

**Functional analysis of Sss1p in *Saccharomyces*
*cerevisiae***

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Engineering

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Abstract

Polypeptides are translocated across the ER membrane into the lumen of the endoplasmic reticulum through gated channels termed translocons. The channel through which polypeptides are translocated is formed by the heterotrimeric Sec61 complex which comprises the polytopic membrane protein Sec61p and two tail anchor proteins Sss1p and Sbh1p. Expression of the Sss1 protein has been demonstrated to be essential for ER translocation and it has been postulated that Sss1p regulates the translocon, functioning as a surrogate signal sequence that occupies the signal peptide binding site prior to the incorporation of signal sequence into the translocation channel. Cysteine scanning mutagenesis has been used to probe the position of Sss1p within the translocon and crosslinking analysis has identified that the endogenous cysteine residue of Sss1p is in close proximity to the C- terminus of Sec61p. Furthermore, this is in agreement with the crystal structure determined from the inactive conformation of an archaeobacterial SecYE β translocon.

Chemical crosslinking has previously detected an 85 kDa Sss1p crosslinked adduct. Using gene repression analysis, this adduct has been found to represent a crosslink between Sss1p and the essential ER membrane component Sec63p. Furthermore, substitution of the transmembrane anchor of Sss1p with that from an unrelated C- terminal anchor protein, Ubc6p, has indicated that this interaction is dependent on the transmembrane domain of Sss1p. This data is consistent with Sss1p recruiting Sec63p to the translocation channel to form functional translocons. Also, it has been identified that Sec63p is phosphorylated at steady state and its phosphorylation would appear to require the expression of *SSS1*.

It has been reported that the Sec61 complex oligomerises to form the translocation channel. In agreement with this, Sss1p can be crosslinked to an adjacent Sss1p molecule via the extreme C- terminus. This region of Sss1p can also be crosslinked to Sec63p and both TM3 and 4 of Sec61p. Furthermore, these crosslinks are retained following the release of ribosomes from membranes. As release of ribosomes from membranes has been proposed to correlate with a switch from an active to an inactive conformation in mammalian microsomes this may imply that the translocon and translocation structures remain assembled whether the translocon is active or inactive.

The extreme C- terminus of Sss1p and its homologues are highly conserved. We have isolated two mutant alleles of *sss1*, *sss1-6* and *sss1-7*, where the residues that have been mutagenised are positioned in this region, have a significant growth and translocation defect at 30°C and are temperature sensitive. However, in contrast to other translocation defective mutants the unfolded protein response (UPR) is induced and ER associated degradation is defective indicating that misfolded proteins are retained in the ER lumen in these mutants. Also, glycosylation is aberrant in these cells. Together this would suggest that protein biogenesis in the ER lumen is perturbed in *sss1-6* and *sss1-7*.

The results presented imply a role for Sss1p regulating both the assembly and function of the ER translocon as well as maintaining ER function.

Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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Dedication

This is dedicated through love and gratitude to my family for their love and support throughout my studies.

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Abbreviations

AEBSF	4-(2-Aminoethyl)benzenesulphonylfluoride
AMP-PNP	Adenosine-5' (β , γ imino) triphosphate
APS	Ammonium persulphate
ATP	Adenosine-5'-triphosphate
BiP	B cell immunoglobulin heavy chain binding protein
BN-PAGE	Blue native polyacrylamide gel electrophoresis
bp	Base pair
BSA	Bovine serum albumin
C-	Carboxyl
CPY	Carboxypeptidase Y
DNA	Deoxyribo nucleic acid
dNTPs	Deoxynucleoside triphosphate
DPAP B	Dipeptidyl aminopeptidase B
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetra-acetic acid
EGTA	Ethylene glycol-bis (β - aminoethylether) - N, N, N', N'- tetraacetic acid
ER	Endoplasmic reticulum
ERAD	ER associated degradation
5'-FOA	5'-Fluoro-orotic acid
Gal	Galactose
GEF	Guanidine exchange factor
Glc	Glucose
GTP	Guanosine-5'-triphosphate
HEPES	N-2-hydroxyethyl piperazine-N'-(2-ethanesulphonic acid)
HRP	Horse radish peroxidase
Hsp	Heat shock protein
IPTG	Isopropyl β -D- thiogalactopyranoside
kb	Kilobase pair
kDa	Kilodalton
LB	Luria-Bertani media
LP-YPD	Low phosphate yeast peptone dextrose
mRNA	Messenger ribonucleic acid
MSB	Membrane storage buffer

N-	Amino
NP40	Nonidet P40
O.D.	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
RNA	Ribonucleic acid
RNase	Ribonuclease
rNTPs	Ribonucleoside triphosphates
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC	Secretion
SP	Signal peptide
SR	Signal recognition particle receptor
SRP	Signal recognition particle
TBS	Tris buffered saline
TEMED	N, N, N', N'-tetramethylethylenediamine
TM/TMD	Transmembrane domain
Tris	Tris-(hydroxymethyl) aminomethane
Ts	Temperature sensitivity
UPR	Unfolded protein response
UPRE	Unfolded protein response element
YP	Yeast peptone
YPD	Yeast peptone dextrose
YNB	Yeast nitrogen base

Chapter 1

Introduction

1.1 The endoplasmic reticulum and the secretory pathway

The eukaryotic cell is divided into functionally distinct membrane bound compartments, or organelles, each containing a defined complement of proteins.

The endoplasmic reticulum on average constitutes approximately half of the total surface area of membrane within the cell and is organised as a labyrinth like network of branching tubules and flattened sacs that interconnect forming a continuous membranous sheet enclosing a highly specialised luminal environment.

The ER is the entry point into the secretory pathway. The ER lumen is a highly controlled environment that has the capacity to modify proteins that enter it. Such modifications can include signal peptide cleavage, N-linked glycosylation, native protein folding and the formation of disulphide bonds. Following their translocation into the ER, proteins can either be retained or targeted to other organelles (figure 1.1). The passage of proteins from the ER to the cell surface via the Golgi, where they can undergo further processing (Herscovics and Orlean, 1993) is the default pathway. However, polypeptides may contain sorting signals for the retention within or passage to a specific compartment, for example the lysosome (the vacuole is the yeast equivalent of this organelle) or the endocytic machinery (Nothwehr and Stevens, 1994).

1.2 Signal peptides

N-terminal signal peptides target proteins into the secretory pathway in both eukaryotes and prokaryotes indicating that such mechanism of protein targeting is conserved through evolution (von Heijne, 1990). Comparative analysis has identified signal peptides to be typically tripartite in structure (figure 1.2) (von Heijne, 1990).

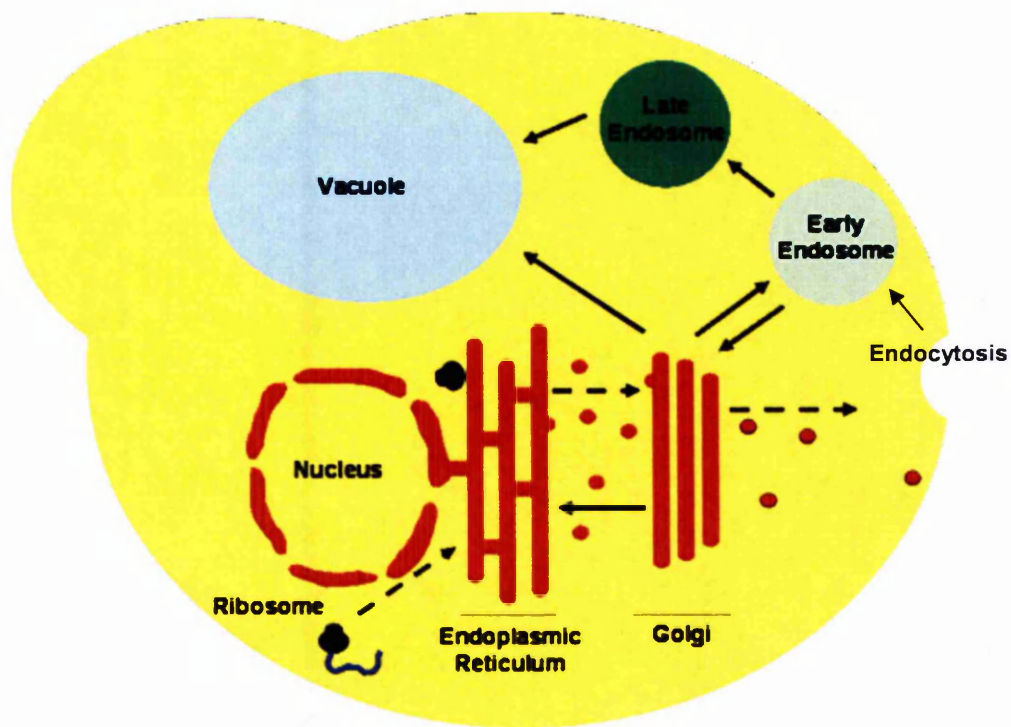


Figure 1.1 The secretory pathway. Proteins targeted to the secretory pathway are translocated from the cytosol into the endoplasmic reticulum. The broken arrow shows the default pathway through the secretory pathway. However proteins can be retained in a specific organelle or targeted to the vacuole or the endocytic machinery.

They comprise a central hydrophobic core (h-domain) that is flanked by an N-terminal extension that is typically positively charged (n-domain) and a hydrophilic C-terminal extension (c-domain). But signal peptides are highly variable as no precise sequence conservation could be identified.

Mutation analysis has revealed the importance of the signal peptide and the necessity of each subdomain. The charged n-domain is required for efficient protein translocation. The net positive charge enables it to interact with the negatively charged phospholipid head group (reviewed in von Heijne, 1990). In eukaryotes it may be the precise order, along with the net positive charge, of the residues in the n-domain that affect the efficiency of translocation (Green *et al.*, 1989). The h-domain is required for efficient protein targeting for entry into the secretory pathway. Charged h-domains or the introduction of mutations that introduce helix-breaking residues reduces the efficiency of translocation but do not completely disrupt this process. The length of this region is also significant as the probability of signal sequence cleavage decreases as the length of the h-domain increases. This is significant for the insertion of proteins into membranes. The hydrophilic c domain has been identified to specify the cleavage site of cleavable signal sequences by signal peptidase (von Heijne, 1986) in particular residues at position -1 and -3 away from the cleavage site.

1.3 Protein translocation into the endoplasmic reticulum

In the yeast *Saccharomyces cerevisiae*, proteins are translocated across the ER membrane by one of two mechanisms. The first occurs co-translationally where a translating ribosome is targeted to the ER enabling the translocation to be coupled to translation. The second occurs post-translationally.

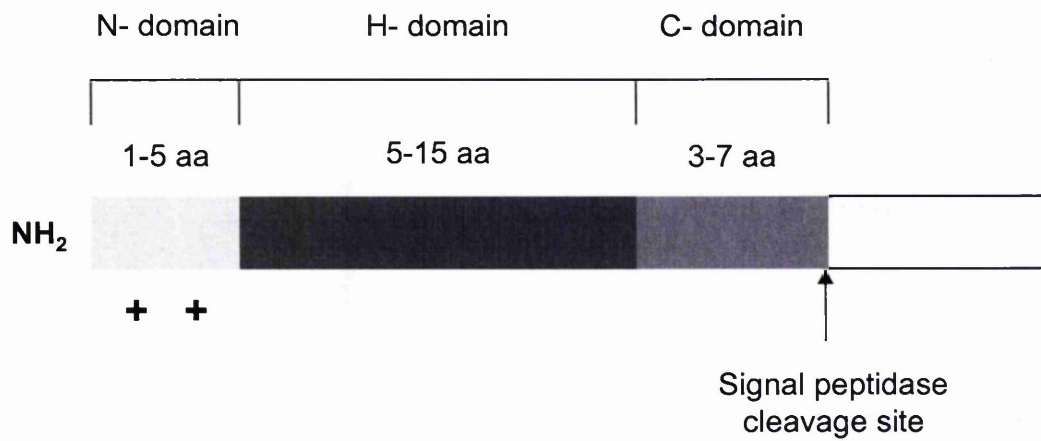


Figure 1.2 Signal peptides are tri-partite in structure. N- terminal signal peptides contain a central hydrophobic core that is flanked by a N- terminal domain that is typically positively charged and a C- domain that specifies the site of cleavage in cleavable signal peptides. The approximate length of each domain is noted accordingly.

mRNA is translated to completion and the protein product is maintained in a translocation competent state by the interaction of cytosolic chaperone proteins. The nascent chain is targeted to the ER and translocated by an alternate mechanism that is ATP dependent.

The signal peptide of a secretory precursor determines the pathway with which it is translocated and it has been predicted that the hydrophobic core determines the mechanism with which it is translocated into the ER. Proteins known to be translocated exclusively co-translationally, for example dipeptidylaminopeptidase B (DPAPB), contain a significantly more hydrophobic signal sequence than proteins that have been shown to be translocated post-translationally, for example carboxypeptidase Y (CPY) (Ng *et al.*, 1996). The h-domain of SRP dependent signal peptides are rich in amino acids containing hydrophobic side chains, particularly leucine, isoleucine and valine, whereas signal sequences of SRP independent precursors are rich in amino acids possessing small or hydroxylated side chains, alanine, serine and threonine, (Ng *et al.*, 1996).

Protein translocation requires two distinct steps. Firstly, proteins must be targeted to the site of translocation in a translocation competent state where it is subsequently translocated across the membrane without disrupting the membranes integrity.

1.4 Co-translational translocation

The mechanism of co-translational translocation couples translation with protein translocation to push the polypeptide through the ER translocon. Proteins that utilise the co-translational translocation pathway are targeted by the signal recognition particle (SRP) (Walter and Blobel, 1980; 1982). SRP binds to the signal sequence of the nascent pre-protein once it has emerged from the exit tunnel of a translating ribosome and the association of SRP with the signal peptide and the ribosome induces

a translational arrest (Gilmore *et al.*, 1982; Walter and Blobel, 1982). The SRP, ribosome and nascent chain complex is then targeted to the membrane of the endoplasmic reticulum through the interaction of SRP with its cognate receptor (Gilmore *et al.*, 1982a). Following this, the arrested ribosome is delivered to the site of protein translocation after which SRP is released from the signal peptide and ribosome (Connolly and Gilmore, 1989). Protein translation is once again re-initiated where the force of protein elongation drives the emerging nascent chain through the translocon.

1.4.1 The signal recognition particle

SRP is required to co-ordinate the targeting of secretory precursors to the ER during their translation. Purification of mammalian SRP from the canine pancreas identified it to be a ribonucleoprotein complex comprising of a 7S RNA (SRP RNA) subunit associated with six polypeptides (Walter and Blobel 1980, 1982). These proteins have been termed SRP9, 14, 19, 54, 68 and 72 by virtue of their molecular weight and they associate with SRP RNA as monomers, SRP19 and SRP54, or as heterodimers, SRP9/14 and SRP68/72 (Walter and Blobel 1980, 1982; Brown *et al.*, 1994).

Sedimentation of cytosol isolated from *Saccharomyces cerevisiae* isolated the yeast homologue (Hann *et al.*, 1992). This was identified as a 16S ribonucleoprotein complex where yeast SRP RNA (scR1 RNA) was found associated to homologues of SRP72, 68, 54, 19 and 14 (Brown *et al.*, 1994). The absence of an SRP9 homologue in yeast is thought to be compensated for by the homodimerisation of Srp14p (Strub *et al.*, 1999; Mason *et al.*, 2000). A 21 kDa SRP subunit has been isolated in yeast, termed Srp21p that appears exclusive to this organism. Although this protein is not related to any mammalian SRP component it is believed to function in a similar manner as mammalian SRP9 (Brown *et al.*, 1994).

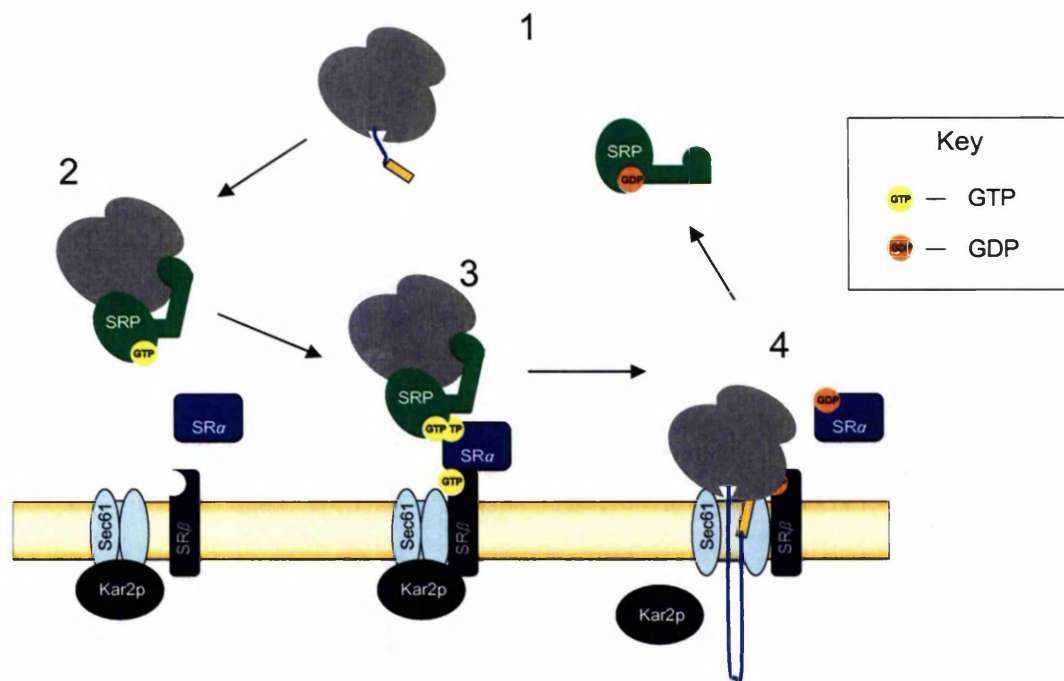


Figure 1.3 SRP dependent ER translocation. Upon synthesis (1), the signal peptide of a nascent chain is bound by SRP and this induces an elongation arrest (2). SRP is then targeted to the ER membrane, in a GTP dependent manner, via an interaction with its cognate receptor, SR (3). In an event requiring the translocon, the signal peptide is incorporated into the translocation channel, this displaces SRP54 (4). By an unknown reaction, dissociation of SRP54 results in the co-ordinated activation of the three GTPase activities. The complex incorporating ribosome, nascent chain, SRP and SR breaks down, the translational arrest is released and the nascent chain is co-translationally translocated. The GTP exchange factors for SRP54 and SR α are unknown but it is postulated that the translocon is the nucleotide exchange factor for SR β .

Genetic evidence in *Saccharomyces cerevisiae* would suggest that SRP is not essential for cell viability as mutations in either *SRP54* or *SEC65* (the gene encoding SRP19 in yeast) do not give rise to lethal phenotypes (Hann and Walter, 1991; Stirling and Hewitt, 1992). However, cells expressing functionally defective SRP undergo severe adaptation, becoming respiratory deficient and prior to this cells are defective in the translocation of a subset of secretory precursors and have a profound growth defect (Hann and Walter, 1991; Brown *et al.*, 1994).

SRP RNA, as well as being functional, provides the scaffold upon the polypeptide chains associate. scR1 RNA is much larger than and shares little sequence homology to SRP RNA in other eukaryotes (van Nues and Brown, 2004). That aside, eukaryotic SRP RNA appears to form rod like structures with conserved motifs.

The functional SRP molecule comprises of two domains. The S domain, or signal peptide binding domain, is the region that binds to the signal peptide of secretory precursors, and consequently targets the SRP, ribosome, nascent chain complex to the site of ER translocation (Siegel and Walter, 1988). The second domain of SRP, termed the Alu domain, is required to initiate the elongation arrest activity (Siegel and Walter, 1986). The contribution of each constituent to either the S-domain or the Alu domain will be described here in.

1.4.1.1 The S domain

The S domain comprises of approximately half of the SRP RNA as well as SRP19, SRP54 and the SRP68/72 heterodimer (Halic *et al.*, 2004). Biochemical analysis has determined that the M-domain of SRP54 constitutes the signal peptide binding activity of SRP (High *et al.*, 1993). SRP54 interacts with a translating ribosome at and around the polypeptide exit tunnel in the immediate vicinity of rpL25 and rpl35 (Pool *et al.*, 2002; Halic *et al.*, 2004). It has been postulated that the initial association of SRP54 with the ribosome induces a conformation in SRP54 that is

competent for scanning the emerging polypeptide chain for a signal sequence of sufficient hydrophobicity to act as substrate. The subsequent association of a signal peptide with SRP54 stabilises an open conformation that results in the high efficiency binding of SRP54 to the ribosome (Halic *et al.*, 2004)

SRP reconstitution experiments with mammalian SRP indicated that the incorporation of SRP54 into the complex requires an interaction between SRP19 and SRP RNA as a pre-requisite (Romisch *et al.*, 1990). This has also been determined in yeast as Srp54p is not incorporated into yeast SRP in a conditional *sec65* allele grown at the restrictive temperature (Hann *et al.*, 1992). Subsequent analysis has shown that the association of SRP19 to SRP RNA induces a conformational change in SRP RNA that results in the presentation of the SRP54 binding site that is otherwise blocked by the RNA chain (Hainzl *et al.*, 2002; Oubridge *et al.*, 2002).

The SRP68/72 heterodimer has been identified to associate with the highly flexible hinge 1 domain of SRP RNA (Halic *et al.*, 2004). Following the binding of SRP54 to signal sequence it has been postulated that the association of SRP68/72 stabilises this region in a conformation that promotes elongation arrest (Halic *et al.*, 2004). In agreement with this model, SRP depleted of SRP68 and SRP72 can no longer induce this activity (Siegel and Walter, 1985). SRP68/72 has been proposed to be required for the association of SRP with its cognate receptor as RNA bound SRP68/72, that had subsequently been partially alkylated, could no longer target precursor to the translocon (Siegel and Walter, 1988). Such findings are consistent with SRP68/72 conferring the correct SRP, ribosome, nascent chain conformation for docking to the ER membrane.

1.4.1.2 The Alu Domain

The Alu domain is required to initiate the elongation arrest activity of SRP (Siegel and Walter, 1986). It consists of both the 5' and 3' ends of SRP-RNA,

containing the Alu sequence, along with the SRP9/14 heterodimer in mammalian SRP or Srp21p and the Srp14p homodimer in yeast (Halic *et al.*, 2004). Although the RNA sequences bound by SRP components are well conserved, the Alu sequence is highly divergent and it is this region in scR1 RNA that is extended (van Nues and Brown, 2004). The region of the ribosome bound by the Alu domain is well conserved and is also the site for binding translation elongation factors, in particular elongation factor 2 (Halic *et al.*, 2004). It is therefore apparent that binding of the Alu domain to the ribosome would directly compete with the elongation factor binding site, thus preventing translation.

Elongation arrest is not a function of SRP that is conserved through evolution as eubacterial SRP does not exhibit this function. However, this function appears to be important in yeast as cells expressing a truncated form of Srp14p are temperature sensitive and appear to show defects in the efficiency of precursor targeting, particularly *in vitro*, (Mason *et al.*, 2000).

1.4.2 SRP Receptor

SRP targets the ribosome and the associated nascent chain to the ER membrane through an interaction with its cognate receptor, SRP Receptor (SR) (Gilmore *et al.*, 1982a). SR was originally identified *in vitro* through its ability to interact with the SRP ribosome complex and relieve the SRP mediated elongation arrest (Gilmore *et al.*, 1982a, 1982b, Meyer *et al.*, 1982). SR has been identified to be a heterodimeric complex anchored in the ER membrane (Gilmore *et al.*, 1982a, 1982b, Meyer *et al.*, 1982, Gorlich *et al.*, 1990), the two subunits being SR α and SR β in mammalian cells. Homologues of these components have subsequently been identified in *S. cerevisiae* and are products of the *SRP101* and *SRP102* genes respectively (Ogg *et al.*, 1992).

SR α is a 70 kDa, peripherally associated membrane protein in tight association with the 30-kDa integral membrane protein, SR β , these are 69 kDa and 27 kDa

respectively in yeast (Ogg *et al.*, 1998). Consistent with SR functioning in the same pathway as SRP, yeast cells depleted of *SRP101* and/or *SRP102* protein (Srp101p and Srp102p respectively) show the same translocation defects and decreased growth rate as cells depleted of SRP (Ogg *et al.*, 1992, 1998). This was confirmed through analysis of the *Δsrp101 Δsrp54* double mutant as the severity of the phenotype is comparable between the double mutant and either of the single mutants (Ogg *et al.*, 1992).

1.4.3 SRP Dependent translocation is coupled to GTP hydrolysis

Both subunits of the SRP receptor as well as SRP54 contain GTPase domains and all three have been shown to bind GTP (Connolly and Gilmore 1986, 1989; Miller *et al.*, 1995). Intriguingly only two of the three GTPase domains are related, the GTPase domain of SRP54 and SR α and crystallographic analysis of their *E. coli* homologues, Ffh and FtsY respectively, has indicated that they define their own subfamily in the GTPase super family (Freymann *et al.*, 1997; Montoya *et al.*, 1997). In contrast the GTPase domain identified in SR β is closely related to the Arf subfamily of GTPases (Fulga *et al.*, 2001).

Early targeting events require GTP binding but not hydrolysis (Connolly *et al.*, 1991). Association of signal peptide with SRP54 stabilises the interaction of SRP54 with the ribosome, elevates the affinity of SRP54 for GTP and inhibits its GTPase activity (Miller *et al.*, 1993; 1994). These activities together lead to stabilisation of an SRP structure that induces elongation arrest (Siegel and Walter, 1985). The SRP, ribosome nascent chain complex (SRP RNC) is then targeted to the ER membrane where SR α bridges this soluble complex with membrane associated SR β (Bacher *et al.*, 1999), the dimerisation of SRP receptor requiring that both the α and β subunit are GTP bound. Therefore, SRP54, SR α and SR β in their GTP bound form induce the stable assembly of SRP RNC, SR ternary complex.

It is following the stable interaction of the three GTPases that the signal peptide is inserted into the translocon in a GTP independent manner (Bacher *et al.*, 1996). Following this the ribosome binds to the aqueous channel and the region of the ribosome that interacts with the pore is the same region that interacts with SRP54 (Pool *et al.*, 2002). This is mutually exclusive suggesting that the integration of signal peptide into the translocation channel is coupled to the release of signal sequence from SRP54 (Pool *et al.*, 2002; Halic *et al.*, 2004).

The signals that trigger the three GTPase activities remains unknown however, it is now apparent that SRP54 and SR α act as mutual GTPase activating proteins (Egea *et al.*, 2004). Furthermore, the translocon has been implicated in controlling the re-association of SR (Helmers *et al.*, 2003). SR is only stably assembled when both SR α and SR β are GTP bound, GTP hydrolysis by SR β dissociates the complex. The translocon, in particular the β subunit, has been proposed to function as the guanine exchange factor (GEF) for SR β (Helmers *et al.*, 2003). Intriguingly, both Sbh1p and Sbh2p have been identified to share homology to the ARF GEF domain of Sec7p and intriguingly, the GTPase domain of SR β is homologous to the GTPase domain of ARF. Sbh1/2p would therefore function in reloading SR β with GTP, controlling the reassociation of SR (Helmers *et al.*, 2003).

1.5 Sec62p Dependent protein translocation

A distinct subset of secretory precursors in *S. cerevisiae* are translocated into the ER lumen by a mechanism that is Sec62p dependent. This mechanism is entirely post-translational as the nascent chain is synthesised and released into the cytosol prior to its translocation. As this pathway is independent of ribosomes the mechanism through which the precursor is targeted to and translocated across the ER membrane is fundamentally different to that of SRP dependent, co-translational translocation.

In vitro analysis has indicated that SRP and the nascent chain associated complex associate with Sec62p dependent precursors during their translation but their association ceases upon release of precursor from the ribosome (Plath and Rapoport, 2000). Following this it is essential that they remain in a conformation that is competent for translocation and this is maintained by preventing the precursor from folding and this is achieved by interacting with the cytosolic Hsp70 chaperones, Ssa1p and Ssa2p (Chirico *et al.*, 1988; Deshaies *et al.*, 1988). In order to function, Hsp70 proteins interact with a co-chaperone that is homologous to bacterial DNAJ. Ydj1p is a cytosolic DNAJ homologue in *S. cerevisiae* that is required to stimulate the ATPase activity of both Ssa1p and Ssa2p (Cyr *et al.*, 1992). Following the interaction of Ydj1p with Ssa1p/Ssa2p, the chaperone nascent chain complex is targeted to the ER membrane where the farnesylated C-terminus of Ydj1p has been found to be required for the efficient translocation of pre-pro alpha factor (pp α f) *in vivo*, presumably facilitating the association of Ydj1p with the ER membrane (Caplan *et al.*, 1992). Once the chaperone-nascent chain complex has reached the ER membrane Ssa1p/Ssa2p undergoes nucleotide exchange and the nascent chain released, the chaperones no longer associate once the nascent chain has engaged the translocon (Plath and Rapoport, 2000).

