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Molecular architecture of the ER translocase probed by chemical crosslinking of Sss1p to complementary fragments of Sec61p

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The heterotrimeric Sec61p complex is a key component of the protein translocation apparatus of the endoplasmic reticulum membrane. The complex characterized from yeast includes Sec61p, a 10-transmembrane-domain membrane protein which has a direct interaction with Sss1p, a small C-terminal anchor protein. In order to gain some insight into the architecture of this complex we have functionally expressed Sec61p as complementary N- and C-terminal fragments. Chemical crosslinking of Sss1p to specific Sec61p fragments in these functional combinations and suppression of *sec61* mutants by over-expression of Sss1p have led to identification of the region which includes transmembrane domains TM6, TM7 and TM8 (amino acid residues L232–R406) of Sec61p as a major site of interaction with Sss1p.

Keywords: chemical crosslinking/ER/protein translocation/Sec61p complex/Sss1p

Introduction

A considerable body of evidence implies a role for integral membrane proteins in the formation of an apparatus required for translocation of proteins across the endoplasmic reticulum (ER) membrane (High, 1995; Schatz and Dobberstein, 1996; Wilkinson *et al.*,1997). The 53 kDa Sec61p is a 10-transmembrane-domain protein of the yeast ER membrane (Wilkinson *et al.*, 1996) required for both growth and protein translocation *in vivo* (Stirling *et al.*, 1992). Mutations in the *SEC61* gene encoding Sec61p result in defects in the translocation of a range of secretory proteins (Deshaies and Schekman, 1987; Stirling *et al.*, 1992; Ng *et al.*, 1996). Furthermore, Sec61p has been shown to be in close proximity to prepro-α-factor at different stages of its translocation through the ER bilayer (Müsch *et al.*, 1992; Sanders *et al.*, 1992; Lyman and Schekman, 1995, 1997). These observations indicate that it is a vital constituent of a protein conducting channel in the ER membrane. The *SSS1* gene, which encodes the 9 kDa C-terminal anchor protein Sss1p, was originally identified as a suppressor of a temperature-sensitive *sec61* mutant and is also essential for growth and protein translocation (Esnault *et al.*, 1993). It forms a stable complex with Sec61p (Esnault *et al.*, 1994; Panzner *et al.*, 1995; Biederer *et al.*, 1996), which may be the basic functional unit of the ER protein translocase (Finke *et al.*, 1996). The trimeric Sec61p complex includes a second C-terminal anchor protein, Sbh1p, encoded by a non-essential gene with no apparent role in translocation (Finke *et al.*, 1996).

A homologous trimeric Sec61 complex, composed of Sec61α (Sec61p), Sec61β (Sbh1p) and Sec61γ (Sss1p) is also found in mammalian ER (Görlich and Rapoport, 1993; Hartmann *et al.*, 1994) The Sec61α component is also in close proximity to polypeptides during their membrane transfer (for a review see Martoglio and Dobberstein, 1996). The Sec61p/Sec61α and Sss1p/Sec61γ components are distantly related to the SecY and SecE proteins of bacteria respectively (Stirling, 1993; Hartmann *et al.*, 1994), which have been widely implicated in protein export across the inner bacterial membrane (for a review see Driessen, 1994) as part of a heterotrimeric SecYEG complex. Neither Sbh1p nor Sec61β is related to the SecG component of the bacterial complex (Hartmann *et al.*, 1994; Finke *et al.*, 1996), although, like Sbh1p, SecG is a non-essential protein under normal growth conditions (Nishiyama *et al.*, 1994).

Sss1p is also found as a component of a second yeast heterotrimeric ER membrane complex together with a Sec61p homologue, Ssh1p, and an Sbh1p homologue, Sbh2p (Finke *et al.*, 1996). Neither Ssh1p nor Sbh2p is essential, suggesting that this second Sss1p-containing complex (the Ssh1 complex) may be functionally redundant with the Sec61p complex. Both the Sec61p and Ssh1p complexes are found associated with ribosomes upon solubilization of microsomes in detergent (Panzner *et al.*, 1995; Finke *et al.*, 1996), indicating that both complexes are involved in the co-translational pathway of translocation. However, the essential nature of the Sec61p complex may be conferred by its role as part of a heptameric complex involved in post-translational translocation in yeast (Panzner *et al.*, 1995).

Electron microscopic studies have shown that both purified mammalian and yeast Sec61 complexes form cylindrical oligomers with a diameter of ~8.5 nm and a central pore of 2.0 nm (Hanein *et al.*, 1996). It has been proposed that these structures, which probably contain three to four Sec61 heterotrimers, represent protein conducting channels. Furthermore, the mammalian Sec61 complex has also been implicated in retrograde translocation of a membrane protein from the ER to the cytosol (Wiertz *et al.*, 1996). A pathway for cytosolic degradation of misfolded ER proteins has also been identified in yeast (Hiller *et al.*, 1996), raising the possibility that the yeast Sec61 complex is also involved in protein dislocation from the ER.

A previous study has reported an interaction between Sec61p and Sss1p (Esnault *et al.*, 1994). In this paper we have dissected the region of interaction between Sec61p and Sss1p. We demonstrate that Sec61p activity can be restored by co-expression of complementary fragments of Sec61p and that, in addition, these functional combinations of fragments can be used to delineate the region of Sec61p which can be crosslinked to Sss1p. The data are consistent with an interaction between Sss1p and residues L232–R406 of Sec61p, which includes transmembrane domains 6–8 (TM6–TM8). This is supported by genetic data, which also suggest an interaction between Sss1p and this region of Sec61p. We discuss the significance of retention of Sec61p function by paired complementary fragments and the possible role of the Sec61p–Sss1p interaction during translocation.

Results

The strategy for expression of Sec61p as complementary polypeptides

A number of transmembrane proteins have been reported to exhibit functional complementation from association of non-overlapping but contiguous inactive fragments, including lactose permease (Zen *et al.*, 1994, and references therein), bacteriorhodopsin (Kahn and Engelman, 1992, and references therein), β_2-adrenergic receptor (Koblika *et al.*, 1988), the yeast a-factor transporter Ste6p (Berkower and Michaelis, 1991), the GLUT1 glucose transporter (Cope *et al.*, 1994), human P-glycoprotein (Loo and Clarke, 1994), rhodopsin (Ridge *et al.*, 1995) and band 3 erythrocyte anion transporter (Groves and Tanner, 1995). We have addressed the possibility that Sec61p may also exhibit this phenomenon in order to map interactions between Sec61p fragments and Sss1p using a chemical crosslinking approach.

Sec61p has been reported to span the ER membrane 10 times, with both the N- and C-termini located on the cytosolic face of the membrane (Wilkinson *et al.*, 1996). Using a series of *Bam*HI sites introduced into the *SEC61* coding sequence, fragments of the gene were subcloned individually or as complementary pairs under separate *SEC61* promoters (see Materials and methods). The *Bam*HI sites had been generated by substitution (Wilkinson *et al.*, 1996), resulting in the mutation of a pair of successive amino acid residues to glycine and serine respectively, where these residues were not already present. All of these mutations were found to complement a *sec61* null mutation (data not shown). Each breakpoint shown in Figure 1A shows two numbered residues which correspond to the pair of residues mutated to glycine and serine in the course of *Bam*HI site introduction. Hydrophilic rather than hydrophobic regions of Sec61p were used as breakpoints in order to guard against the likely loss of function resulting from fragmentation in probable transmembrane domains (Zen *et al.*, 1994). The structure of the Sec61p fragments that were generated is shown in Figure 1B.

