The conserved C-terminus of Sss1p is required to maintain the endoplasmic reticulum permeability barrier

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The endoplasmic reticulum (ER) is the entry point to the secretory pathway and major site of protein biogenesis. Translocation of secretory and integral membrane proteins occurs across or into the ER membrane occurs via the evolutionarily conserved Sec61 complex, a heterotrimeric channel that comprises the Sec61p/Sec61α, Sss1p/Sec61γ, and Sbh1p/Sec61β subunits. In addition to forming a protein-conducting channel, the Sec61 complex also functions to maintain the ER permeability barrier, preventing the mass free flow of essential ER-enriched molecules and ions. Loss in Sec61 integrity is detrimental and implicated in the progression of disease. The Sss1p/Sec61γ C terminus is juxtaposed to the key gating module of Sec61p/Sec61α, and we hypothesize it is important for gating the ER translocon. The ER stress response was found to be constitutively induced in two temperature-sensitive sss1 mutants (sss1ts) that are still proficient to conduct ER translocation. A screen to identify intergenic mutations that allow for sss1ts cells to grow at 37 °C suggests the ER permeability barrier to be compromised in these mutants. We propose the extreme C terminus of Sss1p/Sec61γ is an essential component of the gating module of the ER translocon and is required to maintain the ER permeability barrier.

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This article contains Figs. S1–S3 and Table S1–S4.

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3 The abbreviations used are: ER, endoplasmic reticulum; SRP, signal recognition particle; DSS, disuccinimidyl suberate; ConA, concanavalin A; UPR, unfolded protein response; UPRE, UPR enhancer; OST, oligosaccharyl transferase.

The endoplasmic reticulum permeability barrier is juxtaposed to the key gating module of Sec61p/Sec61α, and we hypothesize it is important for gating the ER translocon. The ER stress response was found to be constitutively induced in two temperature-sensitive sss1 mutants (sss1ts) that are still proficient to conduct ER translocation. A screen to identify intergenic mutations that allow for sss1ts cells to grow at 37 °C suggests the ER permeability barrier to be compromised in these mutants. We propose the extreme C terminus of Sss1p/Sec61γ is an essential component of the gating module of the ER translocon and is required to maintain the ER permeability barrier.

The endoplasmic reticulum (ER) plays a major role in the biosynthesis of about a third of a cell’s proteome (1). Proteins are targeted to and translocated across the ER membrane either cotranslationally or posttranslationally. Cotranslational translocation is initiated upon recognition of the hydrophobic signal sequence by the signal recognition particle (SRP) as it emerges from the ribosome (2–6). The resulting complex is targeted to the ER by association of the SRP with the SRP receptor (3, 5, 6). Interaction with the translocon results in SRP dissociation followed by looped insertion of the nascent chain into the translocation channel (3, 7, 8). Upon resumption of translation the nascent chain moves through the pore and into the lumen of the ER where cleavable signal sequences are processed by the signal peptidase complex (9–12).

Smaller proteins or those containing a moderately hydrophobic signal sequence are completely synthesized in the cytoplasm and translocated posttranslationally (3, 13). Cytosolic chaperones associate with these substrates to maintain them in a translocation-competent state (12, 14, 15). Posttranslational translocation proceeds via the SEC complex, an assembly of the translocon and the Sec62p/Sec63p/Sec71p/Sec72p subcomplex (3, 16–20). Upon association with the SEC complex, chaperones are released and the signal peptide of the secretory precursor is inserted into the translocation channel after which translocation is facilitated by Kar2p (BiP in mammals) (16, 21).

The translocon is formed by the conserved heterotrimeric Sec61 complex. It comprises two essential subunits, Sec61p and Sss1p, and the nonessential Sbh1p subunit (Sec61α, Sec61γ, and Sec61β in mammals, respectively) (3, 16, 22). This complex forms an hourglass-shaped structure with an aqueous pore. At the center lies the pore ring, formed by hydrophobic amino acids, the side chains of which form a gasket through which a polypeptide is threaded during translocation (12, 23, 24). In the closed conformation the pore is sealed by a short helix of TM2 in Sec61p, TM2a, which acts as a plug (23, 25). A Sec61 paralog exists that exclusively functions in the cotranslational pathway (26–28). This paralog is known as Shh1 and Sec61A2 in yeast and mammals, respectively (29). In yeast the Shh1 complex comprises Shh1p and Shb2p, related to Sec61p and Sbh1p, respectively, as well as Sss1p (26, 27).