1.5.1 The SEC complex

Once the nascent chain has been targeted to the ER membrane it interacts with the SEC complex. This complex has been purified from yeast microsomes and found to exist as a heptameric complex of integral and peripheral membrane proteins. Furthermore, proteoliposomes reconstituted with this complex and Kar2p (the yeast equivalent of ER luminal Hsp70, BiP) have been shown to perform post-translational translocation *in vitro* (Panzner *et al.*, 1995). The SEC complex consists of two sub-

complexes (Rapoport *et al.*, 1999), the heterotrimeric Sec61p complex (discussed later) and the tetrameric Sec63p complex (Deshaies *et al.*, 1991; Panzner *et al.*, 1995).

The Sec63p complex has been identified to consist of four ER membrane proteins Sec62p, Sec63p, Sec71p and Sec72p (Deshaies and Schekman, 1989, 1990; Rothblatt *et al.*, 1989; Sadler *et al.*, 1989; Feldheim *et al.*, 1992, 1993; Green *et al.*, 1992). Genetic analysis has revealed that the expression of Sec62p and Sec63p is essential for cell viability whereas expression of Sec71p and Sec72p is not (Deshaies and Schekman 1990; Feldheim *et al.*, 1992; Rapoport *et al.*, 1999). Chemical crosslinking experiments have suggested that Sec62p, Sec71p and Sec72p show signal sequence binding activity suggesting that the Sec63p complex is required in the early stages of post-translational translocation (Lyman and Schekman 1997). Other investigations have shown that the C-terminus of ppαf forms crosslinks with the same proteins once the signal sequence has engaged the translocon (Plath *et al.*, 1998).

In vivo and *in vitro* analysis has demonstrated that *sec62* mutants are defective in the translocation of secretory precursors that are translocated by a mechanism that is independent of SRP (Deshaies and Schekman, 1989; Rothblatt *et al.*, 1989). The Sec62 protein is a 32 kDa integral membrane protein that spans the ER membrane twice with both the N and C terminus extending into the cytoplasm however, the precise function of Sec62p remains unclear. Earlier studies have implied that Sec62p functions during the early stages of translocation as signal sequence was found to be in close proximity to Sec62p prior to translocation (Lyman and Schekman, 1997; Matlack *et al.*, 1997; Wittke *et al.*, 2000) and it has been shown that deletion of 41 residues after TM2 compromises this activity (Wittke *et al.*, 2000). However, recent evidence does not support this notion (Plath *et al.*, 2004). Genetic analysis has implicated an interaction between *SEC62* and *SEC63* (Rothblatt *et al.*, 1989; Deshaies and Schekman, 1990). This is in agreement with the observation that co-expression of specific *sec62* and *sec63* alleles are synthetically lethal (Rothblatt *et al.*, 1989). Further analysis has

demonstrated that the N-terminus of Sec62p interacts with the C-terminus of Sec63p (Wittke *et al.*, 2000; Young *et al.*, 2001) and this interaction is required to tightly align the effector domains of Sec62p and Sec63p for efficient translocation of post translational precursors (Wittke *et al.*, 2000).

Sec63p is a 73-kDa integral membrane protein that spans the ER membrane three times and is orientated such that the C-terminus extends into the cytosol (Feldheim *et al.*, 1992). 70 amino acids of the Sec63p luminal loop between transmembrane domain 2 and 3 have been shown to share 43% sequence identity with the J domain of DNA-J (Sadler *et al.*, 1989; Feldheim *et al.*, 1992). This domain is essential for Sec63p activity as a conditionally lethal point mutation in this domain, *sec63-1*, and the entire deletion of this domain has been shown to be defective in both SRP dependent and Sec62p dependent translocation (Lyman and Schekman, 1995; Willer *et al.*, 2003) and it was concluded that such a mutation inhibits translocation through stalling the precursor at the translocon (Lyman and Schekman, 1995). In *E. coli*, Dna J is required to stimulate the ATPase activity of the *E. coli* Hsp70, Dna-K (Liberek *et al.*, 1991). In yeast, the major ER luminal Hsp70 is Kar2p and genetic and biochemical analysis has indicate that the J-domain of Sec63p is required to drive post translational translocation through interacting with Kar2p and regulating its ATPase activity (Sanders *et al.*, 1992; Lyman and Schekman, 1995). Therefore, one function of Sec63p is to anchor Kar2p to the translocation site, regulating the dynamics of the luminal Hsp70 through ATP hydrolysis (Brodsky and Schekman, 1993; Corsi and Schekman, 1997). The cytosolic domain(s) of Sec63p have been identified to be required for both post and co-translational translocation (Young *et al.*, 2001; Willer *et al.*, 2003; Willer *et al.*, 2004). As previously described, the C-terminus of Sec63p has been identified to be required for the recruitment of Sec62p, this function residing in the C-terminal 28 residues (Young *et al.*, 2001). However, SRP dependent protein translocation is affected in cells expressing Sec63p that contains a deleted C-terminal

cytosolic extension (Young *et al.*, 2001). As described later, Kar2p (BiP) is required to gate the translocon to ensure that both the luminal and cytosolic contents do not mix. Such mutants may indicate a mechanism where Sec63p recognises a requirement for a switch to a translocation competent state and transmits this signal to Kar2p resulting in the opening of the luminal face to the translocating polypeptide (Young *et al.*, 2001).

Sec71p is a 32 kDa glycoprotein that spans the ER once (Deshaies *et al.*, 1991; Feldheim *et al.*, 1993). Mutations in *sec71* were found to confer temperature sensitivity and compromise post translational translocation at this temperature (Feldheim *et al.*, 1993; Brodsky *et al.*, 1993). Analysis of the SEC complex indicated that mutations in *sec71* compromises the assembly of this complex (Feldheim *et al.*, 1993) and further characterisation revealed that these mutants fail to incorporate the Sec72 protein into the complex.

The *SEC72* gene encodes for a 23 kDa polypeptide that is peripherally associated with the ER membrane via an interaction with Sec71p (Feldheim *et al.*, 1993; Feldheim and Schekman, 1994). Like *sec71* mutants, mutations in *sec72* accumulate precursor proteins that are translocated post translationally at 37°C (Green *et al.*, 1992) and it has been postulated that both Sec71p and Sec72p are required for signal peptide recognition in this pathway (Feldheim and Schekman, 1994).

1.5.2 Post translational translocation is mediated by Kar2p

Reconstitution experiments with membranes treated with detergent has demonstrated that the heptameric SEC complex, Kar2p and ATP are all required for post-translational translocation (Panzner *et al.*, 1995; Matlack *et al.*, 1997). It was found that an SRP independent precursor firstly associates with the SEC complex and the subsequent translocation requires Kar2p and ATP. Furthermore, incorporation of a tRNA molecule at the extreme C-terminus of the substrate identified, through cross-

linking, that the tRNA molecule reaches the translocon, implying that Kar2p provides the driving force for translocation (Matlack *et al.*, 1997).

The J-domain of Sec63p stimulates the ATPase activity of Kar2p that regulates the nucleotide dependent peptide-binding activity of Kar2p (Corsi and Schekman, 1997). The peptide-binding pocket of Kar2p is open when ATP is bound, conferring a weak affinity for substrate. However, following the hydrolysis of ATP, the peptide binding site forms a tight seal around a substrate which in turn increases the affinity of Kar2p for substrate (Reviewed in Bakau and Howich, 1998). Two models have been proposed regarding the mechanism of Kar2p interaction with the translocon and the emerging precursor. The first model indicates that ATP bound Kar2p is recruited to the SEC complex via the J-domain of Sec63p and following the hydrolysis of ATP Kar2p is maintained at the translocon in an active, ADP bound form (Corsi and Schekman, 1997). A second model has been postulated where Kar2p.ADP is generated as a final reaction product and not as an active intermediate. This model implies that Kar2p.ATP transiently interacts with the J-domain of Sec63p and then transferred to substrate but, in the absence of precursor, Kar2p binds to the J-domain of Sec63p as if it were substrate (Misselwitz *et al.*, 1999). Kar2p and ATP are required for the post-translational translocation of precursor into the ER lumen, where as many as seven Kar2p molecules were found associated with ppaf *in vitro* (Matlack *et al.*, 1999). A study has suggested that Kar2p alone is sufficient to promote post-translational translocation (Matlack *et al.*, 1999).

Kar2p has been proposed to function as a brownian ratchet where its interaction with precursor prevents it from sliding backwards through the translocation channel and the association of multiple Kar2p molecules further increasing the efficiency with which backward sliding is prevented (Matlack *et al.*, 1999).

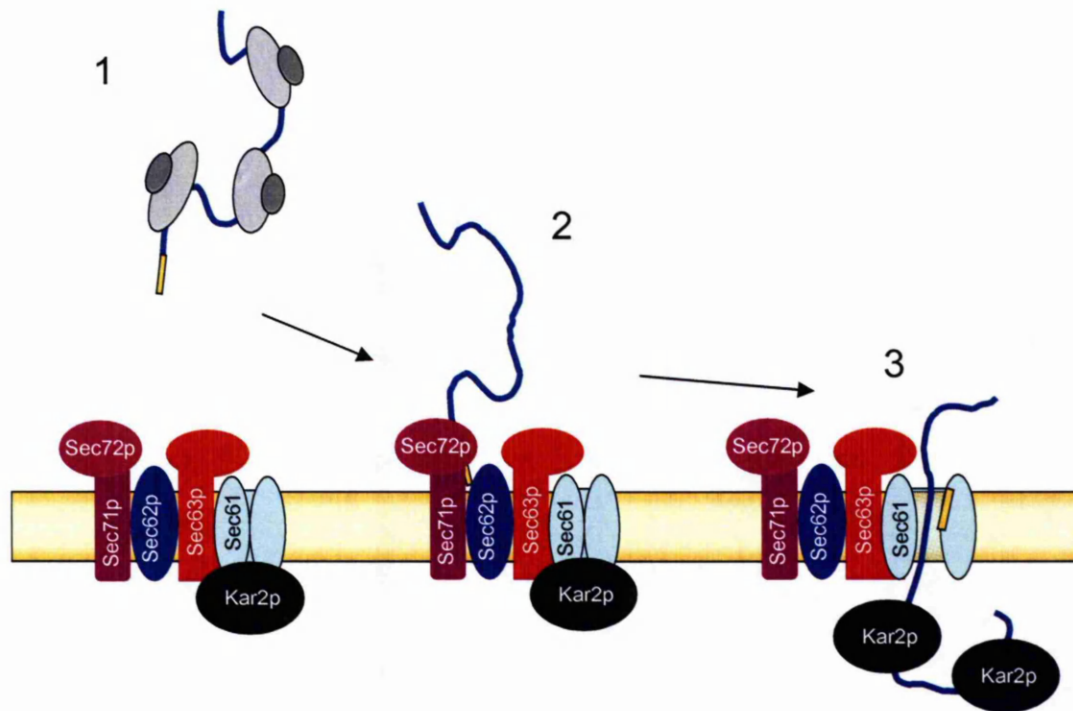


Figure 1.4 Sec62p dependent ER translocation. Secretory precursors that are translocated in a Sec62p dependent manner are translated to completion but maintained in a translocation competent state by cytosolic Hsp70 molecules and their Hsp40 co-chaperones (1). Sec62p dependent precursors are targeted to the ER membrane and associate with the heptameric SEC complex, comprising of Sec61p, Sec62p, Sec63p, Sec71p and Sec72p (2). Precursor is then incorporated into the aqueous channel by an ATP independent manner where it is subsequently translocated in an ATP manner by Kar2p (3). Signal peptides are then cleaved by the signal peptidase complex.

However, in this study the efficiency with which precursor is translocated by Kar2p alone is low. An alternate study has indicated that reconstitution of post-translational translocation with BiP alone is not sufficient and requires reconstitution with a number of ER ATP binding proteins (Dierks *et al.*, 1996). Significantly, two ER luminal, ATP binding proteins were enriched, BiP and GRP170 (Dierks *et al.*, 1996). As previously described, BiP is homologous to Kar2p in yeast, however GRP170 is homologous to a second ER luminal Hsp70 that has been characterised in yeast, Lhs1p (Craven *et al.*, 1996).

Lhs1p has been shown to bind to substrate in a similar fashion to Kar2p (Hamilton *et al.*, 1999), facilitating the translocation of substrate across the translocon. Although *LHS1* is not an essential gene, *lhs1Δ* cells are defective in the translocation of precursors that are translocated by Sec62p dependent translocation (Craven *et al.*, 1996). Furthermore, Lhs1p appears to function in parallel with Kar2p as the *lhs1Δ* mutation is synthetically lethal in combination with certain *kar2* mutants (Craven *et al.*, 1996). Together this would imply that Kar2p alone is not sufficient to drive ER translocation.

Consistent with Lhs1p being required to contribute to the folding environment of the ER lumen, the unfolded protein response is induced in *lhs1Δ* cells (Craven *et al.*, 1996; Tyson and Stirling, 2000). Furthermore induction of the UPR is required to maintain viability in these cells. However, this phenotype is suppressed by the overexpression of Sil1p (Tyson and Stirling, 2000) a nucleotide exchange factor for Kar2p (Kabani *et al.*, 1999). This implies that Lhs1p functions in parallel with Sil1p as well as Kar2p. Consistent with this the *lhs1Δ* mutation is synthetically lethal with the *sil1Δ* mutation and the *lhs1Δ sil1Δ* double deletion is totally defective in ER translocation (Tyson and Stirling, 2000). Subsequent analysis has demonstrated that Lhs1p, like Sil1p, functions as a nucleotide exchange factor for Kar2p (Steel *et al.*,

2004) Therefore, the co-ordinated activity of the two ER luminal Hsp70 molecules, specifically Kar2p and Lhs1p, is required for ER translocation to proceed (Steel *et al.*, 2004) and would indicate that Kar2p alone is not sufficient for ER translocation to proceed.

1.6 The Translocon

For ER translocation to proceed it is required that hydrophilic regions of a secretory precursor pass across the lipid bilayer. In order to maintain the permeability barrier provided by the ER membrane and to allow the passage of hydrophilic domains across the lipid bilayer it was hypothesised that secretory proteins are translocated across the ER membrane via aqueous channels formed by integral ER membrane proteins (Blobel and Dobberstein 1975). The proteinaceous components of the ER membrane required for ER translocation were originally identified by genetic analysis. Following this, the nascent chain was found to be in close proximity to Sec61p during translocation (Müsch *et al.*, 1992; Sanders *et al.*, 1992). The incorporation of photoreactive probes into the nascent chain of precursors led to the identification, through photocrosslinking, of mammalian ER membrane proteins that were adjacent to the nascent chain throughout translocation (Krieg *et al.*, 1989; Wiedmann *et al.*, 1989; High *et al.*, 1991). The mammalian homologue of Sec61p, Sec61 α , was identified to function in the same way as its yeast counterpart (Görlich *et al.*, 1992b) and purification of Sec61 α in non-denaturing conditions led to the purification of two additional proteins, designated Sec61 β and Sec61 γ (Görlich and Rapoport 1993). Reconstitution of proteoliposomes with the Sec61 heterotrimer and the translocon-associated membrane protein (TRAM) has shown that these components of the mammalian translocon are required for protein translocation *in vitro* (Görlich and Rapoport 1993). Purification of Sec61p from the yeast ER membrane by the same method as Sec61 α was purified from mammals identified a homologous heterotrimeric

complex (Panzner *et al.*, 1995). The Sec61p complex has been shown to contain Sec61p, a 53 kDa protein that spans the ER membrane ten times (Wilkinson *et al.*, 1996), and homologues of the 9kDa, C-terminally anchored membrane proteins Sec61 β and Sec61 γ termed Sbh1p (Sec61 beta homologue) and Sss1p respectively (Panzner *et al.*, 1995). A homologous complex required for protein secretion has been identified through genetic analysis in *E. coli*, the SecYEG translocon, indicating that protein translocation occurs by a mechanism that is highly conserved through evolution.

1.7 Evolutionary Conservation Of The Translocon

Certain aspects of the translocon structure and function have been highly conserved in eukaryotes and prokaryotes. 56% sequence identity exists between *S. cerevisiae* Sec61p and mammalian Sec61 α (Görlich *et al.*, 1992b). There is also significant sequence identity between these two components and the *E. coli* homologue SecY. There is 45% sequence identity between Sec61 γ and Sss1p, these two proteins being homologous to TM3 and its flanking regions of *E. coli* SecE. The high degree of conservation in the core translocation machinery is demonstrated via the ability of the homologues from other species to complement certain yeast translocon component deleted mutants. For example, the Sec61p homologue from *Yarrowia lipolytica*, sharing 69% sequence identity with Sec61p, can complement the *sec61* null mutant (Broughton *et al.*, 1997) and mammalian Sec61 γ has been shown to functionally replace Sss1p (Hartmann *et al.*, 1994). This is not always the case, as the Sec61p homologue from *Schizosaccharomyces pombe*, sharing 59% identity with Sec61p, cannot complement a *sec61* null mutant in *S. cerevisiae* (Broughton *et al.*, 1997).

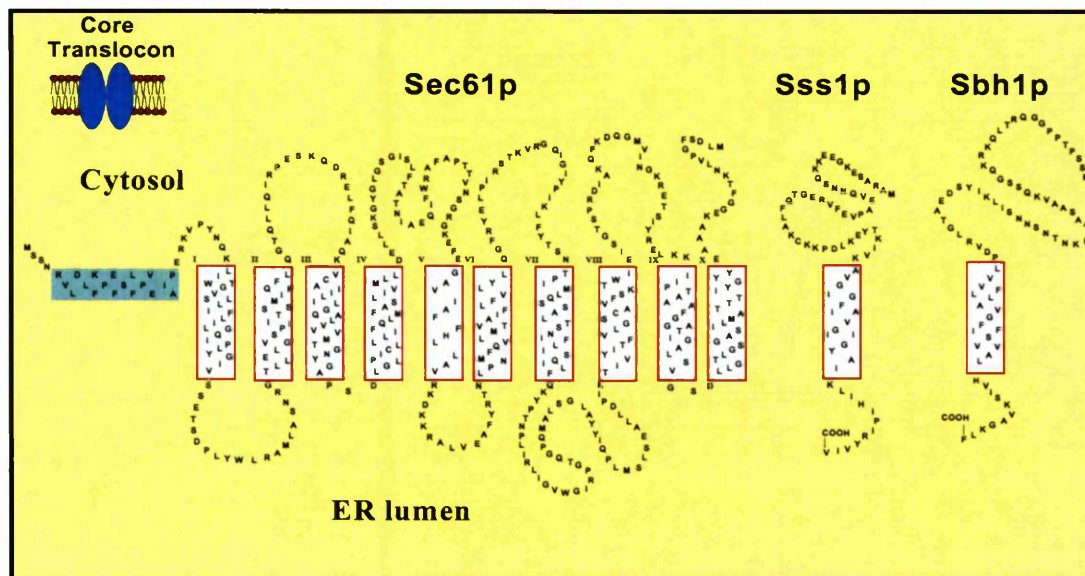


Figure 1.5 The ER translocation channel is heterotrimeric. Purification of the ER translocation channel from ER membranes identified the 53 kDa polytopic membrane protein, Sec61p, to be in association with two 9 kDa type II C-terminal anchor proteins Sss1p and Sbh1p. These proteins correspond to Sec61 α , Sec61 γ and Sec61 β respectively in the mammalian ER.

The degree of homology between Sec61 β and Sbh1p is lower than that observed for the other two components of the translocon, 23% identity and no homology with SecE, the third component of the *E. coli* translocase (Nishiyama *et al.*, 1993, 1994). These components have been found to be non-essential as the $\Delta sbh1$ null mutant and the *secg* null mutant in *S. cerevisiae* and *E. coli* respectively are found to be viable (Finke *et al.*, 1996).

1.8 Structure of the translocon

The translocation channel was first detected using electrophysiological techniques. It was shown that ion conductivity could only occur following the release of the nascent chain from the ribosome by treatment with puromycin (Simon and Blobel., 1991). Furthermore, ion conductivity was dependent on both ribosome and nascent chain. The incorporation of water sensitive fluorescent probes into the nascent chain indicated that it is translocated through an aqueous pore that completely spans the ER membrane (Crowley *et al.*, 1993, 1994). The nascent chain has been identified, by chemical crosslinking, to interact with Sec61p/Sec61 α during its translocation through the channel (High *et al.*, 1993; Mothes *et al.*, 1994; Plath *et al.*, 1998) and this is consistent with the interior of the pore being formed largely by the transmembrane helices of Sec61p/Sec61 α (Wilkinson *et al.*, 1996; Johnson and van Waes, 1999).

Earlier structural analysis of the eukaryotic translocation channel had determined that the complex forms a quasipentagonal structure in both membranes and detergent extracted membranes. It was proposed that three to four copies of the Sec61 heterotrimer associated that generated a central pore that facilitated the translocation of secretory precursors into the ER lumen (figure 1.6 A) (Hanein *et al.*, 1996). Following this, structural analysis of the eubacterial SecYEG translocon and archaeobacterial SecYE β translocon has been obtained to a greater resolution. These studies propose that a single heterotrimeric SecY/Sec61 complex is sufficient to function as the ER

translocase and argues that the indentation observed previously, that had proposed to be the translocation channel, is the consequence of complex oligomerisation (van den Berg *et al.*, 2004). The crystal structure of *Methanococcus jannaschii* SecY ϵ β indicates that SecY is divided into two halves, TM1-5 and TM6-10, that are related by two fold symmetry. The two halves are believed to be anchored together by SecE and the intact structure has been proposed to form an hourglass shaped channel through the membrane possessing a centralised point of constriction (figure 1.6 B). The localisation of conserved residues in this structure has led to the proposition that this structure is representative of secretory translocases of all species.

To date there is much debate regarding the size of the translocation channel. The use of reagents, of different sizes, that quench the fluorescence of a fluorescently labeled nascent chain upon collision has determined the diameter of ribosome bound translocons to be 40-60 Å (Hamman *et al.*, 1997). The overall diameter of a detergent extracted heterotrimeric translocon has been determined, by electron microscopy, to be approximately 110Å (Hanein *et al.*, 1996; Beckmann *et al.*, 1997). But, in contrast to data obtained by fluorescence quenching, electron microscopy and X-ray crystallography has determined the size of the pore to be approximately 20Å rather than 40-60Å (Hanein *et al.*, 1996; van den Berg *et al.*, 2004). It has been suggested that this discrepancy is the consequence of the translocon being in an active or inactive state, the latter accounting for the smaller pore (Hamman *et al.*, 1997).

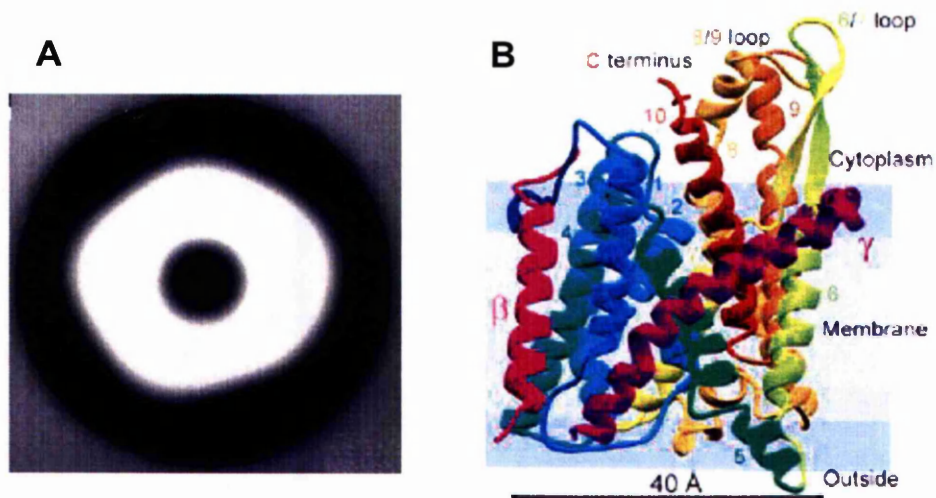


Figure 1.6 Structural analysis of the translocon. (A) (taken from Hanein *et al.*, 1996) Negative stain electron microscopy of the Sec61 complex isolated from yeast membranes. (B) (taken from van den Berg *et al.*, 2004) Crystal structure of the SecYE β in the detergent diheptanoylphosphatidyl choline isolated from *Methanococcus jannaschii* diffracted to 3.2 Å.

1.9 A Homologous Sec61 Complex

The *SSH1*, Sec Sixty-one Homologue, gene was identified to encode a protein that shares 34% identity with Sec61p (Finke *et al.*, 1996; Wilkinson *et al.*, 2001). Like Sec61p, Ssh1p is an ER localised polytopic membrane protein that spans the membrane ten times, with the highest degree of similarity between Sec61p and Ssh1p lying in the cytosolic loops (Finke *et al.*, 1996). Ssh1p has been identified to assemble into a heterotrimeric complex; homologous to the Sec61p complex, containing Sss1p and the Sbh1p homologue, Sbh2p (Finke *et al.*, 1996). The *SSH1* gene was initially suggested to be non-essential, as although $\Delta ssh1$ cells were observed to grow slower than wild type cells, $\Delta ssh1$ cells had no detectable translocation defect (Finke *et al.*, 1996).

A role for Ssh1p in protein translocation into the ER, at least when Sec61p function is impaired, was postulated as a $\Delta ssh1$ mutation is synthetically lethal when combined with a temperature sensitive *sec61* mutant (Finke *et al.*, 1996) and the Ssh1p complex has been found to bind ribosomes with a similar affinity to that of the Sec61p complex (Prinz *et al.*, 2000). Subsequent analysis contradicts the observation that there is no detectable translocation defect in $\Delta ssh1$ cells. $\Delta ssh1$ cells rapidly adapt to the loss of Ssh1p as such cells undergo physiological remodelling, one consequence being that they become respiratory deficient (Wilkinson *et al.*, 2001). Analysis of $\Delta ssh1$ cells prior to their adaptation shows that these cells are defective in the translocation of precursors that utilise the co-translational translocation pathway, specifically DPAP B (Wilkinson *et al.*, 2001). However, following adaptation, $\Delta ssh1$ cells grow extremely slowly with no apparent translocation defect (Wilkinson *et al.*, 2001). It was also found that there was defective translocation in post-translational translocation specific precursor, CPY, but the severity is not as extreme as that observed for DPAP B. These cells were also found to be defective in protein dislocation from the ER (Wilkinson *et*

al., 2001). It was therefore suggested that the Ssh1p complex is primarily required for co-translational translocation of precursor across the ER membrane but the deletion of Ssh1p places a general stress on the translocation apparatus (Wilkinson *et al.*, 2001).

1.10 Gating the translocon

As the translocon is a proteinaceous pore within a lipid bilayer it is essential that the permeability barrier, prohibiting the cross contamination of the cytosol with ER components, is maintained. The mechanism of “gating” has been characterised exclusively regarding co-translational translocation. Fluorescence quenching experiments have indicated that the ribosome forms a tight seal between the cytosol and the cytosolic face of the ER membrane throughout translocation. This would serve two purposes. Firstly it would prevent the lateral movement of a nascent chain into the cytosol during its synthesis and provides a barrier at the cytosolic face that prevents the ER lumen and the cytosol from mixing. The same study has shown that the luminal domain is also tightly sealed. More extensive studies have indicated that BiP, Kar2p in yeast, seals the luminal face of the translocation channel in a non-translocating state, and this seal is released following the synthesis of 70 amino acids (Hamman *et al.*, 1998). However, it is not clear if an interaction between the nascent chain and BiP is required to open the luminal face of the translocon.

Following each cycle of translocation it is unclear whether the translocon undergoes assembly and disassembly but recent evidence suggests that translocation structures remain assembled whether they are active or quiescent (Snapp *et al.*, 2004). Significantly, it has been shown that the translocon remains assembled following the dissociation of ribosomes but appears to undergo a structural rearrangement that results in the pore size decreasing from an average active pore size of 40-60 Å to an average pore diameter of 9-15 Å in a non-translocating state (Hamman *et al.*, 1998). It

has been proposed that this decrease in pore diameter allows the luminal face to be sealed by one molecule of BiP and for the translocon to accommodate this size shift it is likely that the components of the translocon would move laterally into the pore (Hamman *et al.*, 1998).

