Role of the proteasome in membrane extraction of a short-lived ER-transmembrane protein

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Introduction

Secretory proteins, as well as proteins destined for localization at the cell surface or the vesicular system, are synthesized in the cytosol and usually translocated into the endoplasmic reticulum (ER) via the Sec61 translocon (for a review see Matlack et al., 1998). Translocation can proceed either co- or post-translationally and requires the proteins to be unfolded. Commonly coupled with the translocation process, the polypeptides fold into their three-dimensional conformations within the ER where they may assemble with other proteins into oligomeric complexes. Membrane insertion of integral membrane proteins follows the same route as the transport of soluble secretary proteins across the ER membrane. Yet, membrane insertion requires a lateral opening of the translocation channel during the translocation process in order to release the hydrophobic transmembrane segments of the proteins into the lipid bilayer (Martoglio et al., 1995; Matlack et al., 1998). Protein transport and maturation are inevitably prone to errors, but cellular quality control pathways exist which ensure that only correctly folded or assembled proteins are retained in the ER or leave the compartment for their final destinations. Protein folding in the ER is guided by chaperones, and proteolytic pathways have been discovered which clear the compartment from misfolded and misassembled polypeptides (for reviews see Klausner and Sitia, 1990; Bonifacino and Lippincott-Schwartz, 1991; Brodsky and McCracken, 1997; Kopito, 1997).

Recently, it became clear that ER-associated degradation is predominantly mediated by the cytosolic ubiquitin–proteasome system (Sommer and Jentsch, 1993; Jensen et al., 1995; Ward et al., 1995; Biederer et al., 1996; Hampton et al., 1996; Hiller et al., 1996). Degradation by this pathway is initiated by the conjugation of ubiquitin to the proteolytic substrate (for reviews see Ciechanover, 1994; Hochstrasser, 1996; Jentsch and Schlenker, 1996). This reaction requires ubiquitin-activating enzyme (E1) and ubiquitin-conjugating enzymes (E2s) which, sometimes in conjunction with additional ubiquitin ligases (E3), catalyse the covalent attachment of several molecules of ubiquitin to internal lysine residues of the substrate. Multi-ubiquitinated proteins are the preferred substrates of the proteasome.

In yeast two E2 enzymes, UBC6 and UBC7, localize to ER membranes, and indeed these enzymes function in ER-associated proteolysis pathways (Sommer and Jentsch, 1993; Biederer et al., 1996, 1997; Hiller et al., 1996). In order to be completely accessed by the cytosolic proteolytic machinery, membrane proteins and luminal proteins of the ER must be dislocated from the ER to the cytosol. Recent genetic and biochemical data indicate that this retrograde transport process may require components of the Sec61 translocon (Wiertz et al., 1996; Pilon et al., 1997; Plumper et al., 1997). Degradation of integral membrane proteins may be initiated by a lateral assembly of components of the Sec61 complex around the substrate and may proceed by a reversal of the insertion process. Membrane insertion and translocation of proteins into the lumen of the ER are thought to be driven by the translation process (i.e. for co-translational transport) or by the action of ATP-dependent chaperones inside the ER (Matlack et al., 1998). How retrograde transport of proteins and, in particular, the extraction of proteins from the lipid bilayer is achieved has remained an enigma.

Here we investigate the mechanism of ER-membrane protein degradation by the proteasome in vivo. In analogy to previous studies on protein import into organelles, we took advantage of the potency of engineered model substrates. Using substrates which were designed to localize to the ER membrane and to be short-lived, we found that membrane extraction and proteasomal degradation are directly coupled. In fact, we observed that functional proteasomes are required for the efficient extraction of the
short-lived substrate in vivo. This suggests that membrane extraction and retrograde transport of the membrane protein may be driven by the action of membrane-associated proteasomes.

Results

Construction of a short-lived model substrate

Recent studies have shown that natural multispanning proteins may be degraded by multiple proteolytic pathways (Jensen et al., 1995). To avoid unwanted interference by other degradative systems we decided to construct structurally simple model substrates which are degraded exclusively by the ubiquitin–proteasome system. The yeast SEC62 protein (Deshaies and Schekman, 1990) is a structurally simple model substrate. The SEC62 protein’s C-terminal domain contains a short lumenal domain of eight amino acid residues located between the two transmembrane spans. We constructed variants of SEC62, one with a FLAG epitope at the protein’s C-terminus (SEC62FLAG) and another with an additional Deg1 degradation signal, derived from the short-lived yeast transcription factor MATα2 (Deg1–SEC62FLAG), fused to its N-terminus (Deg1–SEC62FLAG). The Deg1 signal, a protein domain of ~70 amino acid residues, mediates ubiquitination by the ER-membrane bound ubiquitin-conjugating enzymes, UBC6 and UBC7. Molecular grafting experiments have shown previously that this signal can be transferred to other proteins where it confers proteasome-dependent degradation on the fusions (Chen et al., 1993). To confirm that the SEC62 variants have received one or two molecules of ubiquitin (possibly due to the activities of other ubiquitin-conjugating enzymes) they do not, however, render the protein unstable.

