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Correspondence and requests for materials should be addressed to A.A.H.

Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation

Richard K. Plemper[*], Sigrun Böhmler[*], Javier Bordallo[*], Thomas Sommer[†] & Dieter H. Wolf[*]

[*] *Institut für Biochemie der Universität Stuttgart, Pfaffenwaldring 55,*
D-70569 Stuttgart, Germany
[†] *Max-Delbrück-Centrum für Molekulare Medizin, Robert-Rössle Strasse 10,*
D-13122 Berlin, Germany

Proteins enter the secretory pathway through the endoplasmic reticulum[1], which delivers properly folded proteins to their site of action[2] and contains a quality-control system to monitor and prevent abnormal proteins from being delivered[3]. Many of these proteins are degraded by the cytoplasmic proteasome[4–8], which requires their retrograde transport to the cytoplasm[5,6]. Based on a co-immunoprecipitation of major histocompatibility complex (MHC) class I heavy-chain breakdown intermediates with the translocon subunit Sec61p (refs 9, 10), it was speculated that Sec61p may be involved in retrograde transport[11]. Here we present functional evidence from genetic studies that Sec61p mediates retrograde transport of a mutated lumenal yeast carboxypeptidase ycsY (CPY*) *in vivo*. The endoplasmic reticulum lumenal chaperone BiP (Kar2p) and Sec63p, which are also subunits of the import machinery[10,12], are involved in export of CPY* to the cytosol. Thus our results demonstrate that retrograde transport of proteins is mediated by a functional translocon. We consider the export of endoplasmic reticulum-localized proteins to the cytosol by the translocon for proteasome degradation to be a general process in eukaryotic cell biology.

Proteins cross the membrane of the endoplasmic reticulum (ER) either co-translationally or post-translationally[9,10]. For both translocation routes, the Sec61 heterotrimeric complex consisting of yeast proteins Sec61p, Sbh1p and Sss1p (ref. 10) constitutes the membrane channel. The post-translational pathway depends on an additional tetrameric complex consisting of the ER transmembrane proteins Sec62p, Sec63p and Sec71p, together with the peripheral membrane protein Sec 72p (refs 10, 12). Sec62p, Sec71p and Sec72p provide a cytosolic binding site for the newly synthesized precursor molecules, whereas Sec63p and the lumenal chaperone Kar2p (BiP) mediate the driving force for import[10,12].

We analysed retrograde transport using yeast strains carrying mutant alleles of translocon components, and used mutated yeast