The interactions that ensure that BiP gates the luminal face of the translocon are unknown. It is possible that Sec61p (Sec61 α), Sss1p (Sec61 γ) or Sbh1p (Sec61 β) provides the site of interaction. Interestingly the nucleotide bound state is an important prerequisite for this function as BiP can only gate the translocon when nucleotide is bound (Hamman *et al.*, 1998). This is true for binding to Sec63p as Kar2p.ADP binds tightly to Sec63p (Corsi and Schekman 1997). Sec63p has been identified to be an essential component in both the SRP dependent and SRP independent translocation pathways (Young *et al.*, 2001) and therefore one of the functions of Sec63p may be to recruit Kar2p to the luminal face of the translocon. The resealing of the translocon appears to follow a defined series of events where following the termination of translocation a conformational change in the ribosome signals for the channel to close (Simon and Blobel 1991; Matlack *et al.*, 1998). This signal would recruit BiP.ATP to the luminal face of the ER membrane and the subsequent ATP hydrolysis to BiP.ADP is coupled to the release of the ribosome from the cytosolic face of the translocon (Hamman *et al.*, 1998).

Structural analysis of an archaeobacterial SecYE β translocon has led to the proposition of an alternate gating hypothesis. The SecYE β translocation channel tapers to a point of closure in the middle of the membrane and this blockage has been identified to be maintained by the luminal half of TM 2, referred to as TM2a, of SecY (van den Berg *et al.*, 2004). It has been proposed that upon the incorporation of signal peptide into the translocon, TM2a is displaced and relocalised, opening the pore. The polypeptide would then translocate through a “gasket” like constriction that further prevents the cross contamination of the two compartments (van den Berg *et al.*, 2004).

1.11 Integration of membrane proteins

Single and multi-spanning integral membrane proteins are incorporated into the ER membrane via the same translocation machinery utilised by soluble secretory precursors. Therefore, it is required that the translocon is directly involved in the recognition and integration of transmembrane domains into the bilayer (Johnson and van Waes, 1999). Integral membrane proteins can be placed into five topologically distinct groups (figure 1.7). Non-cleavable signal peptides, or signal anchors (SA), function as TM domains. Type I SA proteins have a cytosolic C-terminal projection and a luminal N-terminus whereas type II SA proteins possess the opposite orientation. Type I stop transfer integral membrane proteins are targeted to the ER by a cleavable signal sequence and the N-terminus is translocated into the ER lumen until the TM is synthesised, which terminates translocation. Polytopic TM proteins are characterised by possessing multiple membrane spanning segments, interconnected by a series of cytosolic and luminal loops, and can adopt any N- or C- terminal topology. Finally, C-terminal anchor proteins are anchored in the membrane by virtue of a C-terminal TM domain in a type II orientation.

A transmembrane domain is first detected by the ribosome (Liao *et al.*, 1997) and it is following this recognition that the ribosome induces the folding of the nascent chain transmembrane domain into a α -helical conformation, which is otherwise unstable in an aqueous environment, which is retained during its transit through the tunnel (Liao *et al.*, 1997; Woolhead *et al.*, 2004). During the translocation of a nascent secretory or early integration of a transmembrane protein through the ribosome-translocon channel, it can be photo-crosslinked exclusively to a 40 kDa ribosomal protein. However a transmembrane domain can be photocrosslinked to a 18 kDa ribosomal protein, following the closure of the luminal face by BiP and to a 7 kDa

ribosomal protein upon the release of the ribosome-translocon cytosolic seal (these proteins have been proposed to be L17 and L39 respectively) (Liao *et al.*, 1997; Haigh and Johnson, 2002; Woolhead *et al.*, 2004). This is consistent with transmembrane domain recognition being coupled to a structural rearrangement at both the cytosolic and luminal face of the translocon where exposure of a transmembrane domain to L17 signals for the BiP mediated closure of the luminal face of the translocon and the subsequent exposure to L39 induces the opening of the cytosolic seal. It is proposed that this structural rearrangement allows the transmembrane domain to interact with Sec61 α earlier than a secretory precursor would (Woolhead *et al.*, 2004).

In bacteria it has been proposed that the transmembrane domain orientation is determined by the positive-inside rule where the more positively charged of the two regions that flank the transmembrane domain remains on the cytosolic side of the membrane (von Heijne, 1994). However, topological prediction of transmembrane domain integration into the ER membrane is not this simplistic and remains unknown. It is likely that it is the collective properties of the transmembrane domain and flanking regions that dictates the events at the translocon, however this may be regulated by other cytosolic and/or integral membrane factors (Hegde *et al.*, 1998; Johnson and van Waes 1999).

Single spanning membrane proteins are proposed to enter the membrane once the transmembrane domain has exited the ribosome and has been incorporated into the translocon. At this point it has been proposed that the transmembrane domain laterally exits the Sec61 channel and enters the lipid bilayer by partitioning (Heinrich *et al.*, 2000; Heinrich and Rapoport, 2003).

The integration of polytopic membrane proteins is a much more complicated affair. It has been observed that the proper integration requires the presence of

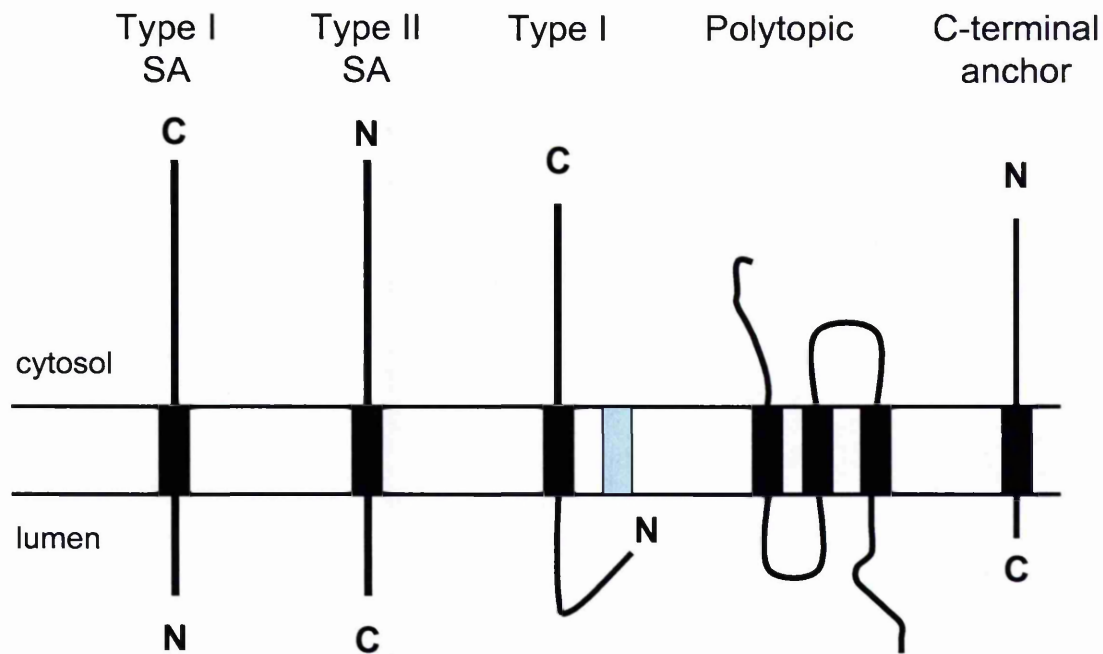


Figure 1.7 Topology of transmembrane proteins. Type I SA proteins have a cytosolic C-terminus and a luminal N-terminus. Type II SA proteins have the opposite topology. Type I proteins are targeted to the ER by cleavable signal sequences. The presence of a TM stops ER translocation. Polytopic membrane proteins span the membrane multiple times and can adopt any orientation. C-terminal anchor proteins possess a C-terminal TM domain that is inserted into a membrane via an unknown, post-translational mechanism.

adjacent transmembrane domains where a transmembrane domain can orientate correctly in the translocon but requires the presence of other transmembrane domains for their efficient integration into the lipid bilayer (Heinrich and Rapoport, 2003; Alder and Johnson, 2004). Two contrasting models have been proposed for the integration of multispanning membrane proteins. The first model proposes that newly synthesised transmembrane domains interact transiently with the translocation machinery (Mothes *et al.*, 1997; Heinrich *et al.*, 2000; Heinrich and Rapoport, 2003). Using *E. coli* leader peptidase as a model protein for *in vitro* insertion it was identified that TM1 exits the translocon and partitions into the lipid bilayer. Following the synthesis of the second transmembrane domain, of a weaker hydrophobicity to TM1, TM1 was found to be re-recruited to the translocon to facilitate the insertion of TM2 into the lipid bilayer (Heinrich and Rapoport, 2003).

An alternative model for transmembrane domain integration proposes that polytopic membrane proteins maintain a prolonged interaction with the translocon and with translocon associated proteins, particularly Sec61 α and/or TRAM (McCormick *et al.*, 2003; Do *et al.*, 1996) as well as PAT-10 (Meacock *et al.*, 2002). This model implies that the translocon and translocon associated membrane proteins play an active role in membrane protein assembly, potentially functioning as chaperones for transmembrane domains, controlling the lateral movement of transmembrane domains into the bilayer (Alder and Johnson, 2004).

C-terminally anchored membrane proteins anchor a functional cytosolic domain to a membrane surface. The N-terminus of a protein can fold before the transmembrane domain is synthesised therefore the insertion of these proteins into the membrane proceeds post-translational. The transmembrane domain and flanking positive charge provides the information for tail anchor insertion however the mechanism with which these proteins are inserted is poorly known. It was identified that the model C-terminal

anchor protein, cytochrome b₅, can insert spontaneously into liposomes *in vitro*.

However, other studies have determined that at least one proteinaceous component in the ER membrane as well as nucleotide is required for their insertion (Kutay *et al.*, 1995; Kim *et al.*, 1999). In yeast, genetic and biochemical analysis has indicated that the classical ER translocation pathway is not required for the *in vitro* and *in vivo* incorporation of C-terminally anchored proteins indicating that this process requires an unknown pathway (Steel *et al.*, 2002; Yabal *et al.*, 2002). But, *in vitro* cross-linking analysis has implicated the mammalian ER translocation pathway to be sufficient for this process (Abell *et al.*, 2003).

1.12 *SSS1*

SSS1 (suppressor of *sec* sixty one) was identified through multicopy suppression of a thermosensitive phenotype of *sec61*, *sec61-2*, and over expression of *SSS1* was also found to directly suppress the translocation defect associated with this mutation (Esnault *et al.*, 1993). Subsequent analysis indicated that over expression of *SSS1* could suppress both the temperature sensitivity and the translocation defect of a second *sec61* mutant, *sec61-3* (Wilkinson *et al.*, 1997). The requirement for *SSS1* in ER translocation was further demonstrated as depletion of Sss1p in a conditional mutant of *sss1* resulted in the accumulation of both SRP dependent and Sec62p dependent precursor proteins (Esnault *et al.*, 1993).

The Sss1 protein is an 8.9 kDa protein that is essential for ER translocation and it has been shown by both immunofluorescence and cell fractionation to localise to the endoplasmic reticulum (Esnault *et al.*, 1993; 1994). Subsequent chemical and proteolytic treatment of crude ER membranes indicated that Sss1p is a C-terminally anchored protein possessing an N- terminal extension of approximately 40-50 residues that would appear to be cytosolic (Esnault *et al.*, 1994). Purification of the Sec61 complex from crude ER membranes indicated that Sss1p directly interacts with Sec61p

(Panzner *et al.*, 1995). Furthermore, Sss1p can be covalently coupled to Sec61p by chemical cross-linking (Esnault *et al.*, 1994). Together this indicates that Sss1p is a component of the core machinery required for ER translocation. Studies on the molecular architecture of the translocon have provided numerous biochemical and genetic evidence for an interaction with Sss1p between TM6-8 of Sec61p. Sec61p can be expressed as complementary fragments where the co-expression of several fragment permutations can complement an otherwise lethal $\Delta sec61$ null mutation (Wilkinson *et al.*, 1997). However co-expression of the complementary fragments containing TM1-7 and TM8-10 cannot complement $\Delta sec61$ unless *SSS1* is overexpressed (Wilkinson *et al.*, 1997). The site of this lethal fragmentation is in the same luminal loop, the loop connecting TM7 and TM8, as the *sec61-3* mutation. In the same study it was reported that Sss1p can be covalently coupled to the cytosolic loop connecting TM6 and 7. Together this suggests that Sss1p interacts with Sec61p between TM6 and 8 (figure 1.8).

The function of Sss1p is unknown. Studies on the yeast Sec62p dependent precursor, pre pro alpha factor (pp α f) has indicated that the hydrophobic core of its signal peptide is α -helical when it is associated with the ER membrane. Cross-linking analysis with a photoactivatable probe incorporated into the signal sequence of pp α f has indicated that the signal peptide of a secretory precursor interacts directly with TM2 and 7 of Sec61p (Plath *et al.*, 1998). The transmembrane domains of Sec61p that interact directly with Sss1p are unknown but evidence suggests that Sss1p interacts with the same region of Sec61p, TM7, as the signal peptide of a secretory precursor. A model has been proposed implying a function for Sss1p in gating the ER translocon. This model predicts that the incorporation of signal peptide into the translocation channel modifies the interaction of Sec61p with Sss1p, displacing Sss1p, revealing the signal peptide binding site of Sec61p, switching the translocon into a translocation competent state (Plath *et al.*, 1998).

Cytosol

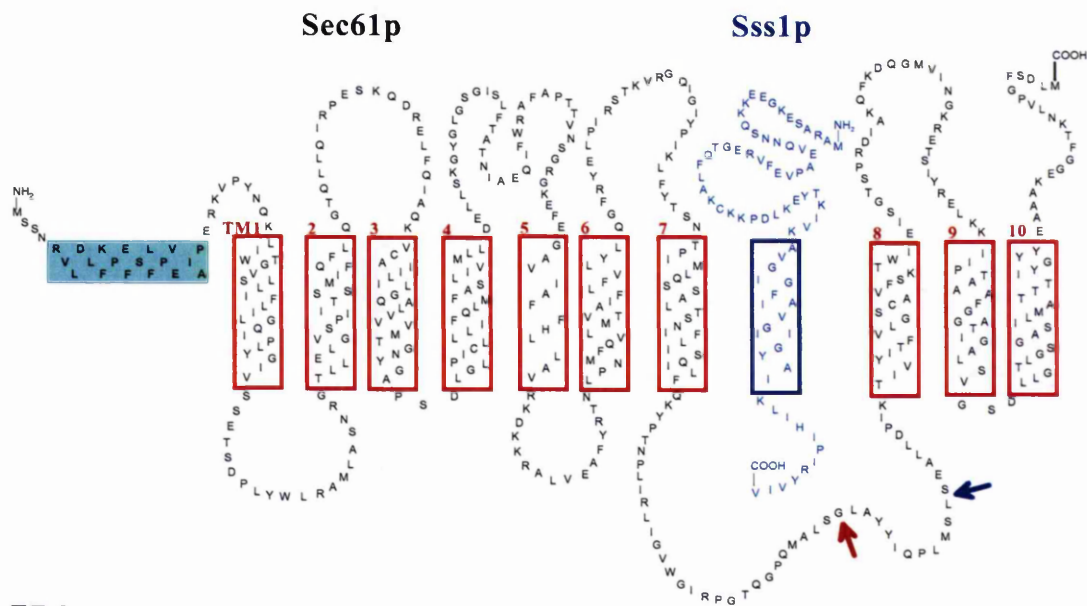


Figure 1.8 Sss1p interacts with defined regions of Sec61p. Genetic and biochemical evidence has indicated that Sss1p interacts with Sec61p between TM6 and TM8. Over expression of *SSS1* suppresses the lethal fragmentation in the luminal loop connecting TM7 and 8 as well as the temperature sensitivity observed in the *sec61-3* mutation.

It has previously been reported that TM3 of the *E. coli* SecE protein (this and the flanking sequences are homologous to Sss1p) is required for the formation of the homologous Sec61 complex, SecYEG (Pohlschroder *et al.*, 1996). Subsequently, cysteine scanning mutagenesis has been used to investigate the architecture of the eubacterial SecYEG complex (Veenendaal *et al.*, 2001). TM3 of SecE was found to interact with TM2 and TM7 of the *E. coli* Sec61p homologue SecY in a 1:1 stoichiometry, and these interactions were maintained upon the addition of precursor into the translocon. The helical face of TM3 opposite to that that was found to interact with TM 2 and 7 of SecE was found to interact with a SecE molecule in an adjacent SecYEG complex (Veenendaal *et al.*, 2001). This data supports the hypothesis that Sss1p functions as a surrogate signal sequence, gating the ER translocon and that interaction between adjacent SecE molecules instigates the formation of a translocation competent oligomeric translocation channel (Veenendaal *et al.*, 2001).

Following this, the crystal structure of an archaebacterial, *Methanococcus jannaschii*, SecYE β translocon has been resolved (van den Berg *et al.*, 2004). This predicts that SecE is required to brace the N and C terminal halves of SecY together where the TM of SecE forms single point contacts, in order from the cytosolic face to the periplasmic face of the bacterial inner membrane, with TM10, 6, 5 and 1 of SecY. These interactions place SecE on the opposite face of the SecYE β translocon to the signal sequence binding pocket and are inconsistent with the previously observed interactions.

1.13 Aims

Although it is known that Sss1p plays an essential role in ER translocation its requirement in this process remains unclear. Therefore, the aim of this study is to

investigate the function of the Sss1 protein during the translocation of secretory precursors across the ER membrane.

Genetic and biochemical analysis has indicated that Sss1p interacts with Sec61p in a similar region to that which has been shown to interact with the signal sequence of secretory precursors. Therefore it has been postulated that Sss1p functions as a surrogate signal sequence that regulates the translocon. To investigate this, the position of Sss1p within the translocon has been examined by cysteine scanning mutagenesis. Also, chemical crosslinking has been used to investigate interactions formed by Sss1p that are important for ER translocation.

The extreme C- terminus of Sss1p is highly conserved. Previous analysis has demonstrated that mutations within this region disrupt the function of Sss1p. In order to elucidate the function of the Sss1p C- terminus the phenotypes associated with these mutants have been further characterised.

Chapter 2

Materials and Methods

2 Materials and Methods

DNA restriction and modification enzymes were purchase from Roche Molecular Biochemicals. [³⁵S] methionine/cysteine TransLabel and [³⁵S] methionine with blue dye was purchased from NEN Life Science. [³²P] orthophosphate, protein purification columns and protein molecular weight markers were obtained from Amersham Pharmacia Biotech. Oligonucleotides were purchased from MWG or Sigma Genosys. All other reagents were from Sigma, British Drug House or Melford Laboratories at analytical grade.

All protocols were performed according to Sambrook *et al.*, 1989 unless otherwise stated.

2.1 Strains and growth conditions

2.1.1 *Escherichia coli* strains used in this study

Table 2.1 *E. coli* strains used in this study

Strain	Genotype	Source/Reference
DH5 α	<i>supE44 ΔlacU169 (Φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Hanahan, 1983
BL21 (DE3)	<i>hsdS gal(λclts857 ind1 Sam7 nin5 lacUV5-T7 gene1)</i>	Studier and Moffat, 1986

2.1.2 Growth and maintenance of *Escherichia coli*

E. coli cells were grown in Luria-Bertani (LB) medium (1% (w/v) Bacto tryptone, 0.5% (w/v) Bacto yeast extract, 1% (w/v) NaCl, pH 7.5) which was supplemented with either/both 100 μ g/ml ampicillin or 34 μ g/ml chloramphenicol where appropriate. Solid media was supplemented with 2% (w/v) agar. IPTG was used at a final concentration of 1 mM.

Glycerol stocks of *E. coli* were used for long term storage. Cultures were grown to late log phase and glycerol was added to the culture to a final concentration of 15% (w/v). Cultures were snap frozen in liquid nitrogen and stored at -80°C.

2.1.3 *Saccharomyces cerevisiae* strains used in this study

Table 2.2 *S. cerevisiae* strains used in this study

Strain	Genotype	Reference
w303 α	<i>MATα ade2 his3 leu2 trp1 ura3</i>	Thomas and Rothstein, 1989
w303d	<i>ade2/ade2 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3</i>	Thomas and Rothstein, 1989
FKY198	<i>MATα, sss1::URA3 pep4::LEU2 leu2 ade2 ura3 his3 trp1 can1-100 p[GAL10::SSS1, ADE2] p[TPI::SUC2, TRP1]</i>	Esnault <i>et al.</i> , 1993
BWY12	<i>MATα ade2 his3 leu2 trp1 ura3 sec61::HIS3 pBW7</i>	Wilkinson <i>et al.</i> , 1996
BWY436	<i>MATα ade2 his3 leu2 trp1 ura3 KanMX4- RPL25 3HA</i>	B. M. Wilkinson, this laboratory
BWY530	<i>MATα ade2 his3 leu2 trp1 ura3 sss1::KanMX4 p[SSS1 2u URA3]</i>	Wilkinson <i>et al.</i> , in preparation
BWY531	<i>MATα ade2 his3 leu2 trp1 ura3 sss1::KanMX4 p[SSS1 2u URA3]</i>	Wilkinson <i>et al.</i> , in preparation
BWY538	<i>MATα ade2 his3 leu2 trp1 ura3 sss1::KanMX4 pJKB2</i>	Wilkinson <i>et al.</i> , in preparation
BWY544	<i>MATα ade2 his3 leu2 trp1 ura3 sss1::KanMX4 pJKB16</i>	Wilkinson <i>et al.</i> , in preparation
BWY585	<i>MATα, sss1::URA3 pep4::LEU2 leu2 ade2 ura3 his3 trp1 can1-100 p[GAL10::SSS1, ADE2] p[TPI::SUC2, TRP1] pRS313</i>	Wilkinson <i>et al.</i> , in preparation
BWY586	<i>MATα, sss1::URA3 pep4::LEU2 leu2 ade2 ura3 his3 trp1 can1-100 p[GAL10::SSS1, ADE2] p[TPI::SUC2, TRP1] pJKB2</i>	Wilkinson <i>et al.</i> , in preparation

BWY591	<i>MATa, sss1::URA3 pep4::LEU2 leu2 ade2 ura3 his3 trp1 can1-100 p[GAL10::SSS1, ADE2] p[TPI::SUC2, TRP1]</i> pBW236	Wilkinson <i>et al.</i> , in preparation
BYY5	<i>MATα ade2 his3 leu2 trp1 ura3 KanMX4-P_{MET3}-SEC63</i>	Young <i>et al.</i> , 2001
BYY7	<i>ade2/ade2 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 KanMX4 P_{MET3}-SEC63/HIS3 sec63Δ14</i>	Young <i>et al.</i> , 2001
BYY8	<i>ade2/ade2 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 KanMX4 P_{MET3}-SEC63/HIS3 sec63Δ28</i>	Young <i>et al.</i> , 2001
AJY1	<i>ade2/ade2 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 KanMX4 P_{MET3}-SEC63/HIS3 3HA-sec63Δ52</i>	A. J. Jermy, this laboratory
JTY33	<i>MATα ade2 his3 leu2 trp1 ura3 KanMX4::lhs1Δ</i>	Tyson and Stirling, 2000
YAF29	<i>MATa pra1ΔSSprc1-1 leu2 der3-1</i>	Bordallo <i>et al.</i> , 1998

Table 2.3 *S. cerevisiae* strains created in this study

Strain	Genotype
CMY1	BWY12 X BWY531
CMY2	<i>MATa ade2 his3 leu2 trp1 ura3 sss1::KanMX4</i> pCM202
CMY3	<i>ade2/ade2 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 sec61::HIS3/SEC61 sss1::KanMX4/SSS1</i>
CMY4	As CMY3 + pCM203
CMY5	<i>MATa ade2 his3 leu2 trp1 ura3 sec61::HIS3 sss1::KanMX4</i> pCM203
CMY6	As CMY5 + pCM202
CMY7	<i>MATa ade2 his3 leu2 trp1 ura3 sec61::HIS3 sss1::KanMX4</i> pCM202 pBW29
CMY8	<i>MATa ade2 his3 leu2 trp1 ura3 sec61::HIS3 sss1::KanMX4</i> pCM202 pCM104
CMY9	<i>MATa ade2 his3 leu2 trp1 ura3 sss1::KanMX4</i> pCM201
CMY10	<i>MATa ade2 his3 leu2 trp1 ura3 sec61::HIS3 sss1::KanMX4</i> pCM202 pCM110

CMY11	<i>MATa ade2 his3 leu2 trp1 ura3 sec61::HIS3 sss1::KanMX4</i> pCM202 pCM111
CMY12	<i>MATa ade2 his3 leu2 trp1 ura3 sec61::HIS3 sss1::KanMX4</i> pCM202 pCM112
CMY13	<i>MATa ade2 his3 leu2 trp1 ura3 sec61::HIS3 sss1::KanMX4</i> pCM202 pCM113
CMY14	<i>MATa ade2 his3 leu2 trp1 ura3 sec61::HIS3 sss1::KanMX4</i> pCM202 pCM114
CMY15	<i>MATa ade2 his3 leu2 trp1 ura3 sec61::HIS3 sss1::KanMX4</i> pCM202 pCM106
CMY16	<i>MATa ade2 his3 leu2 trp1 ura3 sec61::HIS3 sss1::KanMX4</i> pCM202 pCM107
CMY17	<i>MATa ade2 his3 leu2 trp1 ura3 sec61::HIS3 sss1::KanMX4</i> pCM202 pCM105
CMY18	<i>MATa ade2 his3 leu2 trp1 ura3 sec61::HIS3 sss1::KanMX4</i> pCM202 pCM108
CMY19	<i>MATa ade2 his3 leu2 trp1 ura3 sec61::HIS3 sss1::KanMX4</i> pCM202 pCM109
CMY20	<i>MATa ade2 his3 leu2 trp1 ura3 sss1::KanMX4</i> pCM205
CMY21	<i>MATα ade2 his3 leu2 trp1 ura3 sss1::KanMX4</i> pCM205
CMY22	<i>MATa ade2 his3 leu2 trp1 ura3 sss1::KanMX4</i> pJKB16
CMY23	<i>MATα ade2 his3 leu2 trp1 ura3 sss1::KanMX4</i> pCM207
CMY24	<i>MATa ade2 his3 leu2 trp1 ura3 sec61::HIS3 sss1::KanMX4</i> pCM202 pPR22
CMY25	<i>MATa ade2 his3 leu2 trp1 ura3 sec61::HIS3 sss1::KanMX4</i> pCM202 pPR22 pRS316
CMY26	<i>MATa ade2 his3 leu2 trp1 ura3 sec61::HIS3 sss1::KanMX4</i> pCM202 pPR22 pCM115
CMY27	<i>MATa ade2 his3 leu2 trp1 ura3 sec61::HIS3 sss1::KanMX4</i> pCM202 pPR22 pCM116
CMY28	<i>MATa ade2 his3 leu2 trp1 ura3 sec61::HIS3 sss1::KanMX4</i> pCM109 pCM214
CMY29	<i>MATa ade2 his3 leu2 trp1 ura3 sec61::HIS3 sss1::KanMX4</i> pCM109 pCM214 FKp52

CMY30	<i>MATa ade2 his3 leu2 trp1 ura3 sec61::HIS3 sss1::KanMX4</i> pCM109 pCM214 pCM209
CMY31	<i>MATα ade2 his3 leu2 trp1 ura3 sss1::KanMX4</i> pCM217
CMY32	<i>MATa ade2 his3 leu2 trp1 ura3 sec61::HIS3 sss1::KanMX4</i> pCM218 pBW29
CMY33	<i>MATa ade2 his3 leu2 trp1 ura3 sec61::HIS3 sss1::KanMX4</i> pCM218 pCM104
CMY34	<i>MATa ade2 his3 leu2 trp1 ura3 sec61::HIS3 sss1::KanMX4</i> pCM218 pCM101
CMY35	<i>MATa ade2 his3 leu2 trp1 ura3 sec61::HIS3 sss1::KanMX4</i> pCM218 pCM102
CMY36	<i>MATa ade2 his3 leu2 trp1 ura3 sec61::HIS3 sss1::KanMX4</i> pCM218 pCM103
CMY37	<i>MATα ade2 his3 leu2 trp1 ura3 sec61::HIS3</i> pCM108
CMY38	<i>MATα ade2 his3 leu2 trp1 ura3 sec61::HIS3</i> pCM109
CMY39	<i>MATα ade2 his3 leu2 trp1 ura3 sss1::KanMX4</i> pCM201
CMY40	<i>MATα ade2 his3 leu2 trp1 ura3 sss1::KanMX4</i> pCM214

2.1.4 Growth and maintenance of *Saccharomyces cerevisiae*

S. cerevisiae was grown and maintained on YP (1% (w/v) Bacto-yeast extract, 2% (w/v) Bacto-peptone) supplemented with 2% (w/v) glucose (YPD) or galactose (YP Gal) as appropriate. Plasmid selection was maintained on minimal media (0.675% (w/v) yeast nitrogen base (without amino acids)) containing the required supplements. Amino acids, adenine and uracil were added to a final concentration of 20µg/ml and carbohydrate as above. Strains containing the KanMX4 marker were selected on YPD with the addition of 200 µg/ml of G418. Plasmid shuffle was performed on minimal media supplemented with 1g/L 5-FOA and 0.01% (w/v) uracil.

