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The yeast *SSS1* gene is essential for secretory protein translocation and encodes a conserved protein of the endoplasmic reticulum

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The *SEC61*, *SEC62* and *SEC63* yeast gene products are membrane components of the apparatus that catalyses protein translocation into the endoplasmic reticulum (ER). In the hope of uncovering additional components of the translocation apparatus, we sought yeast genes whose overexpression would restore partial thermoresistance in a *sec61* translocation-deficient mutant. The first extragenic Sec sixty-one suppressor, *SSS1*, is an essential single copy gene whose overexpression restores translocation in the *sec61* mutant. Another extragenic suppressor was identified as *TDH3*, which encodes the major isozyme of the most abundant yeast protein, glyceraldehyde-3-phosphate dehydrogenase. *TDH3* overexpression could exert an indirect effect by competitively inhibiting protein synthesis, thereby allowing the impaired translocation apparatus to cope with a reduced flow of newly synthesized secretory proteins. Depletion of the Sss1 protein rapidly results in accumulation of multiple secretory or membrane proteins devoid of post-translational modifications; the normally secreted α-factor accumulates on the cytosolic side of ER membranes. Thus, the *SSS1* gene is required for continued translocation of secretory preproteins beyond their early association to ER membranes. Consistent with its essential role in protein translocation, the Sss1 protein localizes to the ER and homologues were detected in higher eukaryotes.

Key words: endoplasmic reticulum/*Saccharomyces cerevisiae*/secretion/suppressor/translocation

Introduction

The earliest event in the export of secretory proteins from eukaryotic cells is their transport across or insertion into the endoplasmic reticulum (ER) membrane, followed by signal peptide cleavage, glycosylation and folding. How proteins actually cross or are integrated into lipid bilayers remains largely unknown, but is thought to be catalysed by integral membrane proteins. In the past few years, genetic selections uncovered *Saccharomyces cerevisiae* temperature-sensitive lethal mutants (*sec61*, *sec62*, *sec63* and *sec65*) that fail to localize properly a signal peptide bearing cytosolic enzyme chimera to the ER lumen. The corresponding four genes were shown to be required for translocation of several precursor proteins (Deshaies and Schekman, 1987; Rothblatt *et al.*, 1989; Stirling *et al.*, 1992). *SEC65* encodes a component of the yeast signal recognition particle (SRP) (Hann *et al.*, 1992; Stirling and Hewitt, 1992), which catalyses precursor targeting to the ER membrane. The products of the *SEC61*, *SEC62* and *SEC63* genes were found as constituents of the translocation apparatus in a biochemical study that also indicated that this apparatus may comprise other, as yet genetically unidentified, subunits (Deshaies *et al.*, 1991).

The authentic *SEC61* gene was cloned by complementation of the thermosensitive (Ts^-) growth phenotype of the *sec61-2* mutant. Further analysis of *SEC61* revealed an essential gene that encodes a 53 kDa polytopic integral membrane protein (Stirling *et al.*, 1992). In an effort to identify new components of the translocation apparatus, we further isolated from yeast genomic libraries five clones that conferred partial thermoresistance upon the *sec61-2* mutant. We show that these five clones contain two distinct extragenic suppressors, *SSS1* and *SSS2* (for Sec sixty-one suppressor). While overexpression of both wild-type *SSS* genes relieves partially the thermosensitivity associated with a *sec61* mutation, only *SSS1* also restores protein translocation in mutant cells. Identification of *SSS2* revealed its indirect effect on the *sec61* mutant. We found *SSS1* to be a novel gene that is essential for the translocation/insertion process.

Results

Isolation of sec61 extragenic suppressors

Previous work had suggested the existence of genetically unidentified components of the yeast translocation apparatus (Deshaies *et al.*, 1991). We attempted to clone genes involved in protein translocation by complementation of the Ts^- growth phenotype of the *sec61-2* translocation mutant, both from centromeric (i.e. low copy) and episomal (i.e. high copy) genomic libraries. In addition to the authentic *SEC61* gene, cloning from the YCp50-based centromeric library (Stirling *et al.*, 1992) yielded one extragenic suppressor clone, FKp28. Four high copy extragenic suppressor clones were also obtained from a YEp13-based high copy library (Nasmyth and Tatchell, 1980), FKp29, 31, 32 and 33. Based on their restriction maps (Figure 1A) and on cross-hybridization studies, it appeared that FKp28, 29, 31 and 32 contained a common fragment of 4.3 kb. Full *sec61-2* complementing activity was found by subcloning to reside in the 2.5 kb *SalI–EcoRI* fragment. The 1.3 kb minimal complementing region was thus defined as the overlap between the 2.5 and 4.3 kb fragments. The gene responsible for this complementing activity was named *SSS1*. Subsequent manipulations of the *SSS1* gene made use of FKp59, constructed by inserting into pUC19 the 2.8 kb

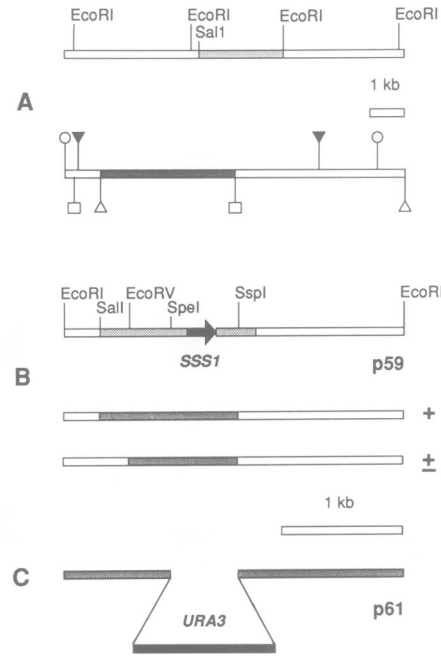

Fig. 1. (**A**) Map of the chromosomal region containing the *SSS1* gene. The FKp28 (△), FKp29 (○), FKp31 (□) and FKp32 (▼) plasmids were analysed by restriction digestion. Their inserts defined together a single chromosomal region of 11 kb whose map is shown above. The 2.5 kb *Sal*I−*Eco*RI fragment (grey box) contains the whole *sec61* complementation activity. The boundaries of the four original inserts are shown below. They overlap along 4.3 kb (dark grey box). (**B**) Detailed map of the central *Eco*RI fragment. The *SSS1* reading frame is highlighted by a black arrow. The sequenced portion is marked by a grey box. FKp59 contains the full 2.8 kb *Eco*RI fragment in pUC19. A 1134 bp *Sal*I−*Ssp*I and a 903 bp *Eco*RV−*Ssp*I fragment were released from FKp59 and inserted into centromeric or episomal vectors. These subclones were tested for complementation of the *sec61-2* Ts⁻ phenotype. The result is summarized on the right. (**C**) Construct used for *SSS1* disruption: a 1.17 kb *URA3*-containing fragment replaced in either orientation the 557 bp *Spe*I−*Ssp*I fragment from FKp59, giving rise to FKp61. The modified *Eco*RI fragment was used for disruption studies.

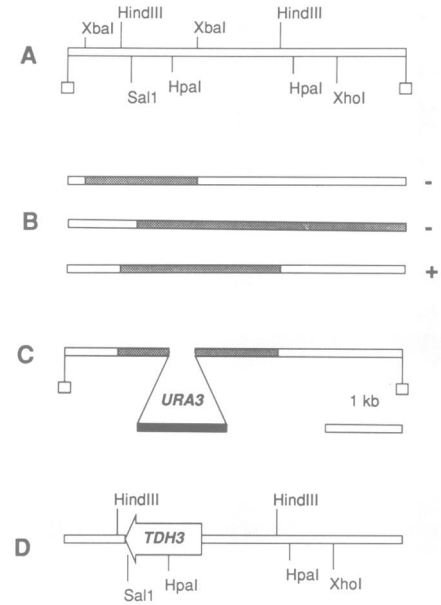

Fig. 2. Map of the FKp33 plasmid containing the *SSS2* gene. (**A**) Restriction map of the 4.4 kb insert of FKp33. (**B**) Subclones in a high copy vector were tested for complementation of the *sec61-2* Ts⁻ phenotype. The result is shown on the right. (**C**) Construct used for *SSS2* disruption: a 1.17 kb *URA3*-containing fragment replaced the 350 bp *Hpa*I−*Xba*I region. The modified *Hin*dIII fragment was used for disruption studies. (**D**) Map of the *TDH3* locus, showing the extent and orientation of this gene (from Holland and Holland, 1979).

*Eco*RI fragment from FKp32. The restriction map of FKp33 indicated that it contained a different gene, named *SSS2* (Figure 2A). Subcloning of FKp33 showed that the minimal complementing region was a 2.1 kb *Hin*dIII fragment, containing an essential ≈140 bp *Hin*dIII−*Sal*I left side (Figure 2B).

