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Degradation of subunits of the Sec61p complex, an integral component of the ER membrane, by the ubiquitin–proteasome pathway

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We have investigated the degradation of subunits of the trimeric Sec61p complex, a key component of the protein translocation apparatus of the ER membrane. A mutant form of Sec61p and one of the two associated proteins (Sss1p) are selectively degraded, while the third constituent of the complex (Sbh1p) is stable. Our results demonstrate that the proteolysis of the multispanning membrane protein Sec61p is mediated by the ubiquitin–proteasome pathway, since it requires polyubiquitination, the presence of a membrane-bound (Ubc6) and a soluble (Ubc7) ubiquitin-conjugating enzyme and a functional proteasome. The process is proposed to be specific for unassembled Sec61p and Sss1p. Thus, our results suggest that one pathway of ER degradation of abnormal or unassembled membrane proteins is initiated at the cytoplasmic side of the ER.
Keywords: ER degradation/membrane proteins/Sec61/ ubiquitin–proteasome pathway/yeast

Introduction

Selective proteolysis is an essential process in every cell. It is required for the rapid breakdown of cellular regulators like cyclins or transcription factors, and for the removal of abnormal proteins. For many proteolytic substrates of the eukaryotic cytosol and the nucleus, the covalent attachment of the small polypeptide ubiquitin (76 amino acids) is an obligatory step prior to their degradation. This degradation pathway shows a high selectivity which is thought to be determined by enzymes that catalyze the attachment of ubiquitin to substrate proteins. After an initial ATP-dependent activation step, catalyzed by the ubiquitin-activating enzyme (E1 or UBA), ubiquitin is transferred to target proteins by a large family of ubiquitin-conjugating enzymes (E2 or Ubc) functioning either with or without a substrate recognition factor (E3 or UBR). The diversity of Ubc enzymes identified so far suggests that they are among the principal determinants of the specificity of the ubiquitin system (reviewed by Ciechanover, 1994).

Ubiquitin is reversibly joined to target proteins by an isopeptide linkage between the C-terminus of ubiquitin and internal lysine residues in the substrates. In successive reactions, a polyubiquitin chain is synthesized through the formation of ubiquitin–ubiquitin linkages. The polyubiquitin chain marks soluble proteins for degradation by the 26S proteasome complex, consisting of the 20S proteasome, probably the proteolytic core, and a 19S cap complex containing a ubiquitin-conjugate binding subunit, isopeptidase activity and ATPase activities (Deveraux *et al.*, 1994; Peters, 1994).

Most ubiquitin-conjugating enzymes investigated so far are soluble proteins of the cytosol or of the nucleoplasm. The only exception is Ubc6p, an integral membrane protein that localizes to the endoplasmic reticulum (ER) and possibly also to the nuclear membrane with the catalytic domain facing the cytosol (Sommer and Jentsch, 1993). Ubc6p acts along the same pathway with Ubc7p in the turnover of the short-lived transcriptional repressor matα2. This repressor is stabilized to the same extent upon disruption of the two genes individually or in combination, suggesting that *UBC6* and *UBC7* belong to the same epistasis group. In support of this genetic evidence, a physical association between Ubc6p and Ubc7p has been demonstrated in the two-hybrid system. Dimerization of Ubc's has been discussed as a possible mechanism for the modulation of the substrate specificity of the ubiquitin system (Chen *et al.*, 1993). For Ubc7p, which is most likely a soluble ubiquitin-conjugating enzyme, another function has been described. It confers resistance to cadmium to yeast cells by eliminating abnormal proteins generated under these stress conditions (Jungmann *et al.*, 1993). In contrast, Ubc6p has no function in this proteolytic process. From these data it is conceivable that the same Ubc may be involved in different proteolytic processes.

Genetic evidence in yeast has also implicated a function for Ubc6p in the degradation of ER membrane proteins. *Ubc6* loss-of-function mutants are specific suppressors of conditional lethal mutations in *SEC61* (Sommer and Jentsch, 1993). It has been speculated that the effect of Ubc6p on the phenotype of *sec61* mutants may be due to the fact that a structurally distorted translocation apparatus is targeted for degradation by components of the ubiquitin system. This assumption is further supported by studies on the overexpression of Sss1p, a protein that is associated with Sec61p (Esnault *et al.*, 1994). But neither the degradation of mutant Sec61p nor a direct role of the ubiquitin–proteasome pathway in the degradation of multispanning membrane proteins, especially of the ER membrane, could be demonstrated. Therefore, we asked whether ubiquitin-conjugating enzymes participate in the turnover of components of the translocation apparatus. These results would have implications for a recently described proteolytic system that provides protein quality control functions to ER resident proteins and to proteins in transit through the ER (reviewed by Klausner and Sitia, 1990; Bonifacino and Lippincott-Schwartz, 1991). Although a number of soluble and membrane-bound substrates of this ER degradation mechanism have been examined, little is known about the components that mediate the degradation.