2.1.5 Sporulation and tetrad dissection of *Saccharomyces cerevisiae*

Diploid yeast strains were constructed by mixing haploid strains of opposite mating types on YPD agar. After incubating over night, diploids were selected for using appropriate auxotrophic markers. Sporulation was induced in diploid *S. cerevisiae* strains by growing the strain to stationary phase, 2 days at 30°C, in YP or selective media containing 4% (w/v) glucose. Cells were washed and then resuspended in 10 ml Sporulation media (1% (w/v) potassium acetate, 0.1% (w/v) Bacto-Yeast extract) containing amino acid and nucleotide supplements to a final concentration of 20µg/ml. Sporulation was induced by growing at 24°C for 3-4 days, after which the efficiency of sporulation was monitored by microscopically examining the cells. Once sporulation had occurred, 10µl of cells adjusted to 200 µl with ddH₂O were digested with 5µl of lyticase (1.5 U/ml) for 5 minutes. 10µl was run vertically down a YPD agar plate and allowed to dry. Once dry, a Narishige micro-manipulator microscope was used to separate the four spores from each asci and arranged 5mm apart. This was typically carried out upon a minimum of 10 full tetrads. Spores were recovered by growth on YPD at 30°C for 3 days.

2.2 Plasmids

Table 2.4 Plasmids used in this study

Plasmid	Description	Reference
pET16b	Vector for the expression of N-terminal 10xHis tagged fusion proteins in <i>E. coli</i>	Novagen
pRS313	Yeast, single copy (<i>CEN6 ARS4</i>) shuttle vector carrying the <i>HIS3</i> marker	Sikorski and Heiter, 1989
pRS314	Yeast, single copy (<i>CEN6 ARS4</i>) shuttle vector carrying the <i>TRP1</i> marker	Sikorski and Heiter, 1989
pRS315	Yeast, single copy (<i>CEN6 ARS4</i>) shuttle vector carrying the <i>LEU2</i> marker	Sikorski and Heiter, 1989
pRS316	Yeast, single copy (<i>CEN6 ARS4</i>) shuttle vector carrying the <i>URA3</i>	Sikorski and Heiter, 1989

	marker	
pBW7	pRS316 derived, Single copy expression of <i>SEC61</i> , <i>URA3</i> selection	Wilkinson <i>et al.</i> , 1996
pBW29	pRS315 derived, Single copy expression of <i>SEC61</i> (T533A silent mutation)	Wilkinson <i>et al.</i> , 1996
FKp52	pUN80 derived (AmpR, <i>URA3</i> , <i>LacZ'</i> , <i>CEN</i>) containing 1.8kb <i>EcoRI Sall SSSI</i> insert	Esnault <i>et al.</i> , 1993
pBW236	pRS313 derived, Single copy expression of <i>SUS</i>	Wilkinson <i>et al.</i> , in preparation
pJKB2	1.8kb <i>Sall/EcoRI</i> fragment containing <i>SSSI</i> derived from FKp52 subcloned into pRS313	J. K. Brownsword, this laboratory
pJKB16	pJKB2 mutagenised to substitute PI74,75 AA, <i>sss1-6</i>	J. K. Brownsword, this laboratory
pJKB18	pJKB2 mutagenised to introduce a stop codon at position 53 after N- terminal 52 residues	J. K. Brownsword, this laboratory
pJT30	UPRE- <i>lacZ</i> cassette, which was ligated into <i>HindIII</i> -digested pRS316	Wilkinson <i>et al.</i> , 2000
pJT31	700 bp <i>Sall/BamHI</i> fragment encoding for the <i>MET3</i> promoter ligated into pRS313	J. R. Tyson, this laboratory
pPR22	pRS315 derived, <i>SEC61</i> under the control of <i>MET3</i> promoter	Wilkinson <i>et al.</i> , 2000
pCT43	<i>prc1-1</i> with a triple HA tag subcloned into pRS316	Taxis <i>et al.</i> , 2002
pEH3	600 bp <i>EcoRI/XbaI</i> fragment encoding prepro-alpha factor in pGEM3Z	E. W. Hewitt, this laboratory

Table 2.5 Plasmids generated in this study

Plasmid	Description
pCM101	Derived from pBW29 except C121A point mutation

pCM102	Derived from pBW29 except C150A point mutation.
pCM103	Derived from pBW29 except C121, 150A point mutations.
pCM104	Derived from pCM103 except C373A point mutation. Single copy expression of cysteineless Sec61p
pCM105	Derived from pCM103 except G371C, S372C point mutations. Single copy expression of Sec61p TM8CCC
pCM106	Derived from pCM104 except M249C, T250C, V251C point mutations. Single copy expression of Sec61p TM6CCC
pCM107	Derived from pCM104 except S301C, N302C, I303C point mutations. Single copy expression of Sec61pTM7CCC
pCM108	Derived from pCM104 except A423C, F424C, G425C point mutations. Single copy expression of Sec61pTM9CCC
pCM109	Derived from pCM104 except I448C, L449C M450C point mutations. Single copy expression of Sec61pTM10CCC
pCM110	Derived from pCM104 except L44C, I45C, L46C point mutations. Single copy expression of Sec61pTM1CCC
pCM111	Derived from pCM104 except I85C, I86C, T87C point mutations. Single copy expression of Sec61pTM2CCC
pCM112	Derived from pCM104 except A130C, L131C, V132C point mutations. Single copy expression of Sec61pTM3CCC
pCM113	Derived from pCM104 except L157C, M158C, F159C point mutations. Single copy expression of Sec61pTM4CCC
pCM114	Derived from pCM104 except F219C, H220C, L221C point mutations. Single copy expression of Sec61pTM5CCC
pCM115	2.5 kb <i>HindIII</i> fragment from pCM107 subcloned into pRS316. Single copy expression of Sec61pTM7CCC with <i>URA3</i> selection.
pCM116	2.5 kb <i>HindIII</i> fragment from pCM112 subcloned into pRS316. Single copy expression of Sec61pTM3CCC with <i>URA3</i> selection.
pCM201	1.8kb <i>Sall EcoRI SSS1</i> fragment from FKp52 ligated into pRS314
pCM201 5'NdeI	<i>NdeI</i> restriction site incorporated into pCM201 at <i>SSS1</i> initiation codon (a ₃ aaatg – c ₃ atatg)
pCM202	Derived from pCM201 except I59A60V61CCC point mutations. Single copy expression of Sss1pTMCCC

pCM203	1.8 kb <i>SalI SmaI SSS1</i> fragment ligated into <i>XhoI, SmaI</i> (4364) site in pBW7
pJKB18 5'NdeI	<i>NdeI</i> restriction site incorporated into pJKB18 at <i>SSS1</i> initiation codon (a ₃ aaatg – c ₃ atatg)
pCM204	441 bp <i>NdeI/EcoRI</i> fragment from pJKB18 5'NdeI ligated into pET16b
pCM205	Derived from pJKB2 except H72K point mutation
pCM207	Derived from pJKB2 except C-terminal cysteine insertion (C81) mutant of <i>SSS1</i> . Single copy expression of Sss1p 81C
pCM208	800bp <i>SalI SmaI</i> fragment containing SUS subcloned into pRS316
pCM214	Endogenous <i>SSS1</i> promoter was excised from pCM201 5'NdeI as a 700 bp <i>SalI NdeI</i> (blunt) fragment and a 700 bp <i>XhoI BamHI</i> (blunt) fragment containing the <i>MET3</i> promoter was ligated into the promoterless pCM201 5'NdeI vector back bone to create a <i>MET3</i> regulatable allele of <i>SSS1</i>
pCM215	435 bp <i>NdeI SspI</i> fragment containing the <i>SSS1</i> coding sequence from pCM201 5'NdeI ligated into pET16b. 10X His- <i>SSS1</i> cloning intermediate.
pCM216	535 bp <i>XbaI SspI</i> fragment from pCM215 ligated into pRS313 cut with <i>XbaI</i> and <i>SmaI</i> . 10X His- <i>SSS1</i> cloning intermediate.
pCM217	A 565 bp <i>NcoI</i> (blunt) <i>EcoRI</i> fragment from pCM216 was ligated into 5.4 kbp pCM201 5'NdeI <i>NdeI</i> (blunt) <i>EcoRI</i> fragment. Single copy expression of 10X His-Sss1p
pCM218	1.8 kb <i>SalI EcoRI</i> fragment containing sss1 81C from pCM207 ligated into pRS314

2.3 Oligonucleotides

Table 2.6 Oligonucleotides used in this study.

Name	Sequence
Sec61p C121A_F	gctcaaaagggtggccgctattattctgac
Sec61p C121A_R	gatcagaataatagcggccaccttttgagc
Sec61p C150A_F	ctcggattgcccacatgccttggttgaatc

Sec61p C150A_R	gattaacaacaaggcgatgggcaatccgcg
Sec61p C373A_F	gttcttggttcagccgcagtattttcc
Sec61p C373A_R	ggaaaataactgcgggtgaaccaagaac
SEC61 TM1CCC_F	ctctactgatcttttgcgtgctgcggccagattccg
SEC61 TM1CCC_R	cggaatctggccgcagcagcaaaagatcagtagag
SEC61 TM2CCC_F	ggtgtttcgcccatctgctgctgctctatgattttc
SEC61 TM2CCC_R	gaaaatcatagagcagcagcagatgggcgaaacacc
SEC61 TM3CCC_F	gatcttgggccaatgctgctgcgtcgtcatgacagg
SEC61 TM3CCC_R	cctgtcatgacgacgcagcagcattggcccaagatc
SEC61 TM4CCC_F	gttaatctttcaatgctgctgcgcacgcgtgattg
SEC61 TM4CCC_R	caatcagcgatgcgcagcagcattgaaagattaac
SEC61 TM5CCC_F	gctgtgattgcatgctgctgccttttggtgtc
SEC61 TM5CCC_R	gacagccaaaaggcagcagcatgcaatcacagc
SEC61 TM6CCC_F	ctaatatgttccaagtgttgctgctgcgccatcttctctttg
SEC61 TM6CCC_R	caaagaggaagatggcgcagcagcacaacacttggaacatattag
SEC61 TM7CCC_F	cagagtgcattgacttgctgctgcttcttgatctctcaaac
SEC61 TM7CCC_R	gatttgagagatcaagaagcagcagcaagtcaatgcactctg
SEC61 TM8CCC_F	catcacatttgttctttgctgctgcgcagtattttcc
SEC61 TM8CCC_R	ggaaaataactgcgcagcagcaaaagaacaaatgtgatg
SEC61 TM9CCC_F	cattccaactgctgcatgctgctgcgggtgctaccatc
SEC61 TM9CCC_R	gatggtagcaccgcagcagcatgcagcagttggaatg
SEC61 TM10CCC_F	ggttctggggcatgctgctgcatggctactaccacc
SEC61 TM10CCC_R	ggtggtagtagccatgcagcagcatgccccagaacc
SSS1 TMCCC_F	gttggtattgggtttttgctgctgcggtatcattggttac
SSS1 TMCCC_R	gtaaccaatgataccgcagcagcaaaaaccaataccaac
pJKB18 (5'NdeI)_F	gtataacattgaaaaatcatatggctagagctagtg
pJKB18 (5'NdeI)_R	cactagctctagccatatgatttttcaatgttatac

SUS_F	cgggatccatggtttatattggtatcgctatTTTTTgttttggttg gcTTTTTatgaagttgattcatattccaatcag
SUS_R	gcagtaatattatatgggaag
sss1 H72K_F	catcaagttgattaagattccaatcag
sss1 H72K_R	ctgattggaatcttaatcaacttgatg
sss1 81C_F	cgttattgtttgctaaaagagataaaaag
sss181C_R	cttttatctcttttagcaaacaataacg

2.4 *In vitro* DNA manipulations

2.4.1 Site directed mutagenesis

Site directed mutagenesis was carried out using the PCR based Quick Change™ Site Directed Mutagenesis protocol (Stratagene). Mutagenic primers were designed and reactions were carried out with 5 and 10 ng of template DNA, 125 ng of both forward and reverse mutagenic primers, 1X Pfu PCR buffer, 200 μ M dNTP's and 2.5 U Pfu DNA polymerase. Each PCR cycle included 95°C for 30 seconds, 45°C for 1 minute and 68°C for 2 X plasmid size (kb) minutes. This cycle was repeated 18 times.

Following this, samples were treated with 10 U *Dpn I* restriction enzyme at 37°C for 2 hours to digest the methylated, non-mutated, parental DNA of which 2 μ l was used to transform *E. coli* by electroporation.

2.4.2 Restriction endonuclease digestion of plasmid DNA

Plasmid DNA was digested using commercially available enzymes with the buffers supplied. Digests were routinely performed in 10-20 μ l volumes containing 1-2 μ g of plasmid DNA and 10 U of enzyme. Digests were incubated at the recommended temperature for 2 hours.

2.4.3 Alkaline phosphatase treatment of linear DNA fragments

After restriction digest, samples were adjusted to 200µl with dephosphorylation buffer and 5U of calf intestinal alkaline phosphatase (Roche). The samples were incubated at 37°C for 2 hours. Following this treatment, samples were incubated at 70°C for 30 minutes in order to inactivate the alkaline phosphatase. DNA was isolated using the PCR purification system (Qiagen).

2.4.4 Filling in DNA cohesive ends

DNA fragments with 5' overhangs were end filled using the large fragment (Klenow) of DNA ligase I. After restriction digest the DNA sample was diluted to 50 µl by the addition of an equal volume of ddH₂O, dNTP's were added to a final concentration of 100 µM and 2 U of Klenow enzyme was added. The sample was incubated at 37°C for 30 minutes after which the DNA was isolated and the Klenow enzyme deactivated.

2.4.5 Ligations of DNA fragments

DNA ligations were performed with an insert to vector ratio ranging from 1:3 to 1:6. Reactions were performed in 30 µl volumes and each reaction contained DNA fragments, 2 U of T4 DNA ligase and 3 µl of 10X ligation buffer supplied with the enzyme (Roche). The reactions were incubated at 24°C for 2 hours or overnight at 16°C. The samples were desalted using the PCR purification system (Qiagen) and eluted in 30 µl ddH₂O of which 5 µl was used to transform *E. coli* by electroporation.

2.4.6 DNA sequencing

DNA sequencing was performed using the ABI PRISM™ BigDye™ terminator cycle reaction mix (Perkin Elmer Applied Biosystems). Reactions were carried out in 10 µl volumes and consisted of 4 µl Big Dye™ terminator mix, 5µl template DNA and 50 pmoles of sequencing specific primer. Each PCR cycle includes 96°C for 30

seconds, 50°C for 30 seconds and 60°C for 4 minutes. This cycle was repeated 30 times.

2.4.7 Phenol/chloroform extraction and ethanol precipitation

Phenol chloroform extraction was routinely used to remove contaminating proteins. An equal volume of phenol equilibrated with TE (10 mM Tris-HCl, 1 mM EDTA, pH 8) was added to the sample and the sample was adjusted to 100 μ l by the addition of TE. After vigorous mixing, the sample was spun at full speed in a microfuge for 2 minutes and the upper aqueous layer retained and placed in an eppendorf tube containing 40 μ l chloroform. After vortexing and microfugation, the aqueous layer was retained and chloroform extraction repeated. Following this, nucleic acids were isolated from the aqueous phase by incubating with sodium acetate (10 mM final concentration) and two and a half volumes of 100% (v/v) ethanol on ice for 30 minutes. Salt pellets were isolated by centrifugation in a microfuge at full speed for 10 minutes, washed in 70% (v/v) ethanol, dried under a vacuum and resuspended in an appropriate of buffer for downstream procedures.

2.4.8 Agarose gel electrophoresis of DNA

DNA fragments were separated on 1% (w/v) agarose gels in 1X TAE running buffer (40mM Tris, 20mM sodium acetate, 1mM EDTA, pH8.2). Gels were run at 60V submerged in 1X TAE running buffer after which gels were soaked in 1X TAE containing 10ug/ml ethidium bromide. DNA was visualised under short wave ultraviolet light in a LAS 1000 imager (Fuji) unless it was required that the DNA be purified from the gel in which case long wave ultraviolet light was used.

2.5 Transformation of *Escherichia coli*

2.5.1 Transformation of *E. coli* by calcium chloride

One ml of fresh overnight culture of the *E. coli* strain DH5 α or BL21 was used to inoculate 100 ml of LB or LB supplemented with 34 μ g/ml chloramphenicol respectively. The culture was grown with shaking at 37°C until the OD_{600nm} was 0.5. The cells were harvested by centrifugation at 3800g at 4°C for 10 minutes and the pellet was gently resuspended in 5 ml of ice cold 100m CaCl₂. The cells were left on ice for 2 or more hours, the competency of the cells increases with time, and were kept at 4°C for a maximum of 2 days.

200 μ l of cells were transformed with 0.5-1 μ g DNA. Tubes were mixed gently and incubated on ice for 30 minutes. The cells were then heat shocked at 42°C for 2 minutes and then immediately incubated on ice for a further 10 minutes. Following this, 800 μ l of LB was added and the transformation recovered at 37 °C for 1 hour. The cells were then plated out onto selective LB media and incubated at 37 °C overnight

2.5.2 Transformation of *E. coli* by electroporation

300 ml of LB media was inoculated with 1 ml of overnight DH5 α *E. coli* culture and grown to O.D._{600nm} \approx 0.4. Cells were chilled on ice for 30 minutes and isolated by centrifugation at 3800g at 4°C for 5 minutes in a pre-cooled rotor. Cells were washed 4 times in 50 ml ice-cooled dH₂O. Cells were recovered and washed with 2ml ice-cooled 10% (w/v) glycerol. Cells were again recovered and resuspended in 900 μ l 10% glycerol and separated into 50 μ l aliquots. Cells were frozen in liquid nitrogen and stored at -80°C until required (each aliquot has a shelf life of approximately 6 months).

For transformation, electrocompetent cells were defrosted on ice and incubated with 2-5 μ l DNA in dH₂O on ice for 1 minute. Each aliquot was then transferred to a pre-chilled 0.2cm path electroporation cuvette (BioRad). Samples were electroporated

using a BioRad Gene Pulser™ at 25 μ F, 2.1 kV, 200 ω , time constant of 4.8-5.0. Cells were recovered for 1 hour in SOC medium at 37°C for 1 hour after which they were plated out onto selective media and incubated overnight at 37°C.

2.5.3 Preparation of plasmid DNA

E. coli cultures were grown overnight at 37°C and plasmids were extracted using Qiagen miniprep according to the manufacturers protocol.

2.6 Transformation of *Saccharomyces cerevisiae*

2.6.1 ONE-STEP transformation of *S. cerevisiae*

0.5 ml of cells (per transformation) from an overnight culture was isolated and washed with 1ml ddH₂O. Cells were resuspended in 100 μ l ONE- STEP buffer (0.2M lithium acetate pH5.5, 40% (w/v) PEG 3350, 100mM DTT) to which 50 μ g of denatured salmon sperm DNA and 1-10ng of transforming DNA was added. The transformation mix was vortexed vigorously, heat shocked at 45°C for 30 minutes and plated out onto appropriate selective media.

2.6.2 Transformation of *S. cerevisiae* by electroporation

100 ml of YPD or 200 ml of YNB plus nutrients was inoculated with a starter culture of yeast and grown to an O.D._{600nm} 1.3 – 1.5 (YPD) or 0.5 (YNB). Cells were then harvested by centrifugation at 3800 g for 5 minutes and resuspended in 10 ml of a solution containing 0.1 M lithium acetate, 10 mM Tris.HCl pH 7.4, 1 mM EDTA and incubated at 30°C for 45 minutes. Following this, cells were incubated for a further 15 minutes after the addition of DTT to a final concentration of 25 mM. Cells were recovered by centrifugation at 3800 g for 5 minutes at 4°C in a pre-cooled rotor. Cells were washed three times with 50 ml of ice cold ddH₂O and once with ice cold 1 M

sorbitol each time harvested as above. Following this cells were isolated and resuspended in 500 μ l ice cold sorbitol with an approximate O.D._{600nm} of 200.

For transformation, electrocompetent cells were incubated with ≤ 100 ng plasmid DNA and incubated on ice for 1 minute. Each aliquot was then transferred to a pre-chilled 0.2cm path electroporation cuvette (BioRad). Samples were electroporated using a BioRad Gene Pulser™ at 25 μ F, 2.1 kV, 200 ω , time constant of 4.8-5.0 and recovered in 1ml of ice cold 1 M sorbitol. Cells were plated out onto selective media supplemented with 1 M sorbitol.

2.7 Protein Biochemistry

2.7.1 SDS-PAGE

Proteins were resolved by migration through SDS-containing polyacrylamide gels. SDS-PAGE resolving gels contained between 8-15% polyacrylamide, depending on the size of the protein to be resolved, and consisted of the required volume of 30% (w/v) acrylamide stock solution (acrylamide:bisacrylamide, 29:1 (Bio-Rad)) in 1X resolving buffer (375mM Tris. HCl pH8.8, 0.1% (w/v) SDS). Gels were polymerised through the addition of 200 μ l of 10% (w/v) ammonium peroxodisulphate (APS) and 40 μ l tetramethylethylenediamine (TEMED (Amersham Pharmacia)). A stacking gel was layered over the resolving gel and consisted of 3.5% (w/v) acrylamide in 1X stacking buffer (125mM Tris. HCl pH 6.8, 0.1% (w/v) SDS). Polymerisation was as above except 60 μ l rather than 200 μ l 10% APS was used to catalyse the reaction. Samples were solubilised in 1X Laemmli sample buffer (10% (v/v) glycerol, 3% (w/v) SDS, 25mM Tris. HCl pH6.8, 0.01% (w/v) bromophenol blue, 62.5mM Tris. HCl pH6.8, 5% (v/v) β -mercaptoethanol). Gels were run at 25mA or 5mA overnight in SDS-PAGE running buffer (25mM Tris. HCl, 192mM glycine, 0.1% (w/v) SDS).

2.7.2 Coomassie staining of polyacrylamide gels

Gels were soaked in 10% (v/v) methanol, 10% (v/v) acetic acid containing 0.5% (w/v) Coomassie Brilliant Blue for 1 hour. Background staining was removed by repeated washes in 10% (v/v) methanol, 10% (v/v) acetic acid. Gels were visualised by digital imaging in a LAS 1000 (Fuji).

2.7.3 Western blotting and immunodetection

Following electrophoresis, proteins were transferred to nitrocellulose (Amersham Pharmacia) or PVDF (Millipore) membrane by western transfer. A sandwich consisting of three sheets of Whatmann 3MM paper, nitrocellulose or PVDF membrane, the gel and three more sheets of filter paper, all previously soaked in transfer buffer, was assembled and the air bubbles removed by rolling the sandwich with a glass rod. PVDF membrane was activated by soaking in 100% Methanol. The sandwich was placed in a transfer tank, the gel adjacent to the cathode, containing transfer buffer (20mM Tris-base, 150mM glycine, 5% (v/v) methanol) and transferred at 100mA overnight or 800mA for 1.5 hours. The membrane was washed in ddH₂O and the efficiency of transfer visualised by soaking the membrane in Ponceau stain (1% (w/v) Ponceau S, 3% (w/v) TCA, 3% Sulphosilylic acid). The molecular weight standards were marked with pencil. Each membrane was washed in blocking solution (2% (w/v) dried skimmed milk powder in TBS-NP40 (130mM NaCl, 2.6mM KCl, 2mM Tris-base pH7.6, 0.1% (v/v) NP40) for a minimum of 30 minutes, changing the blocking solution at least once. The membrane was then incubated in 30ml blocking solution containing primary antibody at a required concentration for 1 hour after which the antibody solution was removed and the membrane washed in blocking solution for a minimum of 30 minutes, changing the solution at least three times. The membrane was then incubated with a secondary antibody conjugated to horse radish peroxidase (HRP), immunoreactive against the species that the primary antibody was raised in,

diluted 1:10000 in blocking solution for 1 hour. Excess secondary antibody was washed away as before. The membrane was washed a further two times, 5 minutes each wash, in TBS-NP40. Protein was identified by incubating the membrane in enhanced chemiluminescence reagent (NEN) for 1 minute and visualised in a LAS 1000 imager (Fuji).

2.7.4 Antibodies used for immunodetection

Table 2.7 Antibodies used in this study

Antigen	Animal immunised	Dilution for blotting	Source
Sec61p (C- terminal peptide)	Rabbit	1:5000	Stirling <i>et al.</i> , 1992
Sss1p (His- tagged cytosolic domain (M1-K52)fusion protein	Sheep	1:3000	This study
Sec63p (GST- cytosolic domain (C- terminus) fusion protein	Sheep	1:10000	Young <i>et al.</i> , 2001
Alpha factor (His tagged fusion protein)	Sheep	1:10000	E. Doherty (this laboratory)
Kar2p (His-tagged fusion protein)	Sheep	1:10000	J. Tyson (this laboratory)
Sil1p (GST- tagged fusion protein	Sheep	1:5000	J. Tyson (this laboratory)
12CA5 (affinity purified anti HA monoclonal)	Mouse	1:20000	University of Manchester
Polyhistidine	Mouse	1:3000	Sigma
Anti Sheep IgG's	Donkey	1:10000	Sigma
Anti Rabbit IgG's	Goat	1:10000	Sigma
Anti Mouse IgG's	Goat	1:10000	Sigma

2.7.5 Preparation of whole *E. coli* cell protein extracts

0.5 O.D._{600nm} units of cells were isolated and solubilised in 1X Laemmli sample buffer. After this each extract was immediately heated at 95°C for 10 minutes and prepared for SDS-PAGE.

2.7.6 Preparation of whole yeast cell protein extracts

Yeast was grown overnight and then diluted to an O.D._{600nm} of 0.1. Cells were grown to an O.D._{600nm} \approx 0.5 and 5 O.D._{600nm} units were harvested by centrifugation at 3800g for 5 minutes and washed with 1 ml of ddH₂O. The cell pellet was resuspended in 200 μ l 1X Laemmli sample buffer (10% (v/v) glycerol, 3% (w/v) SDS, 25mM Tris. HCl pH6.8, 0.01% (w/v) bromophenol blue, 62.5mM Tris. HCl pH6.8, 5% (v/v) β -mercaptoethanol). Glass beads were added to 2/3 volume and the cells were lysed using a Hybaid Ribolyser at maximum speed (6.5) for 30 seconds. Following cell lysis, each extract was immediately heated at 95°C for 10 minutes, cooled and the yeast total extract isolated and prepared for SDS-PAGE and western analysis.

2.7.7 Preparation of crude membranes

Microsomal membranes were prepared according to the spheroplasting/glass bead lysis protocol (Rothblatt and Meyer, 1986). Yeast cultures were grown to an O.D._{600nm} of 2.0-4.0. Cells were harvested by centrifugation at 3800g for 5 minutes and the cell pellet resuspended in 100mM Tris.SO₄, pH 9.4, 10mM DTT at a concentration of 50 O.D._{600nm} units ml⁻¹. Cells were incubated at room temperature for 10 minutes, harvested as above and resuspended in spheroplast buffer (0.75X YP, 0.7M sorbitol, 0.5% (w/v) glucose, 10mM Tris. HCl pH7.4) at a concentration of 100 O.D._{600nm} units/ml. Cells were spheroplasted by the addition of 1.5U/ml of yeast lytic enzyme and incubated at 30°C for 30 minutes. Spheroplasts were harvested at 3800g for 5 minutes and resuspended in ice-cold lysis buffer (0.1M sorbitol, 50mM potassium

acetate, 20mM HEPES pH 7.4, 2mM EDTA, 1mM DTT, 1mM AEBSF) at a concentration of 200 O.D._{600nm} units ml⁻¹. Acid washed beads were added to the meniscus of the suspension (approximately 75% final volume) and lysis was induced through vortexing 4 times for 30 seconds with alternating 30second incubations on ice. Following cell disruption, 1ml of ice-cold lysis buffer was added and the suspension vortexed for a further 5 seconds. Cell debris was removed by centrifugation at 3800g at 4°C for 10 minutes. The supernatant was retained and the centrifugation repeated.