WT yeast. As predicted, FLAG-tagged SEC62 was found exclusively in the membrane fraction, indicating that the protein is correctly inserted into the membrane (Figure 2). However, no FLAG antibody-reactive material was detectable in either cytosolic or membrane fractions when the Deg1-containing derivative was used (Figure 2). This suggested that the latter protein was indeed turned over rapidly. To increase the sensitivity of detection for the short-lived substrate and to identify possible proteolytic intermediates, we replaced the C-terminal FLAG tag by two copies of a protein-A domain (Deg1–SEC62FLAG). Like the previous constructs this new variant retained WT SEC62 topology (see Materials and methods). Pulse–chase studies confirmed that the fusion protein was rapidly degraded with a half-life of ~10 min in WT cells (Figure 3, WT). During the chase period, the protein appeared exclusively in the microsomal pellet fraction, suggesting that the protein was first inserted into the ER membrane and then degraded. To confirm that the substrate is degraded by an ER-associated proteolytic system we took advantage of various yeast mutants. No influence on the protein’s half-life was observed in pep4 mutants, defective in lysosomal function, or in sec18 mutants, which have a block in the transport of proteins from the ER to the Golgi compartment (not shown). Therefore we conclude that
When we expressed Deg1–SEC62 ProtA in yeast after lysis of cells expressing Myc epitope-tagged ubiquitin driven from a copper-inducible promoter, membrane fractions were isolated and the SEC62 derivative was detected by Western blotting using IgG antibodies directed against horseradish peroxidase. In the presence of copper (right lane; +) a ‘ladder’ of larger species of the substrate is detectable, indicating that the substrate is multi-ubiquitinated.

The Deg1 signal of MATα2 is known to mediate ubiquitination by the enzymes UBC6 and UBC7 (Chen et al., 1993). UBC6 is an integral membrane protein of the ER which is anchored to the membrane via a C-terminal hydrophobic sequence, whereas UBC7 is bound to the ER through association with the CUE1 protein (Jungmann et al., 1993; Sommer and Jentsch, 1993; Biederer et al., 1997). When we expressed Deg1–SEC62ProtA in yeast mutants defective in UBC6 and UBC7 (Figure 3, ubc6 ubc7) we observed an apparent complete stabilization of the protein, indicating that degradation of the engineered substrate is indeed mediated by these enzymes. Further evidence for this conclusion comes from genetic studies which showed that complementation of sec62 null mutants by a single copy gene expressing the short-lived SEC62 variant was not observed in WT cells (strain YWO1), but in ubc6 ubc7 mutants.

We analysed further whether the short-lived substrate is ubiquitinated in WT cells. Because ubiquitinated substrates are rapidly turned over, and therefore barely detectable, we used epitope-tagged variants of ubiquitin. It has been shown previously that these variants promote degradation of conjugates at a slower rate than WT ubiquitin (Ellison and Hochstrasser, 1991). In fact, when we performed a Western blot analysis with protein extracts from WT cells overexpressing Myc-epitope-tagged ubiquitin (Ellison and Hochstrasser, 1991), we were able to observe, in addition to the SEC62 derivative itself, a ‘ladder’ of larger variants, typical for the ubiquitinated forms of substrates (Figure 4). The ubiquitinated substrate was exclusively found in the membrane fraction, indicating that the engineered substrate was ubiquitinated by the UBC6 and UBC7 E2 enzymes directly at ER membranes.

