Re-entering the translocon from the lumenal side of the endoplasmic reticulum. Studies on mutated carboxypeptidase yscY species

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Received 7 December 1998

Abstract Misfolded or unassembled secretory proteins are retained in the endoplasmic reticulum (ER) and subsequently degraded by the cytosolic ubiquitin-proteasome system. This requires their retrograde transport from the ER lumen into the cytosol, which is mediated by the Sec61 translocon. It had remained a mystery whether ER-localised soluble proteins are at all capable of re-entering the Sec61 channel de novo or whether a permanent contact of the imported protein with the translocon is a prerequisite for retrograde transport. In this study we analysed two new variants of the mutated yeast carboxypeptidase yscY, CPY*: a carboxy-terminal fusion protein of CPY* and pig liver esterase and a CPY* species carrying an additional glycosylation site at its carboxy-terminus. With these constructs it can be demonstrated that the newly synthesised CPY* chain is not retained in the translocation channel but reaches its ER lumenal side completely. Our data indicate that the Sec61 channel provides the essential pore for protein transport through the ER membrane in either direction; persistent contact with the translocon after import seems not to be required for retrograde transport.

Key words: Endoplasmic reticulum degradation; Carboxypeptidase yscY; Retrograde transport; Endoplasmic reticulum; Yeast *Saccharomyces cerevisiae*

1. Introduction

In eukaryotic cells all secretory proteins enter the endoplasmic reticulum (ER), where they are folded prior to their further transport through the secretory system [1,2]. Since the ER has to guarantee the delivery of only properly folded proteins, it contains a quality control system to proofread and eliminate inactive proteins. Misfolded or unassembled proteins in the ER lumen or the ER membrane are rapidly degraded by the cytosolic ubiquitin proteasome machinery [3–5]. This process, known as ER-associated degradation, requires the retrograde translocation of the substrate molecules from the ER back to the cytosol prior to their hydrolysis [3–5]. Central components of the Sec61 translocon, identified as the highly conserved channel for protein import into the ER in eukaryotic cells [2], have been described to mediate this retrograde transport [6–8]. For membrane proteins the retrograde transport mechanism through lateral gating into the Sec61 channel and extraction is conceptually easy to envisage [6,9]. For soluble, lumenal proteins of the ER different mechanisms seem possible: already during translocation a not properly foldable protein is recognised as such, translocation is arrested, leaving permanent contact with the Sec61 translocon and thereafter its retrograde transport is initiated. Alternatively, the protein leaves the translocon and is completely translocated into the ER lumen, where it is recognised as being misfolded. The latter mechanism would imply a re-targeting mechanism to the translocon for retrograde protein transport.

In this study we wanted to address the question of whether ER-localised soluble proteins like CPY*, a misfolded protein that undergoes retrograde transport from the ER lumen to the cytosol [7], are indeed capable of entering the Sec61 channel from the lumenal side de novo or whether physical contact with the translocon has to be maintained for retrograde transport and cytosolic degradation to occur. Therefore, we generated two CPY* derivatives, one carrying a carboxy-terminal fusion protein, the second having a fifth glycosylation site at the carboxy-terminus that would function as a tool to monitor persistent contact of the nascent chain with the translocon or complete import into the ER lumen.

2. Materials and methods

2.1. Construction of yeast strains and plasmids

Genetic experiments and molecular biological methods were carried out using standard protocols [10]. Yeast strains used in this study are summarised in Table 1.