Microsomal membranes were isolated from the supernatant by centrifugation at 17500g in a bench top centrifuge at 4°C for 15 minutes. The membrane pellet was resuspended in ice cold membrane storage buffer (250mM sorbitol, 20mM HEPES pH7.4, 50mM potassium acetate, 1mM DTT) at 50 O.D._{280nm} /ml, which was determined in 2% SDS.

2.7.8 Chemical crosslinking

Chemical cross-linking reagents were dissolved in DMSO to a stock concentration of 100mM and diluted to a working concentration of 10 mM. Cross-linking reagents were added to a final concentration of 1 mM. For negative controls an equivalent volume of DMSO was added to samples. Samples were incubated at 30°C for 30 minutes after which the cross-linking reactions were quenched by the addition of 100mM (final concentration) lysine and glycine for lysine specific homobifunctional cross-linking reagents (Disuccinimidyl suberate (DSS) (Pierce)) or with 100 mM DTT for cysteine specific homobifunctional cross-linking reagents (BMH or PDM). Samples were incubated for a further 5 minutes at room temperature. Following this samples were prepared for SDS-PAGE.

2.7.9 Blue native polyacrylamide gel electrophoresis (BN-PAGE)

BN-PAGE was carried out according to established protocols (Schagger and von Jagow, 1991). 2 A_{280nm} units of microsomes were isolated and solubilised in

solubilisation buffer (20 mM Tris.HCl pH 7.6, 5 mM magnesium acetate, 2 mM DTT 10 μ g/ml leupeptin/aprotinin, 5 μ g/ml chymostatin/pepstatin, 1 mM AEBSF, 12% glycerol), containing 2% digitonin and 250 mM NaCl, for 30 minutes on ice. Non-solubilised material was isolated by centrifugation at 10,000 g for 10 minutes, after this the sample underwent further centrifugation at 400,000g for 1 hour at 4°C to remove the ribosome associated material and the soluble material was retained. Samples were then adjusted to 200 μ l with solubilisation buffer and BNG loading buffer (0.5% Coomassie G-250, 50 mM 6-aminocaproic acid, 10 mM Bis-Tris, pH 7.0)

During this time a polyacrylamide gel was prepared with a linear gradient from 6 (500 mM 6-aminocaproic acid, 50 mM Tris.HCl pH 7.0, 6% (w/v) acrylamide) to 16% (500 mM 6-aminocaproic acid, 50 mM Tris.HCl pH 7.0, 16% (w/v) acrylamide, 14% glycerol). 0.4-0.8 A280 U of solubilised membranes were loaded per lane and samples were run for 18 hours in blue cathode buffer (50 mM tricine pH 7.0, 15 mM Bis-Tris pH 7.0, 0.02% Coomassie G-250) at 200V. After this, the blue cathode buffer was changed to colourless cathode buffer (50 mM tricine pH 7.0, 15 mM Bis-Tris pH 7.0) and run for a further 2 hours at 500V. The anode buffer (50mM Bis-Tris pH 7.0) remained constant throughout.

Following this, samples were transferred onto PVDF by western transfer and after this the blot was washed with 90% methanol to remove the bound coomassie. Immunoblotting was performed as previously described.

2.7.10 Alkaline phosphatase treatment of microsomes

Crude membrane extracts were prepared as described above and resuspended in dephosphorylation buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 2 mM EDTA, 2 mM AEBSF), and a commercial mixture of protease inhibitors (Roche Applied Science). The crude extracts were either mock treated or incubated with 10 units of calf

intestinal alkaline phosphatase (Roche Diagnostics) for 30 min at 30 °C. Samples were heated at 95°C for 10 minutes in 1 X Laemmli buffer before SDS-PAGE.

2.8 ^{32}P labeling and immunoprecipitation

Cultures were grown in low phosphate YP media (LP-YP), prepared according to Rubin (1973) with appropriate carbohydrate at 30°C to early exponential growth phase and 5 O.D._{600nm} units of cells were isolated and resuspended in 5 ml of LP-YP, pre-warmed to 30°C. Cells were incubated with 25 $\mu\text{Ci}/\text{O.D.}_{600\text{nm}}$ of [^{32}P] as orthophosphate (Amersham) and incubated at 30°C for 60 minutes. Metabolic labelling was stopped by the addition of 500 μl of ice cold 1 M NaN_3 and incubated on ice for 5 minutes. Cells were then isolated by microfugation at 6000 g and resuspended in 1 ml IP spheroplast buffer (1.4 M sorbitol, 50 mM Tris. HCl pH 7.4, 2 mM MgCl_2 , 10 mM NaN_3) including 1.5 U/O.D._{600nm} yeast lytic enzyme and incubated for 30 minutes at 30°C. After this, spheroplasts were isolated by microfugation at 6000 g, resuspended in 100 μl of ^{32}P IP lysis buffer (50 mM Tris.HCl pH 7.4, 1 mM EDTA, 8 M urea, 1% (w/v) SDS, 10 mM NaF, 100 μM sodium metavanadate) and incubated at 95°C for 10 minutes. After cooling, 1 ml of IP buffer (187.5 mM NaCl, 62.5 mM Tris.HCl pH 8.0, 6.25 mM EDTA, 1.25% (v/v) Triton X-100) was added. Extracts were pre-cleared for Protein-A cross reactive species by the addition of 50 μl insoluble Protein-A suspension for 30 minutes at 4°C with rotation. After this, insoluble Protein-A was isolated and the radiolabeled extract decanted and retained.

2 $\mu\text{l}/\text{O.D.}_{600\text{nm}}$ of antibody raised against the C- terminus of Sec63p was added to the cleared extract and the sample incubated at room temperature, with rotation, for 1 hour. Following this, 40 μl Protein-A sepharose (20% (w/v) in IP buffer) was added and incubated with rotation at room temperature for a further 2 hours. Protein-A sepharose beads were isolated and washed four times with 1 ml IP buffer. Proteins were then eluted from the beads by incubating with 1 X Laemmli sample

buffer at 95°C for 10 minutes. Samples were then analysed by SDS-PAGE and detected by phosphorimaging (BAS1800, Fuji).

2.9 ³⁵S labeling and immunoprecipitation

Cultures were grown in low sulphur media at 30°C to an O.D._{600nm} 0.2 – 0.5. 5 O.D._{600nm} units of cells were isolated and resuspended in 0.5 ml of low sulphur media, pre-warmed to 30°C, and incubated at 30°C for 5 minutes. After this, cells were incubated with 20 µCi/ O.D._{600nm} of [³⁵S]-Easy Tag labelling mix (NEN) and incubated at 30°C for 5-30 minutes. Metabolic labelling was stopped by the addition of 500 µl of ice cold 1 M NaN₃ and incubated on ice for 5 mins. Cells were then isolated by microfugation at 6000g and resuspended in 1 ml of IP spheroplast buffer (1.4 M sorbitol, 50 mM Tris.HCl pH 7.4, 2 mM MgCl₂, 10mM NaN₃) including 1.5 U/ O.D._{600nm} Yeast Lytic Enzyme and incubated for 30 minutes at 30°C. After this, spheroplasts were isolated by microfugation at 6000g resuspended in 200 µl of IP lysis buffer (50 mM Tris.HCl pH 7.4, 1mM EDTA, 1% (w/v) SDS) and incubated at 95°C for 10 minutes. After cooling, 1 ml of IP buffer (187.5 mM NaCl, 62.5 mM Tris.HCl pH 8.0, 6.25 mM EDTA, 1.25% (v/v) Triton X-100) was added. Extracts were pre-cleared for Protein-A cross reactive species by the addition of 50 µl insoluble Protein A suspension (Sigma) for 30 minutes at 4°C with rotation. After this, insoluble Protein A was isolated and the radiolabeled extract decanted and retained.

1-2 µl / O.D._{600nm} of antibody were added to the cleared extract and the sample incubated at room temperature, with rotation, for 2 hours. Following this, 40 µl Protein A-Sepharose (20% (w/v) in IP buffer) was added and incubated with rotation at room temperature for a further 2 hours. Protein A-Sepharose beads were isolated and washed four times with 1 ml IP buffer. Proteins were then eluted from the beads by incubating in 1 X Laemmli sample buffer at 95°C for 10 minutes. Samples were then analysed by SDS-PAGE and detected by phosphorimaging (BAS1800, Fuji).

2.10 *In vitro* transcription

In vitro transcription of pp α f from pEH3 was carried out using T7 RNA polymerase (RiboMAX, Promega). Initially 10 μ g of pEH3 was digested by incubating with *Xba*I and subsequently the linearised template isolating using the PCR purification procedure (Qiagen) and eluted in 20 μ l DEPC treated water. Transcription reactions contained 4 μ l of 5 X T7 buffer, 6 μ l rNTP mix (25 mM rATP, rUTP, rCTP and rGTP), 2 μ l linearised DNA template, 1 μ l Cap analogue (m⁷ G(5') ppp (5') G), 5 μ l DEPC treated water and 2 μ l T7 RNA polymerase mix. Reactions were incubated for 2 hours at 37°C and then underwent phenol chloroform and ethanol precipitation. RNA was pelleted in a microfuge for 10 minutes, resuspended in 40 μ l DEPC treated water and stored in 5 μ l aliquots at -80°C following snap freezing in liquid N₂.

2.11 *In vitro* translation and translocation

In vitro translations were carried out using a rabbit reticulocyte lysate ® (Promega). Reactions contained 200 μ l rabbit reticulocyte lysate, 6 μ l amino acid mix minus methionine, 6 μ l RNase inhibitor (Promega), 5 μ l pp α f mRNA, 56 μ l DEPC treated water and 12 μ l ³⁵S labelled methionine and were incubated at 30°C for 2 hours. After this, translation was inhibited by the addition of 1.4 mM cycloheximide (final concentration) and the ribosomes were isolated by ultracentrifugation at 100,000g for 15 minutes at 4°C.

In vitro translocation were carried out by incubating 10 μ l of *in vitro* translated alpha factor with 4 μ l of membranes, 3 μ l membrane storage buffer and 3 μ l ATP regeneration mix (10 mM ATP, 400 mM creatine phosphate, 2 mg/ml creatine phosphokinase) for 20 minutes at 30°C.

2.12 Expression of His-Sss1p_{M1-K52} fusion protein

100 ml of an overnight culture of BL21 (DE3) cells transformed with pCM204 was added to 4L of LB media containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol and grown at 30°C until the culture had reached an O.D._{600nm} of 0.6-0.8. At this point the expression of the pCM204 derived 10 His – Sss1p (M1-K53) fusion protein was induced by the addition of IPTG to a final concentration of 1mM and further incubation at 30°C for 3 hours.

Cells were then harvested by centrifugation at 3500g for 10 minutes and resuspended in 30 ml of Buffer D (50 mM HEPES pH 6.8, 400 mM potassium acetate, 5 mM magnesium acetate, 0.1% (v/v) Triton X-100, 1 µg/ml AEBSF, 2 µg/ml E64, 1 µg/ml Aprotinin, 1 µg/ml Pepstatin A, 1 µg/ml Chymostatin, 1 µg/ml Leupeptin). Cell lysis was induced by T7 lysosyme, expressed in BL21 from the pLysS vector (Invitrogen). Cells were rapidly frozen in liquid nitrogen and thawed on ice three times and the genomic DNA was sheered by sonication. Cell debris was then isolated by centrifugation at 17000g for 10 minutes and the supernatant retained. Soluble and insoluble material was isolated by ultracentrifugation at 45000 rpm in a MLA-80 rotor for 30 minutes at 4°C.

2.13 Purification of His-Sss1p_{M1-K52} fusion protein and antibody production

Recombinant 10X His-Sss1p_{M1-K52} was purified from the soluble *E. coli* fraction. The soluble fraction was passed through a 4µm filter and loaded onto a 50 ml Super Loop (Amersham Pharmacia) and subsequent purification steps were performed using an AKTA purifier-10 HPLC system.

Five 200 µg samples of fusion protein were sent to Diagnostic Scotland for inoculation into sheep. Three bleeds of approximately 800 ml were tested for western

blot detection of Sss1p. The third bleed was found to be suitable at a working dilution of 1 in 3000.

2.14 ONPG assay for β -Galactosidase activity in *S. cerevisiae*

Overnight cultures of *S. cerevisiae* strains transformed with pJT30 were diluted to an OD_{600nm} of 0.1 and incubated at 30°C for 4 hours in media selective for pJT30. Following this, cells were isolated and resuspended in 5 ml of 1 X Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 10 mM MgSO₄, 50 mM BME, pH 7.0). 0.8 ml aliquots were removed and solubilised by the addition of 50 μ l of 0.1% (w/v) SDS and 100 μ l of chloroform. Samples were vortexed for 15 seconds and incubated at 30°C for 30 minutes. 160 μ l of ONPG (4 mg/ml stock solution) was then added and incubated at 30°C for a further 20 minutes. Finally, 400 μ l of 1 M sodium carbonate pH 9 was added to terminate the reaction. The β -Galactosidase activity (U) was calculated by measuring the OD_{420nm}, OD_{550nm} and OD_{600nm} of the sample and calculated by the following equation

$$U = \frac{1000 \times (OD_{420nm} - (1.75 \times OD_{550nm}))}{OD_{600nm} \times \text{Time in minutes} \times \text{Volume}}$$

Where time is the incubation period in minutes and the volume is the volume of culture used in the assay in ml.

Chapter 3

Investigating the molecular architecture of the Sec61 complex by cysteine scanning mutagenesis

3.1 Introduction

Genetic and biochemical data has demonstrated that Sss1p interacts with Sec61p between TM6 and TM8 (Wilkinson *et al.*, 1997). Consequently, it was identified that the signal sequence of a protein that is translocated into the yeast ER via the Sec62p dependent pathway interacts with TM 2 and 7 of Sec61p (Plath *et al.*, 1998). Although the TM domains of Sec61p which interact with that of Sss1p are unknown it was proposed that that the region of Sec61p that interacts with Sss1p overlaps with that that interacts with the signal sequence of secretory precursors. Therefore it was postulated that Sss1p is required to regulate the ER translocon (Plath *et al.*, 1998). This model predicts that incorporation of signal sequence into the translocation channel displaces Sss1p, revealing the signal peptide binding site of Sec61p, switching the translocon into a translocation competent state (Plath *et al.*, 1998). Importantly, this model predicts that following the displacement of Sss1p by signal peptide the translocon undergoes a significant structural remodelling (Plath *et al.*, 1998). In agreement with this model, it has been suggested that the translocon is a gated membrane conduit whereby translocon activation is accompanied with a change in pore diameter from 15-20 Å to 40-60 Å (reviewed in Johnson and van Waes, 1999).

To test this model cysteine scanning mutagenesis has been used to investigate the molecular environment of each of the transmembrane domains of Sec61p as well as the transmembrane domain of Sss1p. A number of studies have used cysteine scanning mutagenesis to identify sites of interaction between transmembrane helices of polytopic membrane proteins and between multiprotein membrane complexes (Frillingos *et al.*, 1998). This is so as cysteine residues are relatively hydrophobic and are readily tolerated in a lipid bilayer (Frillingos *et al.*, 1998). Importantly, the sulphydryl side chain of a cysteine residue is susceptible to modification with reagents that can determine, by chemical cross-linking, the spatial orientation and proximity of adjacent transmembrane domains in a lipid bilayer. Specifically, cysteine scanning mutagenesis

has successfully been utilised to identify the transmembrane domains of SecY that interact with TM3 of SecE (Kaufman *et al.*, 1999; Veenendaal *et al.*, 2001) and to provide structural analysis of the *E. coli* lac permease (Wang *et al.*, 1999; Kwaw *et al.*, 2000).

Hydropathy and topological analysis has indicated that Sec61p spans the ER membrane ten times (Wilkinson *et al.*, 1996) and that Sss1p is a C- terminal anchor protein (Esnault *et al.*, 1994). Based on these data, the cysteine scanning approach requires that each of the ten transmembrane domains of Sec61p and the C- terminal anchor of Sss1p are mutagenised so that three consecutive amino acids are substituted to cysteine. This ensures that a complete turn of the helix has the potential to form sulphydryl dependent interactions with a cysteine residue that is present on an adjacent transmembrane domain that is in both close proximity and correct spatial orientation. A positive interaction between Sss1p and Sec61p would be identified by chemical cross-linking where a chemical compound can covalently couple the sulphydryl side chains of two adjacent cysteine residues and, following SDS-PAGE and western transfer, crosslinked adducts are isolated that contain both Sss1p and Sec61p and are specific to the co-expression of these particular mutations.

3.2 Isolating anti Sss1p antibodies

This assay requires the identification of protein adducts formed by Sss1p and Sec61p by immunoblotting. Although anti-Sss1p antibodies have been generated, its availability is limited. It therefore requires that an anti-Sss1p antiserum is raised for this study. An antigen was generated where by the N- terminal 52 residues of Sss1p were expressed in *E. coli*, isolated and injected into sheep. To facilitate the purification of the Sss1p antigen, the N-terminal 52 residues were fused to a polyhistidine tag enabling the recombinant protein to be purified by nickel affinity chromatography.

The pET series of vectors are designed to allow the regulated expression of fusion proteins in *E. coli* as the expression cassette is under the control of the bacteriophage T7 promoter which can be transcriptionally activated in an *E. coli* strain that produces T7 polymerase. λ DE3 lysogens of BL21 (BL21 (DE3)) possess a genomic copy of the gene encoding T7 RNA polymerase under the control of the lac promoter. Therefore, following the addition of IPTG, a gratuitous inducer of the lac promoter, T7 RNA polymerase is expressed which in turn can activate the expression of the fusion protein.

An Sss1p expression vector was constructed based on the pET16-b vector (Novagen). The sequence encoding the N- terminal 52 residues of Sss1p was fused downstream of the polyhistidine tag (10X His) encoded by this vector. The pJKB18 vector contains the *SSS1* coding sequence that has a stop codon inserted immediately after the codon encoding for residue 52. This plasmid was mutagenised by site directed mutagenesis, using the mutagenic primers pJKB18 5'NdeI_F and pJKB18 5'NdeI_R, to incorporate a novel *NdeI* restriction site immediately upstream of the initiation codon. The *in vitro* mutagenised vector was transformed into DH5 α by electroporation and positively mutagenised clones were isolated following digestion with the *NdeI* restriction endonuclease and DNA sequencing. The coding sequence for the N-terminal 52 residues of Sss1p was then ligated as a 460 bp *NdeI/BamHI* fragment from the cloning intermediate, pJKB18 5'NdeI, into pET16-b giving pCM204.

To express 10X His-Sss1p_{M1-K52}, pCM204 was transformed into BL21 (DE3) by chemical transformation. Following selection of transformants, the expression profile of the 10X His Sss1p fusion protein was investigated. Cells were grown to an O.D._{600nm} of 0.2, time point 0 after which expression of the fusion protein was induced by the addition of 1 mM IPTG. Total *E. coli* protein extracts were prepared at 0, 1, 2, 3 and 4 hours after IPTG induction. Following SDS-PAGE and western transfer, expression of the 10X His-Sss1p_{M1-K52} fusion protein was determined by probing total

protein extracts with anti polyhistidine antibodies. A single band of approximately 8.5 kDa was identified following the addition of IPTG and maximal expression was observed after 3 hours of induction (figure 3.1 A). This indicates that BL21 (DE3) cells transformed with pCM204 express the 10X His-Sss1p_{M1-K52} fusion protein allowing the downstream purification of an Sss1p antigen.

Large scale induction of recombinant proteins in *E. coli* can result in the formation of insoluble aggregates or inclusion bodies and these can be isolated from an *E. coli* total cell lysate by ultracentrifugation. To determine whether the Sss1p fusion protein is in the soluble or insoluble fraction 200 ml of BL21 (DE3) transformed with pCM204 were grown to an O.D._{600nm} of 0.2 and then 10X His-Sss1p_{M1-K52} expression was induced by growing cells for 3 hours at 30°C with 1 mM IPTG. Following this, cells were harvested, lysed by freeze thawing and DNA was sheared by sonication. The extract was centrifuged at 10000 g to isolate the soluble and insoluble fractions and these were prepared for SDS-PAGE and western transfer. Immunoblotting with anti polyhistidine antibodies indicated that the majority of the 10X His-Sss1p_{M1-K52} fusion protein was in the soluble fraction (figure 3.1 B, compare lanes 2 and 3), therefore the subsequent purification of 10X His-Sss1p_{M1-K52} will be from the soluble fraction of the *E. coli* lysate.

The protocol used to express the 10X His-Sss1p_{M1-K52} fusion protein was scaled up so that cells were harvested from a 4L culture. The 10X His-Sss1p_{M1-K52} fusion protein was purified from the soluble fraction of an *E. coli* lysate on a Hi Trap column (Amersham) that had been prebound with nickel ions, using an AKTA purifier-10 HPLC system. Once the lysate had been loaded onto the column the column was washed extensively to remove unbound material.

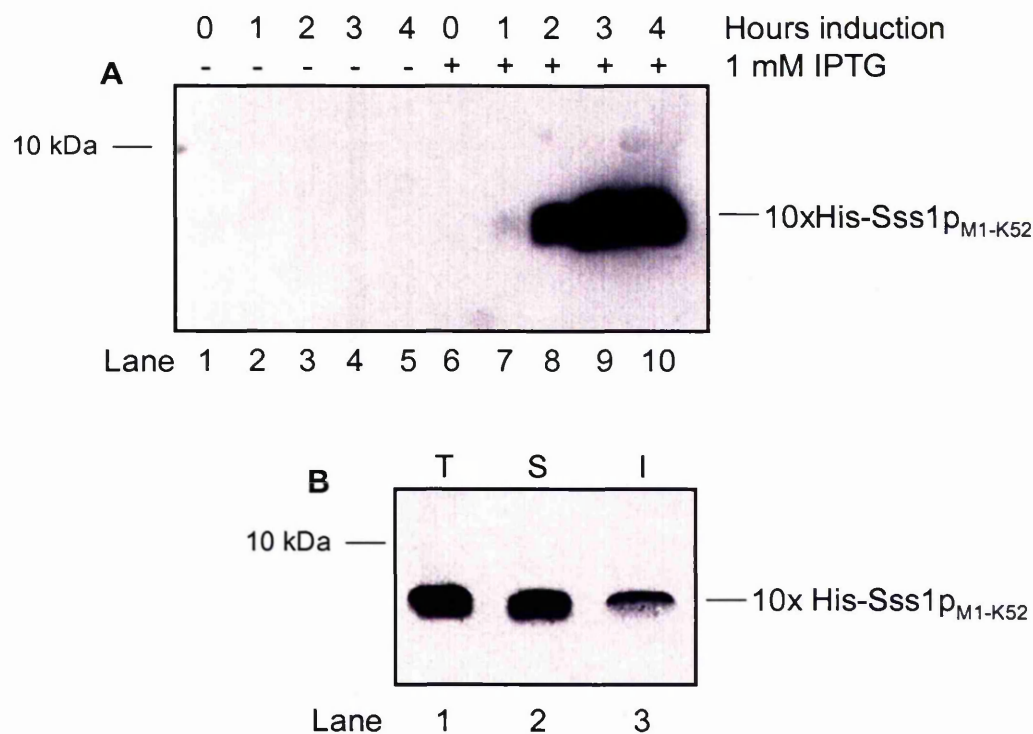


Figure 3.1 Expression of the 10X His-Sss1p_{M1-K52} fusion protein. (A) BL21 (DE3) cells transformed with pCM204 were grown at 30°C to early log phase in LB media supplemented with 34 µg/ml chloramphenicol and 100 µg/ml ampicillin. After this, expression of recombinant protein was induced by incubating cells with 1 mM IPTG for 1-4 hours. Total *E. coli* protein extracts were prepared from 0.5 O.D._{600nm} of cells from each time point and 0.1 O.D._{600nm} equivalents were analysed by SDS-PAGE and after western transfer samples were immunoblotted with anti polyhistidine antibodies. (B) To investigate the solubility of the 10X His-Sss1p_{M1-K52} fusion protein a 200 ml culture of BL21 (DE3) cells transformed with pCM204 was incubated with 1 mM IPTG for 3 hours, lysed by freeze thawing and centrifuged at 10000g to isolate soluble and insoluble fractions. Total (T), soluble (S) and insoluble (I) fractions were prepared for SDS-PAGE and after western transfer samples were immunoblotted with anti polyhistidine antibodies.

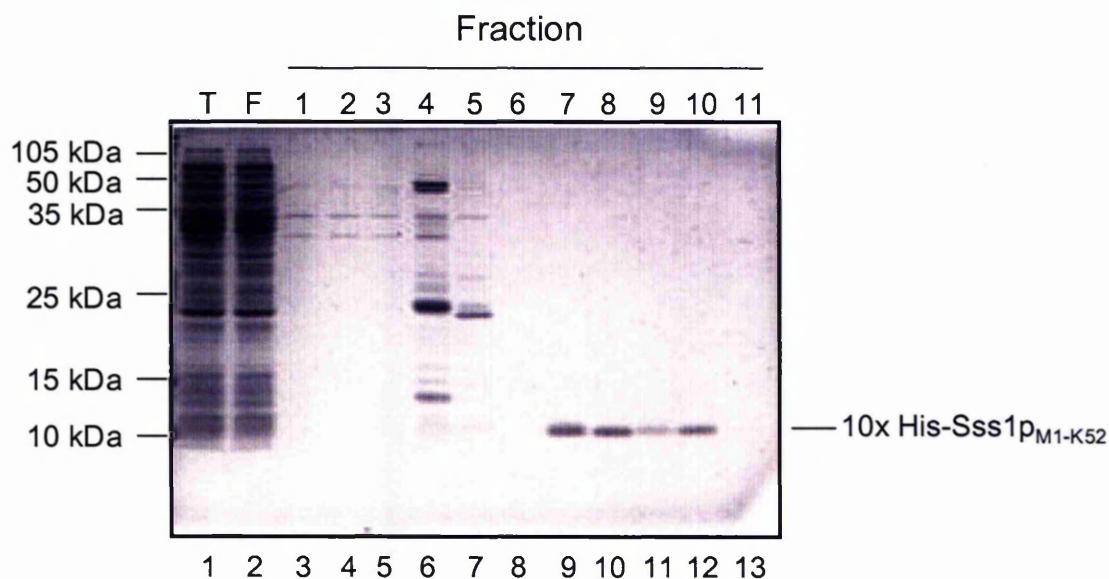


Figure 3.2 Purification of the 10X His-Sss1p_{M1-K52} fusion protein. The soluble fraction of an *E. coli* lysate was isolated from 4 L of BL21 (DE3) transformed with pCM204 that had been incubated with 1 mM IPTG for 3 hours. The extract was loaded onto a HiTrap column (T) that had been prebound with nickel ions and, after washing (F), bound material was eluted with a 20 ml gradient of 0.05-1 M imidazole. 1 ml fractions were collected and 10 μ l of each fraction was prepared for SDS-PAGE. Proteins were visualised by staining with coomassie.

The 10X His-Sss1p_{M1-K52} fusion protein was then eluted with a 20 ml gradient of 0.05-1 M imidazole and 1 ml fractions were collected. The 10X His-Sss1p_{M1-K52} fusion protein was eluted in fractions 7-9, commencing at an imidazole concentration of 350 mM (figure 3.2, lanes 9-11). Fractions 7-9 were pooled, denatured by SDS and dialysed against PBS overnight at 4°C. The protein concentration of the final preparation was determined, using the Bradford assay, to be 1 mg/ml and a final yield of 3 mg of recombinant protein was isolated. This was then used to immunise sheep.

Sheep were tested for their cross reactivity to yeast proteins. A yeast total protein extract was prepared from the strain w303d and subjected to SDS-PAGE and western transfer. Sheep S305B was identified to possess low cross-reactivity to yeast antigens (figure 3.3 A) and was selected for immunisation. The course of immunisation was followed by testing bleeds for their ability to recognise the Sss1 protein by immunoblotting yeast total protein extracts. The third bleed was found to be suitable at a working dilution of 1 in 3000 (figure 3.3 B) and the antigen was determined to be Sss1p as an extract prepared from a strain where *SSS1* had been repressed (discussed in chapter 4) was found to no longer possess the major cross-reacting species of approximately 12 kDa. This indicates that an anti Sss1p antiserum has been generated that can be used for the identification of Sss1p by immunoblotting.