The conformational changes that take place within the translocon upon the initiation of translocation are best understood for cotranslational translocation. Interaction of the ribosome–nascent chain complex with cytosolic loops 6 and 8 of Sec61p is critical for initiating the preliminary conformational changes that facilitate translocation (3, 30–33). This includes partial displacement of the plug and opening of the lateral gate, formed by TM2b and TM7 of Sec61p, to allow the intercalation of the signal sequence (23, 34). The latter promotes further conformational changes that include full displacement of the
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The Sss1p C-terminus is highly conserved. A, a ribbon diagram of the Sec61 homologue complex crystal structure (PDB ID: 2WWA) (32) was composed using Chimera software. Ssh1p, Sbh2p, and Sss1p are colored gray, blue, and red, respectively. The highly conserved KLIHIPI heptapeptide is highlighted in gold. B, the sequence of the extreme C terminus of Sec61γ and Sss1p are aligned using Clustal Omega sequence alignment software and the position of each double alanine scanning mutation indicated. C, BWY530 yeast transformed with either YCp HIS3, YCp SSS1, YCp SSS1P74A,I75A, YCp SSS1L70A,I71A, YCp SSS1H72A,I73A, or YCp SSS1I68A,K69A were streaked onto His selective medium and medium containing FOA and incubated at 30 °C for 2 days. D, cell extracts derived from WT cells or cells expressing either SSS1P74A,I75A, SSS1L70A,I71A, SSS1H72A,I73A, or SSS1I68A,K69A were immunoblotted with anti-Sss1p or anti-Sec61p antibodies.

Results

The C terminus of Sss1p is highly conserved

The Sec61 complex comprises Sec61/Sec61α, Sbh1p/Sec61β, and Sss1p/Sec61γ. Eukaryotes also encode for a second ER translocase which contains Ssh1p/Sec61α2, Sss1p/Sec61γ, and Sbh2p/Sec61β (Fig. 1A). Given that Sss1p/Sec61γ is the only essential protein to be a component of both ER translocons we considered the possibility that its activity may be tightly regulated. The Sss1p transmembrane domain is highly conserved, in particular the C-terminal portion where the K_{69}LIHIPI_{75} heptapeptide is absolutely conserved. We hypothesized this region to be functionally important and performed double alanine scanning mutagenesis throughout it, creating variants Sss1pP74A,I75A, Sss1pI68A,K69A, Sss1pL70A,I71A, and Sss1pH72A,I73A (Fig. 1B) to investigate the role of these residues in Sss1p function. As SSS1 is essential, we tested if expression of these variants could sustain cell viability. YCp SSS1 and each mutant were transformed into BWY530 (sss1::KanMX4 Fk53) and tested for the ability of these strains to grow after loss of Fk5p3 on 5-FOA medium. Strains harboring YCp SSS1 or any of the mutants, unlike cells transformed with vector alone, produced viable colonies that expressed stable Sss1 protein (Fig. 1, C and D).

Mutations in the Sss1p C terminus disrupt ER homeostasis

The growth of cells expressing the sss1P74A,I75A double mutant, referred to as sss1–6 herein, is temperature sensitive as they grow poorly at 37 °C (Fig. 2A). We have isolated a second mutation within the C terminus that is temperature sensitive (Fig. 2A). The Sss1pI723K variant (referred to as sss1–7) was originally generated to enable us to identify potential lumenal interacting proteins by crosslinking. The resulting strain was even more temperature sensitive than sss1–6 cells (Fig. 2, A and B).

The hydrophobicity of the extreme C terminus of tail-anchored proteins is a critical property that allows their stable integration into membranes by the GET complex (37). Hydrophathy analysis shows that the mutations in the C terminus of Sss1–6p and Sss1–7p have little effect on the hydrophobicity of
this region relative to Sss1p (Fig. S1). We used fluorescence microscopy to investigate if these mutations affect the integration of Sss1p into the ER membrane. GFP-Sss1p shows a perinuclear and peripheral locality and colocalizes with the ER resident protein Sec63p (Fig. 2C). As with GFP-Sss1p, we observe GFP-Sss1–6p and GFP-Sss1–7p to localize to the perinuclear and peripheral ER and both entirely colocalize with Sec63p (Fig. 2C).