Suppression activity

Suppression by the *SSS* genes did not extend to the *sec62* or *sec63* mutants. To determine the mode of suppression, restoration of temperature resistance and protein translocation in the *sec61-2* (Deshaies and Schekman, 1987) or *sec61-3* (Stirling *et al.*, 1992) mutants was quantified. *SSS1* overexpression increased the transition temperature of both *sec61* mutants by 1°C in low copy number and by 2.5°C in high copy number. *SSS2* overexpression increased the transition temperature of *sec61-2* by 1°C in high copy number and had no effect in low copy number. No reproducible effect of *SSS2* was observed on *sec61-3*. *SSS2* overexpression was apparently toxic to yeast cells, as *SSS2*-bearing plasmids were very rapidly lost under non-selective growth conditions (not shown).

The translocation capacity exhibited by *sec61-2* at 37°C, in the presence of suppressor plasmids, was evaluated by immunoblot of whole cell proteins (Figure 3). As described earlier (Deshaies and Schekman, 1987), the *sec61-2* mutant accumulated intracellularly the precursor form of the α-factor pheromone (lane 1). This translocation block was relieved entirely by plasmid expression of wild-type *SEC61* (lane 2) and to a great extent by low or high copy number *SSS1* (lanes 3−4). In contrast, high copy number *SSS2* showed very little effect (lane 5).

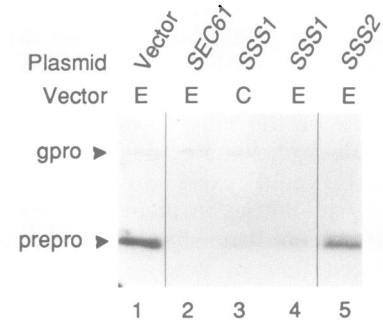

Fig. 3. The *sec61-2* translocation block is relieved by *SSS1* overexpression. Strain RDM 15-5B was transformed with plasmids containing various genes, based either on episomal (E) or centromeric (C) vectors. The transformants were grown overnight at 24°C to early log phase. The cultures were shifted to 37°C for 2 h. Cells were then lysed and the whole protein extracts were loaded on a 12.5% gel. After migration, the proteins were blotted onto nitrocellulose and probed with anti-α-factor antibodies. The immunoblot was visualized with [^{125}I]protein A. The transforming plasmids were, from left to right: vector alone, pCS6−1, FKp28, FKp29 and FKp33. Prepro, unprocessed; gpro, glycosylated pro-α-factor.

Identification of the SSS2 gene

The small to negligible effects of *SSS2* overexpression on the *sec61* mutants both in terms of temperature sensitivity

and of translocation block suggested an indirect mechanism of suppression. Gene disruption analysis (Figure 2C) showed that the *SSS2* gene is not essential for yeast vegetative life, even in a *sec61-2* mutant context, presumably because the genome harbors two highly homologous genes, as seen by high stringency Southern blotting (not shown). Surprisingly, sporulation of diploid cells bearing one disrupted *SSS2* copy resulted in inefficient germination of spores (40% instead of >85%). Half of the germinated spores bore a disrupted *SSS2* copy, indicating that the loss of germination potential was caused by the decreased gene dosage in the original diploid cell.

The *SSS2* gene was identified as *TDH3* by subcloning and partial sequencing (not shown). In particular, note that the 3'-end of the *TDH3* open reading frame (ORF) falls in the ≈140 bp *Hin*dIII−*Sal*I region that is essential for the *sec61* complementing activity of *SSS2* (Figure 2, compare panels A and B with D). *TDH3* is the most expressed of the three yeast *TDH* genes that encode the glyceraldehyde-3-phosphate dehydrogenase isozymes (Holland and Holland, 1979; McAlister and Holland, 1985a). The *TDH* genes are ~90% homologous to each other (Holland and Holland, 1980; Holland *et al.*, 1983). Insertion disruption studies allowed McAlister and Holland (1985b) to conclude that a functional *TDH2* or *TDH3* allele is sufficient for cell growth.

SSS1 gene and transcript

The DNA sequence of the 1.3 kb *SSS1*-containing region (Figure 1A) was determined on both strands, starting at the *Sal*I site (Figure 4A). Computer-aided analysis of the sequence identified an ORF that potentially encodes an 80 amino acid residue polypeptide (Figure 4B) with a predicted molecular mass of 8.9 kDa. This *SSS1* reading frame extends from coordinates 702−941, shows no consensus splicing signal and displays a high codon adaptation index with respect to the *S.cerevisiae* codon usage: 0.44 on average (Sharp and Li, 1987). The only other potentially coding ORF (index 0.18) within the sequenced 1271 bp ends at coordinate 399 and is followed by transcription termination signals. This ORF starts either at coordinate 169 (translating into 77 amino acid residues) or upstream of the *Sal*I site (over 133 residues) and would encode a protein with an extremely acidic carboxyl-terminus, displaying limited homology to a mammalian extracellular matrix glycoprotein called tenascin (Saga *et al.*, 1991; Siri *et al.*, 1991; Weller *et al.*, 1991). Further subcloning demonstrated that this other ORF is not responsible for the *sec61* complementing activity (Figure 1B). Two subclones were derived from FKp59 that extend from the 3'-flanking *Ssp*I site to either the *Sal*I site (1134 bp) or the *Eco*RV site (903 bp). The latter insert contains only the last 165 bp of this second ORF but was found to suppress thermosensitivity in *sec61-2* as did the larger insert and the original clone, when borne on a high copy vector. Surprisingly, under the more discriminating conditions brought about by suppression on low copy vectors, only the larger insert showed the usual level of suppression. Deletion of the 231 bp *Sal*I−*Eco*RV segment resulted in a complete loss of low copy suppression activity.

It appears therefore that promoter elements that are important for full expression of the *SSS1* gene are located upstream of the *Eco*RV site, i.e. >470 bp from the translation start codon (Figure 4B). One candidate upstream activating sequence (UAS) in this region is located at

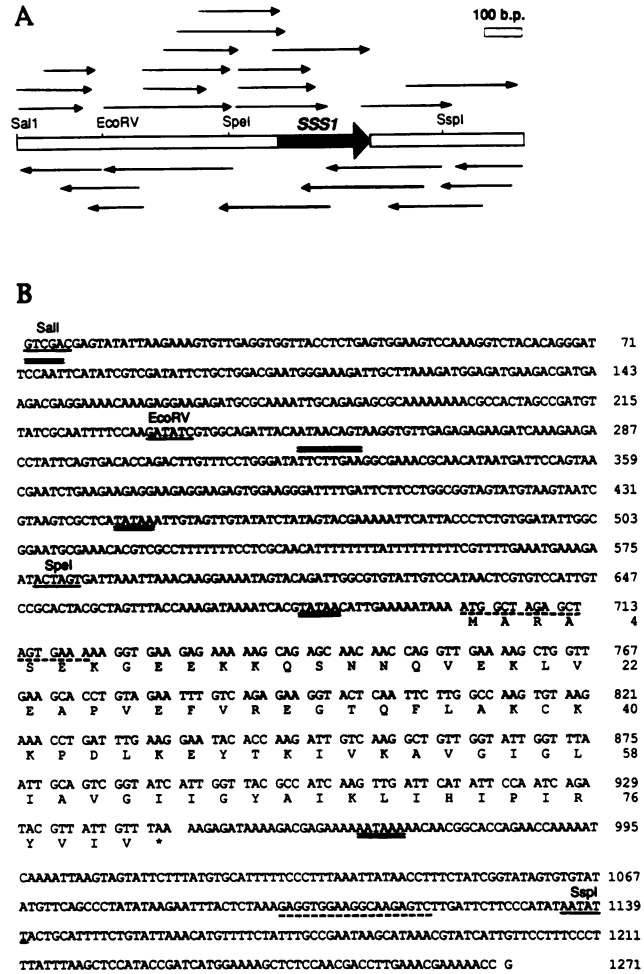

Fig. 4. (**A**) Strategy used for sequencing *SSS1*. The solid arrow represents the extent and orientation of the *SSS1* reading frame. Starting at the *Sal*I site, 1271 bp were sequenced on both strands, as shown by the thin arrows. (**B**) Nucleotide sequence of *SSS1*. A single strand of sequence is shown, with nucleotide coordinates indicated on the right. Restriction sites are underlined. Broken underlines indicate the two 19 bp sequences that served as borders for amplification of the *SSS1* gene. Potential TATA boxes and polyadenylation site are doubly underlined. A putative CCAAT UAS and an 8 bp stretch fitting the heat-shock response element consensus are doubly overlined. The predicted amino acid sequence is shown in single letter code, starting with a translation initiation codon at 702 bp and extending till a termination codon at 941 bp. This sequence is registered with the EMBL gene bank (accession number, X74499).

coordinate 73 (CCAAT), 629 bp upstream of the start codon, a long distance by yeast gene standards (Struhl, 1987). A stretch of eight nucleotides (TTCTtGAA) lies at coordinate 323, i.e. 379 bp upstream of the start codon and matches the consensus heat shock responsive element found 201−383 bp upstream of the start codon in the promoter of yeast heat shock genes (Tuite *et al.*, 1988). However, the intracellular level of the *SSS1* gene product, monitored by immunoblotting, is not significantly altered by a heat shock or by growth temperatures ranging from 24 to 37°C (not shown). Putative transcription initiation elements (TATA boxes) are located at coordinates 443 and 683, respectively 259 and 19 bp upstream of the start codon. TATA boxes are usually found 40−120 bp upstream of the yeast transcription initiation site (Struhl, 1987). A potential polyadenylation signal is located at coordinate 966, 25 bp downstream of

the TAA translation stop codon. Finally, a putative transcription termination element is present between coordinates 1006 and 1091, 65−150 bp downstream of the stop codon. Northern hybridization analysis, using a *Spe*I−*Ssp*I probe derived from FKp59, identified a single RNA species of ≈450 bases, consistent with the transcription elements described above. The abundances of the *SSS1* and *SEC61* transcripts are comparable, and lower than those of the *ACT1* and *URA3* transcripts (not shown).