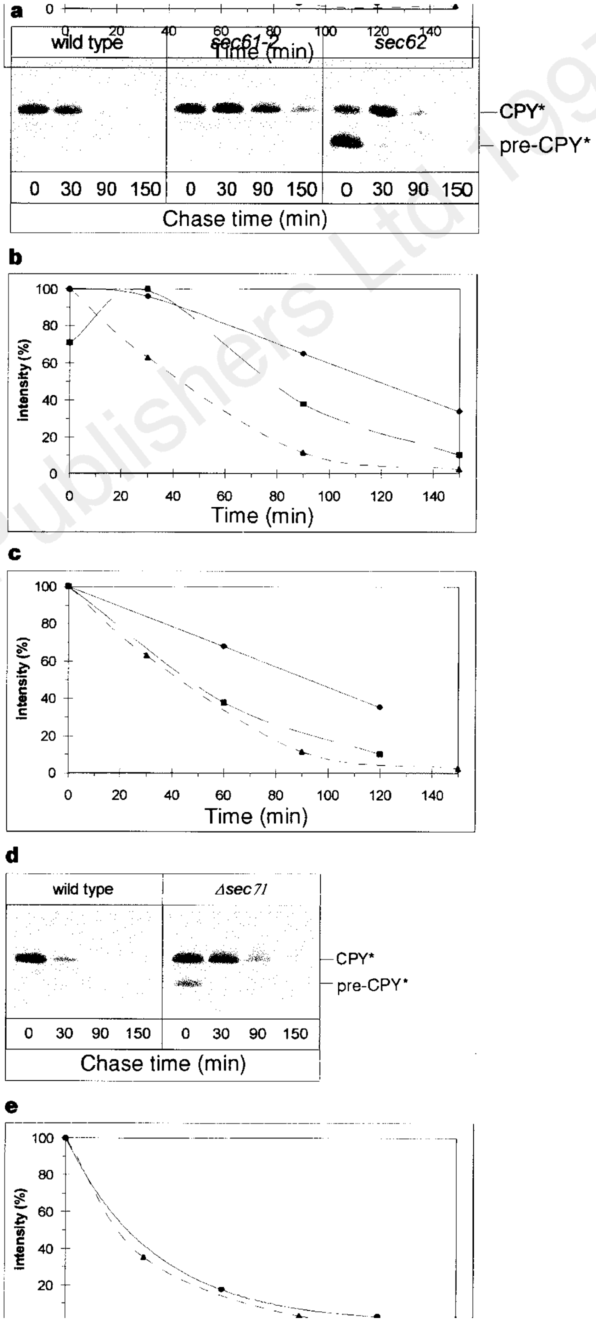

Figure 1 A functional Sec61p is required for ER degradation. **a**–**c**, CPY* is stabilized in the *sec61-2* mutant, pre-CPY* import and not degradation is affected in the *sec62* mutant. Pulse-chase analysis of CPY* was performed using the isogenic strains W303-1C (wild type, triangles), YRP086 (*sec61-2*, diamonds) and YRP087 (*sec62*, squares). Cells were grown at the permissive temperature of 25 °C. After the indicated chase times the cells were lysed, CPY* was immunoprecipitated, and the antigenic material was separated by SDS–PAGE and analysed using a Molecular Dynamics imaging system. **d**, **e**, A *sec71* knockout leads also to accumulation of pre-CPY* but not to a defect in CPY* degradation. Pulse-chase analysis of the isogenic strains W303-1C (wild type, triangles) and YRP091 (*Δsec71*, circles) was done at 30 °C as described in **a**.

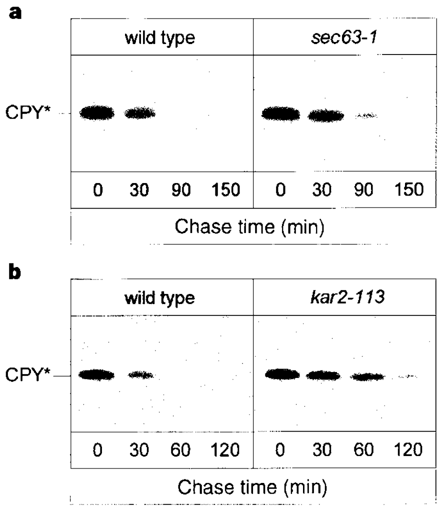

Figure 2 ER degradation of CPY* is affected by the *sec63-1* (**a**) and *kar2-113* (**b**) mutations. Pulse-chase analysis of the isogenic strains W303-1C (wild type), YRP088 (*sec63-1*) and YRP146 (*kar2-113*) at the permissive temperature of 25 °C was performed.