For generating CPY* derivatives, a 2258 bp *Cla*I/*Hpa*I fragment of plasmid bMK150, vector pRS306 [11] containing the *prc1-1* allele, was cloned into a *Cla*I/*Sma*I-linearised pRS313 vector [11] yielding plasmid bRP312. For the carboxy-terminal fusion of pig liver esterase (PLE) to CPY* an *Aat*II site upstream of the *PLE* gene and a *Spe*I site downstream of the *PLE* gene were introduced by PCR using as primers 5′-CCATTTGACGTCGGGCAGCCAGCCTCG-CCG-3′ and 5′-GTCAGACTAGTTCACAGCTCAGCATGCTTTA-TC-3′. The plasmid containing the *PLE* gene was a gift of U. Bornscheuer, Stuttgart. The PCR product was cloned into the *Aat*II/*Spe*I-linearised vector bRP312 after *Aat*II/*Spe*I digestion resulting in plasmid prc1-PLE. To introduce the sequence encoding a fifth glycosylation site into the *prc1-1* allele a recombinant PCR strategy was used. Primer sequences were 5′-TGTGAAGGTGGCAATTTGTGC-3′ and 5′-CGCTTTATAAAGTACTATTGGAGAAACCACCGTG-GATCCATTCG-3′ for PCR I and 5′-CTGGGGTTCTTTGATT-GGGG-3′ and 5′-CGGTGGTTTCTCCAATAGTACTTTATAAA-GCGTGTATGTGTAGGC-3′ for PCR II. The PCR products and the two short primers were used in a third reaction to yield a 970 bp product that was confirmed by DNA sequencing. For generation of plasmid prc1-G5 plasmid bRP312 was linearised with *Not*I, treated with Klenow enzyme, digested with *Aat*II, and ligated with a 404 bp *Aat*II/*Pvu*II fragment of the final PCR reaction. Δ*prc1* deletion strains were generated by transforming yeast strains W303-1B and YRP086 with the *Ksp*I/*Bgl*II-digested plasmid CPY-ko, which was a gift of G. Polhig, Basel. Knockouts were confirmed by CPY activity tests and Western analysis using CPY-specific antiserum.

2.2. Immunodetection

Cells were grown in complete synthetic medium (CM) containing 2% glucose to an OD_{600} of 1.5. For Western analysis the equivalent of 3 OD_{600}/ml of cells were taken and subjected to alkaline lysis and

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PII: S0014-5793(98)01724-4

trichloroacetic acid (TCA) precipitation as described [7]. Precipitates were incubated in sample buffer (8 M urea, 200 mM Tris-HCl, pH 6.8, 5% SDS, 0.1 mM EDTA, 0.03% bromophenol blue, 1.5% DTT) at 45°C for 30 min with vigorous agitation and separated on 8% SDS-polyacrylamide gels. Proteins on immunoblots were visualised by enhanced chemoluminescence detection (ECL, Amersham) according to the manufacturer's instructions.

2.3. Protease protection experiments

Cells were grown as previously described. After spheroblasting the equivalent of 50 OD_{600}/ml of cells [7] these were gently lysed using a tissue grinder. The lysate was cleared of remaining cells and debris by repeated centrifugation for 5 min at $3000 \times g$. The cleared lysate was split into aliquots corresponding to 12.5 OD_{600}/ml. From the time of lysis, all material was kept on ice. Separation of membranes was done by a 30 min centrifugation at $20\,000 \times g$ at 4°C. For protease treatment of the pellet trypsin was added at a final concentration of 0.5 mg/ml after resuspension of the pellet and the samples were incubated for 30 min on ice. If added, Triton X-100 was present at 1%. All treatments were stopped by adding TCA with a final concentration of 10%. After resuspending the pellet in sample buffer at 45°C for 45 min proteins were analysed by SDS-PAGE and immunoblotting.

2.4. Deglycosylation experiments

Cells were grown to an OD_{600} of 2 followed by immunoprecipitation and deglycosylation as described [7]. Briefly, the equivalent of 10 OD_{600}/ml of cells were subjected to alkaline lysis, immunoprecipitation of CPY*, and treatment with 0.06 U endoglycosidase F for 1 h at 37°C. Finally, the samples were resuspended in urea buffer and analysed by SDS-PAGE and immunoblotting.

2.5. Tunicamycin treatment

Cells were grown as described to an OD_{600} of 1.0 and the equivalent of 10 OD_{600}/ml of cells were taken for each sample. Cells were labelled with 25 μCi [^{35}S]methionine for 30 min at 37°C in the absence or presence of 10 μg/ml, 20 μg/ml, and 50 μg/ml tunicamycin followed by cell lysis and immunoprecipitation as described before. Proteins were separated on SDS-polyacrylamide gels followed by fluorography.