SSS1 is an essential single copy gene

Sss1 mutants were required for the functional analysis of the gene. We first examined the phenotype of a null mutant. A null *sss1::URA3* allele was constructed by deleting from FKp59 the 557 bp *Spe*I−*Ssp*I fragment and replacing it with the prototrophic marker *URA3* (1.17 kb) in either orientation. The resulting FKp61 plasmid (Figure 1C) was linearized with *Eco*RI and a diploid W303 strain was transformed with the gel-purified fragment (Rothstein, 1983). Ura$^+$ diploid transformants such as FKY128 were selected and subjected to sporulation. Among 37 tetrads dissected, 30 gave rise to two viable spores and seven produced only one. All 67 viable spores were Ura$^-$; that is, none contained the *sss1::URA3* disrupted allele. Moreover, microscopic examination showed that the inviable spores had not initiated budding.

Southern analysis of FKY128 genomic DNA with a 2.8 kb *Eco*RI probe derived from FKp59 confirmed that this diploid strain possessed one wild-type and one disrupted copy of the *SSS1* gene. Six viable spores obtained from three tetrads were found to possess only the wild-type copy of the gene, in agreement with their Ura$^-$ phenotype (not shown). Southern hybridization at lower stringency failed to detect any additional DNA fragment, suggesting the absence of a second *SSS1*-related sequence in the *S.cerevisiae* genome. In the same experiment, the *SSS1* probe detected homologous sequences in genomic DNA isolated from *Kluyveromyces lactis* and digested with *Eco*RI or *Hin*dIII (not shown). Taken together, these results indicate that the *SSS1* gene is essential for spore germination and may be conserved in phylogenetically related yeasts.

Conditional SSS1 expression

The essentiality of the *SSS1* gene allowed a study of its function. We constructed a yeast strain that conditionally expressed the *SSS1* gene. For this purpose, the reading frame and its 3′-flanking region were amplified by action of the Taq polymerase and cloned by placing its start codon downstream of a regulatable promoter borne on FKp103. This hybrid *GAL10−CYC1* promoter is induced by galactose and repressed by glucose, thus allowing a several hundred-fold decrease of gene expression when cells are shifted from galactose to glucose (Pompon, 1988). By cloning the ORF at various distances from the promoter, different levels of induced or repressed expression were obtained. In the proximal construct FKp107, the start codon is 6 bp away from the cloning site nearest to the promoter; in the distal construct FKp106, it is 21 bp away. Both plasmids were transformed in the heterozygous diploid strain FKY128 which bears one disrupted *SSS1* copy. Sporulation of Ade$^+$ transformants was induced on SPOGal plates. The resulting asci were dissected and the spores were allowed to germinate on YPGal plates. All the analysed transformants gave rise to four viable spores, indicating that both plasmids were able to complement the *sss1::URA3* null allele upon galactose induction. The meiotic progeny of either FKp107 or FKp106 transformants were analysed for the ability to grow on glucose- or galactose-based rich media. On YPD plates, FKp107-transformed haploid cells bearing the disrupted *SSS1* chromosomal copy grew as small white (Ade$^+$) colonies, while those bearing the wild-type copy grew to twice as wide, white colonies with red (Ade$^-$) sectors (not shown). It appeared therefore that residual *SSS1* expression upon glucose repression was sufficient and necessary in this proximal construct to allow nearly normal growth in a chromosomal null background. In contrast, the distal construct FKp106 elicited very dissimilar growth on YPD plates: *sss1::URA3* null mutants (Ura$^+$ Ade$^+$, e.g. FKY198) grew only as a few colonies on a YPD plate and as a full patch on a YPGal plate (Figure 5A, right). A wild-type haploid strain (Ura$^-$ Ade$^+$) obtained from the same tetrad grew as full patches on both media (Figure 5A, left). When the null mutant FKY198 was shifted from liquid YPGal to YPD, after an initial acceleration due to the availability of a better carbon source, growth slowed down ~4 h postshift and by 7 h postshift (4.4 mass doublings) it had stopped (Figure 5B). These data indicate that the growth defect of FKY198 on glucose medium is caused by sufficient repression of *SSS1* expression. That 0.01−0.1% of the population escapes from the glucose block may result from either pseudo-reversion or a very high number of FKp106 plasmids per cell in this subpopulation. In fact, it has been shown that the copy level of 2μ-based plasmids

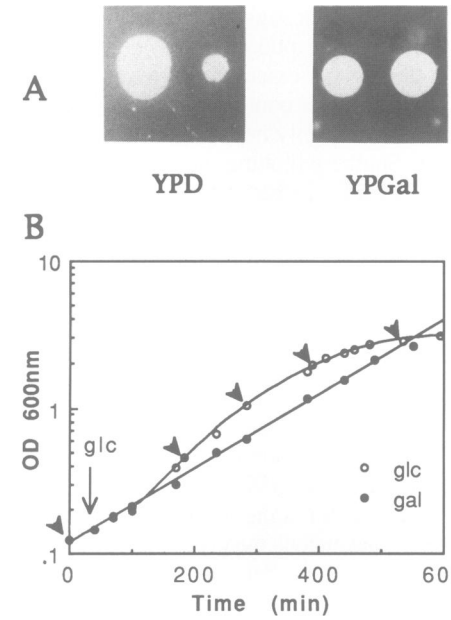

Fig. 5. Conditional *SSS1* expression. **(A)** Meiotic progeny of the *SSS1/sss1::URA3* heterozygous strain FKY128 transformed with FKp106, a plasmid bearing the *SSS1* gene under the control of the *GAL10* promoter. Cultures from one Ura$^-$ spore (*SSS1*, left side) and one Ura$^+$ spore (*sss1::URA3*, FKY198, right side) from the same tetrad were diluted and an equal number of cells was patched on galactose (YPGal) or glucose (YPD). The Ura$^-$ strain grew full patches on both carbon sources, while the Ura$^+$ strain did not grow on glucose, except for a few individual cells. **(B)** Strain FKY198 is glucose-sensitive for growth. The *sss1::URA3* mutant transformed by FKp106 was grown on liquid YPGal at 30°C (●). At OD_{600} ≈0.14 (arrow), half of the culture was collected, washed and resuspended in YPD (○). Both cultures were subsequently grown at 30°C and maintained below OD_{600} 0.5 by successive dilutions. The data were corrected for these dilutions and plotted here in semi-logarithmic coordinates. Arrowheads denote sampling times.

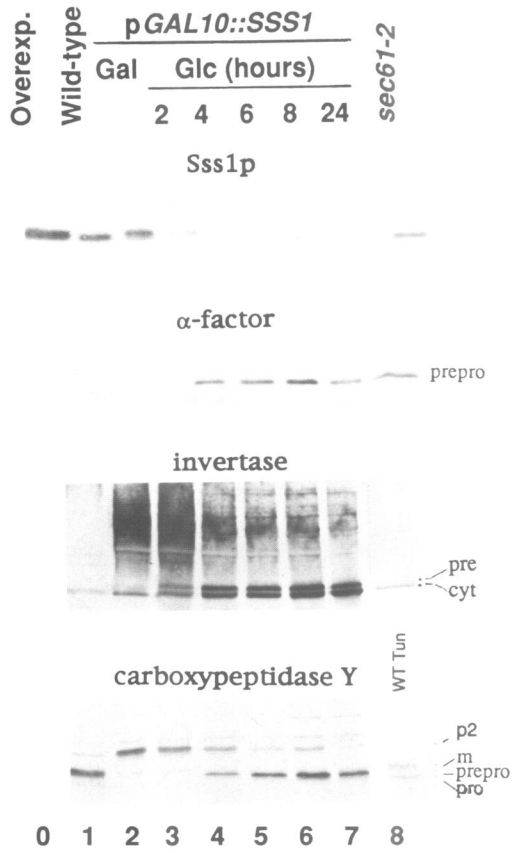

Fig. 6. Depletion of *SSS1* protein causes intracellular accumulation of secretory precursors. Strain FKY198, which contains a chromosomal deletion of *SSS1* and the FKp106 plasmid bearing *SSS1* under the control of the *GAL10* promoter, was grown on galactose. At zero time, it was shifted to glucose to repress the *GAL10* promoter. Samples were taken at ≈2 h intervals (Figure 5). The *SSS1* gene product and three secretory proteins (α-factor, invertase and CPY) were examined by immunoblotting. Controls included the wild-type strain W303 1B (Wild-type), a FKp107 transformant grown on YPGal to overexpress *SSS1* (Overexp.) and a *sec61-2* mutant shifted 2 h at 38°C (*sec61-2*). For the invertase experiment, secretory invertase synthesis in *sec61* was derepressed on 0.1% glucose. Strain W303 1B was also treated with 10 μg/ml tunicamycin for 1 h at 38°C (WT Tun). The corresponding lane is underexposed to precisely show the electrophoretic behavior of the mature and pro forms of CPY. Cyt, cytosolic; prepro, unprocessed; pre, unprocessed; pro, signal peptide cleaved and unglycosylated; p2, outer-chain glycosylated; m, mature.