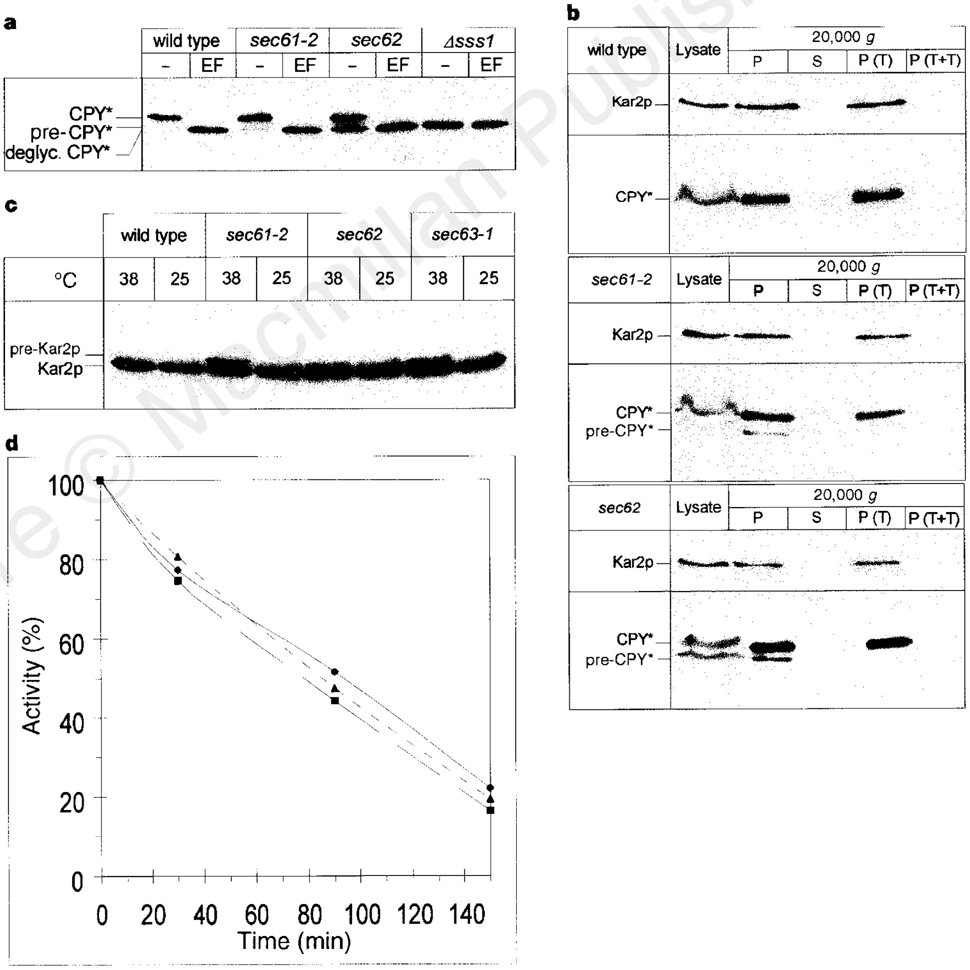

Figure 3 Control experiments demonstrate that CPY* stabilization in the *sec61-2* background is based on a defective export. **a**, CPY* accumulating in *sec61-2* mutant cells is glycosylated, whereas the second antigenic species is unglycosylated, showing that it is pre-CPY*. Western analysis of CPY* and pre-CPY* in the isogenic strains W303-1C (wild type), YRP086 (*sec61-2*), YRP087 (*sec62*) and YRP090 (*Δsss1*, pGAL*SSS1* on plasmid) after growth of cells at 32 °C. Deglycosylation was performed with endoglycosidase F (EF) after immunoprecipitation of CPY* antigenic material. **b**, Although CPY* is membrane protected from proteolysis, pre-CPY* is bound to the cytosolic face of vesicles. Western analysis of CPY*, pre-CPY* and (for control) Kar2p, in the strains W303-1C (wild type), YRP086 (*sec61-2*) and YRP087 (*sec62*), after preparing spheroblast homogenates followed by a 20,000*g* centrifugation. (P, pellet fraction; S, supernatant; P (T), pellet treated with trypsin; P (T + T), pellet treated with trypsin and Triton X-100. **c**, Kar2p is imported without precursor accumulation in *sec61-2* mutant cells at the permissive temperature. Western analysis of Kar2p in the strains W303-1C (wild type), YRP086 (*sec61-2*), YRP087 (*sec62*) and YRP088 (*sec63-1*). **d**, The Ubc6–Ubc7 proteosome degradation system is functional in the *sec61-2* and *sec63-1* mutants. *β*-Galactosidase activity was tested after alkaline lysis of the strains W303-1C (wild type, triangles), YRP086 (*sec61-2*, diamonds) and YRP088 (*sec63-1*, squares). Cells expressing the plasmid-encoded fusion protein Deg1-β-galactosidase were analysed after incubation at the permissive temperature of 25 °C.