Degradation by functionally impaired proteasomes
Ubiquitinated substrates are usually recognized and degraded by the 26S proteasome. We therefore investigated whether the engineered short-lived protein is degraded by this protease and, in particular, we asked how proteasomal degradation of integral membrane proteins might be accomplished. Two possible routes by which membrane proteins could be accessed by the proteasome can be envisaged. One idea, entertained previously (Wiertz et al., 1996), suggests that proteasomal degradation commences after the membrane protein has been completely transferred from the membrane into the cytosol. We also considered a second possibility by which dislocation and proteasomal degradation are directly coupled. To distinguish between these two possibilities we expressed the short-lived fusion protein in yeast proteasome mutants. If the complete transfer of the substrate from the ER membrane is a prerequisite for degradation by the proteasome, the completely dislocated substrate would be expected to accumulate in the cytosol of proteasome mutants. If, however, dislocation and degradation are coupled, we would expect to find, in addition to a stabilization of the substrate, defects in the extraction of the molecule from the membrane. The proteasome mutant we used for this study has a defect in the PRE1 gene, which encodes an essential subunit of the 20S core particle of the proteasome (Heinemeyer et al., 1991). This mutant, prel-1, is viable but expresses functionally attenuated proteasomes (Heinemeyer et al., 1991; Richter-Ruoff et al., 1992; Seufert and Jentsch, 1992; Chen and Hochstrasser, 1996). It has been shown previously that due to this defect a number of otherwise short-lived proteins are stabilized at normal growth temperature (Heinemeyer et al., 1991; Richter-Ruoff et al., 1992; Seufert and Jentsch, 1992; Jungmann et al., 1993). We expressed the SEC62 variant in this strain from a glucose-repressible promoter. Therefore, after shutting off the promoter by adding glucose to the medium, the stability of the initially expressed protein could be followed over time. As shown in Figure 5A significant stabilization of antibody-reactive material was observed in the prel-1
mutant. As a matter of fact, no immunoreactive material was found in the cytosol. Unexpectedly, however, even at early time-points, most material accumulated in the membrane fraction as a collection of apparent proteolytic intermediates within the size range of ~35 kDa. Remarkably, the full-length SEC62 derivative decayed with an apparent half-life of ~15 min, whereas the level of the intermediates initially increased and, after ~1 h, declined with a half-life of ~60 min (Figure 5A). Continued degradation of the intermediates in vivo was evidently not mediated by lysosomal enzymes (Figure 5B), suggesting that they are indeed proteolytic intermediates of a processive reaction (see below). Interestingly, a similar kinetic defect was also observed with a cim5 mutant (Figure 5C), which expresses proteasomes with a defective ATPase subunit of the 19S cap of the 26S proteasome (Ghislain et al., 1993). This indicates that the accumulation of the observed intermediates is indeed specific for proteasomal defects. These fragments are exclusively membrane-embedded as they could only be extracted from membranes in vitro by incubation with detergents, but not by high salt or high pH (Figure 6). We therefore conclude that the substrate is degraded directly at the membrane and that a complete dislocation of the substrate into the cytosol is not necessary for degradation by the proteasome.

Characterization of the proteolytic intermediate

The aforementioned experiments strongly suggest that the accumulating membrane-bound fragments were proteolytic intermediates generated by impaired proteasomes. Consistent with this hypothesis we found that the half-life of the protein was not affected by mutants in signal peptidase (sec11; not shown). Signal peptidase cleaves proteins at the luminal face of the membrane, and previous data have suggested a role for this enzyme in ER-associated degradation pathways (Yuk and Lodish, 1993). To determine the cleavage site which led to the formation of the proteolytic intermediate we isolated the major 35 kDa fragment from membrane fractions of a 30 l culture of prel-1 cells by antibody-affinity chromatography. The fragment was purified by gel electrophoresis, blotted onto a membrane and subjected to Edman degradation. Identification of the first 10 N-terminal residues indicated that the cleavage which produced the 35 kDa fragment occurred on the cytosolic face of the membrane, ~50 residues N-terminal from the first transmembrane domain of the molecule (Figure 7). The remaining part of the polypeptide contained the two membrane spans and the C-terminal cytosolic domain bearing the protein-A tag used for the isolation of the fragment. This finding rules out the participation of ER–luminal proteases, but is consistent with the cytosolic proteasomal activity. To obtain additional evidence for this hypothesis we constructed an N-terminally truncated form of SEC62ProtA resembling the proteolytic intermediate in protein sequence and expressed the protein in WT cells (Figure 7; termed SEC62 fragment mimic). This protein was inserted into the ER membrane and, in contrast to its sequence ortholog, this protein was almost stable and the half-life was not affected by proteasome mutants (Figure 7 and data not shown). This indicates that the turnover of the proteolytic fragment observed in prel-1 mutants was indeed a result of a processive event, most likely mediated by those proteasomes which initiated the proteolytic process.