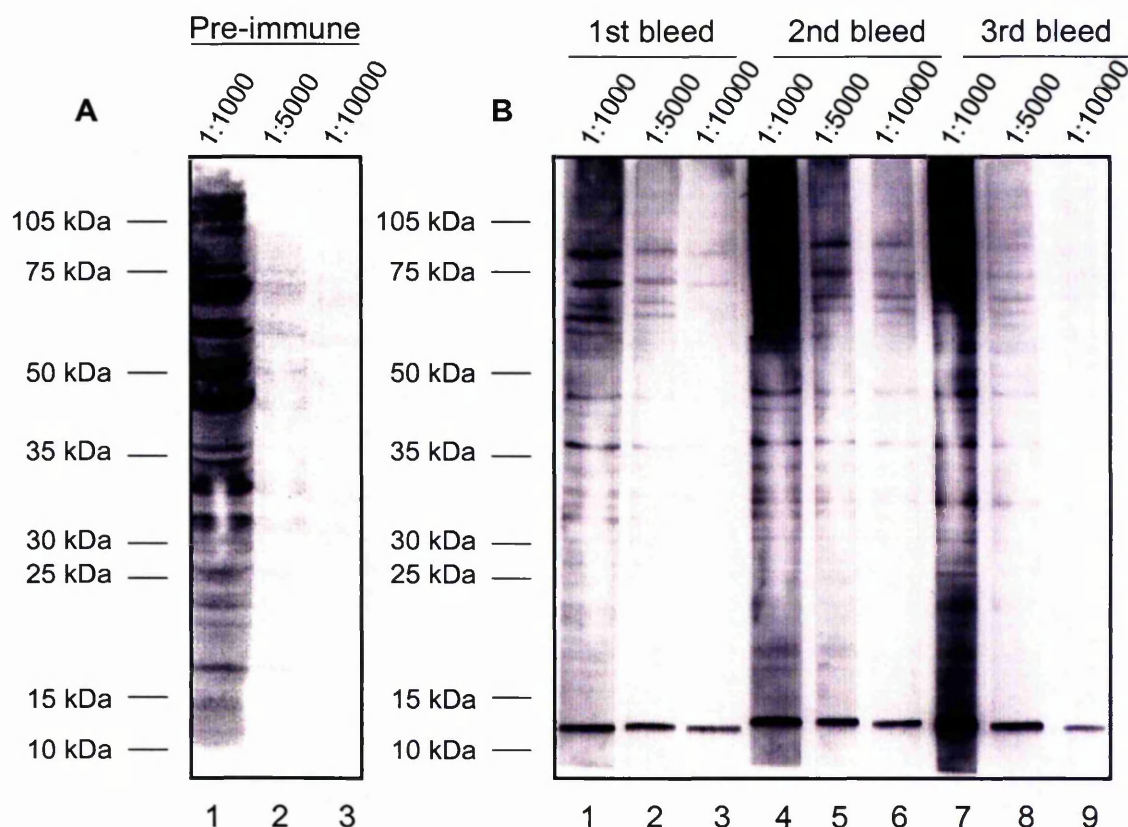


Figure 3.3 Characterising anti Sss1p antibodies. (A) A total cell protein extract was prepared from 5 O.D._{600nm} of w303d. 0.1 O.D._{600nm} equivalents were loaded per lane and analysed by SDS-PAGE on an 8-15% linear gradient. After western transfer each lane was isolated and immunoblotted with pre-immune antiserum isolated from sheep S305B at a dilution of 1:1000, 1:5000 or 1:10000. (B) A total cell protein extract was prepared from 5 O.D._{600nm} of w303d cells and 0.1 O.D._{600nm} equivalents were loaded per lane and analysed by SDS-PAGE on an 8-15% linear gradient. After western transfer each lane was isolated and incubated with antiserum isolated from S305B after the 1st, 2nd and 3rd immunisation with 10X His-Sss1p_{M1-K52} antigen at either 1:1000, 1:5000 or 1:10000. After immunisation, antiserum isolated from S305B is immunoreactive to a protein with a molecular weight of approximately 12 kDa.

3.3 Isolating a strain that allows the co-expression of *sss1* and *sec61* mutants

Mapping the transmembrane domain(s) of Sec61p which interact with that of Sss1p by cysteine scanning mutagenesis requires that membranes are isolated from cells that co-express the site directed mutants of Sss1p and Sec61p. To do this mutants are expressed ectopically from plasmids. Therefore, a host strain was created in which the genomic copy of both *SEC61* and *SSS1* had been deleted. However, expression of both of these genes is essential for cell viability, therefore this was sustained by expression of plasmid borne copies of these genes. The plasmids encoding the mutated genes can then be co-transformed into this strain and, providing that the mutants are functional, the plasmid encoding for the wild type copies of these genes can be counter selected. Following this, the only source of Sss1p and Sec61p is mutant.

Saccharomyces cerevisiae can exist as stable haploid or diploid strains indefinitely in nutrient rich media via mitosis. Haploid cells can be one of two mating types, specifically **a**- or **α**-, depending on the pheromone which they secrete, and diploid cells arise through the mating and fusion of an **a**- and **α**- strain. Following this, under conditions of carbon and nitrogen starvation diploid cells undergo meiosis resulting in the formation of four haploid progeny, or spores, which can be individually isolated. With this in mind, the mating of compatible **a**- and **α**- strains allows the creation of new strains allowing the combination of specific mutations.

The strains BWY12 and BWY531 are haploid strains, *MATa Δsec61* and *MATa Δsss1* respectively, whose growth is sustained by expression of a plasmid born copy of *SEC61* and *SSS1* respectively, that confer uracil prototrophy. The *SEC61* gene in BWY12 has been disrupted by the incorporation of *HIS3* into the *SEC61* locus, by homologous recombination and cells possessing this mutation can be selected for by histidine prototrophy. The *SSS1* locus in BWY531 has been disrupted by the KanMX4

gene and these cells are selected for by their ability to grow on media supplemented with G418 as the protein product of the KanM4 gene detoxifies this compound. These two strains were mated and the resulting diploid, CMY1, was selected by isolating cells that were prototrophic for histidine and could grow on media supplemented with G418 (figure 3.4 A). The mating was confirmed to be successful as BWY12 cells grew only on YNB minus histidine (figure 3.4A) and BWY531 cells on YPD supplemented with G418 (figure 3.4A).

Following mating, CMY1 cells are prototrophic for uracil as they inherit the *URA3* selective plasmids that maintain cell viability in the respective haploid strains. Before CMY1 cells can undergo further genetic manipulation it is necessary to cure these cells of plasmid. CMY1 contains both a functional and a disrupted copy of both *SEC61* and *SSS1* and it is likely that the cells are viable following the loss of plasmid unless they are haploinsufficient for *SEC61* or *SSS1*. Plasmids are unstable in yeast unless selective pressure for the plasmid is maintained and can be lost from cells by mis-segregation or mis-replication at a rate of about 10^{-2} per generation therefore, CMY1 cells were grown in non-selective conditions in order to promote plasmid loss.

The *URA3* gene encodes for orotidine 5'- phosphate decarboxylase and is required for the biosynthesis of uracil. However, this enzyme can also metabolise 5-fluororotic acid (5-FOA) to 5-fluorouracil, a metabolite that is toxic for growth as it inhibits transcription. Therefore, media supplemented with 5-FOA can be used to select for the loss of *URA3* selectable plasmids as only cells that are *ura3* can grow on this media, thus , the *URA3* gene can be used as both a selectable and counterselectable marker (Boeke *et al.*, 1984). CMY1 cells, from single colony on nutrient rich media, and the parental BWY12 and BWY531 cells were plated onto YNB supplemented with 5-FOA and grown for 2 days at 30°C.

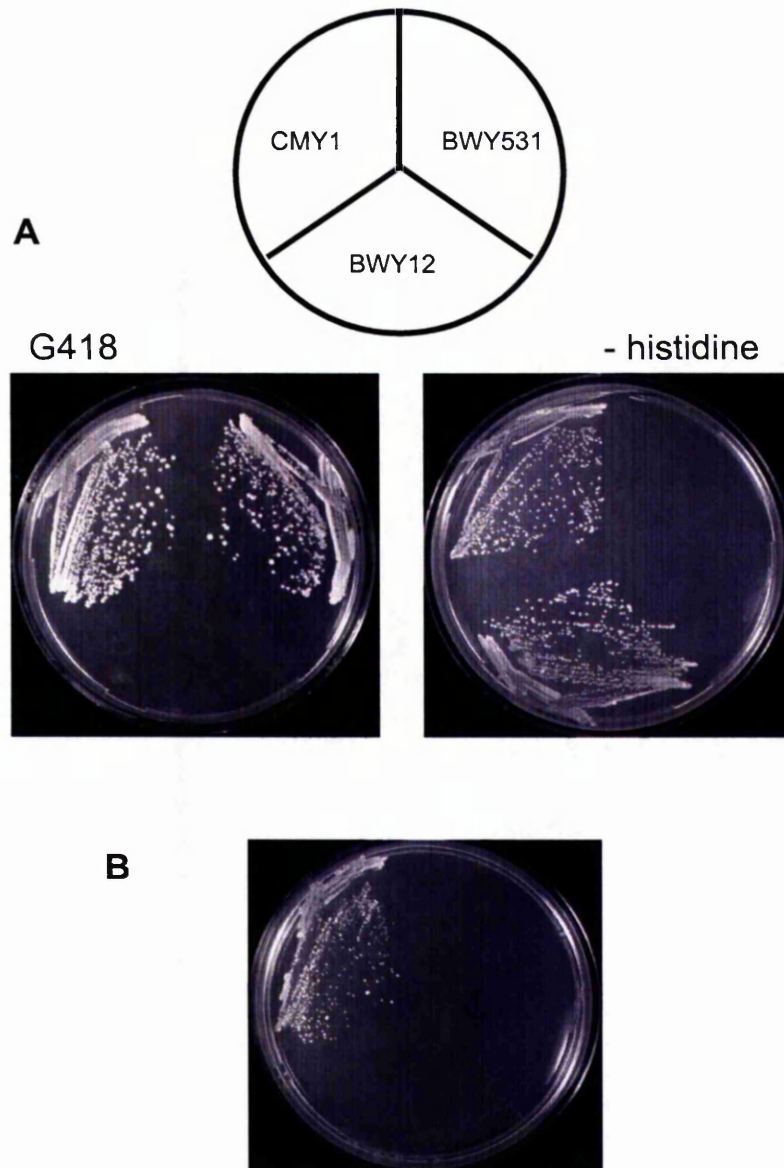


Figure 3.4 Constructing a *SEC61/sec61Δ SSS1/sss1Δ* diploid. (A) BWY12 and BWY531 cells were mated by lightly patching the two strains together on YPD and were incubated at 30°C overnight. After this, BWY12, BWY531 and the mated cells were plated out onto YPD plates containing 200 µg/ml G418 and incubated at 30°C for 2 days. BWY12, BWY531 and mated cells that grew on rich media containing 200 µg/ml G418 were then plated out onto YNB plates depleted of histidine at 30°C for 2 days. CMY1 cells were selected for growth on both media. **(B)** BWY12, BWY531 and CMY1 cells were plated out onto media containing 1 mg/ml 5-FOA and incubated at 30°C for 2 days. Growth of cells derived from CMY1 indicates that these cells have been cured of *URA3* containing plasmids giving CMY3

Both BWY12 and BWY531 did not grow on YNB supplemented with 5-FOA (figure 3.4 B) indicating that these cells cannot tolerate the loss of the *URA3* selectable plasmid as they are required for cell viability. However, cells were isolated from CMY1 under these growth conditions (figure 3.4 B) indicating that these cells, CMY3, are not haploinsufficient for either *SEC61* or *SSS1* as they do not require a second, plasmid borne copy, of either gene to support viability in a diploid state.

Sporulation of CMY3 would give rise to spores with either a *SEC61 SSS1*, *SEC61 sss1Δ*, *sec61Δ SSS1* or *sec61Δ sss1Δ* genotype. However, only the *SEC61 SSS1* spores would be viable as both *SEC61* and *SSS1* are essential for cell viability. Therefore it is paramount that prior to sporulation, CMY3 is transformed with a vector that can sustain cell viability in all mutant spores. A vector was constructed containing *SEC61* and *SSS1* with the *URA3* selectable marker, pCM203, to allow counterselection in downstream genetic manipulations. Plasmid, pBW7, is a *URA3* selective, yeast centromeric vector encoding for *SEC61*. pCM203 was constructed by sub-cloning a 1.8 kb *Sall/SmaI SSS1* fragment (figure 3.5) from pCM201 into the *XhoI/SmaI* (4364) restriction sites present in the multiple cloning site of pBW7.

There are two *SmaI* restriction sites, at 2035bp and 4364bp, in pBW7 that flank the *SEC61* insert. Therefore pBW7 was partially digested with *SmaI*, prior to digestion with *XhoI*, in order to maintain the *SEC61* gene. pBW7 partial digestion was achieved using a serial dilution series of *SmaI* from 0.02 U – 0.0025 U of restriction endonuclease on 10 µl of miniprep plasmid DNA (figure 3.5 A). Cleavage at both *SmaI* restriction sites yields products of approximately 5.1 and 2.3 kb whereas cleavage at either restriction site gives rise to the linearised vector of approximately 7.4 kb. *SmaI* digestion is almost complete when pBW7 is incubated with 0.02 U of *SmaI* at 30°C for 30minutes (figure 3.5 A, lane 1) as the 5.1 and 2.3 kb fragments are the major restriction products. However, a fraction of the products is linearised template. Under higher dilution conditions the 7.4 kb linearised vector is more prevalent (figure 3.5 A,

lanes 2-4) and this was isolated and subsequently digested with *XhoI* restriction endonuclease. Partial digestion at *SmaI* site 4364 followed by cleavage at *XhoI* 4598 gives rise to DNA fragments of approximately 7.2 and 0.2 kb whereas cleavage at *SmaI* 2035 and *XhoI* 4598 produces DNA fragments of 4.8 and 2.6 kb (figure 3.5 B). After this, the 7.2 kb *SmaI/XhoI SEC61 URA3* fragment was isolated and extracted from the 1% agarose gel.

The 1.8 kb *SalI/SmaI SSSI* fragment (figure 3.5 C, lane 2) and the 7.2 kb *SmaI/XhoI SEC61 URA3* fragment were ligated, by virtue of *SalI* and *XhoI* restriction sites being compatible for cohesive end ligation, and ligation reactions were transformed into *E. coli* by electroporation and transformants were isolated and plasmid DNA extracted. pCM203 is anticipated to be approximately 9 kb, therefore plasmids with notably slower migrating supercoiled and relaxed forms, compared to pBW7, were retained and diagnostic restriction digests were used to confirm that pCM203 had been isolated. Ligation of the 1.8 kb *SalI/SmaI SSSI* fragment from pCM201 into *SmaI* (4364)/*XhoI* incorporates a second *SpeI* site in addition to that in pBW7. Incubation of pCM203 with *SpeI* yields two products of approximately 5.4 and 3.6 kb of predicted size (figure 3.5 D, lane 2) whereas incubation of pBW7 with *SpeI* generates a 7.4 kb fragment (figure 3.5 D, lane 1) indicating that pCM203 has been isolated.

CMY3 cells were transformed with pCM203 and transformants were selected for growth on minimal media minus uracil. The resulting strain, CMY4, was then sporulated by starving the cell of nutrients for 4-5 days. After this, spores were dissected from tetrads and allowed to germinate for 3 days at 30°C on YPD. The *SSSI* and *SEC61* genes are not linked so it is anticipated that 1 in 4 spores isolated will possess a $\Delta sec61 \Delta sss1$ genotype. 66 spores were successfully germinated from a possible 80 and 11 spores were determined to possess a *sec61::HIS3 sss1::KanMX4 {p[SEC61 SSSI URA3]}* genotype.

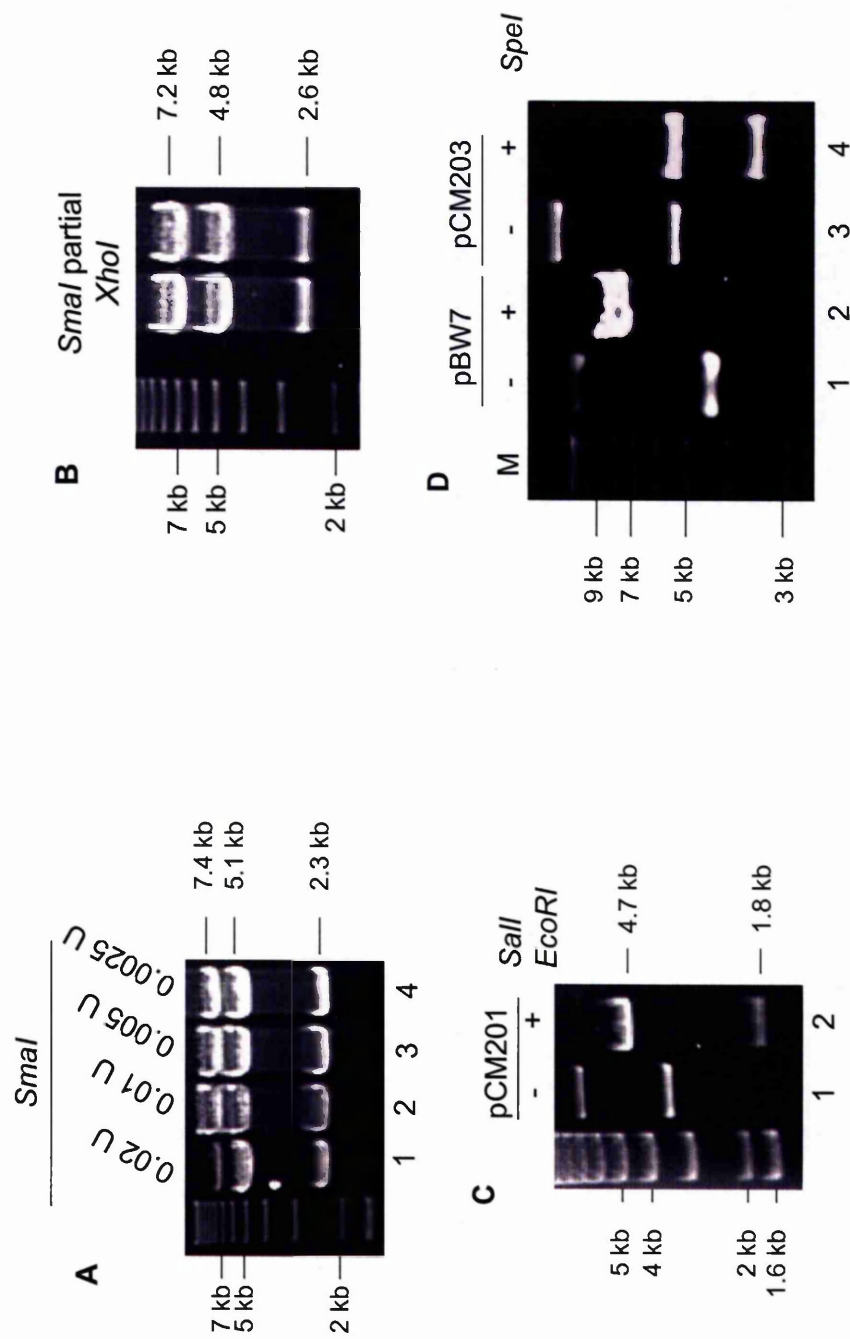


Figure 3.5 Construction of pCM203. (A) pBW7 was partially digested with 0.02 U, 0.01 U, 0.005 U, and 0.0025 U of *SmaI* restriction endonuclease. Digests were incubated at 30°C for 45 minutes and then analysed on a 1% agarose gel. The 7.4 kb linearised pBW7 plasmid was isolated from a 1% agarose gel. (B) Linearised pBW7 isolated following partial digestion with *SmaI* was digested with 10 U *XhoI* restriction enzyme for 2 hours at 37°C. A 7.2 kb *SmaI XhoI* pBW7 fragment was isolated from a 1% agarose gel. (C) pCM201 was incubated with 10 U of *SmaI* and *SmaI* restriction endonuclease at 37°C for 2 hours. A 1.8 kb *SmaI SSSI* fragment was isolated from a 1% agarose gel. (D) The 7.2 kb *SmaI* pBW7 fragment and the 1.8 kb *SmaI SSSI* fragment were ligated giving pCM203. Isolation of pCM203 was verified following incubation with *SpeI* restriction enzyme. Incubation of pBW7 with *SpeI* gives rise to a 7.4 kb linearised product whereas incubation of pCM203 with *SpeI* gives rise to 3.6 kb and 5.4 kb fragments.

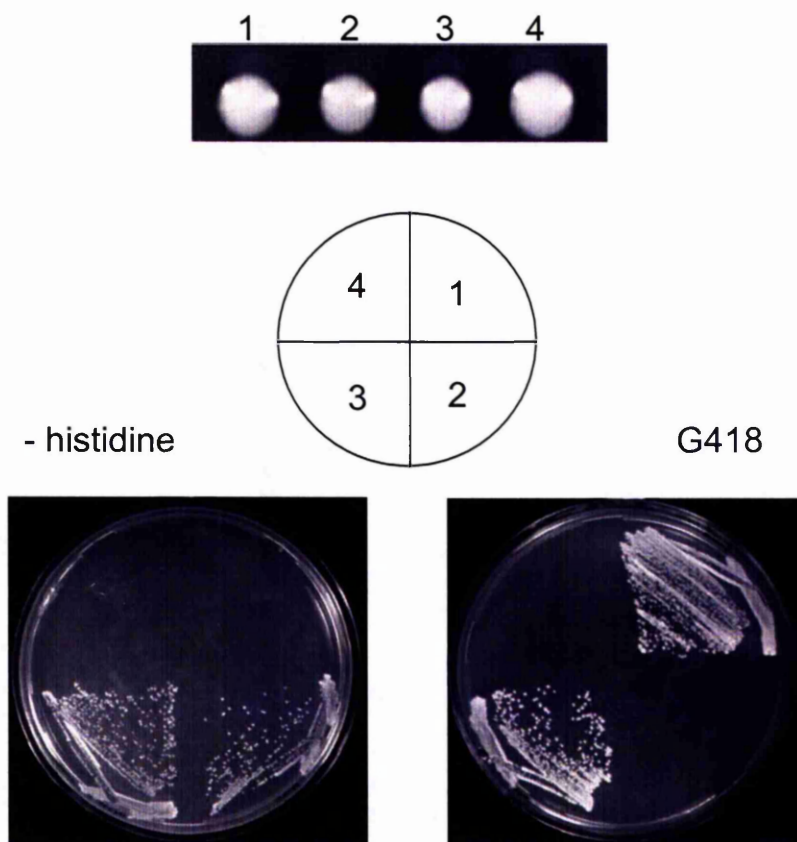


Figure 3.6 Isolating CMY5. (A) CMY3 cells were transformed with pCM203 giving CMY4. CMY4 cells were sporulated, tetrads were isolated and the spores dissected and allowed to germinate at 30°C for 2 days. (B) Each of the spores from a complete tetrad was plated out onto YNB depleted of histidine and nutrient rich media supplemented with 200 µg/ml G418. Spores were incubated at 30°C for 2 days. The *sss1Δ sec61Δ* mutant, spore 3, was selected for its ability to grow on both medium.

Such spores were scored for their ability to grow on both minimal media without histidine and YPD supplemented with G418 (figure 3.6). This indicates that a suitable host strain, CMY5, has been isolated that allows the co-expression of functional *sec61* and *sss1* mutants following the counterselection of the wild type plasmid.

3.4 Incorporating three consecutive mutations into the TMD of Sss1p and each of the ten TMD's of Sec61p

Cysteine scanning mutagenesis requires the expression of plasmid born mutants of *sss1* and *sec61*. Therefore, we require a means with which plasmids expressing these mutants can be selected for after transformation into CMY5. CMY5 is auxotrophic for both tryptophan and leucine biogenesis as the *TRP1* and *LEU2* loci are disrupted in these cells. Therefore, these genetic markers can be used for selection of plasmids that confer either tryptophan or leucine prototrophy by the *TRP1* or *LEU2* genes respectively. Plasmid pBW29 encodes for a functional copy of *SEC61* with the *LEU2* selectable marker and following mutagenesis can be used for the expression of Sec61p mutants. To allow for the isolation of transformants with plasmids encoding *SSS1*, an *SSS1* coding vector was constructed containing the *TRP1* selective marker. This was obtained by ligating a 1.8 kb *SalI/EcoRI* *SSS1* fragment from FKp52 into the multiple cloning site of the yeast centromeric vector pRS314 giving the pCM201 vector. After this pCM201 was mutagenised by site directed mutagenesis with the mutagenic primers SSS1TMCCC_F and SSS1TMCCC_R. These primers incorporate three point mutations, specifically the substitution of I₅₉ A₆₀ V₆₁ with cysteine residues, into the TM of Sss1p, giving pCM202. The reaction was used to transform DH5 α by electroporation and the mutagenised clones were identified by DNA sequencing.

The Sss1p I₅₉C A₆₀C V₆₁C (Sss1pTMCCC) mutation incorporates three consecutive cysteine residues into the middle of the Sss1p TM (figure 3.7 A). Hydropathy analysis

indicates that these mutations incorporated into the Sss1 protein significantly decreases the hydrophobicity of the transmembrane domain predicted by earlier topological analysis (figure 3.7 B) (Esnault *et al.*, 1994). The functionality of this mutant was tested by plasmid shuffle where the strain BWY531 was transformed with pRS314, pCM201 or pCM202.

Transformants were selected for tryptophan prototrophy and then plated onto media containing 5-FOA. Cells transformed with pRS314 vector control could not grow on media containing 5-FOA as this vector does not contain a functional copy of *SSS1* that can complement the loss of the wild type plasmid whereas cells transformed with either pCM201 or pCM202 could grow under these conditions (figure 3.7 C). This demonstrates that the *sss1* *I₅₉ A₆₀ V₆₁ CCC* mutation does not compromise the function of Sss1p.

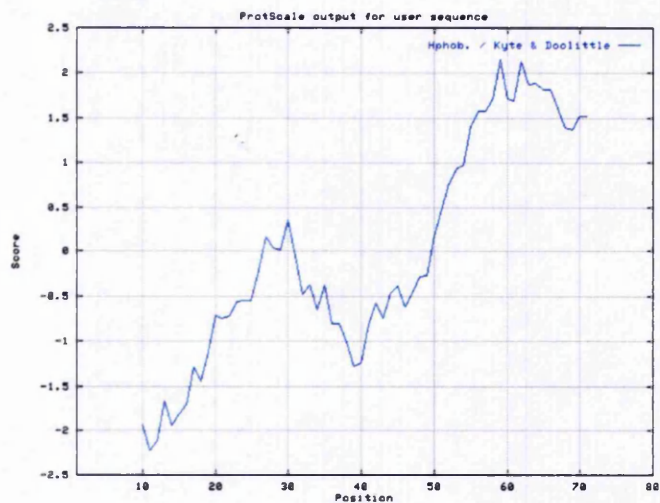
To further characterise Sss1pTMCCC we investigated the level of Sss1 protein at steady state. Total yeast protein extracts were made from cells expressing either *SSS1* or *sss1* *I₅₉ A₆₀ V₆₁ CCC* that were growing logarithmically. Immunoblot analysis of total protein extracted from cells expressing *SSS1* with anti Sss1p antibodies identified a single major cross-reactive species of approximately 12 kDa that has previously shown to be Sss1p (figure 3.7 D, lane 1) (Esnault *et al.*, 1993). The same analysis identified a single major cross-reactive species in cells expressing Sss1pTMCCC of comparable intensity. However, this species had a rMw of approximately 9 kDa indicating that these point mutations in the TM of Sss1p affects the electrophoretic mobility of Sss1p (figure 3.7 D, lane 2). Together these data indicate that although the *I₅₉A₆₀V₆₁CCC* point mutations affect the rMw of Sss1p, when investigated by SDS-PAGE, they do not compromise the level of Sss1p at steady state or the function of the Sss1 protein as this mutant can complement the otherwise lethal *sss1Δ* mutation. This indicates that Sss1pTMCCC is a suitable mutant to investigate the molecular environment of the Sss1p transmembrane domain.