2.6. Cycloheximide chase analysis

Cells were grown as described and then adjusted to approximately 8 OD_{600}/ml. After adding cycloheximide to a final concentration of 0.1 mg/ml, 1 ml of cell suspension was removed at the indicated time points, suspended in a sodium azide solution (final concentration 10 mg/ml) and kept at −80°C. Cell lysis, TCA precipitation, SDS-PAGE, and immunodetection were performed as described before.

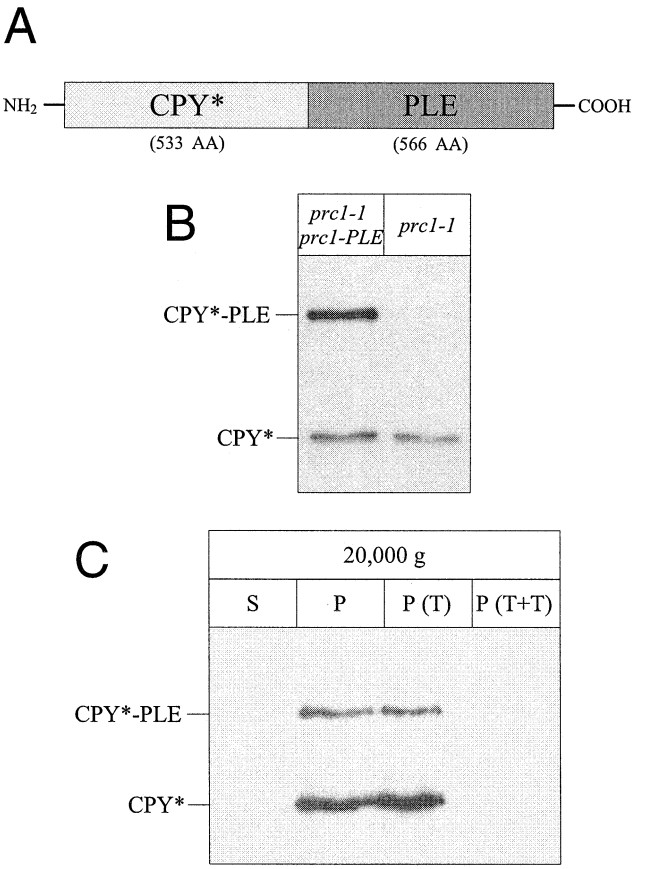

Fig. 1. A fusion protein of CPY* and PLE is imported completely into the ER. A: Scheme of the fusion construct. B: Western analysis of yeast strain W303-1C PLE (*prc1-1 prc1-PLE*) carrying the plasmid-encoded CPY*-PLE fusion protein and W303-1C (*prc1-1*). C: Protease protection analysis of yeast strain W303-1C PLE (*prc1-1 prc1-PLE*) after preparing spheroblast homogenates followed by a $20\,000 \times g$ centrifugation (S, supernatant; P, pellet fraction; P (T), pellet treated with trypsin; P (T+T) pellet treated with trypsin and Triton X-100). To confirm the integrity of the prepared vesicles CPY* was analysed on the same membrane. For immunodetection antibodies directed against CPY were used.

3. Results

3.1. Translocation of a carboxy-terminal fusion protein of CPY into the ER is not affected by the ER quality control system*

Using a mutated carboxypeptidase yscY (CPY*), allele *prc1-1* [12,13], as a substrate we addressed the question whether the translocation of CPY* into the ER lumen is completed or whether CPY* maintains permanent contact with the translocon due to its recognition by the ER quality control system prior to retrograde transport for its proteasomal degradation. Successful termination of CPY* import into the

Table 1
Yeast strains used in this study

Strain	Genotype	Source
W303-1B	MATα *ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1^{oc} can1-100*	[20]
W303-1C	W303 *prc1-1*	[16]
W303-1C PLE	W303 *prc1-1 prc1-PLE*	This study
W303 ΔC	W303 Δ*prc1*::*LEU2*	This study
W303-1C G5	W303 *prc1-1 prc1-G5*	This study
W303 ΔC G5	W303 Δ*prc1*::*LEU2 prc1-G5*	This study
YRP086	W303 *sec61-2 prc1-1*	[7]
YRP086 G5	*W303 sec61-2 prc1-1 prc1-G5*	This study
YRP086 ΔC G5	*W303 sec61-2* Δ*prc1*::*LEU2 prc1-G5*	This study
W303-CD	W303 Δ*der1*::*URA3 prc1-1*	[16]
W303-CD G5	W303 Δ*der1*::*URA3 prc1-1 prc1-G5*	This study
W303-CPQ	W303 Δ*ubc6*::*LEU2* Δ*ubc7*::*LEU2 prc1-1*	[15]
W303-CPQ G5	W303 Δ*ubc6*::*LEU2* Δ*ubc7*::*LEU2 prc1-1 prc1-G5*	This study