Sec61p can be functionally expressed as complementary TM 1–5 and TM 6–10 polypeptides

Initially, the *SEC61* gene was restricted into two approximately equal size fragments corresponding to a breakpoint in the hydrophilic segment ('loop') between TM5 and TM6 (Figure 1). This produced a fragment containing the first five putative transmembrane segments and part of the TM 5–6 intervening loop (fragment 1–5) and a C-terminal fragment containing the last five transmembrane segments (fragment 6–10). The 243 amino acid residue 1–5 fragment consists of the first 229 amino acid residues fused to the 12 most C-terminal residues of Sec61p (T469–M480) via a GS linker corresponding to the sequence encoding the *Bam*HI sites remaining in the fusion (see Materials and methods). Similarly, the 264 residue 6–10 fragment was made by fusion of the 13 most N-terminal residues (M1–F13) of Sec61p to GS–L232–M480. Since both the 1–5 and 6–10 fragments share the extreme C-terminal residues of Sec61p (T469–M480) they can both be detected with the antiserum previously raised against this epitope (Stirling *et al.*, 1992).

Two haploid yeast strains, BWY12 and BWY47 (see Materials and methods), were used in order to examine expression and function of Sec61p fragments. Both of these haploid strains contain the *sec61*::*HIS3* null mutation, complemented in each case by a low-copy *URA3* plasmid encoding a functional form of Sec61p lacking the extreme C-terminus, abolishing recognition by antiserum raised against this region of the protein (Stirling *et al.*, 1992). In the case of BWY12, *SEC61* is under control of its own promoter, whilst the plasmid present in BWY47 has *SEC61* under the control of the *GAL1* promoter, permitting growth of this strain on galactose- but not on glucose-containing medium (Wilkinson *et al.*, 1996). Introduction of a second plasmid expressing a mutant of *sec61* into BWY47 followed by a shift from galactose to glucose medium provided a test for functional complementation of the null mutant (see Wilkinson *et al.*, 1996).

Low-copy-number *LEU2* plasmids expressing either the 1–5 or the 6–10 fragment were introduced into yeast strains BWY12 and BWY47. Neither fragment could provide Sec61p function when expressed in strain BWY47 grown on glucose (Figure 2A), although both fragments were expressed in BWY12 (Figure 2B, lanes 3 and 4). However, whilst the 1–5 fragment was expressed at a level comparable with wild-type Sec61p, the 6–10 fragment was relatively poorly expressed (Figure 2B, lane 4). Expression of either fragment was not apparent in immunoblots of the BWY47 strains grown on galactose (data not shown). Analysis of the combined expression of 1–5/6–10 was performed by introduction of a low-copy-number plasmid encoding both fragments into strains BWY12 and BWY47. This combination of fragments could complement the *sec61*::*HIS3* null mutation in BWY47 upon transfer to glucose medium (Figure 2A). Following passage of this strain on 5-fluoroorotic acid (5-FOA) medium to counter-select the *GAL–SEC61* plasmid (pBW62), only a slight growth defect was apparent (Table I). The level of the 1–5 and 6–10 co-expressed polypeptides (Figure 2B, lane 5) was similar to the individually expressed fragments in the presence of Sec61p (Figure 2B, lanes 2 and 3), although the 6–10 polypeptide was clearly less abundant than the 1–5 species. However, the level of the co-expressed fragments was increased when this combination provided the only source of functional Sec61p in a *sec61* null mutant background (Figure 2B, lane 6).