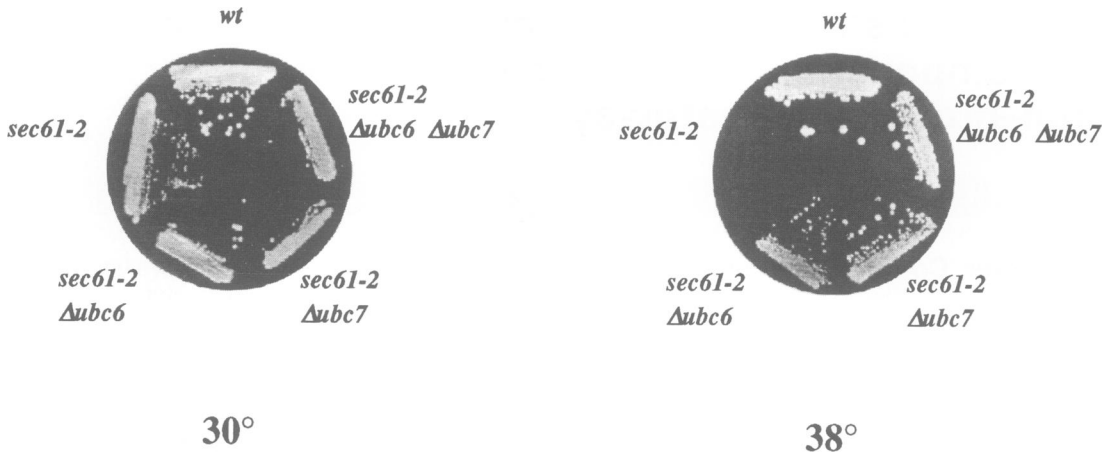

Fig. 1. Disruptions of *UBC6* and *UBC7* are suppressors of the growth deficiency of the *sec61-2* mutant at elevated temperatures. Wild-type (RSY521), *sec61-2* (YFP338), *sec61-2Δubc6::LEU2* (YTX42), *sec61-2Δubc7::LEU2* (YTX93) and *sec61-2Δubc6::ADE2Δubc7::LEU2* (YTX94) strains were tested for growth at 30 and 38°C for 3 days on plates containing rich medium.

Sec61p is found in yeast microsomes as part of a trimeric complex which is highly homologous to the mammalian Sec61p complex (Görlich *et al.*, 1992; Hartmann *et al.*, 1994). The yeast Sec61p complex, consisting of Sec61p, Sbh1p and Sss1p, is in part associated with membrane-bound ribosomes and is therefore presumably involved in the cotranslational translocation pathway (Panzner *et al.*, 1995). This assumption is also based on the analogy with the mammalian system in which an equivalent heterotrimeric Sec61p complex exists which is essential for cotranslational translocation (Görlich and Rapoport, 1993). In addition, Sec61p is found as part of a heptameric Sec complex which is formed by the trimeric Sec61p complex and a tetrameric Sec62p/Sec63p complex, comprising Sec62p, Sec63p, Sec71p and Sec72p. The heptameric Sec complex is involved in post-translational transport of proteins into the ER (Panzner *et al.*, 1995). Conditional mutants in *SEC61* have been isolated in a genetic screen for yeast mutants with translocation defects at elevated temperatures, as indicated by the accumulation of precursor polypeptides in the cytosol (Deshaies and Schekman, 1987; Stirling *et al.*, 1992). The two temperature-sensitive mutations, *sec61-2* and *sec61–3,* reveal different temperature sensitivities. Neither grows at 38°C; in addition, *sec61–3* is cold-sensitive at 17°C. A second component of the trimeric Sec61p complex, Sss1p, has been identified as a multicopy suppressor of *sec61-2* (Esnault *et al.*, 1993). The third component of the Sec61p complex, Sbh1p, is not essential. The analysis of mutants lacking Sbh1p indicates that Sec61p and Sss1p can form a functional dimeric complex, probably the basic unit of the translocation apparatus (Finke *et al.*, 1996).

The results presented in this report demonstrate the involvement of the ubiquitin–proteasome pathway in the proteolysis of key components of the translocation apparatus of the ER membrane. In contrast to wild-type Sec61p, mutant versions of this protein are selectively degraded at non-permissive temperatures resulting in reduced cellular levels of both Sec61p and Sss1p, while Sbh1p is not affected. The reduced amounts of the key components of the translocation apparatus are probably the main cause of the translocation defect of the *sec61* mutants. Our data show that ER degradation of this multispanning membrane protein requires the function of a membrane-bound (Ubc6p) and a soluble ubiquitin-conjugating enzyme (Ubc7p) and the proteasome. The proteolysis takes place at the ER, supporting the suggestion that yeast contains a protein quality control mechanism for abnormal or unassembled ER membrane proteins similar to the one in mammalian cells.

Results

Deletions of two ubiquitin-conjugating enzymes are suppressors of the sec61 mutant

Because *ubc6* loss-of-function mutants are suppressors of the growth deficiency of *sec61* mutants, and Ubc6p and Ubc7p appear to form a complex in at least one degradation pathway (Chen *et al.*, 1993), we tested whether the deletion of *UBC7* (*Δubc7*) is also a suppressor of *sec61*. To this end, we constructed isogenic strains carrying the *sec61-2* allele and disruptions of *UBC6, UBC7* or both. The double mutant *sec61Δubc7* was able to grow at 38°C like the *sec61Δubc6* mutant, while the temperature-sensitive *sec61-2* strain was not (Figure 1). Moreover, the deletion of *UBC7* turned out to be a more efficient suppressor of the *sec61* phenotype than the deletion of *UBC6. sec61* mutants lacking *UBC7* had a generation time comparable with the wild-type at 38°C (~120 min) while *sec61* cells lacking *UBC6* or expressing a non-functional version of *UBC6* still exhibit a slow growth phenotype at 38°C (generation time of ~180 min). A *sec61* mutant lacking both ubiquitin-conjugating enzymes had a generation time identical to that of the *sec61Δubc7* mutant. These findings implicate a function for both Ubc6p and Ubc7p in the degradation of components of the translocation apparatus.

