Figure 6) of cells per time point measured were harvested from a logarithmically growing culture. Growth and labelling medium and the experimental procedures (preparation of crude extracts, antibody precipitation, SDS–PAGE), were performed as previously described (Finger *et al.*, 1993).

Antibodies and immunofluorescence

Monoclonal 12CA5 (a gift of Per Ljungdahl, Stockholm), which recognizes the HA-tag (Wilson *et al.*, 1984; Field *et al.*, 1988) was diluted 1:200 for immunofluorescence and 1:6000 for immunoblotting. Polyclonal antiCPY, antiPrA (Finger *et al.*, 1993) and affinity-purified polyclonal antiKar2 (a gift from B.Chaudhuri) were diluted 1:10 000, 1:15 000 and 1:7500, respectively, for Western blotting. Antibodies specific for α1,6- and α1,3-mannose linkages were a gift of Randy Schekman. The procedure used for immunofluorescence was carried out essentially as described (Finger *et al.*, 1993). NaCl concentrations were 150 mM for the antiCPY and 300 mM for the 12CA5 antiserum. Sterile filtered SC medium, supplemented with 100 mg/l adenine was used. Before fixation, cells were incubated for 3 h on SC medium containing 0.1% glucose at a cell density of 4 OD_{600}/ml. Fixation was carried out for 30 min at 23°C. Cy3-fluorescence was monitored with either a Zeiss Axioskop (Zeiss Oberkochen, Germany) or a Zeiss confocal laser scanning microscope.

Acknowledgements

We thank Per Ljungdahl, Bob Chaudhuri and Randy Schekman for kindly providing antibodies, Fatima Cvrcková and Kim Nasmyth for the *CEN-LEU2* library, Hugh Pelham for the *ERD1*-deletion allele and Joseph Sambrook and Mary-Jane Gething for the UPR-β-gal construct. We also want to thank Theo Brigge for assistance with the confocal microscope and Hans Rudolph and Stefan Schork for helpful comments and many discussions. Special thanks go to Ingrid Haas for her assistance in critically reviewing the manuscript. The accession number of the *DER1* gene is X92435 SCDER1. This work was supported by the BMFT (ZSP grant A18U).

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Received on September 13, 1995; revised on October 13, 1995