Discussion

Several mechanistic models for ER-associated membrane protein disposal have been entertained during the past years (Amara et al., 1989; Klausner and Sitia, 1990; Bonifacino and Lippincott-Schwartz, 1991; Yuk and Lodish, 1993; Brodsky and McCracken, 1997). The finding that proteolysis requires transport of the substrates back into the compartment where they had been synthesized originally was unexpected and raised several important questions. Several lines of evidence now point to a role of the Sec61 translocon in retrograde protein transport (Wiertz et al., 1996; Pilon et al., 1997; Plemper et al., 1997). The evidence suggests that this process may pos-
sibly function as a reversal of the membrane insertion reaction. But how is directionality of retrograde transport ensured and what is the driving force behind the translocation process? Our data suggest a mechanism by which ER-membrane proteins can be degraded directly at membranes involving membrane-bound proteasomes. The data we present are consistent with a model by which substrates are directly dislocated from the membrane into the proteasome. Apparently, degradation of the model substrate starts at its cytosolic N-terminus which carries the Deg1 degradation signal. In WT cells, degradation of the full-length polypeptide proceeds rapidly, with virtually no detectable proteolytic intermediates (see Figures 3 and 5C). In mutants with functionally impaired proteasomes, proteolysis proceeds nearly as rapidly as in WT cells until the proteasome encounters the first transmembrane span. However, further degradation of the membrane protein requires membrane extraction, and during this phase defective proteasomes appear to proceed significantly more slowly than fully active WT proteasomes. This results in the accumulation of similarly sized proteolytic intermediates in proteasome mutants truncated near the first transmembrane span. Importantly, our genetic data demonstrate that functional proteasomes are required for membrane extraction of the substrate in vivo. Otherwise, if proteasome-independent extraction mechanisms were operative, the substrate or its proteolytic intermediates would be expected to accumulate in the cytosol of proteasome mutants.

Although different mechanisms of membrane protein degradation may be operative for other substrates, we speculate that the mechanism we propose is utilized frequently. Indeed, ER-localized proteasomes have been detected previously (Rivett, 1993; Palmer et al., 1996), suggesting that these proteasomes are engaged in ER-associated degradation. Our data also provide clues for how the retrograde transport might be driven and may help establish why the process is unidirectional. It seems attractive to speculate that proteins of the ER membrane, possibly components of the Sec61 translocon, provide an extraction-supportive environment within the membrane. Yet, we propose that the dislocation process itself might be driven by the action of the proteasome. Proteasomes possess narrow openings at both ends which allow only unfolded substrates access to the proteolytic chamber (Löwe et al., 1995; for reviews see Jentsch and Schlenker, 1996; Larsen and Finley, 1997; Baumeister et al., 1998). Probably catalysed by the ATPases present at the entry sites of the proteasome (the 19S cap), the substrates become unfolded and may be actively funnelled through the bottleneck into the central cavity of the proteasome. These processes are thought to be coupled and result in a processive action of the proteasome (Jentsch and Schlenker, 1996; Akopian et al., 1997). It therefore seems especially attractive to speculate that ATP hydrolysis catalysed by proteasome subunits may provide the energy for retrograde transport. Consistent with this model is our finding that sim5 mutants, with one defective proteasomal ATPase, exhibit a similar transport–degradation defect as prel-1 mutants. Interestingly, the proposed mechanism also points to possible parallels to the degradation of mitochondrial membrane proteins which employ proteases (Arlt et al., 1996; Leonhard et al., 1996; Suzuki et al., 1997) bearing ATPase domains resembling those of the proteasome.