carboxypeptidase yscY (CPY*) as a substrate for ER degradation[13]. When following the fate of CPY* in a strain harbouring the temperature-sensitive *sec61-2* allele[14] by pulse-chase analysis at 25 °C, a considerable stabilization of CPY* compared to the wild type was visible (Fig. 1a). The half-life of CPY* increased about threefold in the *sec61-2* background (Fig. 1b), indicating a disturbed delivery of lumenal CPY* to the cytosolic proteasome. In contrast, at the same temperature of 25 °C, a mutation in Sec62p led to an import defect, clearly visible by the accumulation of pre-CPY* (Fig. 1a) and the appearance of an intensity maximum of lumenal CPY* after 30 min chase time (Fig. 1b). A delayed import was also seen in a Sec71p deletion strain (Fig. 1d), resulting in an apparent stabilization of CPY*. However, in the *sec62* (Fig. 1c) and *Δsec71* (Fig. 1e) mutants, the kinetics of CPY* degradation was essentially identical to wild type. This was demonstrated by taking the second chase point (30 min), at which the precursor of CPY* is completely imported into the ER, as the time point from which the half-life was calculated. Applying the same calculation to the *sec61-2* mutant strain, the considerably reduced degradation rate of CPY* compared with wild type was again apparent (Fig. 1c). As the function of Sec72p depends on the binding to Sec71p, the *sec71* deletion strain is also devoid of Sec72p function. Thus Sec72p cannot be involved in CPY* retrograde transport. Also, deletion of *SBH1*, a non-essential subunit of the Sec61 heterotrimeric complex[15,16], did not show any changes in the steady-state level of CPY* (data not shown). Furthermore, the amount of CPY* was unaffected in a strain carrying a deletion in *SSH1*, a non-essential homologue of *SEC61*, which appears to be capable of mediating exclusively co-translational protein translocation[16], as well as in a strain deleted in *SBH2*, which encodes a homologue of *SBH1* (ref. 16, and data not shown).

Sec63p and the chaperone Kar2p regulate the dynamics of the Sec61p channel[12]. Interaction between both proteins occurs through a DnaJ box located within the lumenal tail of Sec63p (refs 17, 18), a common binding motif for members of the Hsp70 family[19]. Because the average diameter of the Sec61p channel is about 20 Å (ref. 20), export of CPY* in a globular state seems unlikely. Kar2p, bound to Sec63p, might unfold CPY* before export. We thus tested the degradation rate of CPY* in a *sec63-1* background carrying a mutation in the DnaJ box which prevents binding of Kar2p[21] by pulse-chase analysis at the permissive temperature of 25 °C. Stabilization of CPY* was observed in the *sec63-1* mutant (Fig. 2a), but no CPY* precursor accumulated. Compared to the wild type, the half-life increased about 1.5-fold, indicating that sec63p functions in the CPY* export. We directly addressed the question of whether the presence of intact Kar2p is required for retrograde transport by examining the influence of the mutated *kar2-113* allele[22] on the degradation rate of CPY*. Although import of CPY* into the ER was unaffected in *kar2-113* mutant cells at 25 °C, a remarkable stabilization of lumenal CPY* of about twofold was observed (Fig. 2b). This effect may be due to a defective regulatory function of the Kar2-113 protein on Sec61p, or a reduced unfolding capacity on CPY*.