varies from cell to cell, assuming a fairly broad distribution around the average value (Rose and Broach, 1990).

Reduction of SSS1 expression causes a translocation defect

To determine whether the *SSS1* gene was essential for protein translocation, we examined the secretory phenotype of the FKY198 strain in which synthesis of the wild-type *SSS1* protein could be repressed. Controls included an isogenic wild-type strain and a *sec61-2* mutant, which had similar levels of *SSS1* protein (Figure 6, lanes 1 and 8). On galactose medium, expression of *SSS1* from the hybrid promoter of FKp106 led to nearly wild-type levels of its product (lane 2), whereas expression from FKp107 led to elevated levels (lane 0). Lower *SSS1* expression by FKp106 than by FKp107 probably holds true on glucose medium as well (not measurable experimentally), consistent with the previous observation that FKp106, but not FKp107, conferred glucose sensitivity upon *sss1* null mutants. After shifting to glucose, aliquots were removed from the FKY198 culture at 2 h intervals, as shown by arrowheads on Figure 5B. The level of the *SSS1* gene product was reduced to undetectable levels in 6 h (~4 mass doublings), suggesting some turnover of this protein, beyond its mere dilution by cellular mass increase (Figure 6, lanes 2–7). The fate of several well-characterized secretory proteins was monitored in this culture by immunoblotting of whole cell extracts.

Carboxypeptidase Y (CPY) is a vacuolar protease that is synthesized as a 59 kDa precursor called prepro-CPY. CPY is directed to the lumen of the ER by a signal peptide that is removed, giving rise to the 57 kDa pro-CPY. Core glycosylation in the ER yields the 67 kDa p1 form (Stevens *et al.*, 1982). Limited outer chain glycosylation in the Golgi body results in the 69 kDa p2 form. Upon subsequent arrival to the vacuole of wild-type cells, the p2 form is cleaved by the *PEP4* gene product, giving rise to the 61 kDa mature CPY (Ammerer *et al.*, 1986; see Figure 6, lane 1). Treatment of wild-type cells with the N-linked glycosylation inhibitor tunicamycin resulted in accumulation of non-glycosylated pro-CPY, in addition to some pre-existing mature form (lane 8). To allow unambiguous electrophoretic resolution of prepro-CPY from mature CPY, the *PEP4* gene was inactivated by chromosomal disruption in the FKY198 strain. The *pep4*-Δ strain was first grown on galactose to allow expression of the *SSS1* gene. Under these permissive conditions of growth, normal formation of p2 CPY was observed (lane 2). A species appeared after 4 h of growth on glucose (lanes 4–7), that migrated just between the mature and pro- forms (lanes 1 and 8), consistent with the position expected of prepro-CPY. Concurrent to depletion of the *SSS1* protein and to accumulation of this CPY precursor, the p2 band intensity decreased, presumably due to dilution of the preexisting vacuolar p2 CPY as cellular mass increased in the culture (lanes 2–7).

Invertase is synthesized in two forms, an inducible secretory form and a constitutive cytosolic form. The secretory form differs from the cytosolic one by the presence of a signal peptide which directs it to the lumen of the ER. This pre-invertase undergoes signal peptide cleavage and core glycosylation in the ER, followed by extensive outer chain mannosylations in the Golgi body, before reaching its final periplasmic location (Esmon *et al.*, 1987). Since expression of secretory invertase is repressed by glucose, it was impossible to express pre-invertase under the control of its own promoter, while repressing *SSS1*. We therefore integrated in the genome of strain FKY198 a construct bearing the *SUC2* gene encoding invertase under the control of the strong and constitutive *TPI* gene promoter. The discrete lower band seen on the invertase immunoblot of control protein extracts (Figure 6, lanes 1, 2 and 8) corresponds to the constitutive cytosolic form. In the p*TPI::SUC2* strain grown on galactose, the expected discrete ER forms and heterogeneous mature forms are present (lane 2). After shifting cells to glucose, a new 61 kDa species appeared within 2 h just above the 59 kDa cytosolic form (lane 3), thus co-migrating with the precursor form of invertase that accumulates in the derepressed *sec61-2* mutant shifted to non-permissive temperature (lane 8). As previously observed, invertase was not fully blocked in this Ts^- mutant (Deshaies and Schekman, 1987). The appearance of the 61 kDa pre-invertase reached a maximum by 4 h (lane 4). A strong, albeit gradual, decrease in the amount of glycosylated forms was also observed (lanes 3–7),

compatible with dilution of the pre-existing periplasmic material by mass increase. Surprisingly, a slow increase of the 59 kDa form was observed between 4 and 24 h postshift (lanes 4–7). Since the ratio of cytosolic invertase over total protein is invariant, this new co-migrating form is likely to result from proteolytic cleavage of accumulated secretory pre-invertase. This interpretation would suggest either that the accumulated pre-invertase is in a second step slowly converted by signal peptidase into invertase, or that a cytosolic protease is capable of performing a similar proteolytic cleavage.

The α-factor mating pheromone is encoded by two genes that translate into identical proteins of 21 kDa, prepro-α-factor (Kurjan and Herskowitz, 1982; Singh *et al.*, 1983). Translocation of this precursor into the ER is followed by cleavage of its signal peptide, giving the 19 kDa pro-α-factor, and core glycosylation, resulting in the 26 kDa ER form. This ER form is transported to the Golgi body where it undergoes outer chain glycosylation and proteolytic maturation. Final processing in a late Golgi compartment produces a mature peptide which is excreted from the cell (Julius *et al.*, 1984). The whole process is normally very rapid and α-factor intermediates could not be trapped in a wild-type strain (Figure 6, lane 1). Concurrent with dilution of the *SSS1* gene product, an α-factor species started to accumulate in the conditional mutant after shifting to glucose (compare lane 2 with lanes 3–7). This species had the same apparent mobility as the 21 kDa prepro-α-factor produced in a *sec61-2* mutant (lane 8). The very weak precursor accumulation seen on galactose (lane 2) may have resulted from the small subpopulation, occurring at each generation that had lost the FKp106 episome (Rose and Broach, 1990).

Whereas immunoblotting allows measurement of the total cellular level of a given protein, radiolabelling/immunoprecipitation allows access to the rate of cellular accumulation of a newly synthesized protein species. Proteins from wild-type and *sec61-2* strains were labelled after 60 min preincubation at 38°C. Proteins from FKY198 cells were labelled at 30°C before or 210 min after transfer from galactose- to glucose-based medium. Whole cell extracts were prepared and α-factor was immunoprecipitated. The immunoprecipitates were resolved by SDS–PAGE, followed by fluorography. Figure 7A clearly demonstrates that the rate of prepro-α-factor accumulation increased markedly in the *SSS1* conditional mutant when shifted for 210 min from permissive to restrictive conditions. Similar results were obtained with CPY and invertase (not shown). However, this elevated rate of precursor accumulation was still 2–3 times lower than that in the *sec61* mutant shifted for 60 min to the restrictive temperature. This discrepancy probably reflects progressive versus rapid imposition of the translocation block in the conditional expression versus thermosensitive mutants. In fact, we noted above that 210 min postshift was approximately the moment when FKY198 growth started to slow down (Figure 5B).