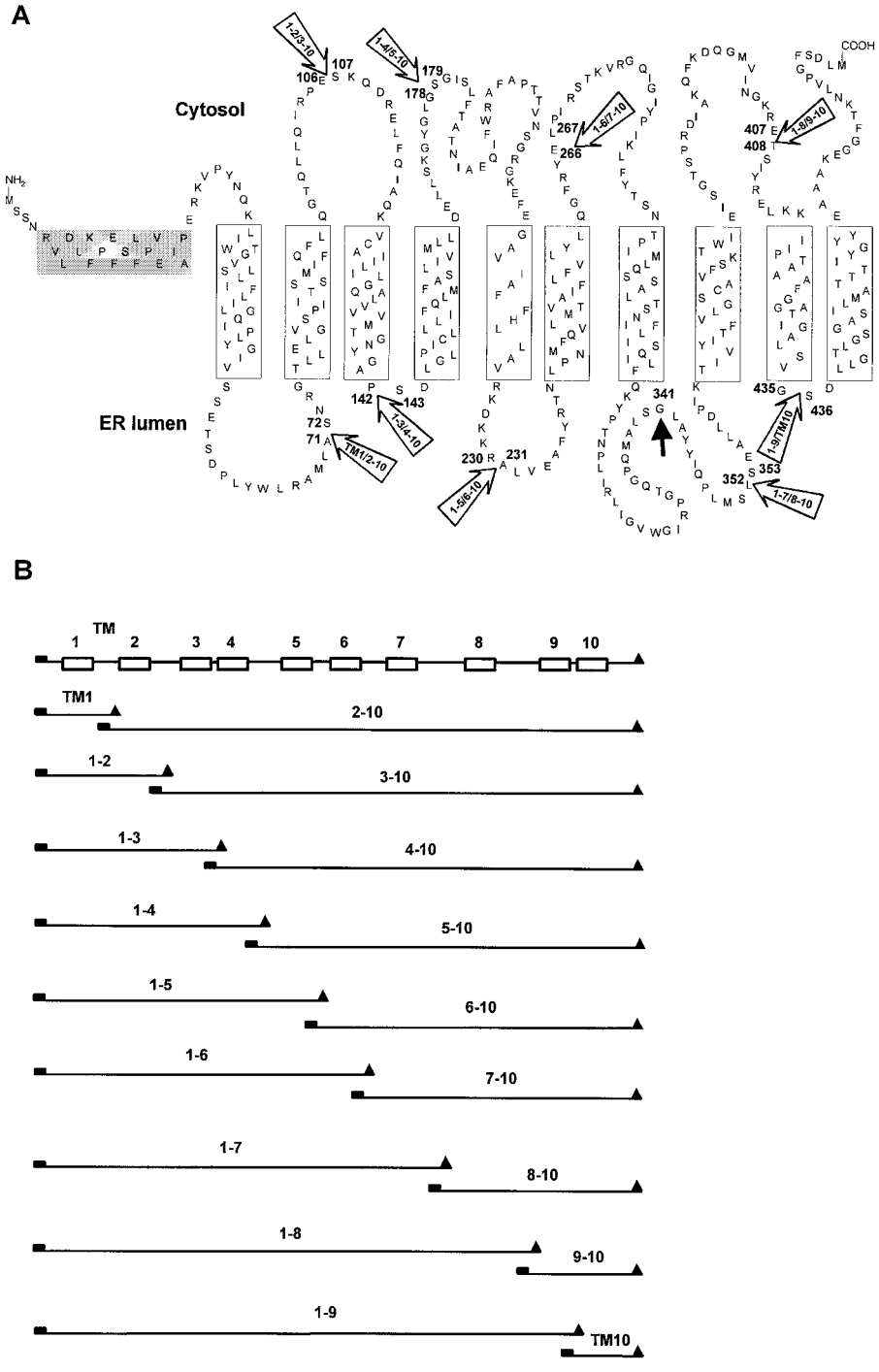

Fig. 1. The structure of Sec61p polypeptide fragments and complementary combinations. (**A**) Sequence and proposed topology of Sec61p in the ER membrane. The single-letter amino acid code is used and the 10 putative transmembrane domains (TM1–10) are shown in boxes. A putative amphipathic helix at the N-terminus is indicated by shading. The model is based on a hydropathy plot of the primary amino acid sequence and empirical analysis of topology (Wilkinson *et al.*, 1996). The positions of the nine breakpoints used in the analysis of complementary Sec61p fragments are shown by the arrows and numbered pairs of residues. A bold arrow marks the position of the residue altered by the *sec61-3* mutation in the TM7–8 hydrophilic lumenal loop. (**B**) Diagrammatic representation of Sec61p polypeptides shown as complementary pairs. All polypeptides were expressed as fragments that included uninterrupted putative transmembrane domains, and shared the same 13-residue N-terminus (residues M1–F13 of Sec61p, shaded rectangle) and the same 12-residue C-terminus (residues T469–M480, shaded triangle). They are named according to the transmembrane domains included. Polypeptide TM1 contains residues M1–L70 fused to GS–T469–M480 (84 residues). Correspondingly, polypeptide 2–10 contains residues M1–F13–GS fused to N73–M480 (423 residues). The number of residues contained in the remaining polypeptides are shown in the following in parentheses; 1–2 (119), 3–10 (388), 1–3 (155), 4–6(352), 1–4 (191), 5–10 (316), 1–5 (243), 6–10 (264), 1–6 (279), 7–10 (288), 1–7 (365), 8–10 (142), 1–8 (420), 9–10 (87), 1–9 (448) and 10 (59). The total number of residues for each complementary pair is 507, with a combined molecular weight of 56 kDa.

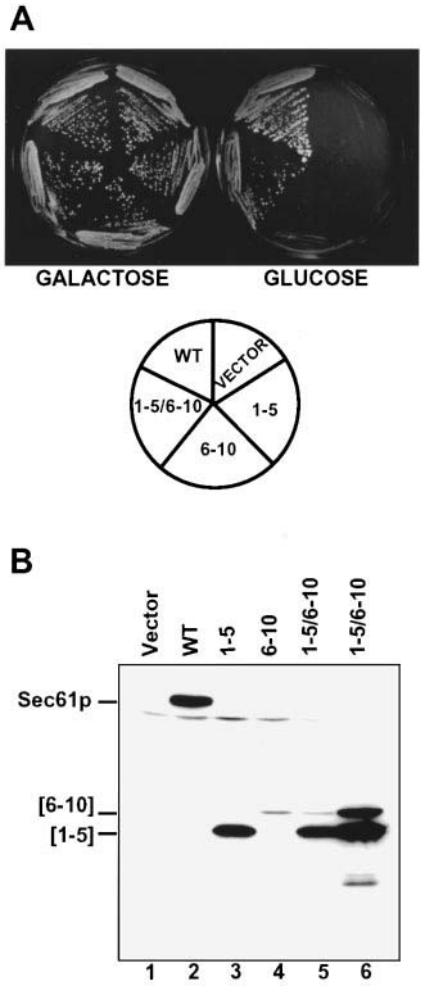

Fig. 2. Examination of function and expression of the 1–5 and 6–10 Sec61p polypeptides. (**A**) The ability of the fragments both individually and in combination to provide Sec61p function was examined by introduction of low-copy-number plasmids expressing these polypeptides into BWY47. Transformants selected on galactose-containing medium were plated to appropriately supplemented minimal medium containing either 2% galactose or 2% glucose. All strains grew normally on galactose medium, but the vector control (pRS315) and the 1–5- and 6–10-expressing strains were unable to grow on glucose-containing medium. However, the ability of the 1–5/6–10 combination to grow on glucose medium demonstrated its ability to provide Sec61p function as shown by the wild-type *SEC61* (pBW11-containing) control strain. (**B**) Whole-cell extracts were prepared as previously described (Wilkinson *et al.*, 1996) from BWY12 strains containing vector (pRS315, lane 1), a wild-type *SEC61* plasmid (pBW11, lane 2) or low-copy plasmids expressing the 1–5 (lane 3), 6–10 (lane 4) or combined 1–5/6–10 polypeptides (lane 5). An extract was also prepared from BWY47 expressing the combination (lane 6) after passage on 5-FOA-containing medium (see text). Aliquots of 1.0 A_{600} cell equivalents of each extract were resolved by 12.5% SDS–PAGE before immunoblotting with antiserum specific for the C-terminus of Sec61p. The positions of Sec61p and, in brackets, the 1–5 and 6–10 polypeptides are shown on the left.

Other combinations of complementary polypeptides can provide Sec61p function

Using the same strategy described above for the 1–5/6–10 combination, plasmids expressing further fragments and another eight combinations TM1/2–10, 1–2/3–10, 1–3/4–10, 1–4/5–10, 1–6/7–10, 1–7/8–10, 1–8/9–10 and 1–9/TM10 were constructed (Figure 1B) and tested for their ability to provide Sec61p function. None of the individual fragments could provide Sec61p function from a low-copy *LEU2* vector when transformed into BWY47 and tested for growth on glucose. As with the 1–5/6–10 combination, the TM1/2–10, 1–2/3–10, 1–3/4–10, 1–4/5–10, 1–8/9–10 and 1–9/TM10 combinations could provide Sec61p function in this assay. The growth rates of strains expressing these polypeptide combinations after passage on 5-FOA medium to counterselect pBW62 were found to vary from a small defect (1–2/3–10) to a 142 min doubling time for the 1–4/5–10 combination (Table I). Expression of these polypeptide combinations was analysed by SDS–PAGE of membranes (Figure 3). The weak or absent detection of the smaller polypeptides (TM1, 1/2, 9/10 and TM10) on immunoblots might be the result of sub-stoichiometric expression compared with their larger partner fragment, but could also be due poor adsorption to membranes during Western transfer. This latter explanation seems more likely, since membranes derived from strains containing fragment combinations treated with amine-reactive homobifunctional agents efficiently yielded an adduct of 44 kDa consistent with the product of the complementary fragments (see following sections). Compelling evidence for the expression of the smaller polypeptide fragments is provided by the fact that their larger complementary fragments cannot provide Sec61p function in their absence.

Table I. Growth rates of yeast strains expressing Sec61p as functional complementary fragments

Sec61p combination	Growth rate (min)
Wild-type	95
TM1/2–10	130
1–2/3–10	102
1–3/4–10	135
1–4/5–10	142
1–5/6–10	110
1–7/8–10[a]	165
1–8/9–10	114
1–9/TM10	105

Each pair of Sec61p complementary fragments was expressed jointly from a low-copy-number plasmid. Functional expression was obtained after introduction of these plasmids into yeast strain BWY47 followed by a plasmid shuffle into a *sec61* null mutant background on 5-FOA-containing medium (see text and Materials and methods). Growth rates were determined by optical density measurement at 600 nm of shaking cultures grown in YPD at 30°C. The wild-type growth rate was determined using strain BWY67.
[a]The 1–7/8–10 Sec61p polypeptide combination was functional only upon over-expression of *SSS1* (see text).