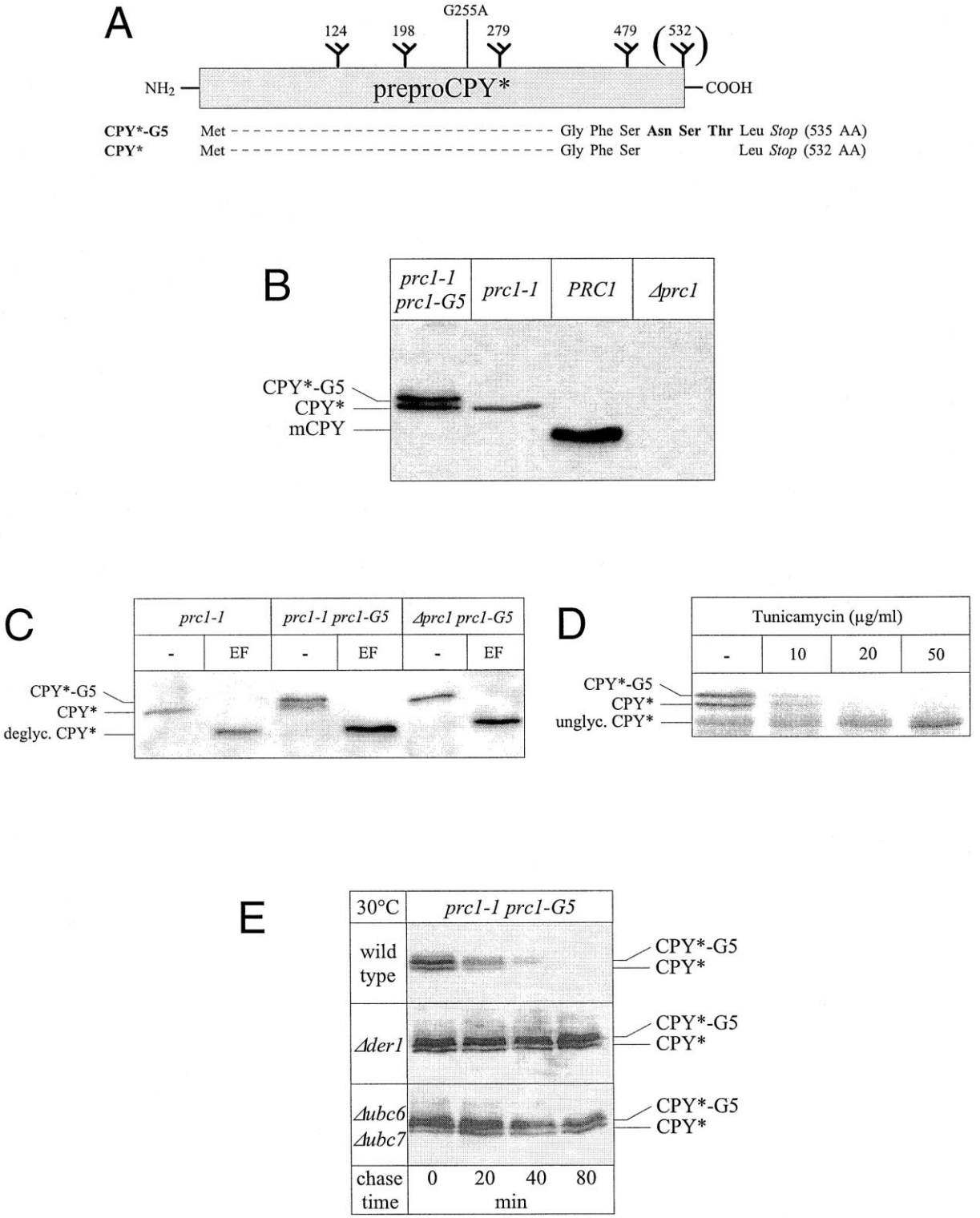

Fig. 2. An additional glycosylation site at the carboxy-terminus of CPY* is recognised by the ER glycosylation machinery: evidence that the entire protein is imported into the ER. A: An additional glycosylation side was introduced at the carboxy-terminus of CPY*. B: Western analysis of W303-1C G5 (*prc1-1 prc1-G5*) carrying the plasmid encoded *PRC1-G5* allele, W303-1C (*prc1-1*), W303-1B (*PRC1*), and W303-ΔC (Δ*prc1*::*LEU2*). C: Endoglycosidase F (EF) treatment of immunoprecipitates of W303-1C (*prc1-1*), W303-1C G5 (*prc1-1 prc1-G5*), and W303-ΔC G5 (Δ*prc1*::*LEU2 prc1-G5*) cell lysates. D: Autoradiographic analysis of strain W303-1C G5 (*prc1-1 prc1-G5*) after incubation in the presence of increasing amounts of tunicamycin. E: The fivefold glycosylated CPY* species is still a substrate of the ER degradation system. Cycloheximide chase analysis was performed in yeast strains W303-1C G5 (*prc1-1 prc1-G5*), W303-CD G5 (Δ*der1*::*LEU2 prc1-G5*), and W303-CPQ G5 (Δ*ubc6* Δ*ubc7 prc1-G5*) at 30°C. After adding cycloheximide, aliquots of cells were lysed at each time point and TCA precipitates were subjected to SDS-PAGE and immunoblotting using CPY-specific antibodies.