A Sss1p

MARASEKGEEKKQSNQVEKLVEAPVEFVREGTQFLAKCKKPD**LKEYTK**

IVKAVGIGF**IA**VG**IIGYA**IKLIHIPIRYVIV

B Sss1p



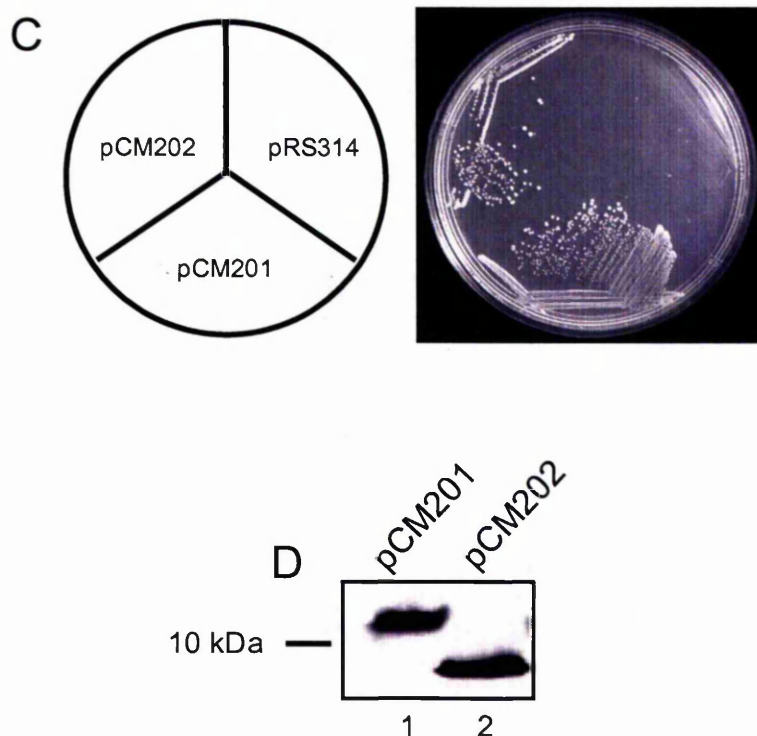


Figure 3.7 Investigating the functionality of Sss1p_{I59C A60C V61C}. (A) The amino acid sequence of Sss1p has been annotated to show the position of the transmembrane domain according to either Esnault *et al.*, 1994 (underlined) or van den Berg *et al.*, 2004 (highlighted in bold). The location of the three residues that had been substituted to cysteine are italicised and are coloured red. (B) The amino acid sequence of Sss1p and the Sss1p_{I59C A60C V61C} mutant was analysed by Kyte Doolittle analysis (www.expasy.ch) to compare the hydrophobicity of both proteins. (C) The functionality of Sss1p_{I59C A60C V61C} was assessed by its ability to complement the otherwise lethal *sss1Δ* mutation. BWY531 cells were transformed with either pRS314 vector control, pCM201 (Sss1p) or pCM202 (Sss1p_{I59C A60C V61C}) by ONE-STEP transformation and transformants were selected for tryptophan prototrophy. After this cells were grown on minimal media supplemented with 1 mg/ml 5-FOA to counterselect for the *URA3* selective, *SSS1* encoding vector. Cells were incubated at 30°C for 2 days. (D) The levels of Sss1p_{I59C A60C V61C} compared to Sss1p were analysed at steady state. Total yeast cell protein extracts were prepared from 5 O.D._{600nm} of cells expressing either *SSS1* (CMY9 (pCM201)) or Sss1p_{I59C A60C V61C} (CMY2 (pCM202)) that were growing logarithmically. After this extracts were analysed by SDS-PAGE and after western transfer samples were immunoblotted with anti Sss1p antibodies.

The Sec61 protein contains three cysteine residues, C121, C150 and C373 that are positioned in TM3, 4 and 8 respectively. The location of these residues is particularly problematic as they could potentially be cross-linked to the TM of Sss1p. It is therefore vital that these residues are mutagenised out of the protein, resulting in the isolation of a cysteineless copy of Sec61p. Each codon encoding for cysteine was mutagenised by site directed mutagenesis and substituted to a codon that encodes for alanine as these two amino acids are of comparable hydrophobicity and size. Each SDM reaction was used to transform DH5 α by electroporation and mutants were identified by DNA sequencing and the functionality of each mutation was assessed by plasmid shuffle. BWY12 cells were transformed with either pRS315 (data not shown), pBW29, pCM101, pCM102, pCM103 or pCM104 (cysteineless Sec61p) and transformants were selected for leucine prototrophy. Cells were then plated out onto media supplemented with 5-FOA to select for the loss of the endogenous *SEC61* encoding vector. Cells transformed with pBW29 could complement the loss of the endogenous plasmid as this encodes a functional copy of the gene. Each of Sec61p cysteine delete mutations were able to complement the loss of *SEC61* indicating that each of these mutant *sec61* alleles encode a functional copy of Sec61p, in particular the cysteineless copy of Sec61p (figure 3.8). Therefore, the plasmid that expresses the cysteineless copy of Sec61p, pCM104, presents an appropriate molecular background for the incorporation of site specific cysteine mutations into each of the ten transmembrane domains of Sec61p.

Plasmid	Form of Sec61p expressed
pBW29	Sec61p
pCM101	Sec61p _{C121A}
pCM102	Sec61p _{C150A}
pCM103	Sec61p _{C121A C150A}
pCM104	Sec61p _{C121A C150A C373A}

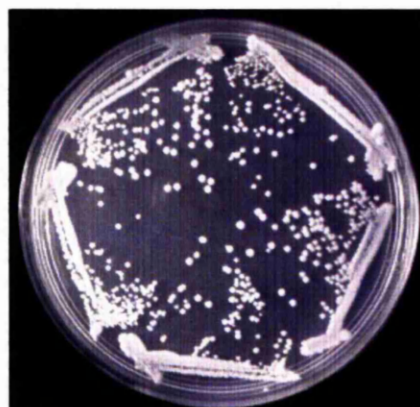
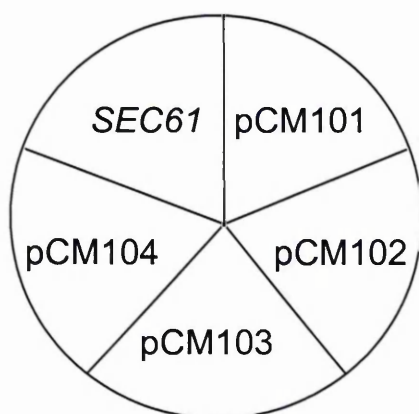


Figure 3.8 Cysteineless Sec61p is functional. The functionality of each of the cysteine deleted mutants was assessed by their ability to complement the otherwise lethal *sec61Δ* mutation. BWY12 cells were transformed with either pBW29 pCM101, pCM102, pCM103 or pCM104 by ONE-STEP transformation and transformants were selected for leucine prototrophy. After this, cells were incubated on minimal media containing 1 mg/ml 5-FOA at 30°C for 2 days to counterselect the *URA3* selective *SEC61* encoding vector.

Each TMD of Sec61p was mutagenised by SDM with forward and reverse primers and each mutation was confirmed by DNA sequencing, the location of the mutagenised residues are presented in figure 3.9A. Hydropathy analysis indicates that although mutations incorporated into the Sec61 protein do not greatly affect the hydrophobicity of the transmembrane domains there are 2 noticeable differences, notably in TM3 and TM7 compared to earlier topological analysis (figure 3.9 B) (Wilkinson *et al.*, 1996). To test the functionality of each of the ten Sec61p triple cysteine mutations that had been individually incorporated into pCM104 by SDM, each of the *sec61* mutants was assessed for their ability to complement the lethal *sec61* null mutation. The residues mutagenised by site directed mutagenesis and the plasmids encoding each of the specific mutations are represented in table 3.1. BWY12 cells were transformed with pRS315 and pCM104-pCM114 and transformants were selected for by leucine prototrophy. Following transformation, cells were plated out onto minimal media containing 5-FOA, to counterselect for *SEC61*, and grown for 2 days at 30°C. Cells transformed with pRS315 negative control plasmid did not grow on this media as this plasmid does not encode a functional copy of *SEC61* whereas cells transformed with pCM104, encoding the cysteineless Sec61p template, could. The plasmids encoding for mutants of *sec61* where three consecutive amino acids had been substituted with cysteine had been incorporated into either TM1, 2, 4, 5, 6, 8 or 9 were all found to complement the *sec61Δ* mutation (figure 3.9 C) indicating that these mutations do not compromise Sec61p function. Cells that had been transformed with Sec61pTM10CCC (pCM109) and counter selected for pBW7 were found to grow much more slowly than those isolated from cells that had been transformed with pCM104 (figure 3.9 C). This growth defect is consistent with this mutation partially compromising Sec61p function. But, incorporation of three consecutive cysteine residues into TM3 or 7 of Sec61p abolished Sec61p function as these mutant alleles could not complement

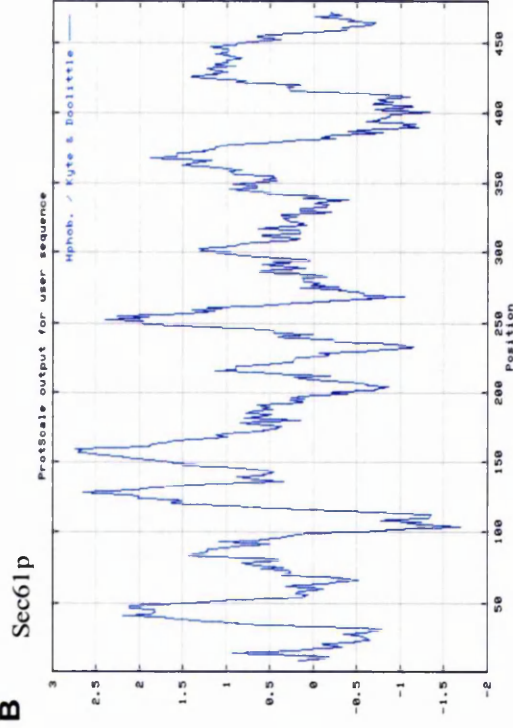
Table 3.1 List of plasmids encoding Sec61p TM mutants.

Plasmid	Sec61p TM mutagenised	Residues substituted to cysteine
pCM105	8	G371 S372
pCM106	6	M249 T250 V251
pCM107	7	S301 N302 I303
pCM108	9	A423 F424 G425
pCM109	10	I448 L449 M450
pCM110	1	L44 I45 L46
pCM111	2	I85 I86 T87
pCM112	3	A130 L131 V132
pCM113	4	L157 M158 F159
pCM114	5	F219 H220 L221

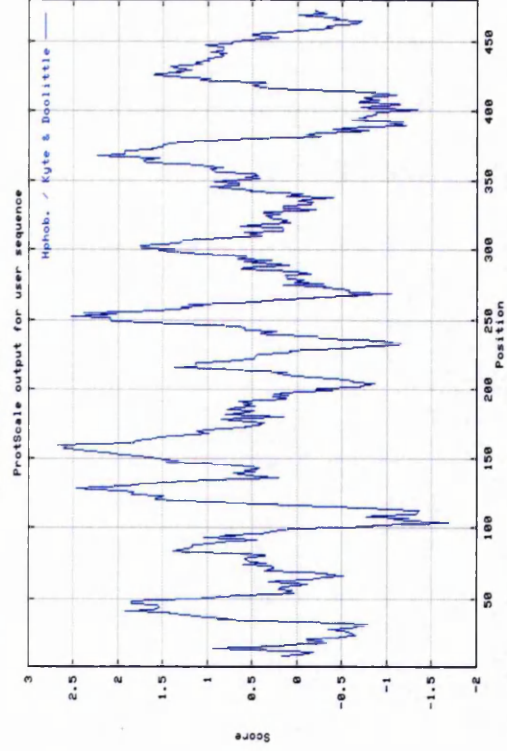
A Sec61p

TM1
 MSSNRVLDLFPKPFESFLPEVIAPERKVPYNQKLIWTGVSLLI**FLIL**GQI
 TM2
 PLYGIVSSETSDPLYWLRAMLASNRGTLLELGVSP**II**TSSMIFQFLQGT
 TM3
 QLLQIRPESKQDRELFIQAQKVCAIILILGQ**ALVV**MTGNYGAPSDDLGL
 TM4
 PICLLLI**FQLMF**ASLIIVMLLDELLSKGYGLSGISLFTATNIAEQIFWR
 TM5
 AFAPTTVNSGRGKEFEGAVIA**FHLL**AVRKDKKRALVEAFYRTNLPNMF
 TM6
 QVL**MTVA**IFLFVLYLQGFYELPIRSTKVRGQIGIYPIKLFYTSNTPIM
 TM7
 LQSALTS**NI**FLISQILFQKYPTNPLIRLIGVWGIRPGTQGPQMALSGLA
 TM8
 YYIQPLMSLSEALLDP**IKTIVYITFVLGSC**AVFSKTWIEISGTSPRDIA
 TM9
 KQFKDQGMVINGKRETSIYRELKKIIP**TAAAFG**GATIGALSVGSDLLGT
 TM10
 LGSGAS**IL**MA**TTTI**YGYEAAAKEGGFTKNLVPGFSDLM

B



All cysteine mutations incorporated into Sec61p C121A C150A C373A



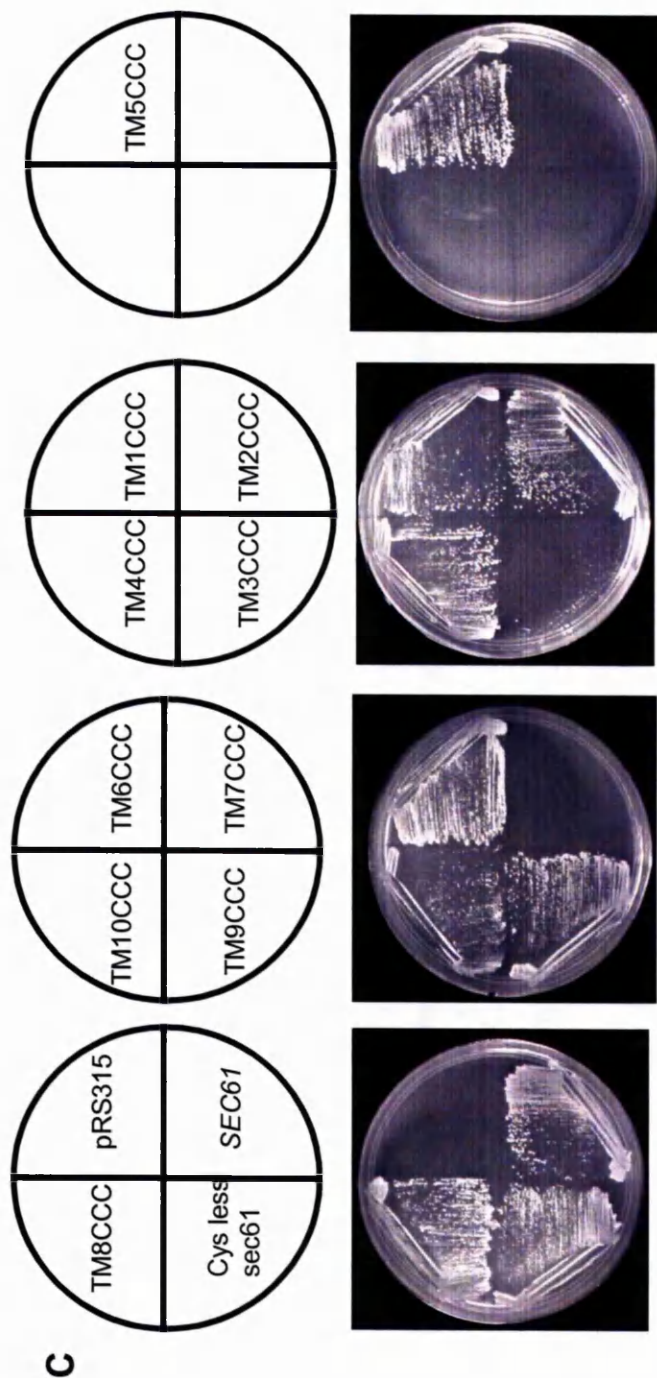


Figure 3.9 Investigating the functionality of each of the Sec61p TM mutants. (A) The amino acid sequence of Sec61p has been annotated to show the position of each transmembrane domain according to either Wilkinson *et al.*, 1996 (underlined) or van den Berg *et al.*, 2004 (highlighted in bold). The location of the three residues that had been substituted to cysteine are italicised and are coloured red. (B) The amino acid sequence of Sec61p and Sec61p sequence containing all of the TM mutations was analysed by Kyte Doolittle analysis (www.expasy.ch) to compare the hydrophobicity of these proteins. (C) The functionality of each of the Sec61p mutants was assessed by their ability to complement the otherwise lethal *sec61Δ* mutation. BWY12 cells were transformed with either pRS315 vector control, pBW29 (*SEC61*), pCM104 (cysteineless Sec61p) or with plasmids encoding each of the ten TM mutants, pCM105-pCM114 by ONE-STEP transformation and transformants were selected for leucine prototrophy. After this cells were incubated on minimal media supplemented with 1 mg/ml 5-FOA at 30°C for 2 days to counterselect the *URA3* selective, *SEC61* encoding vector. Cells were grown at 30°C for 2 days.

sec61Δ (figure 3.9 C). This demonstrates that the plasmids expressing each of the *sec61* mutants are suitable to identify the transmembrane domain(s) of Sec61p that interact with the TM of Sss1p with the exception of pCM107 (Sec61pTM7CCC) and pCM112 (Sec61pTM3CCC).

The plasmids expressing the viable *sec61* mutants were co-transformed with pCM202 into CMY5 via electroporation and transformants were selected for by leucine and tryptophan prototrophy on selective minimal media containing 1M sorbitol. Sorbitol was included in the media to provide osmotic balance so that the electroporated cells did not lyse. Following this transformants were counterselected for pCM203. All of the transformants were found to grow following the loss of pCM203, indicating that no synthetic lethality occurs between Sss1pTMCCC and each of the functional *sec61* mutants (data not shown).

In order to allow the co-expression of Sss1pTMCCC with Sec61pTM3CCC or Sec61pTM7CCC it was necessary to construct a yeast strain that possesses a regulatable allele of *SEC61* that, after repression, would allow the co-expression and characterisation of these defective *sec61* alleles with Sss1pTMCCC. Plasmid pPR22 has previously been constructed in an earlier study and promotes the methionine regulated expression of *SEC61* through the transcription control of the *MET3* promoter (Wilkinson *et al.*, 2000). In the absence of methionine, the *MET3* promoter is transcriptionally activated by the Met4p transcription factor. However, in the presence of methionine this transcription factor is prevented from activating target genes by preventing it from binding DNA sequence and/or is targeted for degradation by the cytosolic proteasome (Rouillon *et al.*, 2000).

CMY5 was firstly co-transformed with pCM202 and pPR22 by electroporation and transformants selected for leucine and tryptophan prototrophy. Then, transformants were counterselected for the endogenous pCM203 plasmid by growth on selective media containing 5-FOA. After this CMY24 can grow normally on selective minimal media depleted of

methionine but is unable to grow on nutrient rich media (figure 3.10 A) indicating that *SEC61* expression in CMY24 is regulated by methionine.

To investigate the expression profile of the *MET3* regulated allele of *SEC61*, Sec61 protein levels were analysed in cells that had been grown in the presence or absence of methionine. Previous analysis has indicated that there is no detectable Sec61 protein, by western blot analysis, following growth of strains containing the *MET3* regulated allele of *SEC61* in media containing 2 mM methionine or in nutrient rich media for 8 hours (P. R. Reid, 1998). Sec61 protein levels were investigated in CMY24 cells that had been grown in either selective media without methionine or in nutrient rich media for 8 hours. Western blot analysis of a total cell protein extract prepared from cells grown in the absence of methionine with anti Sec61p antibodies identified a single immunoreactive species of approximately 38 kDa (figure 3.10 B, lane 1) which has previously been reported to be Sec61p (Stirling *et al.*, 1992). When a total cell protein extract was prepared from cells that had been grown in nutrient media was probed with anti Sec61p antibodies the 38 kDa species was no longer detected (figure 3.10 B, lane 2). The reduction in Sec61 protein levels in a total cell protein extract isolated from cells that had been grown in nutrient rich media is entirely consistent with the methionine dependent regulation of *MET3-SEC61*. These conditions can therefore be used to deplete cells of endogenous Sec61p in order to investigate whether the non-functional triple cysteine mis-sense mutations incorporated into either TM3 or TM7 of Sec61p promote chemical cross-linking with Sss1p TMCCC. To allow for the selection of plasmid encoding for the non-functional mutants of *sec61* in CMY24, the sequence encoding for Sec61pTM7CCC or Sec61pTM3CCC was sub-cloned from pCM107 and pCM112 as *HindIII* fragments and inserted into the multiple cloning site of pRS316, which confers uracil prototrophy, giving pCM117 and pCM116 respectively (data not shown).

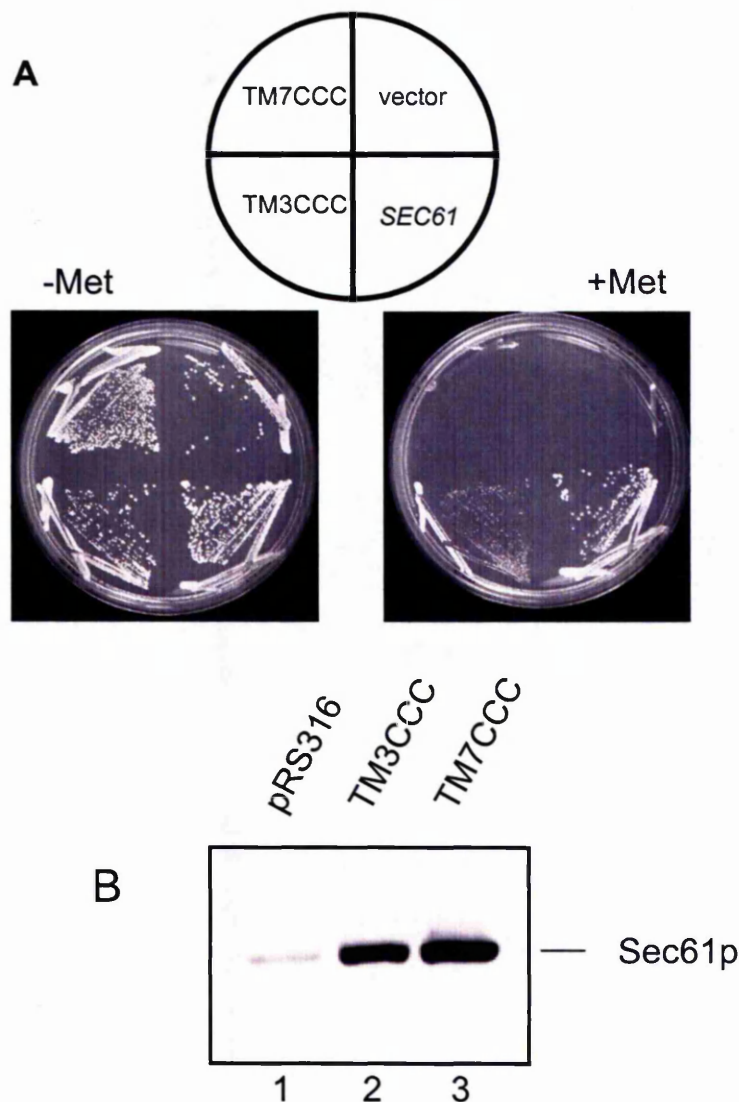


Figure 3.10 Co-expression of Sss1p^{I59C A60C V61C} with non-functional *sec61* alleles. (A) CMY5 cells were transformed by electroporation with pCM202 (Sss1p^{I59C A60C V61C}) and pPR22 (*MET3-SEC61*) and selected for tryptophan and leucine prototrophy. Transformants were then incubated on YNB media containing 1 mg/ml 5'-FOA to counterselect for the *URA3* selective *SEC61 SSS1* encoding vector giving CMY24. CMY24 cells were transformed with either pRS316 (CMY25), pBW7, pCM115 (CMY26) or pCM116 (CMY27) by electroporation and transformants were selected for uracil prototrophy. After this cells were plated out onto either YNB supplemented with only adenine (-Met) or nutrient rich media (+Met) and incubated at 30°C for 2 days. (B) CMY25, CMY26 and CMY27 cells were grown to early exponential phase in YNB supplemented with only adenine and then switched to YPD and incubated at 30°C for 8 hours to repress the *MET3* regulated *SEC61* allele. After this membranes were isolated from these cells and 0.5 A_{280nm} equivalents were solubilised with in 1X Laemmli sample buffer and analysed by SDS-PAGE. After western transfer samples were immunoblotted with anti Sec61p antibodies.

3.5 Investigating the ability of each mutant to form protein complexes required for ER translocation.

Before the ability of Sec61p to be cross-linked to Sss1p in each of the mutants can be investigated it is necessary to determine whether the mutants, when co-expressed, can form protein complexes required for ER translocation, in particular the Sec61 complex. This is so that we can be confident that absence of a cross-link between Sec61p and Sss1p is a consequence of the TMD's not interacting rather than being a failure to form these complexes.

It has previously been shown in extracts prepared from dog pancreatic microsomes that the mammalian Sec61 complex can be resolved by blue native PAGE (BN-PAGE) (Wang and Dobberstein, 1999). BN-PAGE is a non-invasive, charge shift based assay whereby binding of negatively charged coomassie to a protein complex provides sufficient charge for a protein complex to be resolved by electrophoresis (Schagger and von Jagow, 1991). Following solubilisation of dog pancreatic microsomes with digitonin, a protein complex of approximately 140 kDa (figure 3.11, lane 1) was detected with anti Sec61 α , Sec61 β and Sec61 γ antibodies it was concluded that this represents the Sec61 complex (Wang and Dobberstein, 1999). Therefore, we investigated the formation of protein complexes required for ER translocation in wild type and mutant membranes that have been solubilised by digitonin by BN-PAGE.

Immunoblot analysis of digitonin solubilised membranes, prepared from wild type cells, with anti Sec61p antibodies revealed a complex of approximately 280 kDa. This complex is also detected with anti Sss1p and anti Sbh1p antibodies (personal communication, B. M. Wilkinson). In addition to this, two larger complexes of approximately 355 and 380 kDa were also detected with anti Sec61p antibodies (figure 3.11, lane 1). An alternative project in the laboratory has identified both complexes to contain Sec63p but only the 380 kDa complex

to contain the Sec62 protein, that is required for post translational translocation (personal communication, B. M. Wilkinson; A. J. Jermy). It has therefore been postulated that the 380 kDa represents the SEC complex and that the 355 kDa complex either represents a novel protein complex required for ER translocation or a precursor of the SEC complex.

We proceeded to investigate the ability of each of the Sec61p mutants to form these complexes when co-expressed with Sss1p TMCCC by BN-PAGE (figure 3.11). The 280, 355 and 380 kDa protein complexes were detected with anti Sec61p antibodies in digitonin solubilised membranes isolated from all of the mutants with the exception of membranes isolated from cells where Sss1pTMCCC was co-expressed with Sec61p TM3CCC (figure 3.11, lane 5). In these membranes anti Sec61p antibodies identified a protein species of approximately 70 kDa (figure 3.11, lane 5). This data indicates that the mutations incorporated into the TMD of Sss1p and each of the ten TMD's of Sec61p, with exception of TM3, do not affect the ability of these proteins to form protein complexes required for ER translocation. However, substitution of three residues in TM3 with cysteine appears to disrupt the ability to form these complexes and correlates with the observation that this mutation does not complement the lethal *sec61Δ* mutation.