Reduced cellular contents of two components of the mutant Sec61 complex

Assuming that the *sec61* phenotype is based on the enhanced turnover of some components of the translocation complexes, the cellular level of them should be affected by this process. Therefore, we investigated the steady state levels of various proteins with a known function in protein translocation in the *sec61* mutant at the restrictive temperature. Exponentially-growing wild-

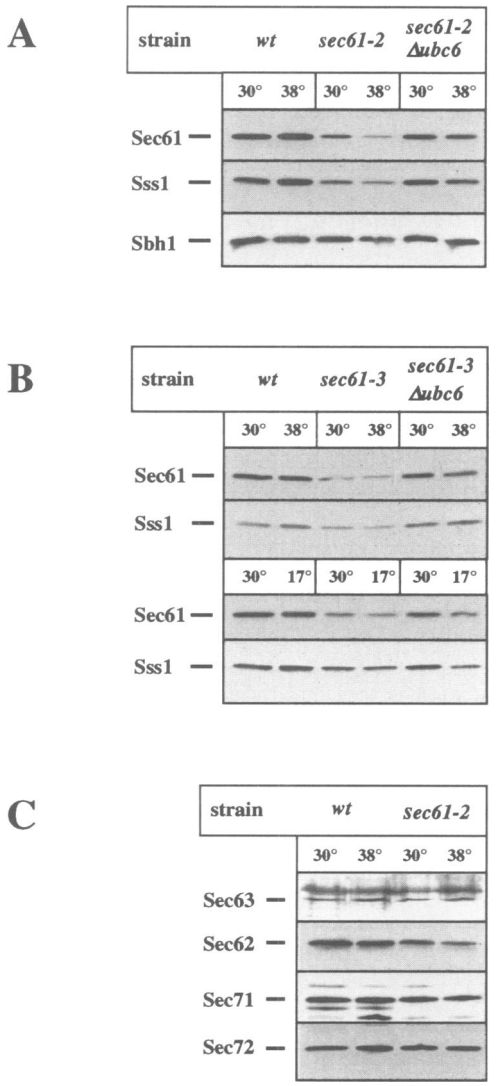

Fig. 2. The amounts of both Sec61p and Sss1p are selectively reduced in both *sec61* alleles at the restrictive temperature while other components of the translocation apparatus remain unchanged. Membranes were prepared from isogenic strains growing exponentially at 30°C or after a shift of 2 h to the restrictive temperatures (38 or 17°C). Equal amounts of solubilized membrane proteins were separated on 18% SDS–polyacrylamide gels and analyzed by immunoblotting. (**A**) Membrane proteins of wild-type (RSY521), *sec61-2* (YFP338) and *sec61-2Δubc6::LEU2* (YTX42) were analyzed with antibodies specific for Sec61p or Sss1p. (**B**) Membrane proteins of wild-type (RSY521), *sec61-3* (RSY455) and *sec61-3Δubc6::LEU2* (YTX43) were analyzed with the same antibodies as in (A). (**C**) Membrane proteins of wild-type (RSY521) and *sec61-2* (YFP338) were analyzed with antibodies specific for Sec62p, Sec63p, Sec71p or Sec72p.

type, *sec61-2*, *sec61-3*, *sec61-2Δubc6* and *sec61-3Δubc6* cells were harvested at 30°C, or after a shift for 2 h to the restrictive temperature (38 or 17°C). After disruption of the cells, membranes were prepared and the proteins contained in them analyzed by immunoblotting with various antibodies (Figure 2).

Sec61p was diminished in the *sec61-2* allele at an elevated temperature compared with the wild-type. We also observed a reduction in the amount of Sec61p in preparations from *sec61-2* cells grown at 30°C, which was expected since the mutant shows a growth defect even at the permissive temperature which led to its identification. The two small subunits of the trimeric Sec61p complex behaved differently: Sss1p followed Sec61 in the reduction of its cellular amounts while the protein level of Sbh1p was not altered. In *sec61* cells lacking Ubc6p (Figure 2A) or Ubc7p (data not shown), the cellular content of both Sec61p and Sss1p was restored to nearly wild-type level. The second conditional allele of *SEC61*, *sec61-3*, behaved similarly at both restrictive temperatures (38 and 17°C). A significant reduction of the level of Sss1p was also observed, although the effect was less pronounced in this allele (Figure 2B). In contrast to Sec61p and Sss1p, no reproducible changes in the levels of Sec62p, Sec63p, Sec71p and Sec72p were observed (Figure 2C).

In conclusion, these experiments revealed that the amount of both essential subunits of the mutant Sec61p complex in yeast is reduced in comparison with Sbh1p or the components of the Sec62p/Sec63p complex. Both degraded subunits are probably a target of Ubc6p function.

Mutant Sec61 protein is short-lived in vivo, but it can be stabilized by deletions of UBC6 or UBC7 or by overproduction of Sss1p

To test whether the observed reduction of some components of the trimeric Sec61p complex in the mutant is due to enhanced protein turnover rather than reduced transcription, we assessed the half-life of the wild-type and mutant Sec61 protein in the membrane in a pulse–chase approach. Exponentially-growing cells were labeled with [^{35}S]methionine at the permissive temperature (30°C) or after a shift for 2 h to the restrictive temperature (38°C). Lysates were prepared immediately or after a chase period at the respective temperatures. Membranes were prepared from each lysate and the proteins contained in them were subjected to immunoprecipitation with antibodies specific for Sec61p.

The wild-type Sec61 protein was stable at 30°C and at 38°C (Figure 3A). The turnover of mutant Sec61p at 30°C was only marginally faster than that of the wild-type, but the half-life at 38°C was drastically reduced (~40–50 min), explaining the reduced levels of mutant Sec61p at elevated temperature in the immunoblot analysis. Next, we looked for a stabilization of the mutant protein at elevated temperatures in strains deleted for *UBC6*, *UBC7* or both. The deletion of either or both Ubc's results in a drastically prolonged half-life of mutant Sec61p. In *sec61Δubc6* cells the stabilization was significant but not complete, while in cells lacking Ubc7p (*sec61Δubc7* or *sec61Δubc6Δubc7*), the mutant Sec61p was as stable as the wild-type protein.

Thus, mutant Sec61p is a metabolically unstable protein at 38°C and both ubiquitin-conjugating enzymes participate in the turnover of the mutant Sec61p at the elevated temperatures. However, the stabilization is more pronounced in cells lacking Ubc7p, which reflects the strength of the suppression of the *sec61* mutant phenotype by deletions of *UBC6* or *UBC7* (see Figure 1).