### Materials and methods

#### Construction of yeast plasmids and strains

Standard protocols were followed for preparation of yeast media, transformation by the lithium acetate method, sporulation, tetrad dissection, and preparation of total yeast DNA (Guthrie and Fink, 1991; Ausubel et al., 1994). Genes for SEC62FLAG, Deg1–SEC62FLAG, Deg1–SEC62ProtA and the SEC62 fragment mimic (Figure 7) were constructed as described below by PCR and placed under GAL1-10 promoter control into YIpplac128 (Gietz and Sugino, 1988; the DNA and protein sequences of the constructed SEC62 derivatives will be provided upon request). For these constructs the SEC62 open reading frame (ORF) was cloned from genomic DNA by PCR. For the construction of SEC62FLAG and Deg1–SEC62FLAG, primers were designed to introduce a FLAG-epitope after the last amino acid codon of the SEC62 ORF. The gene for Deg1–SEC62ProtA encodes a C-terminally doubly ProtA-tagged version of SEC62 and contains an oligonucleotide linker encoding the FLAG-epitope between the DNA encoding the Deg1 signal and the SEC62 coding region. The ProtA tag is as described by Nilsson et al. (1987). The membrane topology of the constructed SEC62 variants was confirmed by a complementation assay for SEC62 function in a sec62 null strain. To this end WT (strain YWO1) and a ubc6 ubc7 mutant strain (in order to stabilize the short-lived SEC62 variants) were transformed with plasmids encoding the SEC62 variants, and the genomic WT SEC62 locus was deleted afterwards which was confirmed by PCR. The SEC62 fragment mimic was cloned by PCR using the gene for Deg1–SEC62ProtA as a template. An initiator methionine codon was introduced to obtain a fusion gene (Chen et al., 1995) and cloned in-frame 5′ from the SEC62 ORF. The gene for Deg1–SEC62ProtA encodes a C-terminally doubly ProtA-tagged version of SEC62 and contains an oligonucleotide linker encoding the FLAG-epitope between the DNA encoding the Deg1 signal and the SEC62 protein. The ProtA tag is as described by Nilsson et al. (1987). The membrane topology of the constructed SEC62 variants was confirmed by a complementation assay for SEC62 function in a sec62 null strain. To this end WT (strain YWO1) and a ubc6 ubc7 mutant strain (in order to stabilize the short-lived SEC62 variants) were transformed with plasmids encoding the SEC62 variants, and the genomic WT SEC62 locus was deleted afterwards which was confirmed by PCR. The SEC62 fragment mimic was cloned by PCR using the gene for Deg1–SEC62ProtA as a template. An initiator methionine codon was introduced to obtain a protein which starts with the sequence MVIPYK (see Figure 7).

Table 1 lists the yeast strains used in this work. Strains YTX99, YTX99 and YFP338 are derivatives of isogenic strains (supplied by R. Ausubel, 1994). Genes for SEC62FLAG, Deg1–SEC62FLAG, Deg1–SEC62ProtA and the SEC62 fragment mimic (Figure 7) were constructed as described below by PCR and placed under GAL1-10 promoter control into YIpplac128 (Gietz and Sugino, 1988; the DNA and protein sequences of the constructed SEC62 derivatives will be provided upon request). For these constructs the SEC62 open reading frame (ORF) was cloned from genomic DNA by PCR. For the construction of SEC62FLAG and Deg1–SEC62FLAG, primers were designed to introduce a FLAG-epitope after the last amino acid codon of the SEC62 ORF. The gene for Deg1–SEC62ProtA encodes a C-terminally doubly ProtA-tagged version of SEC62 and contains an oligonucleotide linker encoding the FLAG-epitope between the DNA encoding the Deg1 signal and the SEC62 coding region. The ProtA tag is as described by Nilsson et al. (1987). The membrane topology of the constructed SEC62 variants was confirmed by a complementation assay for SEC62 function in a sec62 null strain. To this end WT (strain YWO1) and a ubc6 ubc7 mutant strain (in order to stabilize the short-lived SEC62 variants) were transformed with plasmids encoding the SEC62 variants, and the genomic WT SEC62 locus was deleted afterwards which was confirmed by PCR. The SEC62 fragment mimic was cloned by PCR using the gene for Deg1–SEC62ProtA as a template. An initiator methionine codon was introduced to obtain a protein which starts with the sequence MVIPYK (see Figure 7).

Table 1 lists the yeast strains used in this work. Strains YTX99, YTX99 and YFP338 are derivatives of isogenic strains (supplied by R. Schekman) and congenic WT strains were used as controls in the respective experiments. CMY826 and YWO71 were used as the WT controls for CMY765 and YWO74, respectively. All others strains are

### Table 1. Yeast strains used in this work

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<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
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<td>YWO1</td>
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<td>MATα ura3-52 leu2Δ1 hisΔ2-200 cin5-1</td>
<td>C.Mann</td>
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</table>
derivatives of DFS (Finley et al., 1985). A pep5Δ strain (Y0602) and a prep1-1 pep5Δ double mutant (Y0601) was made by replacing the first 25% of the PEP4 ORF (Jones, 1991) by URA3 and introduction into respective genetic backgrounds (YWO1, YWO74). Homologous recombination was verified by Southern hybridization. The SEC62 disruption allele was constructed by inserting the URA3-coding region into the unique AffI site within the SEC62 ORF. Homologous recombination was confirmed by Southern hybridization.