The lethality of the 1–7/8–10 fragment combination is suppressed by Sss1p

The 1–6/7–10 and 1–7/8–10 combinations were unable to complement the *sec61*::*HIS3* mutation when low-copy-number plasmids encoding them were introduced into BWY47 and the resulting transformants were transferred to glucose medium. The combined and individual fragments were very poorly expressed in strain BWY12 (data not shown). These observations suggest that these Sec61p polypeptide combinations are unable to form stable and functional Sec61 complexes. The possibility that these combinations are defective in their interaction with Sss1p was addressed by over-expression of Sss1p. A multicopy *TRP1* plasmid encoding *SSS1* or the vector alone (see

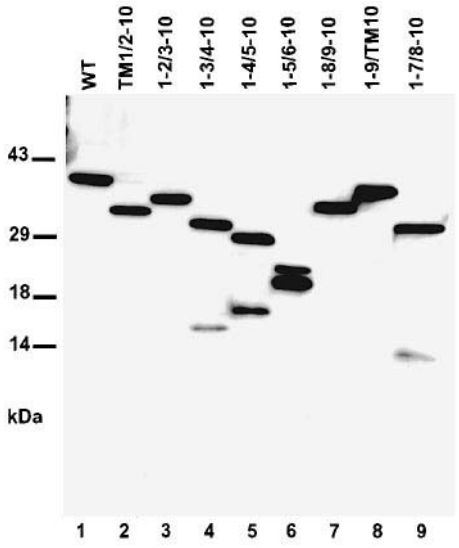

Fig. 3. Expression of functional complementary pairs of Sec61p polypeptides. Microsomes were prepared from BWY47 strains after passage on 5-FOA medium expressing either wild-type Sec61p or complementary fragments of Sec61p. Aliquots of 0.1 A_{280} U membrane were resolved by 10–20% gradient SDS–PAGE before immunoblotting with antiserum specific for the C-terminus of Sec61p. All fragments were detected with the exception of the four smallest (TM1, 1–2, 9–10 and TM10). The 2–10 and 3–10 polypeptides displayed anomalous migration with respect to each other (lanes 2 and 3), since the larger 2–10 fragment migrated as the smaller of the two. The 1–7/8–10 combination was expressed in BWY47 (lane 9) only in the presence of multicopy *SSS1*, which is a requirement for functionality of this fragment pair (see text and Figure 4).

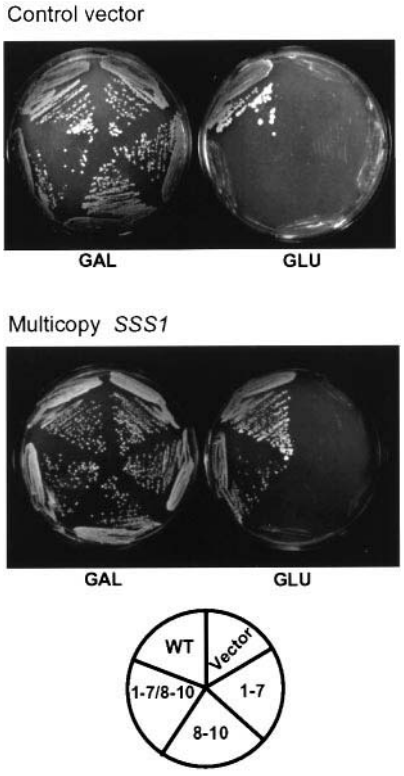

Fig. 4. Functional analysis of the 1–7 and the 8–10 Sec61p polypeptides. Yeast strain BWY47 was transformed with low-copy plasmids expressing wild-type *SEC61* (pBW11), the 1–7 fragment, the 8–10 fragment, the 1–7/8–10 combination or a control vector (pRS315) together with a multicopy *SSS1* plasmid (lower panel) or its control vector pRS424 (upper panel). Transformants selected on galactose medium were analysed on appropriately supplemented minimal medium containing 2% galactose (GAL) or 2% glucose (GLU). The strain containing the wild-type plasmid grew under all conditions, but only the 1–7/8–10 combination of fragments in the presence of over-expressed *SSS1* grew (lower panel), consistent with the ability to provide Sec61p function.

Materials and methods) was introduced into BWY47 together with these fragment combinations. Upon transfer to glucose-containing medium the normally lethal 1–7/8–10 Sec61p was rescued by multicopy *SSS1*, albeit with a substantial growth defect (Figure 4). After passage on 5-FOA medium the resulting strain clearly expressed both fragments (Figure 3, lane 9) and grew with a doubling time of 165 min (Table I). However, the 1–6/7–10 combination was not similarly rescued by multicopy *SSS1*.

Sss1p can be crosslinked to Sec61p

Both Sss1p and Sec61p are good candidates for crosslinking analysis using amine-specific homobifunctional crosslinking agents. Sec61p contains 21 lysine residues, predicted to be located in the hydrophilic loops of the protein (Stirling *et al.*, 1992), and a single lysine residue (K378), predicted to be located in the bilayer (TM8). The 80 amino acid Sss1p contains 10 lysine residues in the hydrophilic cytosolic region and a single lysine (K69), which is predicted to be on the lumenal face of the ER membrane (Esnault *et al.*, 1994). Microsomes prepared from the wild-type strain W303-1B were treated with the membrane permeable, amine-specific homobifunctional agent disuccinimidyl suberate (DSS), which has a spacer arm of 1.2 nm. Treated samples were loaded in duplicate and resolved by SDS–PAGE, then immunoblotted with either Sec61p or Sss1p antiserum. A DSS-specific immunoreactive band of ~50 kDa was detected with Sss1p antiserum (Figure 5, lanes 1 and 5) and also with antiserum directed against the C-terminus of Sec61p (Figure 5, lane 4). These observations would be consistent with formation of an adduct between Sec61p and Sss1p. Microsomes prepared from BWY12, a strain expressing a truncated Sec61p lacking the C-terminal 10 residues as the only source of the protein, were also treated with DSS. A smaller product of ~48 kDa detected with this mutant Sec61p using Sss1p antiserum (Figure 5, lane 6) provided further evidence that these adducts are between Sec61p and Sss1p.

Sss1p can be crosslinked to specific Sec61p fragments

Microsomes prepared from the strains expressing the TM1–2/3–10, 1–3/4–10, 1–4/5–10, 1–5/6–10, 1–8/9–10 and 1–9/TM10 Sec61p combinations were treated with DSS and analysed as described in the previous section in order to examine Sss1p crosslinking more precisely. Analysis of the 1–2/3–10 pair yielded a DSS-specific product of 41 kDa detected with both Sec61p and Sss1p antisera, consistent with Sss1p crosslinking to the 3–10 fragment (Figure 6A, lanes 2 and 3). Crosslinking between the 1/2 and 3–10 fragments was also apparent with the appearance of a 44 kDa adduct detected with Sec61p antiserum (Figure 6A, lane 3). Similarly, a 41 kDa DSS adduct was formed between Sss1p and the 4–10 fragment of the 1–3/4–10 pair (Figure 6B, lanes 2 and 3). The two fragments also formed an efficient adduct with each other

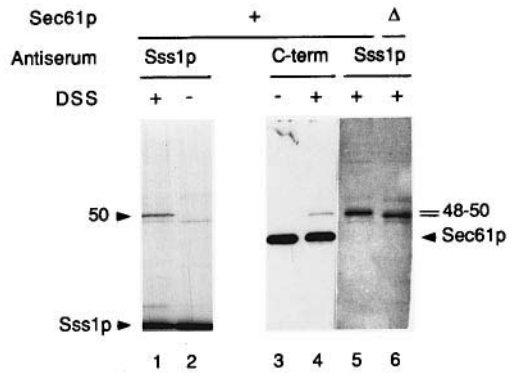

Fig. 5. Chemical crosslinking of Sss1p to Sec61p. Microsomes prepared from W303-1B, a wild-type strain, and BWY12, which expresses a functional Sec61p truncated at the C-terminus, were treated with DSS or mock treated. Reaction products were resolved by SDS–PAGE on either a 14% (lanes 1–2) or a 12% (lanes 3–6) polyacrylamide gel then immunoblotted with either Sss1p antiserum or a C-terminal Sec61p antiserum (C-term) as indicated. In the case of lanes 3–6 duplicate samples were resolved on the same gel and the blot appropriately cut after Western transfer in order to probe reaction products with different antisera. Note that the samples in lanes 1, 4 and 5 are from a single reaction, as are those in lanes 2 and 3. The absence of detectable Sss1p signal in lanes 5 and 6 is due to the lower percentage gel required to resolve the molecular weight shift of the adduct. Molecular weights are shown in kDa. The positions of Sss1p and the 50 kDa product are shown on the left. The positions of Sec61p and the 48 kDa adduct formed by truncated Sec61p (Δ) are shown on the right. Truncated Sec61p (Δ) and any crosslinked products it formed could not be detected with the C-term Sec61p antiserum because it lacks the epitope against which this antiserum was raised.