Sss1p has been identified as a multicopy suppressor of both conditional *sec61* alleles, which restores cell viability and the translocation defect at elevated temperature (Esnault *et al.*, 1993). In a pulse–chase experiment similar to those described above, we tested whether this suppres-

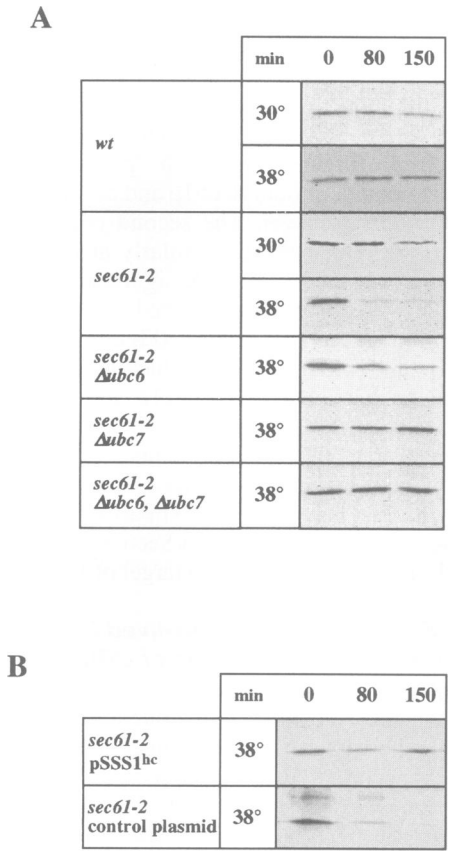

Fig. 3. *Sec61* mutant cells express a short-lived version of Sec61p which is stabilized in cells lacking UBC6p, UBC7p or both as well as by overproduction of Sss1p. (**A**) Pulse–chase analysis of exponentially growing wild-type (RSY521), and *sec61-2* (YFP338) cells which were labeled with [^{35}S]methionine and [^{35}S]cysteine at 30°C or after a shift for 2 h to 38°C. The chase period was done at the respective temperature. At time intervals of 0, 80 and 150 min, membrane proteins were prepared and immunoprecipitation with Sec61p specific antibodies was performed. The precipitated proteins were separated on 18% SDS–polyacrylamide gels. The pulse–chase analysis of double and triple mutants [*sec61-2Δubc6::LEU2* (YTX42), *sec61-2Δubc7::LEU2* (YTX93) and *sec61-2Δubc6::ADE2 Δubc7:: LEU2* (YTX94)] was performed only after a shift for 2 h to 38°C. (**B**) Exponentially-growing *sec61-2* (YFP338) cells either overproducing Sss1p from a 2μm vector (pSEY8; Ausubel *et al.*, 1991) or transformed with a control vector were analyzed in a pulse–chase approach at 38°C followed by immunoprecipitation of Sec61p.

sion is due to a stabilization. The half-life of the mutant Sec61 protein is gradually prolonged when Sss1p is overproduced at 38°C (Figure 3B). In contrast to Sss1p, the overproduction of Sbh1p from a 2μm vector does not suppress the lethality of the *sec61-2* allele at 37°C (data not shown).

The proteasome and polyubiquitination are required for the degradation of mutant Sec61p

To investigate further the function of the ubiquitin–proteasome pathway in the selective degradation of mutant Sec61p, we made use of a mutation in one of the essential genes encoding proteasomal proteins, *PRE1*, which affects the chymotryptic activity of the 20S proteasome. The mutation we included in our analysis, *pre1-1,* leads to decreased protein degradation (Heinemeyer *et al.*, 1991). Moreover, specifically engineered model substrates for the ubiquitin system (ubiquitin–β-galactosidase fusion proteins) are stabilized by these mutations (Seufert and Jentsch, 1992).

For the construction of a *sec61-2pre1-1* strain, the *Δpre1::TRP1* disruption was introduced into a diploid strain heterozygous for *sec61-2*. After tetrad analysis of the diploid strain *sec61-2Δpre1* double mutants were rescued with the mutant *pre1-1* gene on an ARS-CEN plasmid (pTX49). The *pre1-1* mutation cannot be expected to be a suppressor of the *sec61* phenotype, since the *pre1-1* mutation leads to severe growth defects at elevated temperature. In similar pulse–chase experiments to those described above, we measured the turnover of the mutant Sec61 protein at elevated temperature in the wild-type and *pre1-1* background (Figure 4A). Cells with deficiencies in the activity of the proteasome were also deficient in the degradation of the mutant Sec61 protein; the turnover was comparable with that of the wild-type protein.

Degradation by the 26S proteasome complex usually requires polyubiquitination of the proteolytic substrate. In most cases, polyubiquitination is achieved by isopeptide linkage between the C-terminus of one ubiquitin moiety and the internal Lys48 of the previously attached ubiquitin. The co-expression of a derivative of ubiquitin in yeast in which Lys48 is replaced by arginine (UbK48R) leads to a partial inhibition of the cellular protein turnover because such a mutation interferes with the formation of a polyubiquitin chain (Finley *et al.*, 1994). We used such a mutant ubiquitin to test whether the formation of a polyubiquitin chain is required for the degradation of mutant Sec61p and Sss1p. Wild-type, *sec61* and *sec61* cells overproducing UbK48R under control of the CUP-promoter were grown at permissive temperature in the presence of Cu^{2+} to induce the expression of UbK48R. Membranes were prepared after a shift for 2 h to the restrictive temperatures. The membrane proteins were analyzed by immunoblotting with specific antibodies directed against Sec61p and Sss1p (Figure 4B). The overproduction of UbK48R corrected the reduced amounts of both Sec61p and Sss1p observed in *sec61* mutant to nearly wild-type levels. To confirm that the degradation is indeed inhibited in these cells, we determined the half-life of mutant Sec61p directly in a pulse–chase experiment (Figure 4C). The turnover in cells expressing UbK48R is reduced although mutant Sec61p is not as stable as in wild-type cells. Consistently, we found overproduction of UbK48R to be a weak suppressor of the lethality of *sec61-2* mutants (data not shown).