Given that Sss1p is an essential translocon component, we investigated if the integrity of the translocation apparatus was affected in Sss1p variants by measuring the stability of other essential translocon-associated proteins. We find the stability of both Sec61p and Sec63p to be unaffected in sss1–6 and sss1–7 at 30 °C or 37 °C (Fig. 2B). Next, we investigated the integrity of the translocon. The interaction between Sss1p and Sec61p required to form the translocon can be stabilized by the crosslinking reagent disuccinimidyl suberate (DSS) (38, 39).

We detected a DSS-dependent immunoreactive band of ~46 kDa with both anti-Sss1p– and anti-Sec61p–specific antibodies in membranes isolated from WT cells (Fig. 3A). This adduct was also detected in membranes isolated from either sss1–6 or sss1–7 cells treated with DSS, regardless of whether cells were grown at 30 °C or 37 °C (Fig. 3A).

Binding of the Sec71p glycoprotein with the lectin concanavalin A (ConA) enables the affinity purification of the heptamer SEC complex (20, 40). Digitonin solubilized membranes isolated from WT, sss1–6, and sss1–7 cells were incubated with ConA-coupled Sepharose beads, the bound fraction retained, and Sec61p, Sss1p, and Sec63p were visualized by immunoblot analysis (Fig. 3B). As expected, Sec61p, Sss1p, and Sec63p were associated with the ConA-bound fraction of digitonin-solubilized membranes isolated from WT cells. Likewise, these proteins were associated with the ConA-bound fraction of digitonin-solubilized membranes isolated from sss1–6 and sss1–7.
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Figure 3. ER translocation is not affected in sss1–6 and sss1–7. A, membranes derived from WT sss1–6 or sss1–7 yeast incubated with and without 1 mM DSS were immunoblotted with anti-Sss1p and anti-Sec61p antibodies. B, membranes prepared from WT sss1–6 or sss1–7 yeast were subject to ConA chromatography. An equal portion of each fraction was analyzed by immunoblotting with either anti-Sss1p, anti-Sec61p, or anti-Sec63p specific antibodies. The bound fraction is shown. C, cell extracts derived from WT sss1–6 or sss1–7 yeast were immunoblotted with anti-Sss1p, anti-Sec61p, anti-α factor, and anti-DPAP B antibodies. Secretory mutants, sec63–1 and sec65–1 (sects), were included as a negative control for α factor and DPAP B, respectively.

Figure 4. ER homeostasis is perturbed in sss1–6 and sss1–7. A, WT, sss1–6, and sss1–7 yeast transformed with pJT30 (UPRE-LACZ) were grown in Ura selective medium and β-galactosidase activity determined. WT cells treated with 5 mM DTT for 2 h were used as a positive control. B, WT, SEC63420D, and sss1–6 yeast transformed with Yepl HGT1 were grown in Ura selective medium with increasing concentrations of GSH. The relative growth of each strain determined and the GSH sensitivity (1/relative growth) presented. C, WT, sss1–6, and sss1–7 yeast were spotted on YPD agar containing 1 μg/ml terbinafine in a 10-fold dilution series and incubated at 30 °C or 34 °C for 2 days.

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The Sec61 complex has been shown by Toledano and colleagues (42) to facilitate the diffusion of reduced GSH into the ER. Furthermore, it has also been suggested that the Sec61 complex may facilitate the diffusion of other small molecules across the ER membrane. We wanted to determine whether Sss1p C-terminus mutants disrupted the ability to form protein complexes required for ER translocation.

The biogenesis of DPAP B, which is translocated cotranslationally, as well as Lhs1p and α-factor, which are translocated by posttranslational translocation (41), were monitored to determine whether ER translocation is compromised in sss1–6 and sss1–7. There was no obvious translocation defect in sss1–6 and sss1–7 mutants, irrespective of growth conditions, as the levels of both pDPAP B and Lhs1p did not exceed that which accumulates in WT cells (Fig. 3C). This was also the case for a second posttranslational translocation substrate prepro-α factor (Fig. 3C). To further validate this we reasoned that the mating efficiency of MATα variants of both sss1–6 and sss1–7 would be comparable to WT cells at both permissive and semi-permissive temperatures. Indeed, we observed no difference in the mating efficiency of these sss1 mutants to mate as compared with WT cells (Fig. S2A).