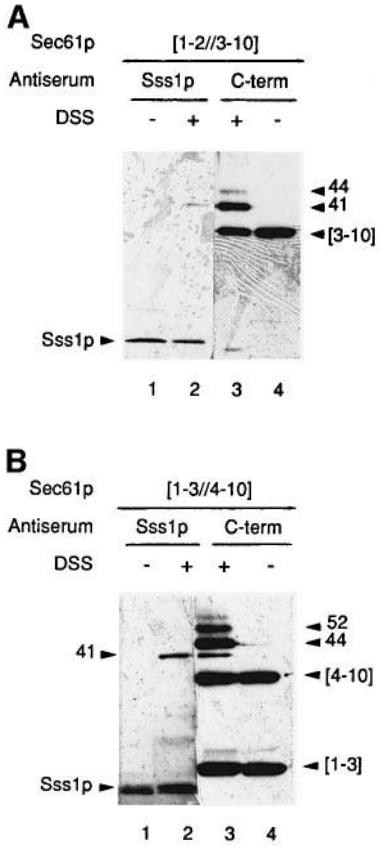

Fig. 6. Chemical crosslinking of Sss1p to the 1–2/3–10 and the 1–3/4–10 combinations of Sec61p polypeptides. (**A**) Microsomes prepared from a yeast strain expressing the 1–2/3–10 combination were treated with DSS and duplicate samples were resolved on a 14% SDS–PAGE gel before immunoblotting individually with Sss1p and Sec61p (C-term) antisera. The 3–10 polypeptide is indicated in brackets. (**B**) Microsomes prepared from a strain expressing the 1–3/4–10 combination were treated with DSS and analysed as described above. The 1–3 and 4–10 polypeptides are shown in brackets. Molecular weights are indicated in kDa.

of 44 kDa (Figure 6B, lane 3). In addition, further products of 52 kDa and a less intense slightly larger species were also detected with anti-Sec61p serum only (Figure 6B, lane 3). The identity of these adducts is unknown, although they may be the result of combinations of Sec61p polypeptides other than a complementary pair.

Analysis of the 1–4/5–10 combination revealed a crosslink of 38 kDa detected with both Sec61p and Sss1p antisera, indicative of a crosslink between Sss1p and the 5–10 fragment (Figure 7A, lanes 2 and 3). Again, a Sec61p antiserum-specific adduct of 44 kDa was formed consistent with crosslinking of complementary fragments and a 58 kDa adduct of unknown identity was also detected (Figure 7A, lane 3). Sss1p antiserum-specific products of 22 and 26 kDa were also detected after crosslinking (Figure 7A, lane 2). The 22 kDa product or a product of similar size, depending on the gel conditions, was detected with all Sec61p combinations analysed upon longer exposures (data not shown). In contrast, the 26 kDa product is unique to the 1–4/5–10 combination and is unlikely to represent a crosslink between Sss1p and the 1–4 polypeptide, since this product was not detected with Sec61p antiserum (Figure 7A, lane 3). The 1–8/9–10 combination revealed a doublet of 43/44 kDa upon immunoblotting the crosslinked products with Sec61p antiserum (Figure 7B, lane 3). A 43 kDa adduct was also detected with Sss1p antiserum, consistent with a crosslink between Sss1p and the 1–8 polypeptide (Figure 7B, lanes 2). Crosslinking of the 1–8 and 9–10 polypeptides would explain the slightly larger 44 kDa adduct detected with Sec61p antiserum only (Figure 7B, lane 3). A crosslink between Sss1p and the 1–9 polypeptide of the 1–9/10 combination of co-expressed polypeptides was also obtained (unpublished data).

Analysis of the 1–5/6–10 combination showed a single adduct detected with both Sec61p and Sss1p antisera, consistent with crosslinking to a single fragment (data not shown). Although the apparent molecular weight of the adduct was most compatible with crosslinking to the 6–10 fragment, it was not possible to reach a firm conclusion because of the similar migration of the 1–5 and 6–10 polypeptides (Figure 3, lane 6). The data from the 1–8/9–10 and 1–9/10 combinations indicate that the crosslink observed with the 1–5/6–10 combination probably occurred to the 6–10 polypeptide. In this case the data demonstrates that Sss1p crosslinks with Sec61p polypeptides 3–10, 4–10, 5–10, 6–10, 1–8 and 1–9, identifying a common domain defined by residues L232–R406, the 5–6 breakpoint to the 8–9 breakpoint (Figure 1A). It was not possible to map the Sss1p interaction using the 1–7/8–10 combination rescued by over-expression of Sss1p. Although the experimental conditions were varied (crosslinking reagent, time and temperature), no crosslink was observed between Sss1p and either the 1–7 or the 8–10 Sec61p polypeptides, while a 44 kDa adduct was efficiently formed consistent with crosslinking of these complementary Sec61p fragments (data not shown).

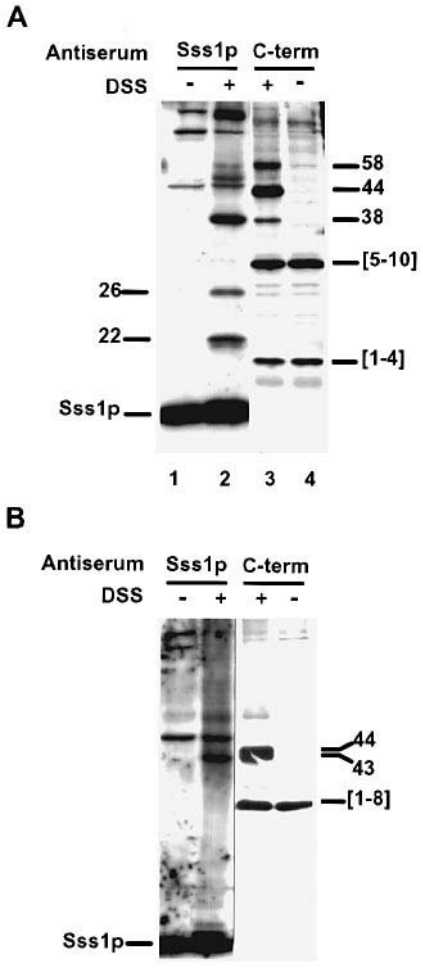

Fig. 7. Chemical crosslinking of Sss1p to the 1–4/5–10 and the 1–8/9–10 combinations of Sec61p polypeptides. (**A**) Microsomes prepared from a yeast strain expressing the 1–4/5–10 combination were treated with DSS and duplicate samples were resolved by 12% SDS–PAGE before immunoblotting individually with Sss1p and Sec61p (C-term) antisera. Molecular weights are indicated in kDa. Sec61p polypeptides are shown in brackets. (**B**) Microsomes prepared from a yeast strain expressing the 1–8/9–10 combination were treated with DSS and duplicate samples were resolved by 10% SDS–PAGE on a gel with a 15% phase at the bottom in order to resolve the 43/44 kDa bands shown on the right and to retain Sss1p (shown on the left) on the gel. Higher molecular weight (>44 kDa) Sss1p-immunoreactive species were occasionally detected both with and without DSS treatment and are probably the result of non-specific cross-reactivity.