The degradation occurs in a pre-Golgi compartment

The role of the secretory pathway in the degradation of mutant Sec61p was investigated to distinguish whether the observed proteolytic process occurs in a cellular compartment earlier or later than the pre-Golgi. In these experiments we took advantage of the *SEC18* gene, which codes for a protein required for formation of the vesicle fusion complex in the ER–Golgi transport (Sogaard *et al.*, 1994). The *sec18-1* mutant grows normally at a temperature of 25°C, but the transport of soluble and membrane proteins is blocked within minutes of a shift to 30°C (Graham and Emr, 1991). An isogenic *sec61sec18* strain was obtained after tetrad analysis of a cross between *sec18-1* and *sec61-2* (Sommer and Jentsch, 1993). We

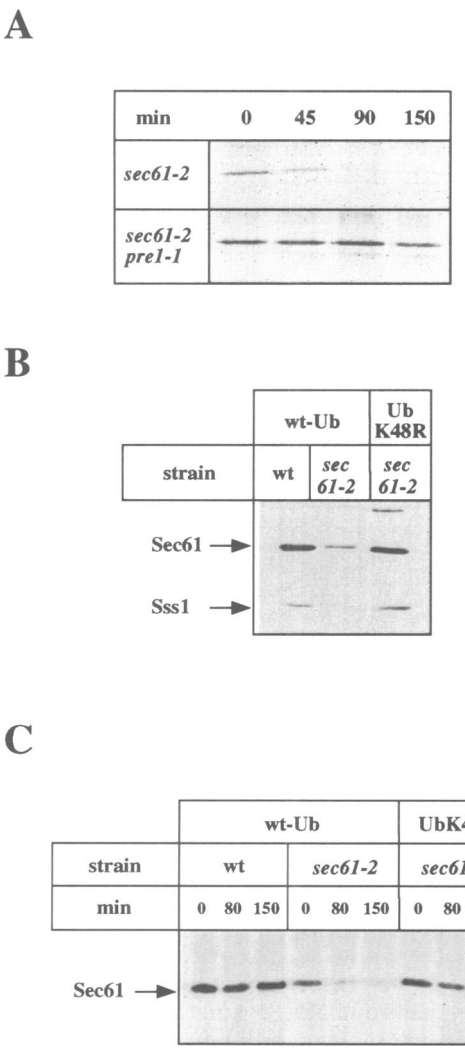

Fig. 4. The turnover of the mutant Sec61 protein is reduced in cells with a deficiency in the chymotryptic activity of the proteasome or in cells that co-produce a mutant version of ubiquitin. (**A**) The turnover of mutant Sec61p in *sec61-2* (YFP338) and *sec61-2pre1-1* (YTX72) cells was measured by a pulse–chase analysis. Cultures were grown to exponential phase at 30°C and pulse labeled after a shift for 2 h to 38°C. During the chase period, the cells were kept at 38°C. At each time point of the chase period membrane proteins were prepared and subjected to immunoprecipitation with Sec61p specific antibodies. (**B**) The steady state levels of Sec61p and Sss1p were investigated by immunoblotting. Wild-type, *sec61-2* (YTX66) cells and *sec61-2* cells overproducing UbK48R (pUB203) were grown at 30°C and shifted for 2 h to 38°C. Equal amounts of membrane proteins of these cells were separated on 18% SDS–polyacrylamide gels and analyzed by immunoblotting with Sec61p and Sss1p specific antibodies. (**C**) The same strains as in (B) were analyzed in a pulse–chase approach. Exponentially growing cells (30°C) were labeled with [^{35}S]methionine and [^{35}S]cysteine after a shift for 2 h to 38°. During a chase period at 38°C membrane proteins were prepared at the times indicated and immunoprecipitation with Sec61p specific antibodies was performed. The precipitated proteins were separated on 18% SDS–polyacrylamide gels.

determined the half-life of mutant Sec61 protein in the membrane by pulse–chase experiments, as described above. Due to the temperature sensitivity of both mutant alleles, cells were grown at 25°C and only shifted to 38°C 1 h prior to the labeling.

The wild-type Sec61 protein showed no altered turnover

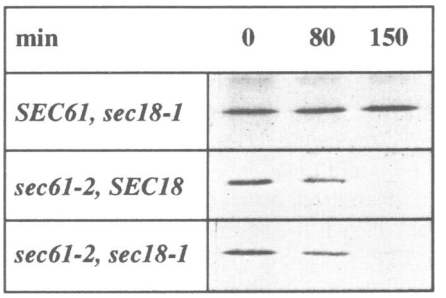

Fig. 5. The proteolysis of mutant Sec61p takes place in a pre-Golgi compartment. The half-life of Sec61p was determined in *sec18-1* (YTX49), *sec61-2sec18-1* (YTX45) and of *sec61-2* (YFP338) strains by a pulse–chase experiment. Cells were grown to exponential phase at 25°C and were labeled after a shift for 1 h to 38°C, followed by a chase period of 0, 80 and 150 min at 38°C. At each time point membrane proteins were prepared and subjected to immunoprecipitation with Sec61p specific antibodies.

in the *sec18-1* mutant background (Figure 5). The turnover of mutant Sec61p in the *sec18* mutant background was identical to the turnover in a *SEC18* wild-type background, confirming that the degradation does not require transport beyond the pre-Golgi. Due to differences in the permissive and non-permissive temperatures between *sec18* and *sec61* mutants, the temperature shifts preceding the pulse-labeling have to be different from those described for the experiments in Figure 3, which may explain the slight difference in the turnover rates of mutant Sec61 protein.