**Extraction of yeast lysomes**

Yeast lysosomes (for the experiment shown in Figure 6) were prepared from cells grown at 28°C as described previously (Lyman and Schekman, 1995). Microsomes were stored at OD_{280} = 30–40 in 50 µl aliquots at –80°C. For immunoblotting 13.5 OD_{280} of exponentially growing yeast cells (0.8 OD_{405nml}) were harvested and resuspended in a Buffer A (50 mM Tris–HCl, pH 7.2, 150 mM NaCl, 50 mM N-ethylmaleimide (NEM) and Complete™ protease inhibitors (Boehringer Mannheim)). Cell disruption was carried out with 1 vol of glass beads by vortex mixing for 5 min at maximum speed at 4°C. Lysates were cleared by low speed centrifugation (750 g) yielding total protein extract. Membranes were collected from the supernatant by centrifugation at 16 000 g. The sedimented membranes and the supernatant cytosolic fraction were harvested as samples (2% SDS, 50 mM dithiothreitol, 10% glycerol, 76.5 mM Tris–HCl, pH 7.5) for 5 min at 88°C and separated on 9 or 12% SDS–PAGE for immunoblotting. Standard techniques for SDS–PAGE and Western blotting were used (Ausubel et al., 1994). Immunoprecipitations were carried out with IgG-Sepharose (IgG Sepharose 6 Fast Flow, Pharmacia) after dissolving the sedimented membranes in Buffer B (Buffer A containing 0.1% SDS and 1% Triton X-100). Immunoprecipitations were performed as described previously (Biederer et al., 1996).

**Pulse–chase and promoter shut-off experiments**

WT and mutant yeast cells were grown to an OD_{600} of 1–2 at 28°C in synthetic dextrose (SD) media without methionine, then diluted in the same medium to OD_{600} = 0.15 and were further incubated at the growth temperature. When the cultures reached an OD_{280} of 0.6, growing cells of 3 OD_{280} were harvested for each time-point of the pulse–chase experiment. Cells were resuspended in pre-warmed, fresh SD media and labelled with 50 µCi/3S-methionine (120 Ci/mm, Amersham) for 5 min. The chase period was performed in pre-warmed, fresh SD media supplemented with 0.06% methionine and the required amino acids. Cells were incubated during the chase period at the growth temperature with agitation. Cells of 3 OD_{280} were removed at 0 time and at the time intervals indicated in Figure 3, and were resuspended in 110 µl ice-cold Buffer A. Cells were disrupted with glass beads for 3 min as described above. For each time-point of the promoter shut-off experiment 11.5 OD_{280} of exponentially growing cells were harvested. The galactose-inducible promoter was repressed by adding glucose to the medium (2% final concentration). The promoter shut-off experiment was performed as described previously (Seuffert et al., 1995). Detection of ProA-tagged SEC62 proteins was performed with IgG-antibodies directed against horseradish peroxidase (DAKO).

**Protein sequencing**

The degradation intermediate was isolated as follows: yeast spheroplasts from 30 l YPGal of prep1-1 cells expressing Deg1–SEC62ProtA were suspended in Buffer A and lysed in a dounce homogenizer (Hurt et al., 1988). The lysate was centrifuged at 5000 g and membranes were collected from the supernatant by centrifugation at 16 000 g. The pellet was resuspended in Buffer B and applied to an IgG–Sepharose column (Pharmacia). The bound fragment was eluted with acetic acid, pH 3.4, freeze-dried, concentrated and blotted onto polyvinylidene difluoride (PVDF) membrane after SDS–PAGE. The major 35 kDa fragment was excised from the membrane and subjected to Edman degradation using the Applied Biosystems model 473A sequencer.

**Acknowledgements**

We thank Armin Bosserhoff and Rainer Frank for protein sequencing; Petra Hubbe for technical assistance; Bernhard Dobberstein, Jörg Höhfeld, Manfred Koegel, and Kai Matuschewski for discussions and comments on the manuscript; Thomas Sommer, Dieter Wolf, Carl Mann, Randy Schekman and Mark Rose for providing strains and plasmids. This work was supported by grants of Deutsche Forschungsgemeinschaft to S.J.

**References**


Received March 20, 1998; revised April 8, 1998; accepted April 9, 1998.