The sec61-3 mutation is located in the TM7–8 loop

The *sec61-3* allele was recovered from the genomic DNA of yeast strain CSY150 (*sec61-3*) by PCR amplification and the entire nucleotide sequence was determined on both DNA strands from five independent amplifications (see Materials and methods). The nucleotide sequence data were uniformly consistent for each PCR product. Only a single point mutation located at position 1467 of the *SEC61* nucleotide sequence (Stirling *et al.*, 1992) was found in each case, corresponding to a GGG→GAG transition that introduces a non-conservative G341E amino acid change responsible for the *sec61-3* mutation. According to the 10 transmembrane spanning model for Sec61p this places the mutation in the TM7–8 loop (Figure 1A). Over-expression of Sss1p suppresses the temperature-sensitive phenotype of *sec61-3* by stabilizing the mutant protein at the non-permissive temperature (Esnault *et al.*, 1993, 1994), consistent with an interaction between the lumenal TM7–8 loop of Sec61p and Sss1p.

Discussion

We have previously determined the topology of Sec61p and presented a model in which it spans the ER bilayer 10 times (Wilkinson *et al.*, 1996). In this report we have used this model to 'fragment' Sec61p into inactive polypeptides that restore Sec61p function when co-expressed with their complementary fragment. Furthermore, we have used these mutants to probe the interaction between the Sec61p and Sss1p components of the Sec61 complex. The resulting data and a number of genetic observations have led to identification of a specific region of Sec61p in contact with Sss1p.

The ability of several Sec61p fragment pairs to function implies the existence of multiple signal sequences within Sec61p directing correct assembly of each fragment into the ER membrane. However, it should be noted that assembly of any given fragment might not be independent of its partner. For example, we have previously reported that downstream sequences are required for correct membrane integration of TM5 and that these can be supplied *in trans* (Wilkinson *et al.*, 1996). This model predicts a direct interaction between the 1–5 and 6–10 polypeptides in order to achieve an active conformation in the bilayer. Intriguingly, the 6–10 fragment is present at low levels when expressed either singly (Figure 2B, lane 4) or when in combination with the 1–5 fragment in a strain also expressing intact Sec61p (Figure 2B, lane 5). In contrast, the levels of both the 1–5 and 6–10 fragments are significantly enhanced when expressed as the sole source of Sec61 function (Figure 2B, lane 6), suggesting stabilization of fragments within a functional translocation complex. These results indicate that co-expression of the 1–5 fragment is insufficient to stabilize 6–10 and suggest that other components of the translocation complex are also required. In the presence of intact Sec61p, the 1–5/6–10 combination is presumably unable to compete efficiently for these other components. A reduced affinity for components of the translocation machinery might explain the slight growth defect observed when the 1–5/6–10 combination complements a *sec61* null mutant (Table I).

The inability of any individual polypeptide fragment to complement the *sec61* null mutation demonstrates that Sec61p cannot tolerate deletion of even a single TM domain from either its N- or C-terminal ends. However, the integrity of hydrophilic loops between transmembrane domains is clearly not essential for Sec61p function, with the exception of the TM6–7 loop. A disjunction in the TM7–8 loop is also non-functional, but in this case function can be restored by over-expression of Sss1p (see below). The failure of the 1–6/7–10 combination to rescue the *sec61* null mutation indicates that the TM6–7 loop must remain intact in order to carry out an essential role either in the function and/or assembly of Sec61p into the ER bilayer. The breakpoint at L266 (Figure 1A) is close to a region which is particularly important for assembly of TM6 into the bilayer (Wilkinson *et al.*, 1996) and this may interfere with stable integration of TM6 into the ER membrane. Alternatively, it is possible that an intact

TM6–7 loop interacts with an essential component of the translocation machinery and that a break in this region disrupts this interaction. In either scenario, the failure of the 1–6 and 7–10 fragments to form a stable complex would account for the relatively low steady-state levels of these fragments detected in Western blots. In general terms, the rescue of a *sec61* null mutation by co-expression of complementary fragments might reflect assembly of those fragments into a functional molecular complex or dissection of Sec61p into independent biochemical activities. The efficiency with which each Sec61p fragment could be crosslinked to its complementary partner strongly suggests an intimate interaction between partners in the functional translocon.

The interaction between Sec61p and Sss1p was probed by chemical crosslinking and assayed by co-migration of adducts detected with both Sss1p and Sec61p specific antisera. In wild-type microsomes the major crosslinked species detected with Sss1p antibodies was shown to be marginally smaller when a truncated derivative of Sec61p was used, thus confirming identification of this species as a Sec61p–Sss1p adduct. When the same criteria were employed in the interpretation of adducts formed between Sss1p and various Sec61p fragments, the data indicate that Sss1p becomes crosslinked to Sec61p in the region between Sec61p residues L232 and R406. This 175 residue region within Sec61p contains eight lysine residues located within either TM8 (K378) or in the extramembranous loops located between TM6–7 (K273, K284), TM7–8 (K314, K361) and TM8–9 (K393, K396, K405) (see Figure 1), from which we conclude that one or more of these domains is in close proximity to Sss1p. Given the absence of lysine residues in the single transmembrane domain of Sss1p, then it seems highly probable that the observed crosslinking between Sss1p and Sec61p occurs between extramembranous domains.

Sss1p is also found in a complex with the Sec61p homologue Ssh1p (Finke *et al.*, 1996). Sequence alignment of Sec61p and Ssh1p revealed two particularly well-conserved regions corresponding to Sec61p residues E266–T288 (70% identical) and I383–A422 (59% identical), compared with 32% identity overall (Finke *et al.*, 1996). These regions correspond to the TM6–7 loop (Q261-N290), the TM8–9 loop (K378-K416) and part of TM9 of Sec61p. Both of these conserved cytosolic loops lie within the region of Sec61p responsible for the major DSS crosslinks to Sss1p. This would be consistent with these conserved loops being involved in direct interactions with the cytosolic domain of Sss1p in both the Sec61 and Ssh1 complexes. Interestingly, we did not detect any adducts between Sss1p and Ssh1p in our crosslinking assay. This was probably the result of lability of Ssh1p in both whole-cell extracts and microsome preparations (data not shown). The TM1/2–10, 1–2/3–10, 1–3/4–10, 1–4/5–10, 1–5/6–10, 1–8/9–10 and 1–9/TM10 Sec61p fragment combinations are functional in a Δ*sec61*, Δ*ssh1* strain background, indicating that these mutants were not rescued by *SSH1* (data not shown). This suggests that although Sec61p and Ssh1p may overlap in function, these Sec61p mutants have retained the essential function of Sec61p.