Discussion

Unassembled subunits of the Sec61p complex may be the targets for degradation

The function of the ubiquitin–proteasome pathway in the degradation of short-lived or abnormal proteins of the cytosol and the nucleus has been widely established (Ciechanover, 1994). Our results now demonstrate that this pathway is also involved in the degradation of components of the Sec61p complex, a key component of the protein translocation apparatus of the ER membrane. The trigger for the proteolysis of these membrane proteins seems to be the disassembly of the trimeric Sec61p complex.

The reduced amounts of Sec61p and wild-type Sss1p in the *sec61* mutant strain and the fact that the third component of the trimeric complex, Sbh1p, remains unchanged suggest that only Sec61p and Sss1p are targets for proteolysis, while Sbh1p is not. It can be speculated either that unassembled Sec61p and Sss1p are degraded or that an associated dimeric assembly of Sec61p and Sss1p is a target for proteolysis. Since such a dimer is stable and functional in cells lacking Sbh1p (Finke *et al.*, 1996), we favor the first assumption, implying that the mutations in Sec61p result in an increased dissociation of the complex. In conjunction with an enhanced degradation of unassembled Sec61p and Sss1p, this would result in reduced amounts of both membrane proteins. Our assumption is also consistent with the observed stabilization of the mutant Sec61 protein by overproduction of Sss1p. Under suppressing conditions, the dissociation equilibrium of the mutant Sec61p complex may be shifted to the side of the intact complex. Further support comes

from the analysis of a protein complex required for protein export in *E.coli*, consisting of SecY, SecE and SecG. SecY is related to Sec61p (Görlich *et al.*, 1992) and SecE is homologous to Sss1p (Hartmann *et al.*, 1994). Overproduced SecY protein is rapidly degraded unless it is stabilized by simultaneous overexpression of SecE. In addition, a decreased level of SecE destabilizes SecY (Taura *et al.*, 1993). In the case of Sec61p and Sss1p this may be similar since Sec61p can be overproduced only 2-fold (C.Volkwein, unpublished observation).

Sbh1p seems to be a stable membrane protein as a separate entity or it may be stabilized by the association with other membrane proteins.

The function of the ubiquitin system in ER degradation

We present evidence that a mutation in Sec61p results in a metabolically unstable protein compared with the wild-type. The mutant Sec61 protein is stabilized *in vivo* by mutations in several components of the ubiquitin–proteasome pathway, manifesting a role for this pathway in degradation of membrane proteins of the yeast ER. Moreover, the complete stabilization observed in mutants in the ubiquitin–proteasome pathway suggests that the selectivity of this proteolytic process is mediated by this system. The proteolysis takes place in a pre-Golgi compartment, since the breakdown of mutant Sec61p is not impaired by the obstruction of the transport between ER and Golgi.

We have shown that the amounts of two subunits of the Sec61p complex, Sec61p and Sss1p, are selectively reduced in the *sec61-2* mutant allele, while the third subunit, Sbh1p, was not affected. The reduced levels of both membrane proteins were corrected by deletions of either *UBC6* or *UBC7*. It may be possible that the effect on the amount of Sss1p is indirect, mediated by its association with the stabilized Sec61p. Moreover, a pulse–chase analysis revealed that the mutant Sec61p is stabilized in either disruption of the two ubiquitin-conjugating enzymes. The deletion of *UBC6* results in only a partial stabilization of mutant Sec61p, most likely because *UBC7* is still present. In contrast, after disruption of *UBC7*, Ubc6p has no function in the degradation of Sec61p, suggesting that Ubc6p functions exclusively in a complex with Ubc7p. The complete stabilization of mutant Sec61p after *UBC7* deletion can be explained by the assumption of a second ubiquitin-conjugating enzyme besides Ubc6p, which also recruits Ubc7p either to the membrane or into a soluble complex in the cytosol. The deletion of *UBC7* would disturb both complexes, while the deletion of *UBC6* affects only one of the two complexes.

Consistent with a function of the ubiquitin system in this degradation process, we found that a mutation in ubiquitin (UbK48R), which prevents the formation of a polyubiquitin chain, corrected the reduced amounts of both Sec61p and Sss1p and increased the half-life of mutant Sec61p. Overproduced UbK48R is also a weak suppressor of the lethality of the *sec61-2* mutant at elevated temperatures.

Moreover, we demonstrate a stabilization of mutant Sec61p in a *pre1-1* strain in which the chymotryptic activity of the proteasome is affected. From our experiments we cannot decide whether the 26S proteasome

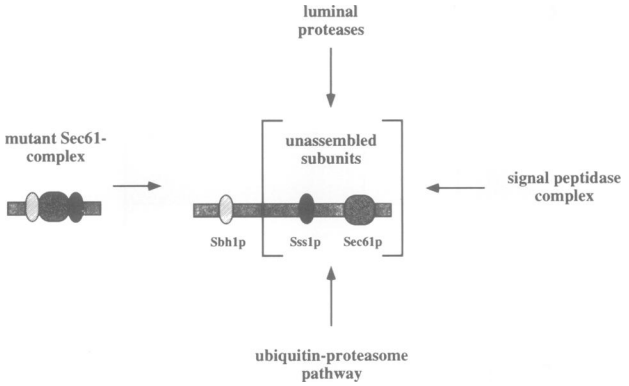

Fig. 6. Model for the degradation of mutant Sec61p. The degradation of two components of the mutant trimeric Sec61p complex is initiated at the cytosolic side of the ER membrane by the ubiquitin–proteasome pathway while the third subunit is stable. This initiation of the degradation may result in unstable breakdown intermediates which are degraded by other proteolytic systems, such as the signal peptidase complex and lumenal proteases.