We observed significant ER distension and expansion in sss1–6, in particular, and sss1–7, indicating the ER to be stressed in these mutants (Fig. 2C). Given this, we tested if the unfolded protein response (UPR) was induced in sss1–6 and sss1–7 mutants. For this we used a lacZ reporter placed under transcriptional control of a yeast UPR enhancer (UPRE) (Wilkinson et al. 36). WT cells were treated with the reducing agent dithiothreitol (DTT) to gauge a typical UPR response. UPR-dependent LacZ activity was significantly elevated in DTT-treated cells compared with WT (Fig. 4A), ensuring the range of response expected from these controls. LacZ activity in both sss1 mutants at 30 °C and 37 °C was up to 11-fold greater than that of WT. This confirms that the UPR is constitutively induced in sss1–6 and sss1–7 cells.

Together, these experiments confirmed that despite their large effects on cell viability at 37 °C and causing constitutive induction of the UPR, these Sss1p point mutations did not affect the abundance, overall integrity, or general transloca-tional activity of the translocon. This suggested that the role of this conserved section of Sss1p is independent of protein translocation.

The ER is more permeable in sss1–6 and sss1–7

The Sec61 complex has been shown by Toledano and colleagues (42) to facilitate the diffusion of reduced GSH into the ER. Furthermore, it has also been suggested that the Sec61 complex may facilitate the diffusion of other small molecules across the ER membrane. We wanted to determine whether sss1–6 and sss1–7 cells possessed phenotypes consistent with altered ER permeability. WT cells that overexpress Hgt1p, the plasma membrane high-affinity GSH transporter (↑HGT1 cells hereafter), accumulate high levels of GSH when supplied with exogenous GSH, which becomes cytotoxic because of hyperoxidation of the ER (42, 43). HGT1 is overexpressed in ↑HGT1 cells by placing the HGT1 ORF under transcriptional control of the constitutive and robust PMA1 promoter on a
multicopy plasmid (YEp). In our hands, WT Hgt1p cells easily tolerate up to 10 μM GSH (Fig. 4B) whereas the growth of SEC61N302D Hgt1p cells is hypersensitive (Fig. 4B). sss1–6 Hgt1p growth is also hypersensitive to GSH as it was severely perturbed by 2.5 μM GSH and 5 μM GSH and completely arrested by 10 μM GSH (Fig. 4B).

Mn²⁺ is an essential cofactor for cytoplasmic farnesyl pyrophosphate (FPP) synthetase (Fpp1p) (44–46). Schuldiner and colleagues (44) have shown that Fpp1p activity is elevated upon deletion of the ER resident Mn²⁺ transporter Spf1p because of a failure to store Mn²⁺ in the ER which gives rise to elevated Mn²⁺ levels in the cytosol. Elevated Fpp1p activity leads to increased squalene synthesis, which inhibits cell proliferation if cells cannot remove it through metabolism, such as when cells are challenged with the squalene epoxidase inhibitor terbinafine (44). We used this system to test if Sss1p mutants were also defective in maintaining normal Mn²⁺ homeostasis and possessed increased Fpp1p activity. sss1–6 and sss1–7, in particular, cell growth is extremely sensitive to terbinafine as, unlike WT, 1 μg/ml terbinafine completely inhibited the growth of sss1–7 and sss1–6 at 30 °C and 34 °C, respectively (Fig. 4C). Importantly, neither sss1–6 nor sss1–7 cells are hypersensitive to the 14α-sterol demethylase inhibitor monacolone (Fig. S2B). Therefore, the hypersensitivity of both sss1–6 and sss1–7 cells to terbinafine is not simply because of these mutants being hypersensitive to small molecules that inhibit ergosterol biosynthesis.

**Mutations in residues of Sec61p located in important gating modules suppress sss1–6 and sss1–7 temperature sensitivity**

To understand how mutations in the C terminus of Sss1p disrupt ER homeostasis we sought to identify mutations in the major interacting partner of Sss1p, Sec61p, which could suppress sss1–7 temperature-sensitive growth. Missense mutations were incorporated into the SEC61 gene by error-prone PCR, and mutagenized SEC61 genes were then transfected into sss1–7 yeast and integrated into the SEC61 genetic locus by homologous recombination. In total, 203 Ts¹ colonies were isolated and the complete nucleotide sequence of 50 was determined. On average EP-PCR yielded 3.5 mutations per 1 kb and isolated and the complete nucleotide sequence of 50 was determined. On average EP-PCR yielded 3.5 mutations per 1 kb and