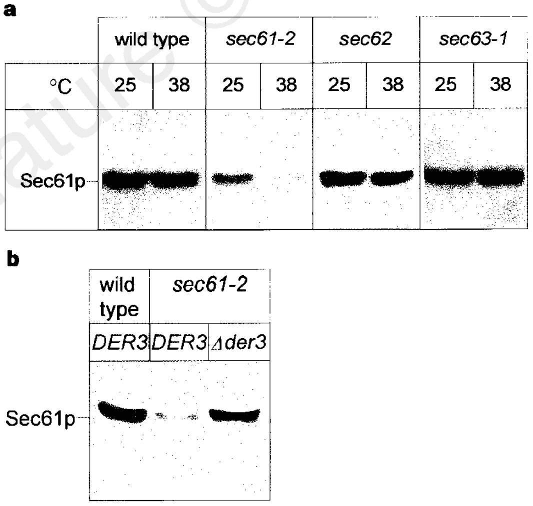

Figure 4 Mutant Sec61p is a substrate of ER degradation. **a**, The Sec61-2p steady-state level is reduced at the permissive temperature of 25 °C. Western analysis of Sec61p in the strains W303-1C (wild-type), YRP086 (*sec61-2*), YRP087 (*sec62*) and YRP088 (*sec63-1*) at permissive (25 °C) and restrictive (38 °C) temperature. cells were grown at 25 °C and portions were shifted to the restrictive temperature. After 60 min, crude extracts of cells incubated at 25 °C or shifted to 38 °C were analysed. **b**, Der3, a gene product involved in CPY* degradation, participates in the degradation of mutant Sec61p. Western analysis of Sec61p in the isogenic strains W303-1C (wild-type), YRP086 (*sec61-2*) and YRP105 (*Δder3 sec61-2*). Cells were analysed after a shift to the restrictive temperature of 38 °C for 45 min.

It was important to demonstrate that the molecular form of CPY* seen in the *sec61-2* mutant represents glycosylated, lumenal CPY*, and that the additional molecular form seen in the *sec62* mutant was cytosolic pre-CPY* and not some already exported degradation intermediate. A CPY* species, once imported into the ER lumen, is expected to be glycosylated and thus accessible to deglycosylation by endoglycosidase F, whereas non-imported pre-CPY* should be devoid of carbohydrate. We induced the accumulation of CPY* precursor molecules in the *sec61-2* and the *sec62* mutant by incubating cells at 32 °C. In *sec63-1* and *kar2-113* mutant cells, no pre-CPY* was visible at this temperature (data not shown). The antigenic material of higher relative molecular mass (M_r) appearing in all strains is completely accessible to endoglycosidase F treatment (Fig. 3a). In contrast, the band of lower M_r seen in the *sec61-2* and *sec62* mutants does not undergo a shift upon endoglycosidase F treatment as expected for non-imported pre-CPY*. We confirmed the stability of this band against deglycosylation by analysing the CPY* species in a strain devoid of Sss1p, an essential subunit of the translocon[23]. This strain was kept alive with the *SSS1* gene under the control of the GAL promoter. After repressing the promoter by adding glucose, the protein import into the ER was completely blocked. In these cells only pre-CPY* accumulated, which, as expected, was completely resistant to endoglycosidase F treatment (Fig. 3a).

We then determined the intracellular localization of the CPY* species in *sec61-2*, *sec62* and in wild-type cells at 32 °C. After destruction of cells, the lysate was separated into a pellet fraction containing the microsomes and a supernatant fraction. The fractions were run on an SDS–polyacrylamide gel and immunoblotted with specific antibodies against CPY* and Kar2p as a control for the integrity of the ER vesicles. The *sec61-2* and *sec62* mutants accumulate pre-CPY* and CPY* species, which fractionate with the pellet (Fig. 3b). Trypsin treatment of the pellet leads to disappearance of the predicted pre-CPY*, whereas the band expected to represent lumenal CPY* remained stable. Only after treatment with Triton X-100 was the latter species accessible to trypsin digestion. This indicates that pre-CPY* was attached to the cytosolic face of the ER membrane, probably to the Sec62–Sec71–Sec72 complex[12]; CPY* was imported into the ER lumen in both mutant strains.

Investigation of the import of two additional proteins, Kar2p wild-type protein and proteinase yscA, showed that the decreased degradation rate of CPY* seen in the strains carrying the mutated translocon subunits *sec61-2* and *sec63-1* was not due to some general import defect that might affect components of the ER degradation system. Although, as expected, pre-Kar2p accumulated at the restrictive temperature of 38 °C in the mutant cells (Fig. 3c), no accumulation was seen at the permissive temperature (25 °C), as had been found for CPY* (Figs 1a, 2a). There was also no accumulation of prepro-proteinase yscA in the mutants at 25 °C (data not shown). In *sec62* mutant cells, only a very weak accumulation of pre-Kar2p could be observed at 25 °C, suggesting that Kar2p was mainly imported through the co-translational translocation pathway[24]. These findings suggest that the import machinery into the ER lumen in the *sec61-2* and the *sec63-1* mutants was unimpaired at the permissive temperature of 25 °C.