complex or the 20S proteasome is required for this degradation process, but the dependence on the formation of a polyubiquitin chain suggests the involvement of the 26S proteasome, which is specific for ubiquitin-conjugated targets (Peters, 1994). A function of this protease in the degradation of mutant Sec61p is unexpected. It is reasonable that large cytosolic N- or C-terminal domains of certain membrane proteins are degraded by the proteasome, because these domains can enter the central chamber in which the catalytic sites are buried (Löwe *et al.*, 1995). In the case of Sec61p, only small loops and the short N- and C-termini are located in the cytosol. For steric reasons, it would be surprising if the 26S proteasome complex can cleave those parts of the protein that are in close proximity to the membrane. It seems more plausible that the cleavage by the proteasome is incomplete and that after a few cuts which initiate the proteolysis the process is continued by other proteolytic systems. Breakdown intermediates that may be targets of other proteolytic systems or stable subfragments of Sec61p may not be detected since the antibodies used in this study recognize only the extreme C-terminus of Sec61p (Panzner *et al.*, 1995).

These results have several implications for the degradation of membrane proteins of the ER which are summarized in the following model (Figure 6). Abnormal or unassembled membrane proteins, in our study represented by mutant Sec61p and Sss1p, may be recognized by components of the ubiquitin system and a polyubiquitin chain is formed on the proteolytic target. The conjugated membrane protein is then recognized by the proteasome and the degradation of the cytoplasmic parts of the target proteins results in unstable breakdown intermediates consisting of the membrane anchors and the luminal parts of Sec61p. We further propose that the membrane anchors are cleaved from the luminal parts, which may be done by a specific peptidase, perhaps the signal peptidase. The remaining membrane anchors may subsequently become a target for a signal peptide peptidase. The residual soluble parts may be degraded by luminal proteases which are also involved in the turnover of soluble targets of the ER degradation pathway.

Our model is supported by several observations. Results

Table I. Yeast strains

Strain	Genotype	Reference
YFP338	*MATα, sec61-2, leu2-3, 112, ura3-52, ade2-3, pep4-3*	Rose *et al.*, 1989
RSY455	*MATa, sec61-3, leu2-3, 112, ura3-52, trp1-1, his4-401, HOL1-1*	R.Schekmann
RSY521	*MATα, leu2-3, 112, ura3-52, trp1-1, his4-401, HOL1-1*	R.Schekmann
HMSF176	*MATa, sec18-1, SUC2, mal, gal2, CUP1*	Yeast genetic stock center
YTX42	*MATα, sec61-2, Δubc6::LEU2, leu2-3, 112, ura3-52, ade2-2, pep4-3*	Sommer and Jentsch, 1993
YTX43	*MATa, sec61-3, Δubc6::LEU2, leu2-3, 112, ura3-52, trp1-1, his4-401, HOL1-1*	Sommer and Jentsch, 1993
YTX45	*MATα, sec61-2, sec18-1, ura3-52, leu2-3, 112*	Sommer and Jentsch, 1993
YTX49	*MATα, sec18-1, ura3-52, leu2-3, 112*	this study
YTX66	*MATa, sec61-2, ura3, trp1-1, ade2–1, pep4-3*	this study
YTX72	*MATα, sec61-2, Δpre1::TRP1, ura3-52, his3-11, 15, leu2-3, 112, ade2-3, pTX49(ARS/CEN, URA3)*	this study
YTX93	*MATα, sec61-2, Δubc7::LEU2, leu2-3, 112, ura3-52, ade2-3, pep4-3*	this study
YTX94	*MATα, sec61-2, Δubc7::LEU2, Δubc6::ADE2, leu2-3, 112, ura3-52, ade2-3, pep4-3*	this study

with soluble targets of an ER degradation mechanism suggest the existence of luminal proteases (Gardner *et al.*, 1993). Yuk and Lodish (1993) have detected a luminal breakdown intermediate that occurs during the proteolysis of the H2 subunit of the ASGP receptor in the ER. From the amino acid sequence of the cleavage site, it seemed likely that the signal peptidase is involved in the generation of the fragment. Further support comes from results showing that the luminal portion of a type 2 transmembrane protein is probably cleaved by the signal peptidase from the membrane anchor after removal of the cytoplasmic parts (Lipp and Dobberstein, 1986; Schmid and Spiess, 1988) and from results on an artificial membrane protein which is stabilized by a mutation in *SEC11*, a subunit of the signal peptidase complex of yeast (Mullins *et al.*, 1995). Most recently, studies with inhibitors of the ubiquitin–proteasome pathway have indicated an involvement of this system in the degradation of the CFTR protein in mammalian cells (Jensen *et al.*, 1995; Ward *et al.*, 1995).

Although it seems unlikely, an alternative hypothesis would be that the whole ubiquitinated membrane protein is pulled out of the membrane by a reverse translocation process and degraded completely in the cytosol by the ubiquitin–proteasome pathway. It can be speculated that such a process would require proteins with chaperone functions to keep the hydrophobic segments soluble and ATPases to provide the required energy. In the case of *E.coli* SecY, the involvement of an ATPase in the degradation of this membrane protein seems likely. Oversynthesized SecY is stabilized by mutations in FtsH, a membrane-bound member of a family of ATPases which is widely distributed among eukaryotes and prokaryotes. Most interestingly, two subunits of the 19S subcomplex of the 26S proteasome, Cim3p and Cim5p, also belong to this family (Kihara *et al.*, 1995).

In some cases of ER degradation, which may include well known targets of this process such as unassembled subunits of the T-cell receptor and the H2 subunit of the ASGP receptor (Klausner and Sitia, 1990; Bonifacino and Lippincott-Schwartz, 1991), it is possible that only luminal proteases are involved. Thus, ER degradation may be a misleading term, as different proteolytic pathways may be operating in the degradation of misfolded proteins of this compartment.