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**Figure 5. Mutations in residues of Sec61p located in important gating modules suppress sss1–6 and sss1–7 temperature sensitivity**

A, WT, sss1–6, or sss1–7 yeast transformed with either YCp SEC61, YCp SEC61V82F, YCp SEC61N302K, YCp SEC61N302Y, or YCp SEC61T379A were spotted on YPD agar in a 10-fold dilution series and incubated at 30 °C or 37 °C for 2 days. B, WT, sss1–6, or sss1–7 yeast transformed with either YCp SEC61, YCp SEC61V82F, YCp SEC61N302K, YCp SEC61N302Y, or YCp SEC61T379A and with pJ30 (UPRE-LacZ) were grown in Ura selective medium and β-galactosidase activity determined. As a positive control WT cells were treated with 5 mM DTT for 2 h.

**Figure 6. SEC61-dependent suppressors of sss1–6 and sss1–7 are functional**

A, WT, SEC61V82F, SEC61S289F, SEC61N302K, SEC61N302Y, SEC61T379A, SEC61N302D, or SEC61N302D yeast were spotted on YPD agar in a 10-fold dilution series and incubated at 30 °C or 37 °C for 2 days. B, cell extracts derived from WT SEC61V82F, SEC61S289F, SEC61N302K, SEC61N302Y, SEC61T379A, or SEC61N302D yeast were immunoblotted with anti-Sss1p, anti-Sec61p, and anti-Sec63p antibodies. C, cell extracts derived from WT SEC61V82F, SEC61S289F, SEC61N302K, SEC61N302Y, SEC61T379A, or SEC61N302D yeast grown at 30 °C or 37 °C were immunoblotted with anti-Lhs1p, anti-DPAP B, and anti-α factor antibodies.

(Fig. 6C). Also, neither SRP-independent translocation precursors pLhs1p nor prepro-α factor accumulated in these SEC61 mutants at 30 °C and 37 °C, unlike the sec62–1ts control (Fig. 6C).

Mapping each mutation on the crystal structure of the Sec61 homologue Shs1 revealed them to be positioned at critical sites for translocon gating including the pore ring (V82F) and the...
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lateral and lumenal gate (S289F, N302K/Y, and T379A) (Fig. S3). Residue Asn-302 is part of a network that is responsible for the opening and closing of the lateral and lumenal gates of the translocon. That the SEC61N302K and SEC61N302Y mutations suppress both sss1–6 and sss1–7 suggests that dynamics of the lateral and lumenal gate of the translocon is altered somehow in these sss1 mutants. Substitution of Asn-302 to a polar (N302D) or hydrophobic residue (N302L) destabilizes the closed or open conformation of the Sec61 complex, respectively (47).

To investigate if sss1H72K and sss1P74A, I75A mutations destabilize either the open or the closed conformation of the translocon, we tested if the SEC61N302L or the SEC61N302D mutation suppressed sss1–6 and sss1–7 temperature sensitivity. The SEC61N302L mutant suppressed sss1–6 and sss1–7 temperature-sensitive growth (Fig. 7A) and reduced UPR induction (Fig. 7B) like SEC61N302K and SEC61N302Y. In contrast, co-expression of SEC61N302D with either sss1–6 or sss1–7 exacerbated the growth defects of these mutants (Fig. 7C, C and D). Furthermore, we were unable to isolate sss1–7 sec61N302D double mutants (Fig. 7C), suggesting the possibility that these mutations, when co-expressed, are synthetically lethal. Together, this suggests that the sss1–6 and sss1–7 mutations may destabilize the closed conformation of the translocon.

**Mutations in residues located in important Sec61p gating modules suppress the elevated ER permeability observed in sss1–6 and sss1–7**

Given the impressive manner in which our SEC61 mutants were able to diminish the level of ER stress in both sss1–6 and sss1–7, we were keen to determine whether these mutants were also able to suppress phenotypes associated with altered ER permeability. Co-expression of each of the SEC61 suppressors of sss1–7 temperature sensitive growth were able to suppress the hypersensitivity of the sss1–6 mutant to GSH (Fig. 8, A and B). Furthermore, co-expression of the SEC61 mutants that suppressed sss1–6 and sss1–7 temperature-sensitive growth also suppressed their hypersensitivity to terbinafine, albeit with varying strength (Fig. 8, C and D).