It was also of interest to test the effect of *SSS1* repression on the insertion into the ER of a membrane protein, in view of the relative resistance of such insertion to *sec* mutations (Stirling *et al.*, 1992). Dipeptidylaminopeptidase B (DPAPB) is an integral membrane glycoprotein whose carboxyl-terminus is lumenally oriented (Roberts *et al.*, 1989). The unglycosylated precursor migrates as a 96 kDa species, and the mature vacuolar form migrates as a 120 kDa species. Radiolabelling and immunoprecipitation of DPAPB from

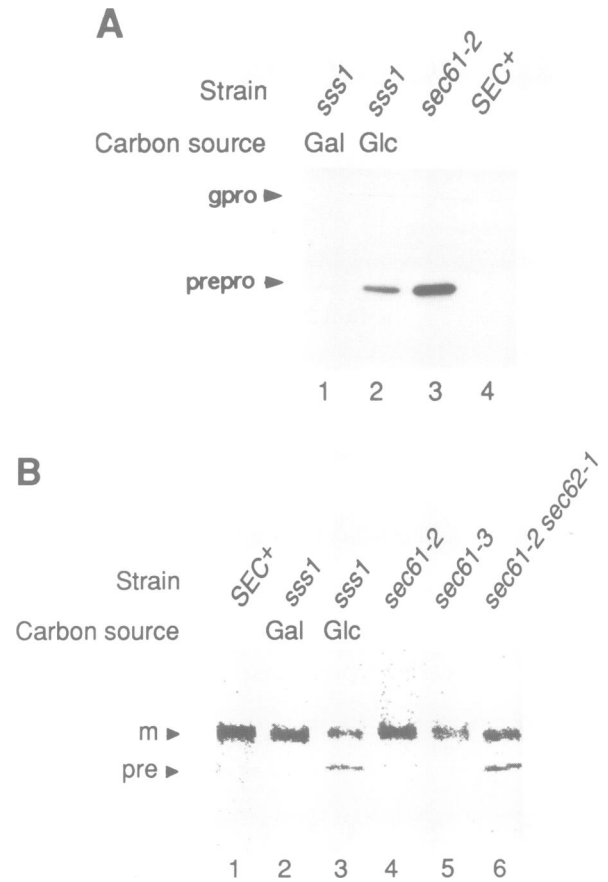

Fig. 7. Rate of accumulation of protein precursors in the *sss1* conditional expression mutant. All the cultures were grown overnight in minimal medium supplemented with 100 μM ammonium sulfate. Strain FKY198 was grown on galactose or shifted for 210 min to glucose. The *sec61* mutants and the wild-type strain were grown on glucose at 24°C and shifted for 60 min to 37°C. The *sec61-2 sec62-1* double mutant was grown at 17°C and shifted for 60 min to 37°C. 10 OD_{600} × ml of each culture were harvested at an OD_{600} of 0.2 and radiolabelled with 0.3 mCi Tran-^{35}S label in 0.4 ml sulfate-free minimal medium for 60 min. Cells were transferred on ice, washed with 10 mM NaN_3 and resuspended in 0.3 ml lysis buffer (1% SDS, 50 mM Tris–Cl pH 7.4, 1 mM EDTA and 1 mM PMSF). They were then lysed by strong agitation with one volume of glass beads for 90 s and samples were heated at 95°C for 5 min. The beads were washed with 0.9 ml washing buffer (17 mM sodium phosphate pH 7.4, 270 mM NaCl and 1% Triton X-100) and pelleted with unbroken cells by a 2 min centrifugation in a Microfuge. The supernatants were immunoprecipitated with suitable primary antibodies. Protein A–Sepharose was added. (**A**) Mutants accumulate α-factor precursor. SDS–PAGE was followed by fluorography. Prepro, unprocessed; gpro, glycosylated pro-α-factor. (**B**) Mutants accumulate DPAPB precursor. SDS–PAGE was followed by analysis with a PhosphorImager (Molecular Dynamics). Pre, unprocessed; m, mature.

FKY198 cells after a shift to restrictive conditions revealed the accumulation of precursor forms (Figure 7B, lane 3) to an extent that was comparable with that seen in a *sec61-2 sec62-1* double mutant (lane 6). In contrast, wild-type cells or FKY198 cells grown under permissive conditions, accumulated no DPAPB precursor (lanes 1 and 2). The *sec61* simple mutants (lanes 4 and 5) showed only a marginal accumulation of the precursor species, which was visible upon longer exposure (not shown). These results are in keeping with those of Stirling *et al.* (1992) and further demonstrate that the assembly of the integral membrane

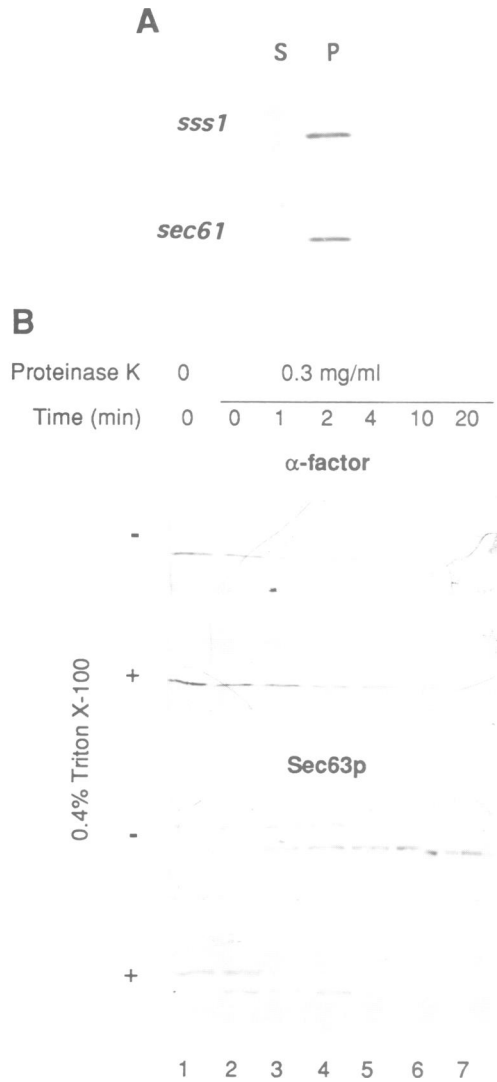

Fig. 8. Fractionation and protease sensitivity of α-factor precursor accumulated at the *sss1* block. After overnight growth in liquid YPGal followed by 4 h *SSS1* repression in YPD, 30 OD_{600} × ml of FKY198 cells were collected at an OD_{600} of 0.3. Cells were washed with 10 mM NaN_3 and resuspended in 0.56 ml spheroplast buffer (1.5 M sorbitol, 50 mM Tris−Cl pH 8, 2 mM $MgCl_2$, 10 mM NaN_3 and 60 mM 2-mercaptoethanol). They were incubated with 250 units of lyticase for 30 min at 30°C. Spheroplasts were harvested on a cushion of spheroplast buffer adjusted to 1.8 M sorbitol by a 5 min 6000 *g* centrifugation and resuspended in 1.6 ml lysis buffer (0.3 M sorbitol, 0.1 M KCl, 50 mM Tris−Cl pH 7.5 and 1 mM EGTA) as described (Deshaies and Schekman, 1987). The spheroplasts were gently lysed in a 5 ml Potter-Elvejem homogenizer (Bioblock Scientific, France) by 15 manual strokes of the tight fitting pestle. Unbroken cells and spheroplasts were pelleted by a 4 min centrifugation at 660 *g*. The cell free supernatant was collected. (**A**) This supernatant was further fractionated by a 60 min centrifugation at 100 000 *g*. The high speed supernatant 'S' was decanted and the pellet 'P' was resuspended in an equal volume of lysis buffer. Proteins from each fraction were separated by SDS−PAGE and immunoblotted with anti-α-factor antibodies. A *sec61-2* mutant shifted 2 h at 38°C was used as a control (bottom). (**B**) Fractions of the low speed supernatant were treated with 0.3 mg/ml proteinase K, in the absence (−) or presence (+) of 0.4% Triton X-100 for 0−20 min on ice. The reactions were stopped by TCA and evaluated by SDS−PAGE and immunoblotting with either anti-α-factor (top) or anti-Sec63p (bottom) antibodies.

protein DPAPB is partially blocked by *SSS1* protein depletion.

Secretory proteins accumulated at the *sss1* block are not glycosylated; their signal peptides have not been cleaved off, except perhaps for a subpopulation of invertase after long incubation times. These properties are expected for polypeptides that either have not yet engaged into or have only partly penetrated through the ER membrane. To distinguish between these two possibilities, the sedimentation behaviour of prepro-α-factor was examined in FKY198 cells after 4 h of *SSS1* repression. The cells were converted to spheroplasts and gently lysed. Half of the cell-free lysate was subjected to high speed centrifugation. After SDS−PAGE and immunoblotting, most of prepro-α-factor was found with the particulate pellet (Figure 8A). In agreement with Deshaies and Schekman (1987), a similar observation was made with *sec61-2* cells shifted 2 h at 38°C (Figure 8A). To test whether pellet-associated prepro-α-factor was exposed to the cytosolic compartment, the second half of the cell-free lysate was treated with protease in the absence and presence of detergent. Aliquots were quenched with TCA, subjected to SDS−PAGE and immunoblotted with anti-α-factor antibodies (Figure 8B). Upon exposure to proteinase K for increasing lengths of time, the amount of *sss1*-accumulated prepro-α-factor decreased gradually with similar kinetics in the presence or absence of detergent. Membrane integrity was confirmed by an internal control, the integral ER membrane protein Sec63p. In the absence of detergent, only the cytosolic carboxy-terminal fragment of Sec63p was protease-sensitive, resulting in a smaller polypeptide, as described previously by Feldheim *et al.* (1992). In the presence of detergent, this smaller polypeptide was further degraded, indicating that this form is not intrinsically protease-resistant (Figure 8B).