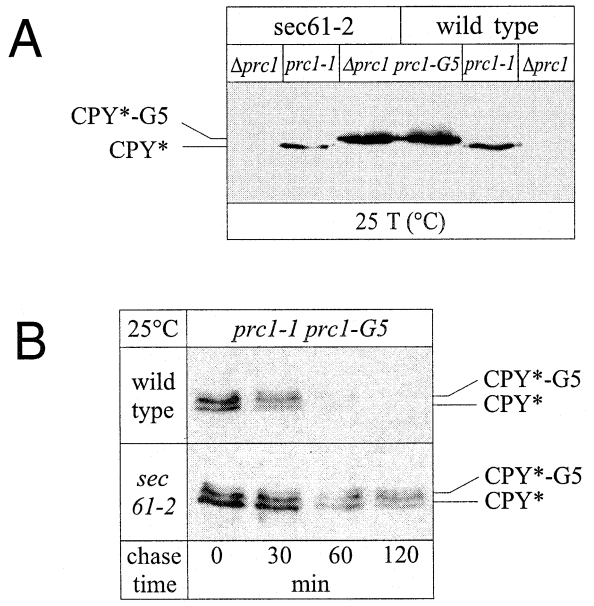

Fig. 3. The *sec61-2* mutation does not affect termination of CPY* import into the ER. A: Western analysis of yeast strains YRP086 ΔC G5 (*sec61-2 Δprc1::LEU2 prc1-G5*) and W303-ΔC G5 (*Δprc1::LEU2 prc1-G5*) after incubation at 25°C. No translocation intermediate with only four or fewer glycosylation chains could be detected. B: Degradation but not import of CPY* and CPY*-G5 is retarded in *sec61-2* mutant cells at 25°C. Cycloheximide chase analysis of yeast strains YRP086 G5 (*sec61-2 prc1-1 prc1-G5*) *G5*), W303-1C G5 (*prc1-1 prc1-G5*) was performed as described in Fig. 2D.

ER would subsequently require a re-targeting event back to the Sec61 translocon followed by re-entering the channel from the ER lumenal side de novo prior to its retrograde transport and degradation. This would imply that opening of the translocation channel can be initiated from both sides of the ER membrane, the cytosol and the lumen.