**Discussion**

Sss1p/Sec61γ is an essential and highly conserved subunit of the ER translocon yet its function is poorly understood. A hep-
Figure 8. Mutations in residues of Sec61p located in important gating modules suppress ER permeability defects in sss1–6 and sss1–7. A, WT, or sss1–6 yeast transformed with either YCp SEC61, YCp SEC61T379A, YCp SEC61V82F, YCp SEC61S289F, or YCp SEC61N302K were transformed with YEp HGT1 and were grown in Ura selective medium with increasing concentrations of GSH. The relative growth of each strain determined and the GSH sensitivity (1/relative growth) presented. B, WT, sss1–6 or sss1–6 yeast transformed with either YCp SEC61N302K or YCp SEC61N302L and YEp HGT1 were grown in Ura medium with increasing concentrations of GSH, the relative growth of each strain determined and the GSH sensitivity (1/relative growth) presented. C, WT and sss1–6 or sss1–7 yeast transformed with either YCp SEC61, YCp SEC61SRPΔ, YCp SEC61V82F, YCp SEC61S289F, YCp SEC61N302K, or YCp SEC61T379A were spotted on YPD agar or YPD agar containing 1 μg/ml terbinafine in a 10-fold dilution series and incubated at 30 °C or 34 °C for 2 days. D, WT, sss1–6, sss1–7 or sss1–6 and sss1–7 yeast transformed with either YCp SEC61V82F or YCp SEC61T379A were spotted on YPD agar or YPD agar supplemented with 1 μg/ml terbinafine in a 10-fold dilution series and incubated at 30 °C or 34 °C for 2 days.

The Sss1p C-terminus is important for ER homeostasis

The extreme Sss1p/Sec61γ C terminus influences ER permeability

Cells expressing sss1 mutants with defective TM domains have been shown to be defective in ER translocation (22, 35). We were surprised to find no ER translocation defect in both sss1–6 and sss1–7 cells even though the UPR was highly induced in both mutants. Sss1p, like Sec61p, is essential for both SRP-dependent and SRP-independent ER translocation. That we find no evidence of the accumulation of secretory precursor proteins at steady state suggests that mutation of the highly conserved C terminus of Sss1p does not result in the gross perturbation in the formation of protein complexes that are required to conduct ER translocation. Given that Sss1p/Sec61γ is a C-terminally anchored protein it is possible that both Sss1–6p and Sss1–7p may not be efficiently targeted to and incorporated into the ER membrane. Our data do not support this notion. We do not observe a decrease in the membrane-associated pool of Sss1–6p and Sss1–7p nor do we observe a decreased ability to crosslink both Sec61p and Sss1p with DSS. The latter point is crucial to this conclusion as the insertion of C-terminal–anchored proteins proceeds via the GET complex and not the ER translocon. Therefore, the crosslinking of Sec61p with both Sss1–6p and Sss1–7p reflects a functional interaction rather than a trivial interaction of Sec61p with a translocation intermediate. In further support, we observe no reduction in the ability to isolate components of the ER SEC complex by ConA pulldown.

Those secretory proteins that are N-glycosylated are modified by the oligosaccharyl transferase (OST) as they are translocated through the translocation channel. Sss1p is physically located at the interface between the translocon and OST. However, we find no evidence of N-glycosylation being perturbed in sss1–6 and sss1–7, the N-glycosylation status of Lhs1p and DPAP B is indistinguishable from WT. Furthermore, MAT α derivatives of both sss1–6 and sss1–7 secrete sufficient quantity of α factor to enable their mating with MAT a strains. We therefore have to consider other possibilities to explain the severe ER stress in sss1–6 and sss1–7 mutants.

A directed evolution approach was therefore used to obtain SEC61 mutants that suppressed the temperature-sensitive growth defect of sss1–7 cells to understand what aspect of translocon function was disrupted in these sss1 mutants. We isolated two different classes of suppressor mutation: class one mutations (V82F) are located in the pore ring and class two mutations (S289F, N302K/Y, and T379A) are located in the lateral and luminal gate that coordinates the opening and closing of the protein-conducting channel. A common feature of both classes of suppressor mutation is that they are situated in

tapeptide, KLIHIPI, located toward the C terminus of the protein is absolutely conserved in all eukaryotes studied to date. Herein, we discover the KLIHIPI peptide to be an important factor that influences ER permeability.