The ubiquitin-conjugating enzymes Ubc6 and Ubc7 act at the ER membrane. Together with the proteasome they are essential components of the CPY* degradation process[5]. We had therefore to exclude the possibility that this degradation system was impaired by the mutated transmembrane proteins *sec61-2* or *sec63-1*. Another protein exclusively degraded through the Ubc6, Ubc7 proteasome system is a fusion between the Deg1 domain of the MATα2 transcriptional repressor and β-galactosidase[25]. We therefore tested degradation of this fusion protein in *sec61-2*, *sec63-1* and in wild-type cells at 25 °C. The decrease of β-galactosidase activity was equal in all strains (Fig. 3d). Also, when analysed in a pulse-chase experiment, the fusion protein was degraded in both mutants as in wild-type cells (data not shown). These experiments demonstrate that the proteolytic machinery was intact in the *sec61-2* and *sec63-1* strains. This result provides further support for the idea that the stabilization of lumenal CPY* in the *sec61-2* and the *sec63-1* background is due to a delayed export of the protein into the cytosol.

The *sec61-2* allele codes for a mutated Sec61 protein, which itself undergoes ER-associated degradation at the restrictive temperature of 38 °C (ref. 4). We examined the Sec61p steady-state level at this temperature and at 25 °C, at which degradation of CPY* is already dramatically retarded (Fig. 1a). Even at 25 °C, the steady-state level of Sec61p was considerably reduced as compared with the wild type, but no reduction of Sec61p was seen in *sec62* or *sec63-1* mutant cells (Fig. 4a). Therefore, even at the permissive temperature of 25 °C, proteasome-dependent degradation of the mutated Sec61 protein seems to occur. We cannot completely exclude the possibility that the *sec61-2* mutation directly affects the binding of a protein required for the export process, but the finding of a lowered steady-state level of Sec61p in the *sec61-2* mutant cells might provide a more likely explanation for our results: that the retrograde transport of CPY* is affected at 25 °C in the mutant background, whereas import is not. In wild-type cells, about 40–60% of the total Sec61p population was found to be either associated with ribosomes or assembled with the Sec62p/Sec63p subcomplex. About 30–50% of Sec61p remains assembled in the trimeric complex, but is probably not involved in protein translocation[15]. This latter portion of Sec61p may be recruited easily for the export of ER degradation substrates without affecting import in wild-type cells. However, in the *sec61-2* mutant at 25 °C, the free pool of Sec61p may be reduced, but the absolute amount of Sec61p transloci involved in co- or post-translational transport is not affected, as protein translocation is essential for cell viability. In contrast, ER-associated degradation is not essential for viability[4,5,26]. Because priority is given to protein import, the cell must have tools to tightly regulate import as well as export. Taking this model into account, we cannot completely exclude the possibility that the export defects of CPY* seen with the *sec63-1* and *kar2-113* mutants also have their basis in a reduced number of transloci available for retrograde transport. However, given that *sec63-1* and *kar2-113* mutant cells do not accumulate CPY* precursor molecules even at 30 °C (data not shown), we assume that there is a large amount of free transloci in these mutants at 25 °C, the temperature at which the experiments were done. We therefore suppose that Kar2p and Sec63p are directly involved in the retrograde transport of CPY*.

Like the degradation of CPY* (ref. 5), proteolysis of unassembled Sec61p occurs through the Ubc6–Ubc7 proteasome degradation pathway[4]. We investigated whether export and degradation of Sec61-2p also depends on additional components known to be required for CPY* degradation. The *DER3* gene product, which is identical to that of *HRD1* (ref. 27), has been shown to reside in the ER membrane, and is essential for degradation of CPY* (J.B., R.K.P., A. Finger and D.H.W., unpublished data). We introduced a null allele of *DER3* into the *sec61-2* mutant strain. Although at the restrictive temperature of 38 °C the steady-state level of Sec61-2p is dramatically lowered in *DER3* cells, Sec61-2p reaches wild-type levels in the *der3* deletion strain (Fig. 4b). Together with the finding that, in the absence of Der3p, *sec61-2* cells are able to grow at 38 °C (J.B., R.K.P., A. Finger and D.H.W., unpublished data), these results indicate that Der3p is also required for the degradation of mutated Sec61p. This finding demonstrates that an intact ER-degradation machinery is present in *sec61-2* mutant cells, even at 38 °C. Indeed, Der3p is present in the *sec61-2* background in wild-type amounts (data not shown). It seems likely that under restrictive conditions, and to a lesser extent under the permissive conditions of 25 °C, unassembled Sec61p chains enter, probably through lateral gating, still intact transloci to become accessible to the proteasome.