Thus, both immunoblotting and radiolabelling experiments indicate that *SSS1* protein depletion results in severe defects in intracellular transport of several secretory and membrane proteins, and accumulation of the unmodified precursor species. The precursor proteins appear to be exposed to the cytoplasm and to fractionate with the particulate pellet.

SSS1 encodes a highly conserved protein that localizes to the ER

The *SSS1* gene product (Sss1p) is an 8.9 kDa protein with 26 hydrophobic, 15 basic, nine acidic and 13 other polar residues (Figure 9). The longest stretch containing an excess of hydrophobic residues, bordered by Lys52 and Lys69, is only 16 residues long. With four Gly and one Tyr, this stretch does not exhibit the usual features of a membrane-spanning domain. Using the BLAST algorithm to find locally optimal alignments (Altschul *et al.*, 1990), Sss1p was found to be 36% identical and 60% similar to a 69-residue polypeptide predicted from a rice cDNA sequence that was recently deposited in the databases (T.Sasaki and Y.Minobe, Rice Genome Research Program, unpublished). The similarity extends over the entire length of the shorter protein (Figure 9), but it is higher in the stretches flanking the hydrophobic region. Compared with the rice protein, the yeast Sss1p has an additional 14-residue polar amino-terminus.

Another argument for the evolutionary conservation of Sss1p through the eukaryotic world came from antibody cross-reactivity studies. Polyclonal antisera raised against both the N-terminal and medial peptides ('T + M') of Sss1p detected a single 9 and 8 kDa protein in cellular extracts prepared from the yeast *K.lactis* and from canine pancreas, respectively (Figure 10).

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αααααααααααααααααααααααααααααααααααααααααααααααααα        ßßßßßßßßßßßßßßßßßßßßßßßßßßßßßß
h + p-+ --++ppppph-+hh-  h-hh+- pphh +p++ -h+-pp+hh+ h h hh h hh p h+hh+h h+phhh
        10        20        30        40        50        60        70        80
MARASEKGEEKKQSNNQVEKLVEAPVEFVREGTQFLAKCKKPDLKEYTKIVKAVGIGLIAVGIIGYAIKLIHIPIRYVIV      Yeast

                :. .:.   :: ..  . . .: ::: ::.::.     :: . .: .:. .::: ::: .::
MDAVDSVVDPLREFAKDSVRLVKRCHKPDRKEFTKVAARTAIGFVVMGFVGFFVKLIFIPINNIIVGSG   Rice
          10        20        30        40        50        60
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Fig. 9. Comparison of the yeast Sss1 protein with a rice homologue. The algorithm of Chou and Fasman (1978) predicts for yeast Sss1p a tendency to form α-helices in the amino-terminal 46 residues (α), and β-sheets in the carboxy-terminal 28 residues (β). The polarity of the amino acids in yeast Sss1p is indicated above the primary sequence: h, hydrophobic; +, basic; −, acidic; p, polar and uncharged. Two oligopeptides that were used as synthetic antigens are underlined. The longest hydrophobic stretch is italicized. The rice sequence has been translated from a 362-nucleotide sequence stored in the EMBL database (accession number, D15706). Double dots denote amino acid identities between the aligned sequences, whereas single dots denote similarities.

Immunofluorescence microscopy was performed to determine the intracellular localization of Sss1p in wild-type or *SSS1*-overexpressing diploid cells (Figure 11). Fixed and permeabilized cells were probed with affinity-purified anti-Sss1p immune serum (left) and with the DNA binding dye DAPI (right). Sss1p immunoreactivity in overexpressing cells was localized mostly in a perinuclear area, with some peripheral staining. Occasionally, threads could be seen extending from the perinuclear area to the peripheral one. The peripheral staining was not homogeneous, however, and could therefore be ascribed to subplasmalemmal ER rather than to plasma membrane. This staining was Sss1p-specific, since (i) it decreased to background level if the primary antibodies had been pre-incubated with the immunizing peptide (not shown) and (ii) the immunoreactivity was much weaker in cells that do not overproduce Sss1p (not shown). Control samples were probed with serum directed against Kar2p, an ER lumenal protein, resulting in basically the same staining pattern (not shown), as described previously by Rose *et al.* (1989), and Sweet and Pelham (1993). The Sss1p staining pattern could not be distinguished from that of Sec63p, an ER membrane protein (Feldheim *et al.*, 1992). Hence Sss1p is localized to the nuclear envelope-ER network.

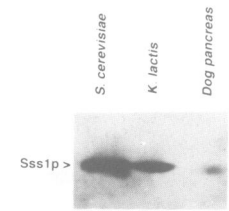

Fig. 10. Anti-Sss1p antibodies cross-react with a protein in the yeast *K.lactis* and in dog pancreas. Microsomal fractions were prepared from yeast cultures by agitation of whole cells with glass beads, followed by a 10 min centrifugation at 10 000 *g* and resuspension of the pellet. Canine pancreatic microsomes were purchased from Amersham France. Equal amounts of microsomal proteins were submitted to SDS−PAGE and probed by immunoblotting with anti-Sss1p polyclonal antiserum.

Discussion

Genetic and biochemical data indicate that the functions of *SEC61, SEC62* and *SEC63* are required *in vivo* for translocation (Deshaies and Schekman, 1987; Rothblatt *et al.*, 1989; Deshaies *et al.*, 1991; Stirling *et al.*, 1992). Recently it was demonstrated that *SEC61* encodes a key component of the eukaryotic translocation apparatus, since it is in close proximity to translocating polypeptide chains (Müsch *et al.*, 1992; Sanders *et al.*, 1992) and to ribosomes (Görlich *et al.*, 1992). In an attempt to identify novel components of the translocation apparatus, we sought yeast wild-type genes whose overexpression would restore partial thermoresistance in a *sec61* mutant. This high copy suppression scheme has often proved fruitful in the past, as an alternate way to gene isolation that does not require a mutation in this gene (Rine, 1991). We isolated two such extragenic Sec sixty-one suppressors.

Based on subcloning, sequence and inactivation data, the *SSS2* gene was identified as *TDH3*, one of the three yeast genes encoding glyceraldehyde-3-phosphate dehydrogenase. This dehydrogenase catalyses interconversion of glyceraldehyde-3-phosphate and 1,3-*bis*phosphoglycerate. It has also been implicated in a number of unrelated cellular activities, such as interaction with microtubules or membranes, protein phosphorylation, DNA repair and tRNA binding (an updated list and references can be found in Singh and Green, 1993). To interpret the surprising effect of this glycolytic enzyme on the *sec61* mutation, two facts must be kept in mind. First, the effect is likely to be indirect because suppression of thermosensitivity was poor and was dependent on a high *SSS2/TDH3* copy number and because this suppressive effect did not extend to the primary defect of the mutant, namely translocation. Second, this dehydrogenase makes up ~10% of total soluble protein in glucose-grown cells, with *TDH3* representing 50−60% of this amount (Holland and Holland, 1979; McAlister and Holland, 1985a). Expectedly, we found *TDH3* overexpression to be toxic to yeast cells; *TDH3* inactivation resulted in a loss of germination potential for sporulated heterozygous diploids (this study) and in slower growth on glucose (McAlister and Holland, 1985b). Consistent with the above remarks, one interpretation could be that overexpression of the *SSS2/TDH3* gene may reduce transcription and translation of other genes, including those encoding secretory protein precursors. The *sec61* translocation mutant could keep in pace with the reduced flow of precursors. This hypothesis recalls the observation that treatments which slow down protein synthesis suppress many *Escherichia coli* mutations affecting protein export (Shatz and Beckwith, 1990). In *S.cerevisiae* also, partial suppression was obtained by treating the *sec65* translocation-deficient mutant with sublethal doses of cycloheximide (D.Feldheim, personal communication). These three observations strengthen the assumption that cytosolic accumulation of secretory precursors is responsible