In addition to the adducts observed between Sec61p fragments and Sss1p, several unassigned adducts were also detected with both Sec61p and Sss1p antibodies. These may represent crosslinks to Sbh1p or to members of other proteins complexes located at the translocation site (e.g. the signal peptidase, oligosaccharyl transferase or Sec62p–Sec63p complexes). The Sec61p polypeptide combinations may be sufficiently altered in structure as to change the location of some lysine residues relative to adjacent proteins, allowing formation of crosslinks not detected with the intact Sec61p molecule. Alternatively, assembly of Sec61p into the ER bilayer may normally require molecular chaperones. Some of the Sec61p fragments may have a prolonged association with such chaperones during their ER assembly, which might account for some of the unassigned adducts. The 19–22 kDa band detected with antiserum directed against Sss1p in DSS-treated membrane extracts may correspond to a homodimer of the protein. The presence of Sss1p in more than one membrane complex and possible dimeric forms may account for the reported Sss1p/Sec61p stoichiometry of 6.3 ± 1.3 (Esnault *et al.*, 1994). Alternatively, this species could be an adduct of Sss1p with Sbh1p or Sbh2p. The role of Sbh1p in the architecture of the Sec61 complex has not been addressed in this report and crosslinking between Sec61p and Sbh1p may not be apparent in this study since such adducts may co-migrate with the Sec61p–Sss1p adduct. The existence of Sbhp homologues specific to either the Sec61p or Ssh1p complexes (Finke *et al.*, 1996) does, however, suggest a direct interaction between Sbh proteins and their polytopic partner.

Purified yeast and mammalian Sec61 complexes have been shown to form oligomeric structures, consisting of three to four Sec61p heterotrimers, upon reconstitution into lipid or detergent films (Hanein *et al.*, 1996). Given the efficiency with which Sec61p fragments can be crosslinked, then it is perhaps surprising that we do not detect any DSS-induced Sec61p dimers in wild-type microsomes. Of course, this may be due to the formation of high molecular weight adducts which fail to enter the gel system. Alternatively, Sec61p monomers may not be in direct contact with another within the observed oligomeric rings, but rather may interact only via Sss1p and/or Sbh1p. Such a role for Sss1p might explain its essential role and its affect on stability of various mutant derivatives of Sec61p.

Identification of the TM6–TM8 region within Sec61p as a major site of interaction with Sss1p is supported by two lines of genetic evidence. Firstly, the 1–7/8–10 fragment combination is unable to function unless Sss1p is over-expressed. This would be consistent with the discontinuity in the TM7–8 lumenal loop resulting in a reduced affinity for Sss1p. Over-expression of Sss1p might then drive the equilibrium towards formation of a Sec61p–Sss1p complex, thus rescuing the defect. Even under circumstances where Sss1p is over-expressed we have been unable to detect DSS-specific crosslinks to either the 1–7 or 8–10 fragments, consistent with a more labile interaction between the proteins. Secondly, the *sec61-3* point mutation results in a thermolabile protein and a temperature-sensitive growth phenotype which can be rescued by increased expression of Sss1p (Esnault *et al.*, 1994). We have mapped the *sec61-3* mutation to a Gly→Glu change at residue 341 within the lumenal TM7–8 loop, again implicating this domain in the interaction between Sss1p

and Sec61p. These data would be consistent with a direct interaction between the TM7–8 loop of Sec61p and the lumenal 12 residue domain of Sss1p (Esnault *et al.*, 1994). Alternatively, mutations in the TM7–8 loop might alter the conformation of the two cytosolic loops implicated in Sss1p crosslinking, possibly via changes in the relative orientations of transmembrane domains 7 and 8.

Studies on interaction between the SecY and SecE proteins of the SecYEG complex of *Escherichia coli*, which are distantly related to the Sec61p/Sec61α and Sss1p/Sec61γ proteins (Stirling, 1993; Hartmann *et al.*, 1994), has revealed some parallels with our findings. Deletion analysis has shown that a region containing the third transmembrane domain of SecE, a membrane protein which is essential for both growth and translocation, is sufficient for activity (Schatz *et al.*, 1991). It is this region of the protein from a number of bacterial species that show some identity with Sss1p/Sec61γ (Hartmann *et al.*, 1994) and which also interacts with SecY (Nishiyama *et al.*, 1992). SecY has been proposed to span the cytoplasmic membrane 10 times (Akiyama and Ito, 1987) in a model similar to Sec61p (Wilkinson *et al.*, 1996). Genetic studies have provided evidence that SecE interacts with the TM6–7 loop (Baba *et al.*, 1994) and domains TM1, TM7 and TM10 (Flowers *et al.*, 1995) of SecY.

The experimental approaches described in this report may be widely applicable in analysis of subunit interactions and architecture of membrane protein complexes. A more detailed analysis of the Sec61p–Sss1p interaction might be possible by systematic removal of the Sec61p lysine residues implicated in Sss1p crosslinking. In addition, isolation of mutants defective in this interaction and subsequent isolation of suppressor mutations will allow an analysis of functional aspects of this interaction. Furthermore, using functional co-expressed Sec61p polypeptides it may be possible to analyse interactions between Sec61p and other components of the targeting/translocation machinery and with a translocating polypeptide chain.

Materials and methods

Materials

DNA restriction and modification enzymes were purchased from Boehringer Mannheim. Sequenase© version 2.0 and sequencing reagents were from United States Biochemicals. Detergents and lyticase were from Sigma (St-Quentin Fallavier, France). DSS was purchased from Pierce Chemicals (Touzart and Matignon, Vitry, France). 5-FOA was purchased from PCR Inc. (California). All other chemicals and reagents were purchased from Boehringer Mannheim, Sigma or British Drug House at analytical grade.

Strains and growth conditions

Bacterial and yeast strains used in this study are listed in Table II. *Escherichia coli* cells were grown in LB (1% tryptone, 0.5% yeast extract, 1% NaCl) at 37°C. Where appropriate, ampicillin was added to 100 μg/ml. Yeast strains were grown at 30°C in YP medium (2% peptone, 1% yeast extract) containing either 2% glucose (YPD) or 2% galactose (YPGAL) or in 0.67% Bacto yeast nitrogen base with 2% glucose or 2% galactose, plus appropriate supplements for selective growth. Solid media were supplemented with 2% Bacto agar. Culture media were obtained from Difco laboratories. Growth rates were determined in shaking YPD cultures at 30°C. Cell density was monitored by measuring optical density at 600 nm using a Series 2 CE292 spectrophotometer (Cecil Instruments, Scientific and Medical Supplies, Cambridge, UK). Yeast transformations were carried out using the one-step procedure (Chen *et al.*, 1992). Counterselection of pBW62 (*GAL1-SEC61*, *CEN*, *URA3*) in BWY47-derived strains was performed on medium containing 5-FOA according to standard procedures (Sikorski and Boeke, 1991). The haploid strain BWY12, which contains the *sec61*::*HIS3* lethal mutation covered by a rescuing plasmid pBW7 (see below), was derived by tetrad dissection of CSY110 which had been transformed to Ura^+ by pBW7. The haploid strain BWY67 provided a wild-type control for determination of growth rate defects. It was derived from BWY47 by means of a plasmid shuffle by transformation to Leu^+ by pBW11 and passage on 5-FOA to remove pBW62.