For this purpose we constructed and analysed two kinds of CPY* mutants. We fused a PLE to the carboxy-terminus of CPY* (Fig. 1A) resulting in a fusion protein of about 130 kDa molecular mass (Fig. 1B). Cell extracts were prepared and separated into a soluble and a microsomal fraction. When treated with antibodies directed against CPY, the antigenic material was exclusively found in the pellet fraction, indicating that its post-translational targeting to the ER membrane after synthesis was unaffected (Fig. 1C). Moreover, the entire molecule including its carboxy-terminal PLE part was imported into the ER lumen as could be demonstrated by protease treatment of intact microsome preparations (Fig. 1C). We thus conclude that import of the CPY* chain into the ER lumen is not blocked by the ER quality control system after the full-length mutated CPY* molecule has appeared in the lumen. We cannot exclude, however, that a stretch containing about 20 amino acids of the carboxy-terminus of the fusion protein is retained in the translocation channel. They could escape degradation by trypsin because they are protected by Sec61p.

3.2. The carboxy-terminus of CPY* reaches the ER lumen

Therefore, we generated another variant of CPY* (CPY*-G5) carrying a fifth glycosylation site at the very end of the carboxy-terminus in addition to the four glycosylation sites present in CPY* (Fig. 2A). Only after completion of protein import into the ER this additional glycosylation site would be recognised by the ER glycosylation machinery. Indeed, Western analysis revealed an increased molecular weight of the CPY*-G5 species as compared to the authentic fourfold glycosylated CPY* suggesting glycosylation at all five sites (Fig. 2B). Treatment with endoglycosidase F and tunicamycin confirmed that the increased molecular mass of the CPY*-G5

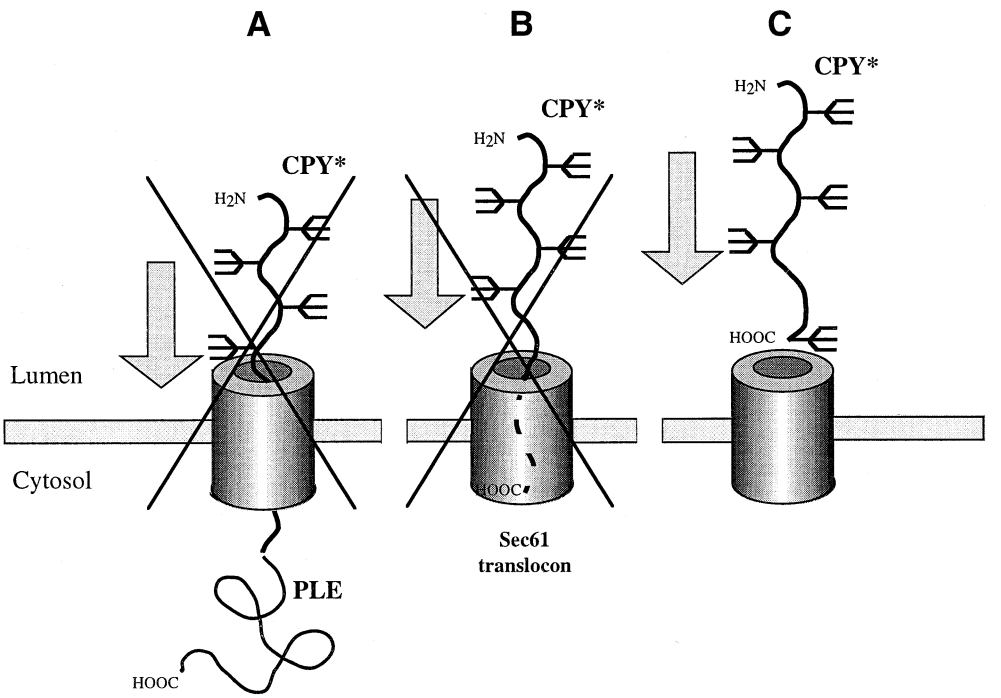

Fig. 4. Schematic representation of three different scenarios conceivable as a basis for retrograde transport of CPY* by the Sec61 channel: permanent physical contact (A/B) or de novo re-entering (C).

species was due to glycosylation and not to the altered amino acid sequence at the carboxy-terminus of the molecule (Fig. 2C,D). This experiment demonstrated full translocation of the CPY*-G5 molecule through the Sec61 channel.