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A directed evolution approach was therefore used to obtain SEC61 mutants that suppressed the temperature-sensitive growth defect of sss1–7 cells to understand what aspect of translocon function was disrupted in these sss1 mutants. We isolated two different classes of suppressor mutation: class one mutations (V82F) are located in the pore ring and class two mutations (S289F, N302K/Y, and T379A) are located in the lateral and luminal gate that coordinates the opening and closing of the protein-conducting channel. A common feature of both classes of suppressor mutation is that they are situated in
regions of the Sec61 protein that are required to gate the channel. The lateral gate, formed by TMs 2 and 7 of Sec61p, enables the incorporation of signal peptides or transmembrane helices into the lipid bilayer once they have emerged from the ribosome. Thus, the opening of the “lateral gate” is a critical process that allows protein translocation to proceed while maintaining the ER permeability barrier to ensure that ER luminal equivalents do not leak into the cytosol and vice versa. Apolar residues in the luminal gate and a cluster of polar residues within the lateral gate form a highly conserved gating motif that regulates the opening and closing of the Sec61 complex (47). One critical residue is Sec61p Asn-302. Importantly, mutations that reconfigure the hydrogen bonding network have been shown to elicit a Prl phenotype (47). In contrast, mutations that increase the hydrophobicity enhance the nonparallel interactions between the lateral and luminal gates and stabilize the closed conformation. Enhancing the nonparallel interaction network suppressed both sss1–6 and sss1–7 temperature sensitivity.

Taken together this suggests the possibility that the ER is more permeable in sss1–6 and sss1–7. We consider that our panel of SEC61 mutations in the luminal and lateral gate negate the ability of sss1–6 and sss1–7 to destabilize the closed conformation of the Sec61 complex. It is possible that these mutations suppress sss1–6 and sss1–7 by reducing the rate of ER translocation to such an extent that a translocating intermediate can seal the channel by clogging the translocon. However, we consider this to be highly unlikely as each mutant is fully functional with no detectable defect in either SRP-dependent or SRP-independent translocation.

The Sec61 complex has been shown by Toledano and colleagues (42) to facilitate the diffusion of reduced GSH into the ER. It is important to note that the SEC61N302L mutation in the luminal-lateral gate was also utilized in this study to verify that the translocon forms the channel that facilitates the diffusion of GSH into the ER. Our panel of SEC61 suppressors as well as SEC61N302I suppresses sss1–6 hypersensitivity to GSH. These SEC61 mutations also rescue the hypersensitivity of both sss1–6 and sss1–7 to terbinaine, a phenotype that has previously shown to arise in mutants defective in ER Mn2+ storage (44). We appreciate that the hypersensitivity of both sss1–6 and sss1–7 growth to terbinaine is itself an indirect measure of increased ER permeability in these mutants. However, both sss1–6 and sss1–7 are not hypersensitive to the 14α-sterol demethylase inhibitor, miconazole ruling out that this phenotype is because of these sss1 mutants being hypersensitive to reagents that inhibit ergosterol biosynthesis. Together we hypothesize that the C terminus of Sss1p/Sec61γ constitutes an important component of the gating module that coordinates the opening and closing of the translocon, both sss1–6 and sss1–7 destabilizing the closed state. Future work will provide a mechanistic insight to the role of this domain. The importance of the C terminus of Sss1p as a key component of the translocon gating module is reflected in the absolute conservation of the KLIHIPII heptapeptide throughout the eukaryota.

Materials and methods

Yeast strains and growth

Saccharomyces cerevisiae strains are listed in Table S1 and plasmids are listed in Table S2. Yeast strains were grown routinely at 30 °C in YP medium (2% peptone, 1% yeast extract) containing 2% glucose (YPD) or in minimal medium (0.67% yeast nitrogen base; YNB) with 2% glucose plus appropriate supplements for selective growth. All media were from Formedium (Hunstanton, UK). For growth assays, yeast were spotted in a 10-fold dilution series on YPD agar and grown at either 30 °C, 34 °C, or 37 °C for 2 to 3 days. 1 μg/ml terbinafine or DMSO was added to YPD agar where indicated.