Vectors, plasmids and nucleic acid manipulations

DNA manipulations were carried out according to standard protocols (Sambrook *et al.*, 1989). *Escherichia coli* strains DH5α and DK1 were used as hosts (Table II). The *E.coli–S.cerevisiae* shuttle vectors pRS316 and pRS424 have been previously described (Sikorski and Hieter, 1989; Christianson *et al.*, 1992). The *GAL1*::*SEC61* encoding plasmid (pBW62) present in BWY47 has been previously described (Wilkinson *et al.*, 1996). Plasmid pBW7 was constructed by cloning a 2.4 kbp *Hin*dIII fragment encoding *SEC61* from pCS43 (Stirling *et al.*, 1992) into *Hin*dIII-linearized pRS316. Both pBW7 and pBW62 express a truncated version of the *SEC61* gene encoding a functional form of Sec61p lacking the C-terminal 10 amino acid residues and consequently the epitope to which the C-terminal antiserum was raised (Stirling *et al.*, 1992). Plasmid pBW171 provided multicopy, *TRP1* selectable expression of *SSS1*. It was constructed by cloning a 1.80 kb *Sal*I–*Eco*RI fragment encoding *SSS1* (Esnault *et al.*, 1993) into *Sal*I/*Eco*RI-digested pRS424. The single copy *LEU2* plasmid pBW11, encoding *SEC61*, and the mutant derivatives containing *Bam*HI sites introduced into the *SEC61* gene have been described previously (Wilkinson *et al.*, 1996).

Construction of plasmids encoding fragments of the Sec61p

Low-copy-number *LEU2*-selectable plasmids encoding fragments of Sec61p were constructed using a series of *Bam*HI sites introduced by substitution into the *SEC61* gene (see Wilkinson *et al.*, 1996). Construction of a plasmid (pBW102) encoding the 264 residue TM6–10 polypeptide fragment ($_{NH2}$M1–F13–GS–L232–M480$_{COOH}$) has been described previously (Wilkinson *et al.*, 1996). A plasmid (pBW103) encoding the TM1–5 polypeptide was constructed using the same strategy by digestion of pBW-688 jointly with *Xho*I and *Bam*HI, removing a 1.3 kb fragment

Table II. Yeast and bacterial strains

Strain	Genotype	Source or reference
Saccharomyces cerevisiae		
W303-1B	MATα *leu2-3,-112, ade2-1 ura3-1 his3-11,-15, trp1-1 can1-100*	Thomas and Rothstein (1989)
CSY110	MAT**a**/MATα *sec61*::*HIS3*/*SEC61 his3-11,-15*/*his3-11,-15 LEU2*/*leu2-3,-122 ura3-1*/*ura3-1 ade2-1*/*ade2-1 trp1-1*/*trp1-1 can1-100*/*can1-100*	Stirling *et al.* (1992)
BWY12	MATα *sec61*::*HIS3 leu2-3,-112 ade2-1 ura3-1 his3-11,-15 trp1-1 can1-100* [pBW7]	this study
BWY47	MATα *sec61*::*HIS3 leu2-3,-112 ade2-1 ura3-1 his3-11,-15 trp1-1 can1-100* [pBW62]	Wilkinson *et al.* (1996)
BWY67	MATα *sec61*::*HIS3 leu2-3,-112 ade2-1 ura3-1 his3-11,-15 trp1-1 can1-100* [pBW11]	this study
CSY150	MATα *sec61-3 leu2-3,-112 ura3-52 trp1-1*	Stirling *et al.* (1992)
Escherichia coli		
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan (1983)
DK1	Δ(*srl-recA*)*306 araD139* Δ(*ara*, *leu*) Δ*lacX74 galU*$^-$ *galK*$^-$ *hsdR*$^-$ *strA mcrA*$^-$ *mcrB*$^-$	Kurnit (1989)

including the majority of the C-terminal region. This was replaced by ligation with a 600 bp gel-purified *Xho*I–*Bam*HI fragment from pBW11-1399. The resulting fragment encoded a 243 residue polypeptide ($_{NH2}$M1–K229–GS–T469–M480$_{COOH}$). Both the 1–5 and 6–10 polypeptides share the same 13 residue N-terminus (residues M1–F13) and the same 12 residue C-terminus (residues T469–M480). The GS residues in each pair correspond to the numbered residues in Figure 1A defining the breakpoint for pairs of complementary polypeptides. A plasmid encoding both fragments was constructed by digestion of pBW102 with *Pst*I and end-filling by Klenow treatment followed by cloning of a 1.7 kb *Hin*dIII end-filled fragment encoding the 1–5 polypeptide from pBW103. Exactly the same strategy using appropriate previously inserted *Bam*HI sites (Wilkinson *et al.*, 1996) was used to construct a series of plasmids expressing fragments both individually and in combination. The exact sequences of these polypeptides are shown in Figure 1B.

Amplification and sequence analysis of the genomic sec61-3 locus

Genomic DNA was prepared from strain CSY150 essentially as described by Philippsen *et al.* (1991). The *sec61-3* locus was amplified between nucleotides 1 and 1940 of the published *SEC61* sequence (Stirling *et al.*, 1992) using AAGCTTGCTATAAGCTAGAA and GGGTGTGGCTA-AATGCG primers synthesized for this purpose. Five independent amplification reactions were carried out using *Taq* polymerase and 30 cycles in a Techne PHC-3 thermal cycler. The reaction products were agarose gel purified and sequencing was carried out by dye terminator cycle sequencing using AmpliTaq DNA polymerase FS (Perkin Elmer) and a series of primers complementary to both strands of *SEC61*. The reaction products were resolved on an ABI Prism 377 DNA sequencer (Perkin Elmer).

Preparation of yeast microsomes

Yeast cultures (200 ml) were grown in YPD to an A_{600} of 2–4. Cells were collected and spheroplasts were prepared according to Sanders *et al.* (1992) using 20 U lytic enzyme/A_{600} U cells. Spheroplasts were collected by centrifugation at 2000 *g* for 5 min at 4°C, resuspended at 100 A_{600} U/ml in lysis buffer (25 mM $NaPO_4$, pH 7.4, 0.2 M sorbitol, 0.1 M NaCl, 1 mM $MgCl_2$, 1 mM phenylmethanesulfonyl fluoride) and lysed by agitation in the presence of 0.2 µm glass beads. Unbroken cells were removed by centrifugation at 370 *g* for 10 min at 4°C. Microsomes were collected by centrifugation of the supernatant at 50 000 *g* for 12 min at 4°C (TLA100.3 Beckman rotor). The microsomes were resuspended at 1.0 A_{280} equivalent/10 µl lysis buffer.

Chemical crosslinking

For each reaction, 8 µl fresh 1% DSS solution prepared in dimethylsulfoxide (DMSO) were added to a 40 µl (0.4 A_{280} U) aliquot of membranes followed by incubation at room temperature for 15 min. Mock samples were treated with 8 µl DMSO. Reactions were terminated by addition of an equal volume of 0.2 M ammonium acetate with incubation on ice for 10 min. Membranes were harvested by centrifugation at 50 000 *g* for 15 min at 4°C (TLA100.1 Beckman rotor) and resuspended in 60 µl sample buffer (Laemmli, 1970), followed by incubation at 65°C prior to SDS–PAGE.

Immunoblot analysis of proteins

After migration on SDS–PAGE gels, proteins were transferred electrophoretically to nitrocellulose membranes (Schleicher & Schuell). Equal loading per lane was confirmed by a brief staining with Ponceau S. Preincubation, antibody incubations and washes were performed in TBS (10 mM Tris–HCl, pH 7.6, 2 mM KCl, 150 mM NaCl) plus 0.05% Tween 20 and 2% non-fat dried milk. Immunodetection was carried out using enhanced chemiluminescence (Amersham) with a peroxidase-conjugated anti-rabbit IgG (Sigma). The antiserum directed against an N-terminal peptide from Sss1p (the 'T' antiserum) has been described previously (Esnault *et al.*, 1993). Antiserum raised against the C-terminal peptide of Sec61p has also been previously described (Stirling *et al.*, 1992).

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