It has also been proposed that retrograde transport requires Sec61p in higher eukaryotic cells[11]. In cells expressing the human cytomegalovirus protein US2, or treated with dithiothreitol, the MHC class I heavy chain is rapidly degraded. When proteasome inhibitors were applied, a non-glycosylated cytosolic breakdown intermediate accumulated in US2-expressing cells. This intermediate was co-immunoprecipitated with Sec61p. In cells treated with dithiothreitol, full-length glycosylated heavy chain was found to be complexed with Sec61p. Our results provide functional support for the idea that components of the translocon are involved in retrograde protein translocation. Although we cannot exclude categorically some indirect effects, our data strongly suggest that Sec61p, and probably also Sec63p and Kar2p, are components of a universally acting subcomplex mediating retrograde transport out of the ER for subsequent degradation of proteins that are not properly folded. It is possible that the direction of transport through the ER membrane is determined by the subunit composition of the translocon. Several transmembrane proteins involved in ER degradation have been described, but their detailed function is still unknown[26,27]. These proteins could interact with the central components of the Sec61p translocon, resulting in a complex that is then programmed for retrograde transport. The nature of this complex may be probed once it becomes possible to isolate transloci in the state of export. □

Methods

Construction of strains and plasmids. Genetic experiments were performed using standard methods[28]. The wild-type strain used in this study was W303-1C (*MATα, ade2-loc ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 prc1-1*)[26]. W303-1Ca (*MATa, ade2-loc ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 prc1-1*) was provided by M. M. Hiller. YFP338 (*MATα, sec61-2 leu2-3,112 ura3-52 trp1-1 his4-401 HOL1-1*) was provided by M. D. Rose. RSY529 (*MATα, sec62 leu2-3,112 his4-401ura3-52*), RSY151 (*MATα, sec63-1 leu2-3,112 ura3-52 pep4-3*) and RSY926 (*MATα, Δsec71::LEU2 ura3-52 lys2-801 his3Δ200 leu2Δ1 trp1Δ63 ade2-101*) were provided by R. Schekman. YRP086 (*MATα, sec61-2 ade2-loc ura3his3-11,15 leu2-3,112 trp-1 can1-100 prc1-1*), YRP087 (*MATα*, sec62 *ade2-loc ura3 his3-11 leu2-3,112 trp1-1 can1-100 prc1-1*), YRP088 (*MATα, sec63-1 ade2-loc ura3 his3-11,15 leu2-3,112 trp1-1 can1-100 prc1-1*) and YRP090 (*MATα, Δsss1::ADE2* pGAL*SSS1* (*LEU2 ARS/CEN* (pKF31)) *ade2-loc ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 prc1-1*) were derived from 4-fold crosses of YFP338, RSY529, RSY151 and YTX138 (*Δsss1::ADE2* (*LEU2 ARS/CEN* (pKF31)) *his3-11-15 trp1-1 ura3-1ade2-1 can1-100*) with W303-1C and W303-1Ca, respectively, followed by selection for the temperature-sensitive phenotype and the W303-1C markers. The plasmid pKF31 was a gift from K. Finke. YRP091 (*MATα, Δsec71::LEU2 ade2-loc ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 prc1-1*) was constructed by transforming W303-1C with the *sec71::LEU2* construct isolated from RSY926 by PCR. YRP146 (MATα, kar2-113 *ade2-loc ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 prc1-1*) was constructed as described[29], and plasmid MR780 was provided by M. D. Rose. YRP105 (*MATα,, Δder3::HIS3 sec61-2 ade2-loc ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 prc1-1*) was constructed by transforming YRP086 with plasmid puc-del3 (*der3::HIS3*). That all homologous recombination events were correct was confirmed by Southern blotting.

Pulse-chase experiments and immunoprecipitations. For pulse-chase experiments, 2.5 A_{600} cells were taken from a logarithmically growing culture for each time point and were labelled with 62.5 μCi [^{35}S]-methionine. Growth, labelling, chase conditions and other experimental procedures, such as cell lysis, immunoprecipitation and SDS–PAGE, were performed as described[13].