Since it had been reported previously that a completely unglycosylated CPY* variant remains stable in the ER lumen [14], it was important to demonstrate that the degradation process of CPY*-G5 was unaffected by the additional carbohydrate chain. Degradation of CPY* by the proteasome depends on ER membrane proteins as Der1p and the ubiquitin conjugating enzymes Ubc6p and Ubc7p. We therefore determined the degradation kinetics of CPY*-G5 as compared to CPY* in wild type cells and in cells carrying mutations which affect the degradation process as Δ*der1* and Δ*ubc6*/*ubc7* [15,16] (Fig. 2E). In wild type cells, the half-life of CPY*-G5 was almost identical to CPY*. Moreover, degradation of CPY*-G5 was also dependent on Der1p and Ubc6/Ubc7 (Fig. 2E) suggesting that this CPY* species is still a substrate of the ER degradation system.

3.3. *The sec61-2 mutation does not affect the termination of CPY* import into the ER*

Mutant strains carrying the temperature sensitive *sec61-2* allele, which causes rapid degradation of unassembled Sec61-2p molecules at the restrictive temperature of 37°C [17], are defective in retrograde transport of CPY* and of a mutated ABC transporter Pdr5 (Pdr5*) at 25°C [7,9]. To further exclude the possibility that also the termination of protein translocation into the ER lumen is affected by the *sec61-2* mutation, we analysed the fate of the CPY*-G5 protein in yeast cells carrying the *sec61-2* allele (Fig. 3A). We observed no translocation intermediates of CPY*-G5 carrying less than five carbohydrate chains in *sec61-2* mutant cells. This demonstrates that once initiated import of CPY*-G5 into the ER lumen goes to completion both in wild type and in *sec61-2* mutants. This finding was confirmed by analysing the degradation kinetics of CPY* and CPY*-G5 in wild type and *sec61-2* mutant cells at 25°C (Fig. 3B). Consistent with previous results for CPY* the half-life of both CPY* species was increased about threefold in *sec61-2* mutant cells, indicating that CPY* re-entering of the Sec61 channel and retrograde transport is affected by the *sec61-2* mutation and not termination of protein import into the ER lumen.

4. Discussion

Using two different CPY* constructs, a carboxy-terminal PLE fusion protein and a species carrying a fifth glycosylation site at the carboxy-terminus, we were able to distinguish between three different scenarios conceivable for retrograde transport of CPY*:

1. The ER quality control system recognises misfolding of CPY* already at the state of import and prevents further translocation. In this case the carboxy-terminus would never reach the translocon. Thus the carboxy-terminally attached PLE protein would remain cytosolic (Fig. 4A). Protease treatment of isolated microsomes demonstrates membrane protection of the entire CPY*-PLE fusion protein and therefore argues against this possibility.
2. The ER quality control system recognises misfolding of CPY* and prevents termination of translocation into the ER. In this case the last about 20 amino acids would be trapped in the translocon (Fig. 4B). Here, a glycosylation site at the very carboxy-terminus of CPY* would not be recognised by the ER lumenal glycosylation machinery.
3. Termination of translocation of CPY* is not affected by the ER quality control system. Here, a glycosylation site at the very carboxy-terminus of CPY* would be used by the ER lumenal glycosylation machinery (Fig. 4C). Our data support this latter possibility. The carboxy-terminally introduced glycosylation site is recognised and a fifth carbohydrate chain is added onto the CPY* molecule.

Our experiments indicate full translocation of CPY* to the lumenal side of the ER membrane. We have thus to conclude that CPY* is able to re-enter the Sec61 channel from the lumenal side for retrograde transport and degradation to occur. This implies a novel ER-localised targeting machinery for retrograde transport of misfolded proteins. Whether previously discovered components of the ER degradation system such as Der1p [16], Der3p/Hrd1p [18,19], or Hrd3p [19] are part of this machinery has to be shown in the future.

Acknowledgements: We thank U. Bornscheuer, M.M. Hiller, R. Hitt, M. Knop, G. Pohlig, and R. Schekman for providing yeast strains and plasmids. This work was supported by the Deutsche Forschungsgemeinschaft, Bonn, and the Fonds der Chemischen Industrie, Frankfurt.

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