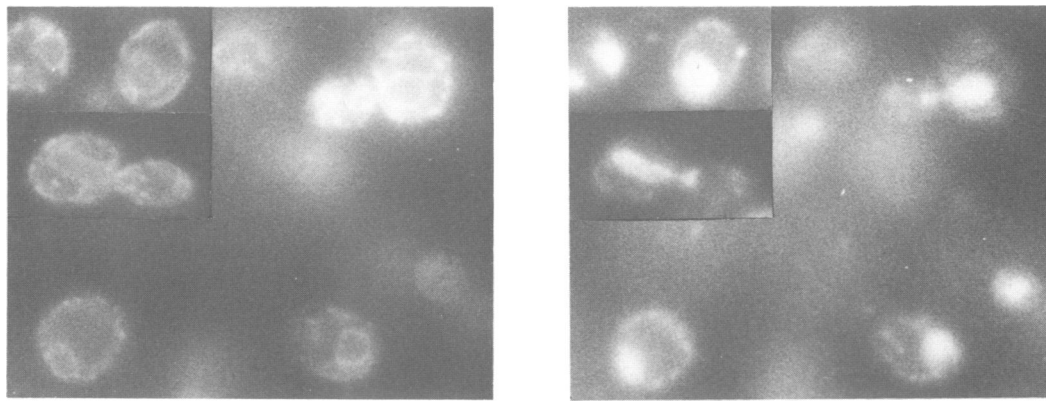

Fig. 11. Localization of yeast Sss1p to the ER. FKp107-transformed FKY128 cells grown at 30°C in rich galactose medium were fixed with formaldehyde, probed with affinity-purified anti-Sss1p antibodies and then decorated with TRITC-conjugated secondary antibodies. Magnification, 1000 × . (**Left panel**) Cells probed with anti-Sss1p antibodies; (**Right panel**) DAPI staining of nuclei.

for the early toxic effects associated with a translocation block, before the expected effects on cell growth and organelle assembly.

In contrast to the *SSS2* case, the positive effect of *SSS1* overexpression on the *sec61* mutants is dependent on gene dosage and it repairs the translocation defect. The *sec61* complementing activity can be ascribed to a small ORF on the basis of subcloning, sequence analysis, disruption/complementation and Northern hybridization. This essential single copy *SSS1* gene is transcribed into a 450 base mRNA of average abundance, which in turn encodes an 80-residue protein, Sss1p. Like Sec61p (Görlich *et al.*, 1992), Sss1p appears to have been conserved along the evolution, since homologues of the *S.cerevisiae* protein were detected in rice by sequence alignment, in *K.lactis* and in dog by antibody cross-reactivity. The yeast Sss1p and its plant homologue are small proteins with relatively polar amino-termini. They are deprived of a signal peptide or of an ER retention signal for soluble proteins (Pelham, 1989), thus suggesting that Sss1p is not a soluble protein in the lumen of secretory organelles. Their longest hydrophobic stretch (16 or 15 residues) is found close to their carboxyl-termini and does not fit the usual standards for transmembrane domains. These features did not allow a prediction of Sss1p localization in the cell. Immunofluorescence microscopy demonstrated, however, that the yeast Sss1p, even when overproduced, localizes to the ER.

In contrast to Sss1p, the known components of the yeast translocation apparatus, the *SEC61*, *SEC62* and *SEC63* gene products, are large proteins with several obvious transmembrane domains (Deshaies and Schekman, 1989; Sadler *et al.*, 1989; Stirling *et al.*, 1992). This contrast raised the question of the direct involvement of Sss1p in the translocation process. A conditional *SSS1* expression mutant was constructed. Repression of *SSS1* expression blocked translocation of three secretory and vacuolar proteins and insertion of an integral membrane protein within 4 h. The accumulated polypeptides were mostly unmodified. Fractionation and protease protection experiments demonstrated that the precursors accumulated on the cytosolic aspect of cellular membranes. This membrane association of *sss1*-blocked secretory preproteins could result from non-specific binding to membranes, for instance via the hydrophobic signal peptide. It could alternatively be caused by a translocation arrest occurring after establishment of a specific contact between the precursor and the translocation apparatus on the cytoplasmic side of the ER membrane. The latter hypothesis is supported by the similarity of the *sss1* and *sec61* mutant phenotypes (Deshaies and Schekman, 1987) and by the genetic interaction between these two genes, in view of the observation that translocating polypeptides can be cross linked to Sec61p (Müsch *et al.*, 1992; Sanders *et al.*, 1992). In particular, *SSS1* repression affects the same spectrum of protein precursors as the cold-sensitive *sec61-3* allele (Stirling *et al.*, 1992), including the soluble periplasmic invertase and the vacuolar membrane protein DPAPB.

Given that the *SSS1* and *SEC61* genes are essential for protein translocation, what is the basis for their genetic interaction? Low copy overexpression of the *SSS1* gene does not suppress the lethality associated with disruption of the *SEC61* gene (C.Stirling, personal communication). Therefore, the observed suppression of *sec61* mutants cannot be caused by a simple bypass of the *SEC61*-mediated function, but is more likely to reflect a physical interaction between the *SSS1* and mutated *sec61* gene products. The question of a physical interaction between the ER proteins Sss1p and Sec61p will be addressed in the future.

Materials and methods

Strains, growth conditions and materials

Bacterial and yeast strains used in this study are listed in Table I. *E.coli* cells were grown at 37°C in LB medium (2.5% Luria broth base). Where appropriate, ampicillin was added at 100 μg/ml. Yeast strains were usually grown at 30°C in YP medium (2% Bacto peptone, 1% Bacto yeast extract) containing 2% of either glucose (YPD) or galactose (YPGal) for derepression of the *GAL10–CYC1* promoter, or in 0.67% yeast nitrogen base with 2% glucose, plus appropriate supplements for selective growth. Solid media were supplemented with 2% Bacto agar. Yeast strains were constructed by usual genetic techniques (Sherman *et al.*, 1986). To induce meiosis and sporulation in diploid cells, colonies were replica-plated on 0.1% Bacto yeast extract, 1% potassium acetate and 2% Bacto agar, containing 0.05% of either glucose (SPO) or galactose (SPOGal). Thermosensitive *sec* mutants were grown either at 24°C (permissive temperature, except for RDM 52-7C, which was grown at 17°C) or at 37–38°C (restrictive temperature). Cell density was monitored in liquid cultures by measuring optical density at 600 nm (OD_{600}) using a DU68 spectrophotometer (Beckman Instruments France, Gagny). Culture media were obtained from Difco laboratories (OSI, Paris).

The following reagents were obtained as indicated. Protein A, EDTA, EGTA, Trizma buffers, NADPH, lyticase, proteinase K, PMSF, BSA, Ponceau S, Triton X-100, ampicillin and diethylpyrocarbonate (DEPC) were obtained from Sigma Chimie (St-Quentin-Fallavier). Restriction

Table I. Bacterial and yeast strains

Strain	Genotype	Source or reference
S.cerevisiae		
RDM 15-5B	*sec61-2 leu2-3,-112 ade2 ura3-52 pep4-3 MATα*	Deshaies and Schekman (1987)
CSYa42	*sec61-3 leu2-3-112 ura3-52 his4-Δ* trp1-1 HOL1-1 MATa	Stirling *et al.* (1992)
RDM 52-7C	*sec61-2 sec62-1 ura3-52 pep4::URA3 MATα*	Rothblatt *et al.* (1989)
W303 1B	*leu2-3,-112 ade2-1 ura3-1 his3-11, -15 trp1-1 can1-100 MATα*	Rothstein and Thomas (1989)
W303 2n	*leu2-3,-112/leu2-3,-112 ade2-1/ade2-1 ura3-1/ura3-1 his3-11,-15/his3-11,-15 trp1-1/trp1-1 can1-100/can1-100 MATα/MATa*	Rothstein and Thomas (1989)
FKY128	Same as W303 2n and *sss1::URA3/SSS1*	This study
FKY198	*sss1::URA3 pep4::LEU2 leu2-3,-112 ade2-1 ura3-1 his3-11,-15 trp1-1 can1-100 MATα* p[*GAL10::SSS1*, *ADE2*] p[*TPI::SUC2, TRP1*]	This study
E.coli		
DH5α	*F' endA1 hsdR17 supE44 thi1* λ- recA gyrA96 relA1 (Φ80 dlacZΔM15)	Hanahan (1983)

endonucleases, DNA modification enzymes, the DIG system of non-radioactive nucleic acid detection and tunicamycin were from Boehringer Mannheim France (Meylan). Agarose and GTG SeaPlaque agarose were from GIBCO BRL (Cergy-Pontoise). Protein A−Sepharose was from Pharmacia France (St-Quentin-en-Yvelines). Tran^{35}S-label ([^{35}S]-labelled mix of methionine and cysteine) was from ICN Biomedicals France (Orsay). ^{125}I-Na, [α-^{32}P]dATP and [^{35}S]dATP were obtained from Amersham France (Les Ulis). The reagents for denaturing polyacrylamide gel electrophoresis (SDS−PAGE) of proteins were purchased from Bio-Rad S.A. (Ivry-Sur-Seine).

Affinity-purified anti-Sec63p IgG and polyclonal antisera against DPAPB and against Kar2p (BiP) were kind gifts from D.Feldheim (University of California, Berkeley), Dr T.Stevens (University of Oregon, Eugene) and Dr M.D.Rose (Princeton University), respectively.