Materials and methods

Yeast and bacterial media and methods

Standard protocols were followed for preparation of yeast and *E.coli* media, yeast transformation by the lithium acetate method, yeast sporulation and tetrad dissection, preparation of total yeast DNA, Southern analysis and construction of plasmids (Ausubel *et al.*, 1991; Guthrie and Fink, 1991). The *E.coli* strain used was XL1blue. Immunoblots were visualized by Enhanced Chemiluminescence (ECL, Amersham Corporation).

Construction of yeast strains and plasmids

Table I lists the yeast strains used and constructed in this work. YTX49 was obtained by a cross of HMSF176 and YFP338 and a haploid *sec18-1* mutant (YTX49) was selected after tetrad dissection. The identity of the *sec18-1* mutation was verified by detection of accumulated gp-α-factor at the non-permissive temperature. YTX66 was obtained after tetrad analysis of a cross between YFP338 and YTX59 (T.Sommer, unpublished data). The identity of *sec61* was verified by detection of accumulated prepro-α-factor at 38°C. YTX72 was constructed by a procedure similar to that described by Seufert and Jentsch (1992). The *Δpre1::TRP1* (Seufert and Jentsch, 1992) null allele was introduced into a diploid heterozygous *sec61-2* strain (C.Volkwein and T.Sommer, unpublished data) and homologous recombination was verified by DNA hybridization. Haploid spores of the constructed heterozygous diploid strain carrying the *PRE1* disruption were unable to germinate. The diploid strain was transformed with a plasmid expressing the *pre1-1* mutant gene (pTX49) and after tetrad dissection of the sporulated strain a *sec61-2Δpre1::LEU2pTX49* mutant was selected. The strain used in this study was verified by back-crossing. The plasmid pTX49 was constructed by inserting the 1.1 kb *Eco*RI–*Hin*dIII fragment of pSE362pre1-1 (Seufert and Jentsch, 1992) carrying the mutant *PRE1* gene into pDP38 (ARS/CEN) (Ausubel *et al.*, 1991). YTX93 and YTX94 are isogenic strains obtained after transformation of YFP338 with the deletion constructs *Δubc6::ADE2* and *Δubc7::LEU2*. The *Δubc6::ADE2* null allele was constructed by replacing the first 604 bp of the coding region of *UBC6* with a 2.6 kb *Bgl*II fragment carrying the *ADE2* gene. The *UBC7* disruption was described previously (Jungmann *et al.*, 1993). Homologous recombination was monitored by DNA hybridization techniques. Plasmids overproducing Sss1p (Hartmann *et al.*, 1994), Sbh1p (Finke *et al.*, 1996) and UbK48R (pUB203; Finley *et al.*, 1994) have been obtained after transformation.

Preparation of membranes for immunoblotting

Ten OD_{600} of exponentially growing yeast cells (0.7 OD_{600}/ml) were harvested with or without a shift of 2 h to elevated temperatures and resuspended in 50 mM Tris–HCl, pH 7.5, 10 mM EDTA and protease inhibitors (Hartmann *et al.*, 1994). Cell disruption was done with 1 vol of glass beads by three repeated cycles of mixing on a Vortex for 30 s at maximum speed, interrupted by 30 s incubation on ice. Lysates were diluted with cold extraction buffer to 1 ml and were cleared by a low speed centrifugation (370 *g*). Membranes were collected from the supernatant by centrifugation at 16 000 *g*. All components of the translocation apparatus investigated in this study were sedimented completely by this procedure as verified by immunoblotting (data not shown). The sedimented membranes were heated in sample buffer (2% SDS, 50 mM DTT, 10% glycerol, 67.5 mM Tris–HCl, pH 7.5) for 15 min at 40°C and separated on 12% or 18% SDS–polyacrylamide gels for immunoblotting. For immunoprecipitation, the sedimented membranes were dissolved in 100 µl of 150 mM NaCl, 50 mM Tris–HCl (pH 7.5) and diluted with buffer.

Pulse–chase experiments

Yeast cells were grown at 30°C in SD media with the appropriate supplements to 0.7 OD_{600}/ml. One aliquot of the culture was shifted to

the restrictive temperature for an additional 2 h and a control aliquot was kept at permissive temperature for the same time. Ten OD_{600} of growing cells were then harvested for each time point of the pulse–chase experiment. The cells were resuspended in pre-warmed, fresh SD medium and labeled with 80 μCi/10 OD_{600} of a mixture of [^{35}S]methionine and cysteine (*in vivo* cell labeling mixture, Amersham Corporation) for 10 min. The chase period was performed in pre-warmed, fresh SD medium supplemented with 0.004% methionine and 0.003% cysteine and the required amino acids. Cells were incubated during the chase period at normal or elevated temperature with agitation. Ten OD_{600} were removed at zero time and at time intervals, chilled on ice and washed with ice-cold 10 mM NaN_3 and resuspended in 100 μl of ice-cold 50 mM Tris–HCl (pH 7.5), 10 mM EDTA containing a mixture of protease inhibitors. Cells were disrupted with glass beads (Sigma) for 3 min as described above.

Immunoblots and immunoprecipitation

Immunoblots were done as described (Görlich *et al.*, 1992). Affinity purified antibodies were kindly supplied by T.A.Rapoport (antibodies specific for Sec61p, Sss1p, Sec62p, Sec72) and R.Schekman (antibodies specific for Sec63, Sec71).

Immunoprecipitations were done in 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 10 mM EDTA overnight at 4°C with agitation. For collecting antibodies, 20 μl of protein-A–Sepharose were added and the extracts were incubated for 30 min at 4°C with agitation. The beads were collected by centrifugation, washed twice with immunoprecipitation buffer, resuspended and heated in sample buffer (see above). Proteins were separated on 12% or 18% SDS–polyacrylamide gels followed by fluorography.

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