Mutagenesis and selection for SEC61 suppressors of sss1–7 temperature sensitivity

EP-PCR was carried out with TaqDNA polymerase in the presence of 500 μM MnCl2 and run for 10 cycles. Reactions were also performed with inverse dNTP concentrations. Samples were pooled after EP-PCR and digested with HindIII for 1 h at 37 °C. sss1–7 yeast were transformed with restricted PCR product and transformants were plated on YPD agar and incubated at 37 °C for up to 5 days. The SEC61 locus of suppressor colonies was sequenced and individual mutations were introduced in pBW11 by site-directed mutagenesis using the Q5® Site-Directed Mutagenesis Protocol (New England Biolabs Inc.); oligonucleotides used are listed in Table S3.

Fluorescence microscopy

The GFP ORF was amplified with oligonucleotides flanked with 5’ PacI and 3’ SphI and ligated into pHKB2 in which nucleotides −8 to −1 and 1 to 6 were mutated to encode PacI and SphI restriction sites, respectively, giving pLB4 (Ycp GFP-SSS1). The sss1–6 and sss1–7 mutations were incorporated into pLB4 using the Q5® Site-Directed Mutagenesis Protocol (New England Biolabs Inc.) giving pLB5 and pLB6 respectively. Oligonucleotides used are listed in Table S3. Static images were collected of live cells attached to a concanavalin A–coated slide using an Ultraview Spinning Disk Confocal Microscope (Perkin Elmer Life Sciences).

DSS crosslinking

Yeast microsomes were prepared according to Rothblatt and Meyer (48). Membranes were treated with DMSO or 1 mM DSS at 30 °C or 37 °C for 30 min and then quenched by the addition of 10 mM lysine and 100 mM Tris for 10 mins.

Immunoblotting

Antibodies used are listed in Table S4.

ConA dependent fractionation of SEC proteins

The fractionation of SEC proteins by ConA was performed according to Pilon et al. (40). Briefly, microsomes were isolated and resuspended in 100 μl of solubilization buffer (50 mM HEPES/KOH, pH 7.4, 400 mM KAc, 5 mM MgAc, 10% (w/v) glycerol, 0.05% (v/v) β-mercaptoethanol) on ice containing protease inhibitors (5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 1 mM amino-benzamidine, 2.5 μg/ml chymostatin, and 0.1 mM...
PMSF). Membranes were solubilized by the addition of 400 µl solubilization buffer containing 3.75% (w/v) digitonin. Next, samples were centrifuged at 100,000 × g in a Beckman TLA100.3 rotor for 60 min at 4 °C to isolate the ribosome attached membrane proteins. The supernatant fraction was added to 100 µl of a suspension of ConA-Sepharose equilibrated in 50 mM HEPES/KOH (pH 7.4), 10% (w/v) glycerol, 0.05% (v/v) β-mercaptoethanol, 1% (w/v) digitonin, and protease inhibitors, and incubated for 60 min at 4 °C. The beads were recovered by centrifugation at 2500 × g and the supernatant fraction was cleared from any remaining beads at 12,000 × g (free fraction). The ConA beads were washed three times with 1 ml of equilibration buffer. Equal aliquots of both fractions were analyzed by SDS-PAGE and immunoblotting with the indicated antibodies.

β-Galactosidase assays

β-Galactosidase assays were performed according to Tyson and Stirling (49). Briefly, yeast cells were grown at 30 °C in minimal medium containing 2% glucose and appropriate supplements. Cultures were diluted to 1.5 × 10^6 cells/ml and grown for a further 4 h. Cells were harvested and resuspended in 2 ml of 0.1% (w/v) SDS and 100 µl of CHCl₃, and samples were equilibrated to 30 °C. Assays were initiated by addition of 160 µl of o-nitrophenyl-galactopyranoside (4 mg/ml stock solution in Z buffer) and incubated at 30 °C for 20 min. Reactions were terminated by addition of 400 µl of 1 M Na₂CO₃, pH 9.0, the A₄₂₀ nm was measured, and LacZ activity (U) was calculated by multiplying A₄₂₀ nm/A₆₈₀ nm by 1000. Three independent biological replicates and at least two technical replicates were performed.

GSH sensitive growth assay

Cells harboring YEp HGT1 were grown to mid logarithmic phase and then subcultured to 0.01 A₆₀₀ nm in SC media without uracil containing 0–10 µM GSH and the growth rate recorded. The growth rate of cells cultured in the absence of GSH was set to 100% and relative growth rates plotted. Three independent biological replicates and at least two technical replicates were performed.

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