Anti-peptide Sss1p antibodies

Two peptides were chemically synthesized: the first 18 amino acids of Sss1p with an added cysteine ('T' oligopeptide: MARASEKGEEKKQSNNQVC) and a medial sequence comprising 12 amino acids ('M' oligopeptide: CKKPDLKEYTKI). Both synthetic peptides were separately conjugated to Keyhole Limpet Hemocyanin with SMPB. The conjugates were extensively dialyzed and subsequently stored at −20°C. Peptide synthesis and conjugation were carried out at the IBV, Rhône-Poulenc Rorer, Vitry. Three New Zealand female rabbits were immunized subcutaneously with antigen in phosphate-buffered saline and Freund's complete adjuvant and then boosted every 4 weeks using Freund's incomplete adjuvant. Blood was collected 10 days after each boost. For each injection, the three rabbits received respectively the T (75 mg), M (200 μg) or T + M (75 + 200 μg) conjugates. The sera were tested and used at 1/1000 dilution for immunoblots. They recognized a protein migrating at ~9 kDa, which was amplified by *SSS1* overexpression and which disappeared when incubation with the primary antibodies was carried out in the presence of the immunizing peptide. To affinity purify antiserum, 2 mg of T oligopeptide was coupled to 0.5 ml of Affigel-10 agarose beads according to the supplier's instructions (Bio-Rad). 0.5 ml of serum collected after boost 7 in response to antigen T was applied to the affinity column. After extensive washing, bound antibodies were eluted at low pH and the eluate was immediately neutralized.

Immunofluorescence

Immunofluorescence microscopy was performed essentially as described by Pringle *et al.* (1991). Affinity-purified anti-Sss1p antibodies or anti-BiP crude immune serum were used at dilutions of 1/2 or 1/25, respectively. Bound primary antibodies were decorated with 3 μl of tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG. Slides were incubated in 1 μg/ml DAPI, mounted in one drop of 'mounting medium' (45 ml glycerol, 5 ml phosphate buffered saline and 50 mg *p*-phenylenediamine) and examined at 1000 × magnification on a Zeiss Axioplan microscope and shutter controller. Images were recorded on Ilford HP5$^+$ black and white film with a Zeiss MC100 camera.

Vectors, plasmids and nucleic acids manipulations

E.coli plasmids pUC19 (Yanisch-Perron *et al.*, 1985) and pBS (Bluescript, from Stratagene) and the *E.coli*−*S.cerevisiae* shuttle vectors YEp13, YCp50 (Broach *et al.*, 1979), YEp352 (Hill *et al.*, 1986), pUN80 (Elledge and Davis, 1988), pFL38 (Bonneaud *et al.*, 1991), YCplac33 and YIplac204 (Gietz and Sugino, 1988) have been described previously. pCS6-1 was a gift from C.Stirling; it contains the whole *SEC61* gene on a ≈3 kb *Eco*RI−*Sac*I fragment inserted in vector YEp352 (Stirling *et al.*, 1992).

Common RNA and recombinant DNA techniques were carried out as described in Sambrook *et al.* (1989) and according to the suppliers' instructions. Unless otherwise noted, yeast transformations made use of the lithium acetate procedure (Ito *et al.*, 1983). Yeast DNA was extracted as described previously by Sherman *et al.* (1986). Generation of subclones for sequencing was performed in pBS either by restriction digestion or by unidirectional exonuclease III deletions (Henikoff, 1984), using the Erase-a-Base system (Promega Biotech, COGER, Paris). Clones that still contained the M13 polylinker after exonuclease III digestion were identified by hybridization with a M13 biotinylated probe. DNA sequencing was performed by the dideoxy chain termination method (Sanger *et al.*, 1977) on double-stranded DNA, using a Sequenase version 2.0 kit (USB, Touzart and Matignon, Vitry-sur-Seine). The resulting sequences were evaluated with the assistance of the programs DNA Strider™ (Marck, 1988), BLAST (Altschul *et al.*, 1990) and BISANCE (Dessen *et al.*, 1990).

Conditional SSS1 expression mutant

A null allele of *SSS1* was constructed by deletion of the 557 bp *Spe*I−*Ssp*I fragment from FKp59. A *URA3*-containing 1.17 kb fragment was prepared from pFL38 by *Bgl*II digestion. All the junctions were filled-in by the Klenow enzyme to allow blunt-end ligation of the *URA3* prototrophic marker into the *SSS1*-deleted FKp59. The resulting FKp61 plasmid (Figure 1C) was linearized with *Eco*RI. The 3.6 kb fragment thus generated was purified on a low melting agarose gel and integrated by transformation and homologous recombination at the *SSS1* chromosomal site of a diploid W303 2n strain. Correct integration was assessed by Southern analysis. The resulting *SSS1/sss1::URA3* heterozygous diploid was named FKY128.

The coding sequence of the *SSS1* gene (Figure 4B) was amplified between nucleotides 702 (translation start codon) and 1118 (177 bp downstream of the translation stop codon and presumably downstream of the transcription terminator). Two 34 bp oligonucleotides were synthesized for that purpose. They were complementary over 19 bp with the borders of the sequence to be amplified. The non-homologous 15 bp parts were designed to introduce artificial restriction sites (*Kpn*I and *Bam*HI on the 5'-side, *Eco*RI and *Kpn*I on the 3'-side) beyond the *SSS1* sequence. The *Bam*HI site was placed just ahead of the initiation codon ATG. The DNA amplification was carried out during 30 cycles in a PREM III LEP (Perkin Elmer−Cetus) on 10 ng of FKp59 with 2 units of Taq polymerase (Pharmacia) and 0.5 μg of each oligonucleotide. The resulting 447 bp fragment was digested either by *Kpn*I or by *Bam*HI and *Eco*RI. It was then cloned at the corresponding sites of FKp103, a conditional expression vector derived from pYeDP60. The latter vector (Pompon, 1988) was a gift from D.Pompon. This vector bears *URA*3 and *ADE*2 markers, and a *GAL10−CYC1* hybrid promoter that allows glucose repression and galactose induction of the gene inserted downstream. FKp103 is a derivative of pYeDP60 in which *URA3* has been inactivated by *Apa*I digestion, filling in with the Klenow enzyme and re-ligation. Correct orientation of the *SSS1* gene with respect to the hybrid promoter was assessed by restriction analysis and the absence of introduced mutations was checked by sequencing. The resulting plasmids were named FKp106 (*Kpn*I) and FKp107 (*Bam*HI−*Eco*RI).

The heterozygous diploid strain FKY128 was then transformed by FKp106 or FKp107. Transformants were sporulated on SPOGal. The resulting tetrads were dissected and the meiotic progeny were allowed to germinate on YPGal. Spores were screened for the Ura$^+$ Ade$^+$ phenotype, indicating a disrupted *sss1::URA3* allele complemented by expression of wild-type *SSS1* under the control of the hybrid promoter. One such FKp106-containing haploid strain was further transformed with a *pep4::LEU2* construction derived from pTS17 (Ammerer *et al.*, 1986) in order to disrupt the non-essential *PEP4* gene which is responsible for the vacuolar maturation of carboxypeptidase Y. Complete *pep4* inactivation was confirmed by a plate assay (Jones, 1977).

The resulting strain was finally transformed in order to constitutively express invertase. The 5 kb *StuI* fragment from pDB31 (Brada and Schekman, 1988) contains the invertase-encoding *SUC2* gene under the control of the constitutive promoter of the triose phosphate isomerase (*TPI*) gene. This fragment was inserted at the *SmaI* site of *TRP1*-bearing vector YIplac204. The resulting plasmid was linearized and integrated in the genome of the FKp106-containing *pep4::LEU2* strain, finally yielding strain FKY198, which strongly and constitutively expresses secretory invertase.

Immunoblot of proteins from whole cell extracts

Yeast cultures were grown overnight to early log phase and 2 OD_{600} × ml were collected. The cells were washed with 10 mM azide and resuspended in 100 μl of 2 × SDS–PAGE sample buffer containing 1 mM PMSF. After 1 min vortexing at top speed in the presence of 100 μl glass beads (0.5 mm diameter), the samples were heated 5 min at 95°C and loaded on SDS–polyacrylamide gels (from 7.5 to 14.5% acrylamide, as indicated). After migration, proteins were transferred to nitrocellulose filters. Equal loading per lane was confirmed by a brief staining with Ponceau S. Preincubation, antibody incubations and washes were conducted in Tris-buffered saline (10 mM Tris–Cl pH 8 and 150 mM NaCl) plus 0.05% Tween 20 and 2% nonfat dry milk. Antisera were used at 1/1000 dilutions, except for invertase (1/500). Either [^{125}I]protein A, obtained by the chloramine T/NaI method (Hunter and Greenwood, 1962) or the ProtoBlot System (Promega Biotech, COGER, Paris), were used to detect primary antibodies on immunoblots. The [^{125}I] blot was visualized by exposure to preflashed film with an intensifying screen at −80°C.

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