Deglycosylation experiments and western analysis. Cells were grown at the indicated temperature in complete synthetic medium containing 2% glucose to an A_{600} of 3.0. Immunoprecipitation and deglycosylation of CPY* was performed as described[5]. Immunoprecipitated material was boiled in 50 μl UREA buffer before SDS–PAGE using a 8% gel and blotting. For western analysis, detection of the indicated proteins was performed using the respective antibodies.

Protease protection experiments. Spheroplasting and cell breakage were done as described[5]. For protease treatment of the pellet, trypsin was added to a final concentration of 0.5 mg ml^{-1} after resuspension of the pellet. The samples were incubated for 30 min on ice. If added, Triton X-100 was present at 1%. All treatments were stopped by TCA precipitation. After resuspending the pellet in 100 μl UREA buffer, CPY* was analysed by SDS–PAGE and immunoblotting.

β-Galactosidase activity test. After adding cycloheximide to a final concentration of 0.5 mg ml^{-1} at zero time ($t = 0$) to the logarithmically growing culture, 0.3 A_{600} of cells were taken for each time point, mixed with lysis buffer (0.6% Triton X-100, 0.75% ONPG, 2.25% β-ME, 0.15 M Tris-BCl, pH 7.5) and kept at −80 °C for 30 min. After incubation for 60–90 min at 37 °C, 75 μl of 1 M $NaHCO_3$ was added to the samples, debris was removed by centrifugation (20,000*g*, 3 min) and A_{405} was determined.

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Correspondence and requests for materials should be addressed to D.H.W. (e-mail: dieter.wolf@po.uni-stuttgart.de).

Marking of active genes on mitotic chromosomes

Emil F. Michelotti*, **Suzanne Sanford & David Levens**

Gene Regulation Section, Laboratory of Pathology, National Cancer Institute,
National Institutes of Health, Building 10, Room 2N105, Bethesda,
Maryland 20892, USA

During development and differentiation, cellular phenotypes are stably propagated through numerous cell divisions[1]. This epigenetic 'cell memory' helps to maintain stable patterns of gene expression[2]. DNA methylation[3] and the propagation of specific chromatin structures may both contribute to cell memory[4]. There are two impediments during the cell cycle that can hinder the inheritance of specific chromatin configurations: first, the pertinent structures must endure the passage of DNA-replication forks in S phase[5]; second, the chromatin state must survive mitosis, when chromatin condenses, transcription is turned off, and almost all double-stranded DNA-binding proteins are displaced[6,7]. After mitosis, the previous pattern of expressed and silent genes must be restored. This restoration might be governed by mass action, determined by the binding affinities and concentrations of individual components. Alternatively, a subset of factors might remain bound to mitotic chromosomes, providing a molecular bookmark to direct proper chromatin reassembly. Here we analyse DNA at transcription start sites during mitosis *in vivo* and find that it is conformationally distorted in genes scheduled for reactivation but is undistorted in repressed genes. These protein-dependent conformational perturbations could help to re-establish transcription after mitosis by 'marking' genes for re-expression.

DNase I-hypersensitive sites in chromatin are useful indicators of gene activity and may persist during mitosis, despite cessation of transcription and dissociation of most transcription factors from mitotic chromosomes[6]. Many DNase I-hypersensitive sites are also sensitive to S1 nuclease and hence may have single-stranded features[8–10]. Melted regions of the human c-*myc* gene have been mapped in unsynchronized cells and shown to bind transcription factors *in vitro*[10,11]. During mitosis, the single-stranded properties of chromatin increase ~10-fold[12]. To test whether any mitosis-specific increase in single-stranded character might occur in previously characterized S1-sensitive regions of the human c-*myc* gene, bases reactive to potassium permanganate[10,11] were mapped by using ligation-mediated polymerase chain reaction (LM-PCR). Factors binding to such melted regions during mitosis may contribute to the inheritance of the c-*myc* gene chromatin structure through this

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* Present address: Genelabs Technologies, 505 Penobscot Drive, Redwood City, California 94043, USA