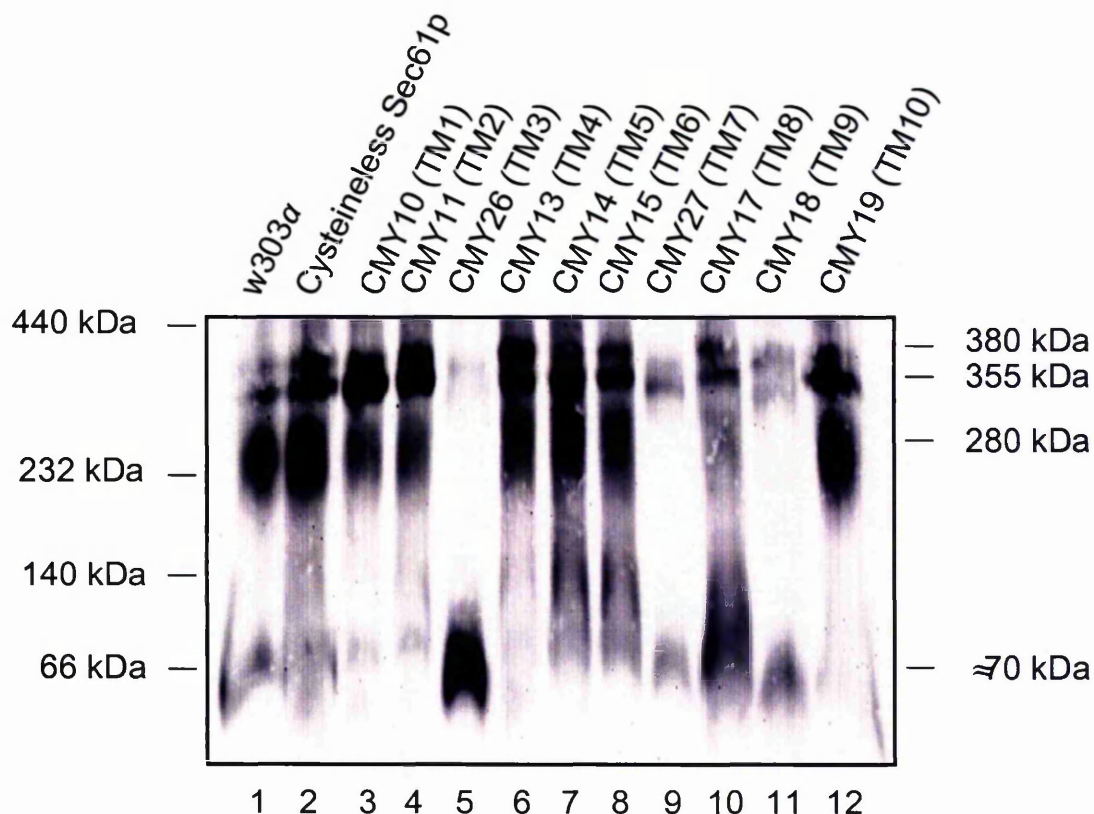


Figure 3.11 Analysis of protein complexes required for ER translocation by BN-PAGE. 2

$A_{280\text{nm}}$ equivalents of membranes isolated from **w303 α** , **CMY8** (Sss1pTMCCC and cysteineless Sec61p) and **CMY10** (Sss1pTMCCC and Sec61pTM1CCC), **CMY11** (Sss1pTMCCC and Sec61pTM2CCC), **CMY13** (Sss1pTMCCC and Sec61pTM4CCC), **CMY14**(Sss1pTMCCC and Sec61pTM5CCC), **CMY15** (Sss1pTMCCC and Sec61pTM6CCC), **CMY17** (Sss1pTMCCC and Sec61pTM8CCC), **CMY18** (Sss1pTMCCC and Sec61pTM9CCC) and **CMY19** (Sss1pTMCCC and Sec61pTM10CCC) as well as from **CMY26** (Sss1pTMCCC and Sec61pTM3CCC) and **CMY27** (Sss1pTMCCC and Sec61pTM7CCC) cells grown in nutrient rich media to repress *MET3-SEC61* were isolated and solubilised with 2% digitonin and non solubilised material was isolated by centrifugation at 10000 g. The ribosome associated material was then isolated by ultracentrifugation at 400000 g and the non pelleted material was retained, prepared for BN-PAGE and analysed on a 6-16% polyacrylamide gel. After this, samples were transferred to PVDF membrane by western transfer and then immunoblotted with anti Sec61p antibodies.

3.6 Investigating the transmembrane domain(s) of Sec61p that interact with that of Sss1p by cysteine dependent chemical crosslinking

As each of the Sec61p mutants, with the exception of TM3, form protein complexes required for ER translocation when co-expressed with Sss1p^{TMCCC} we investigated the ability of each of the Sec61p TMD's to form an adduct with the TMD of Sss1p by crosslinking. Membranes were isolated from cells co-expressing each of the ten Sec61p mutants as well as the cysteineless copy of Sec61p with Sss1p^{TMCCC}. Membranes were harvested from cells co-expressing the non-functional mutants of Sec61p with Sss1p^{TMCCC} after shifting cells from selective media to nutrient rich media for 8 hours. Membranes prepared from each strain were then either mock treated with DMSO or incubated with (1 mM final concentration) bismaleimido-hexane (BMH) or N, N'-ortho-phenylenedimaleimide (o-PDM). Cross-linking between Sec61p and Sss1p would indicate that that particular TMD of Sec61p is in close proximity to that of Sss1p. However, each cross-linking reagent has specific chemical properties. BMH has a flexible spacer arm between the 2 cysteine reactive maleimide groups can covalently couple 2 sulphydryl groups within 15 Å apart whereas that of o-PDM is rigid and can cross-link residues up to 6 Å apart. Therefore, isolation of adducts with o-PDM implies closer proximity between residues.

As expected, when membranes isolated from cells co-expressing a cysteineless mutant of Sec61p and Sss1p^{TMCCC} were treated with both BMH and o-PDM no cross-linking was detected between Sec61p and Sss1p (figure 3.12 A, lanes 2 and 3) as these samples were identical to that that had been mock treated (figure 3.12 A, compare lane 1 with lanes 2 and 3). This indicates that identification of cross-links between Sec61p and Sss1p^{TMCCC} mutants will be dependent on the Sec61p TMD mutant that is co-expressed with Sss1p^{TMCCC}.

Sss1p^{TMCCC} could not be crosslinked to Sec61p when three residues in either TM1, 2, 3, 4, 6, 7 and 8 were substituted to cysteine as these samples were appeared to be identical to membranes isolated from cells expressing cysteineless Sec61p (data not shown). When membranes isolated from CMY18 (TM9CCC) were incubated with both BMH and o-PDM a 46 kDa and 48 kDa Sec61p containing adduct was detected (figure 3.12 B, lane 2 and 3). Furthermore, the efficiency with which these cross-links are generated is comparable between the two cross-linking reagents used (figure 3.12 B, compare lane 2 and 3). It is likely that the difference between the size of this adduct generated by either BMH or o-PDM is a consequence of the different chemical properties of the cross-linking reagents.

A 46 kDa Sec61p containing adduct was also detected in membranes isolated from both CMY14 (TM5CCC) and CMY19 (TM10CCC) that had been incubated with BMH (figure 3.12 C and D). However, there was little o-PDM dependent crosslink detected in membranes harvested from CMY19 after they were incubated with this reagent. The efficiency with which these crosslinks are formed appears to be much lesser in either of these membranes compared to that detected in membranes containing the Sec61p TM9CCC mutation.

As the adducts identified in membranes isolated from either CMY14, CMY18 and CMY19 were detected by anti Sec61p antibodies we wanted to confirm that they also contain Sss1p. Membranes isolated from CMY14, CMY18 and CMY19 cells were again incubated with either BMH, o-PDM or mock treated, prepared for SDS-PAGE and then immunoblotted with anti Sss1p antibodies. The adducts identified by anti Sec61p antibodies in CMY18 and CMY19 were detected by anti Sss1p antibodies (figure 3.13 B and D) implying that these represent crosslinked adducts between Sss1p and Sec61p.

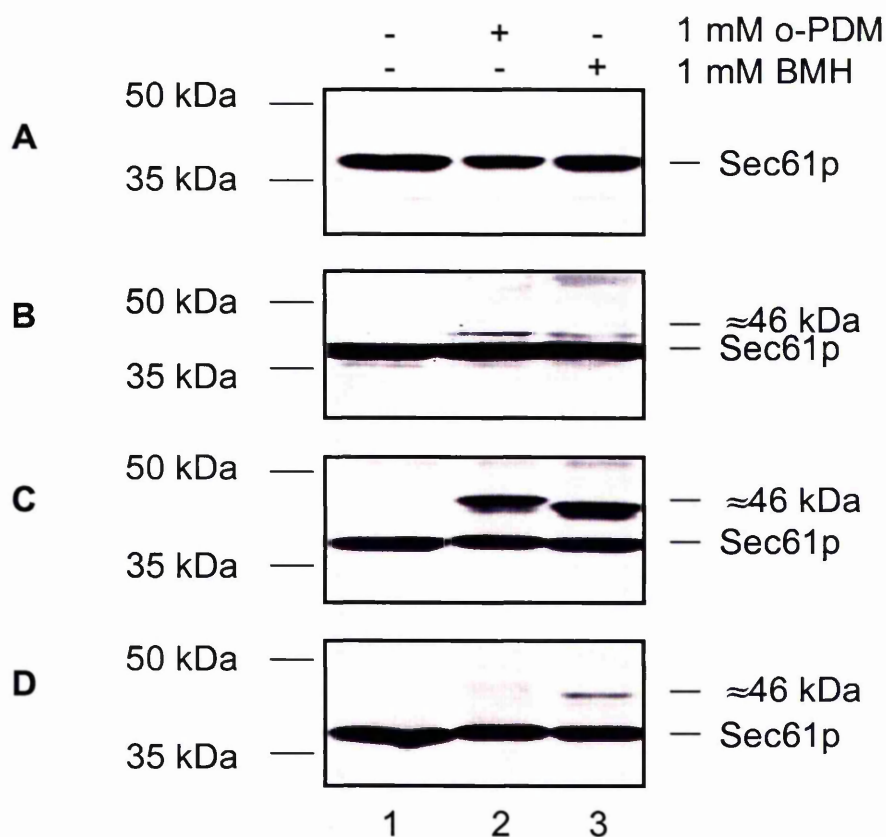


Figure 3.12 Probing the molecular architecture of the Sec61 complex by cysteine scanning mutagenesis. 1 A_{280nm} equivalent of membranes isolated from CMY8 (A) CMY14 (B) CMY18 (C) and CMY19 (D) were either mock treated with DMSO or incubated with either 1 mM BMH or 1 mM o-PDM at 30°C for 30 minutes. After this, crosslinking was quenched by incubating samples with 100 mM DTT for 5 minutes, incubated with an equal volume of 2X Laemmli sample buffer and analysed by SDS-PAGE on a 12.5% acrylamide gel. After this, samples were transferred to nitrocellulose membrane by western transfer and then immunoblotted with anti Sec61p antibodies. Sec61p and Sec61p crosslinked adducts are indicated.

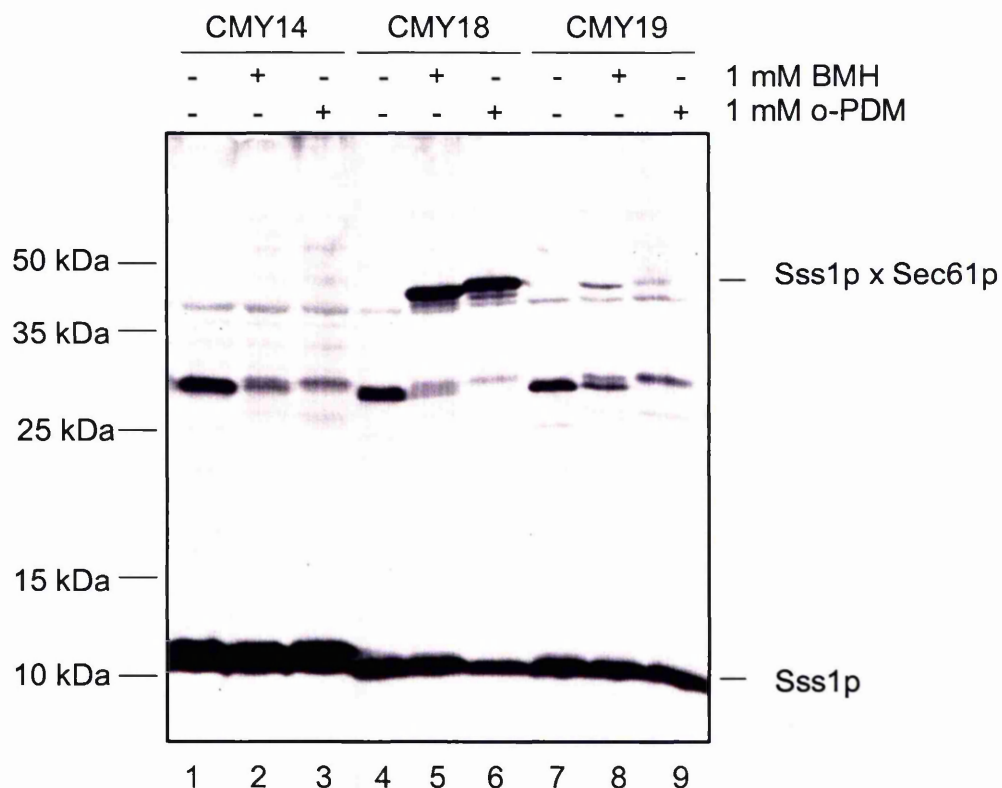


Figure 3.13 TM9 and TM10 of Sec61p can be crosslinked to Sss1p. 1 $A_{280\text{nm}}$ equivalent of membranes isolated from CMY14 (lanes 1-3), CMY18 (lanes 4-6) and CMY19 (lanes 7-9) were either mock treated with DMSO or incubated with either 1 mM BMH or 1 mM o-PDM at 30°C for 30 minutes. After this, crosslinking was quenched by incubating samples with 100 mM DTT for 5 minutes, incubated with an equal volume of 2X Laemmli sample buffer and analysed by SDS-PAGE on a 12.5% acrylamide gel. After this, samples were transferred to nitrocellulose membrane by western transfer and then immunoblotted with anti Sss1p antibodies. Sss1p and Sss1p crosslinked adducts are indicated.

However, the crosslink detected by anti Sec61p antibodies in membranes isolated from CMY14 were not detected with anti Sss1p antibodies (figure 3.13 C). Together this indicates that Sss1p can be covalently coupled to mutants of *sec61* where three consecutive cysteine mis-sense mutations have been incorporated into either TM9 or TM10 of Sec61p. The observed cross-linking efficiencies would imply that TM9 is the principal TMD of Sec61p with which Sss1p can be crosslinked to. Also, a cysteine dependent crosslink was identified between Sec61pTM5 and a protein other than Sss1p.

3.7 Crosslink dependent adducts between Sss1p and Sec61p requires the endogenous cysteine residue of Sss1p

The Sss1 protein contains an endogenous cysteine residue, C39, in a region of Sss1p that has previously been proposed to be cytosolic (Esnault *et al.*, 1994). However, more recent analysis has indicated that the N- terminal 40-50 residues forms an amphipathic helix where one face of the helix is basic and the opposing face is hydrophobic (Beswick *et al.*, 1998; van den Berg *et al.*, 2004) and it has been proposed that the hydrophobic face can protrude into the lipid bilayer (van den Berg *et al.*, 2004). Therefore, it is possible that the cross-links observed is the consequence of the covalent coupling between TM 9 or 10 of Sec61p to the endogenous cysteine residue, C39, rather than the residues incorporated into the TMD of Sss1p by site directed mutagenesis.

To investigate this possibility, membranes were isolated from cells co-expressing either Sec61p TM9 or 10 CCC with Sss1p and incubated with either BMH or o-PDM. In all cases the same cross-linking profile was observed as was detected when the mutagenised derivative of Sss1p was co-expressed with each of these Sec61p mutations (figure 3.14). This indicates that that C39 in the N- terminal helix, rather than the transmembrane domain, of

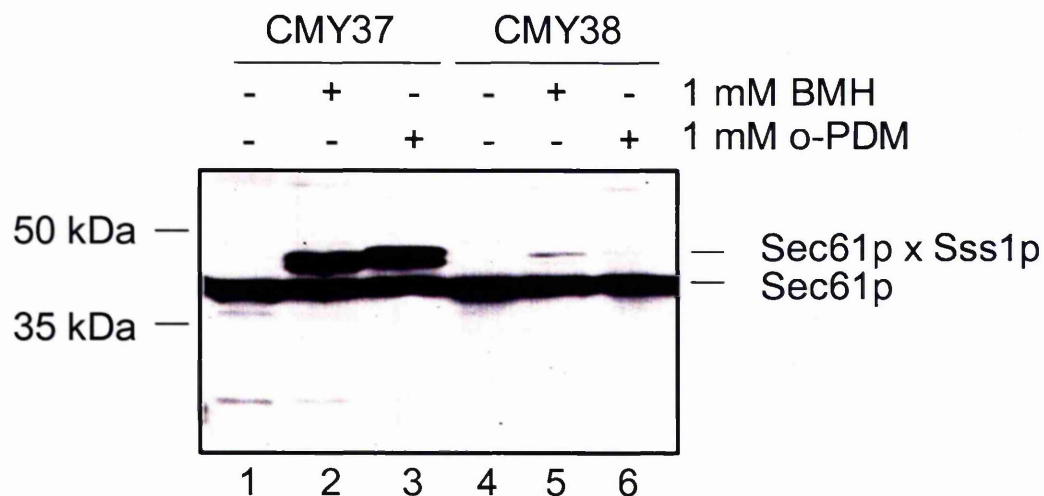


Figure 3.14 TM9 and TM10 of Sec61p are crosslinked to the endogenous cysteine residue of Sss1p. 1 $A_{280\text{nm}}$ equivalent of membranes isolated from CMY37 (Sss1p, Sec61p TM9CCC) and CMY38 (Sss1p, Sec61p TM10CCC) were either mock treated with DMSO or incubated with either 1 mM BMH or 1 mM o-PDM at 30°C for 30 minutes. After this, crosslinking was quenched by incubating samples with 100 mM DTT for 5 minutes, incubated with an equal volume of 2X Laemmli sample buffer and analysed by SDS-PAGE on a 12.5% acrylamide gel. After this, samples were transferred to nitrocellulose membrane by western transfer and then immunoblotted with anti Sec61p antibodies. Sec61p and Sec61p crosslinked adducts are indicated

Sss1p is positioned such that it can be covalently coupled to mutants of Sec61p where three consecutive cysteine mis-sense mutations have been incorporated into either TM9 or 10 of Sec61p.

3.8 Discussion

Biochemical and genetic analysis has indicated that Sss1p interacts with Sec61p between TM6 and TM8 (Wilkinson *et al.*, 1997). Following the identification that the signal sequence of a secretory precursor interacts with both TM2 and TM7 of Sec61p it was postulated that Sss1p functions as a surrogate signal sequence and is required to regulate the function of the translocon (Plath *et al.*, 1998). This model makes two major predictions. Firstly, in an inactive state, Sss1p occupies the signal sequence binding site of Sec61p. Therefore, this predicts that Sss1p directly binds to TM7, and maybe TM2. In agreement with this, it has been identified in *E. coli* that TM3 of SecE, the *E. coli* homologue of Sss1p, is in close proximity to both TM2 and TM7 (Veenendaal *et al.*, 2001). Secondly, the incorporation of signal peptide into the translocon displaces Sss1p, revealing the signal peptide binding site of the Sec61 complex.

In order to investigate this model cysteine scanning mutagenesis has been used to investigate whether the TM of Sss1p can interact with any of the TMD's of Sec61p. An interaction between the two proteins is determined when two cysteine residues are positioned such that allows the covalent coupling of the two sulphydryl side chains upon incubation with cysteine dependent crosslinking reagents.

A yeast strain has been constructed that allows the co-expression of *sss1* and *sec61* mutants following plasmid shuffle. This strain, CMY5, contains genomic disruptions of the *SEC61* and *SSS1* loci and cell viability is maintained by expression of both of these genes from a single *URA3* selective plasmid. Therefore, following the transformation of this strain

with plasmids that express a functional copy of *sec61* and *sss1*, the wild type plasmid can be counterselected by virtue of the strains ability to grow on media supplemented with 5-FOA.

Three consecutive mis-sense mutations were incorporated into the TM of Sss1p and each of the ten TMs of Sec61p by site directed mutagenesis. The three point mutations that were introduced were positioned towards the centre of each of the TM helices, based on protein topology predictions according to Esnault *et al.*, 1994 (Sss1p) and Wilkinson *et al.*, 1996 (Sec61p), in order to restrict bias on the experiment. The mutations introduced did not appear to grossly effect the hydrophobicity of either protein therefore, it is unlikely that these mutations are going to effect the ability of either protein to be integrated into the ER membrane. However hydropathy analysis indicated that the mutations incorporated into the TMD of Sss1p and TM3 of Sec61p appeared to decrease the hydrophobicity of these domains whereas incorporation of three cysteine residues into TM7 of Sec61p appeared to increase the hydrophobicity of this domain.

We investigated the functionality of the *sss1* mutant and each of the ten *sec61* mutants alone by their ability to complement either the *sss1Δ* or *sec61Δ* mutation. Incorporation of three cysteine residues into the TM of Sss1p did not effect the function of this protein and incorporation of three mis-sense mutations into either TM1, 2, 4, 5, 6, 8 or 9 did not disrupt Sec61p function. Substitution of three residues in TM10 with cysteine did partially effect *sec61* function as, although this mutant could complement the *sec61Δ* mutation, cells expressing this mutant grew much slower than wild type. However, substitution of three cysteine residues into either TM3 or TM7 of Sec61p completely disrupted Sec61p function as these mutants could not complement the lethal *sec61Δ* mutation.

Previous analysis has indicated that deletion of TM3 from Sec61p severely perturbs the ability of the resulting Sec61pΔTM3 complex to interact with the Sec63p complex, therefore compromising the ability to form the SEC complex (Wilkinson *et al.*, 2000). In agreement

with this, we have found that the substitution of three consecutive residues in TM3 to cysteine is detrimental to the formation of complexes required for ER translocation, particularly the Sec61 complex and this would imply that the TM 3 is required for the formation of these complexes. Sec61p Δ TM3 was found to disrupt Sec61p structure through topological frustration of TM2. As TM2 is fairly weakly hydrophobic it was postulated that TM3 may facilitate the integration of this domain into the ER membrane (Wilkinson *et al.*, 2000). Interestingly the mutations incorporated into TM3 decrease the hydrophobicity of TM3 and may support this hypothesis. Although the ability form the Sec61 complex is grossly perturbed in Sec61pTM3CCC, we proceeded to continue with this investigation as this region has not been implicated to interact with Sss1p.

Transmembrane domain 7 of Sec61p has been defined to comprise the signal peptide binding activity of Sec61p. Therefore, we postulated that incorporation of three mis-sense mutations into TM7 may disrupt the signal peptide binding activity of Sec61p. *In vitro* analysis indicated that the mutation incorporated into TM7 disrupts the ability of this mutant protein to bind signal peptide (personal communication B. M. Wilkinson). Therefore, the Sec61pTM7CCC mutant protein maybe non-functional as the Sec61 complex no longer has signal peptide binding activity to allow the translocation of secretory precursors to proceed. Interestingly, incorporation of three cysteine mis-sense mutations into TM7 appeared to increase the hydrophobicity of this TMD. Whether this has an effect on the signal peptide binding activity of the translocon remains to be seen.

To investigate whether the transmembrane domain of Sss1p can interact with any of the TMs of Sec61p, membranes isolated from cells co-expressing Sss1pTMCCC with each of the ten mutants of Sec61p were incubated with the cysteine dependent homobifunctional cross-linking reagents BMH and o-PDM. Chemical crosslinking has identified a Sec61p containing adduct of approximately 46 kDa in membranes isolated from cells where either

TM5, 9 or 10 had been mutagenised. However, analysis of these Sec61p containing adducts with anti Sss1p antibodies indicated that only the adducts identified in membranes isolated from cells where either TM9 or TM10 had been mutagenised were found to contain Sss1p. Although it is possible that the cross-link identified in membranes where TM5 was mutagenised could mask the epitope(s) detected by anti Sss1p antibodies it is more likely that this adduct represents the cross-linking of Sec61p TM5 to a small cysteine containing protein. It would be of interest to identify the protein that can be crosslinked to TM5 as it may provide a greater understanding of the recruitment of known translocon associated proteins to the translocon. Alternatively it may identify a new component that is required to function in the translocation of proteins into the ER. But, the efficiency with which this adduct is formed would suggest that this is a minor interaction of the translocon as the efficiency of crosslinking was weak with a longer crosslinking reagent.

The nature with which Sss1p could be covalently coupled to TM9 and TM10 indicates that the major interaction is between Sss1p and TM9. Although Sss1p could be covalently coupled to TM9 and 10 with both BMH and o-PDM, the efficiency with which Sss1p could be crosslinked to TM9 was far greater than that with TM10. Also, Sss1p could be crosslinked to TM9 with BMH and o-PDM with comparable efficiency whereas crosslinking Sss1p to TM10 was more efficient with BMH than o-PDM. In fact the latter is barely detectable with anti Sec61p antibodies. However, neither crosslinking between Sss1p and TM9 nor TM10 was dependent upon the mutations incorporated into the transmembrane domain of Sss1p but required the cysteine residue, C39, in the N- terminal domain of Sss1p. This indicates that cysteine scanning mutagenesis has been unsuccessful in identifying the transmembrane domain(s) of Sec61p that interact with that of Sss1p. This data may imply that Sss1p does not interact with any transmembrane domain of Sec61p. However, there is a significant body of

evidence implying that this is not the case. Determination of the crystal structure of the SecYEB translocon in *Methanococcus jannaschii* has predicted SecE to make single site contacts with TM1, 5, 6 and 10 of SecY. Following this it has been postulated that these interactions are sufficient to brace the N- and C- terminal halves of SecY to form the translocation channel.

The cysteine substitutions that were incorporated into the transmembrane domain of Sss1p and each of the ten transmembrane domains of Sec61p by SDM were done so according to the topology proposed by Esnault *et al.*, 1994 (Sss1p) and Wilkinson *et al.*, 1996 (Sec61p). These topological predictions closely resemble the topology of these proteins predicted from the crystal structure. However the subtle variations have a great effect on the location of the mutations incorporated into these proteins. Therefore, it is possible due to the nature of the interactions predicted from the crystal structure that the mutations that have been incorporated into Sss1p and Sec61p are not in the correct spatial orientation and proximity to promote cross-linking between these two proteins. The transmembrane domain of SecE has been postulated to be inserted at a 35° angle where it first contacts TM6 towards the cytosolic face of the ER membrane followed by TM10, 5 and finally TM1 at the luminal face of the bilayer. Therefore according to the sequence alignment proposed following the determination of the crystal structure it is unlikely that the residues substituted are going to promote cross-linking between Sss1p and Sec61p.

How can the endogenous cysteine residue of Sss1p be covalently coupled to both TM9 and TM10 of Sec61p? The N- terminal domain of Sss1p has been shown to comprise an amphipathic α - helical structure with the hydrophobic face pointing towards the membrane (Beswick *et al.*, 1998; Satoh *et al.*, 2003; van den Berg *et al.*, 2004) and it has been proposed that this helix lies on the ER membrane and interacts with the C- terminal half of SecY (van den Berg *et al.*, 2004). This helix is followed by a short β - strand, that precedes the

transmembrane domain, and sequence alignment predicts the endogenous cysteine residue of Sss1p to reside in this β strand. The refined alignment predicts that the cysteine residues incorporated into TM9 and TM10 are positioned towards the cytosolic face of these transmembrane domains and are positioned in immediate proximity to C39 of Sss1p. This would explain why the endogenous cysteine residue of Sss1p can be covalently coupled to TM9 and TM10 of Sec61p.

The data obtained in this analysis would appear to suggest that there is a certain degree of structural conservation between the yeast Sec61 complex and the archaeobacterial SecY complex.

Chapter 4

Sss1p recruits Sec63p to form functional translocons

4.1 Introduction

ER translocation requires the collective function of a large number of proteins. The heterotrimeric Sec61 complex forms the pore through which substrate is translocated, however this alone is insufficient to mediate ER translocation and genetic analysis has shown that *SEC63* is essential for both SRP dependent and Sec62p dependent translocation to proceed (Young *et al.*, 2001). As it has been demonstrated that Sec63p is a component of the heptameric SEC complex required for Sec62p dependent translocation it would imply that the SRP dependent translocon requires other factors to be recruited to the translocation channel. The mechanism by which translocation complexes assemble is poorly understood. Therefore, we proceeded to investigate the molecular architecture of the ER translocon by chemical cross-linking. Incubation of microsomes isolated from wild type cells with the lysine specific homobifunctional cross-linking reagent disuccinimidyl suberate (DSS) yields two major Sss1p adducts with a relative molecular weight of approximately 46 and 85 kDa (figure 4.1, lane 2). The anti-Sss1p antibodies do appear to detect a species of approximately 85 kDa in the absence of DSS (figure 4.1, lane 1) however, the intensity of this band is significantly lower than that detected upon treatment with DSS (figure 4.1, compare lanes 1 and 2) and is consistent with this being non-specific. It has previously been shown that the 46 kDa species represents the covalent coupling of Sss1p to Sec61p (Esnault *et al.*, 1994; Wilkinson *et al.*, 1997). However, the 85 kDa Sss1p containing adduct has not been characterised. Based on the size of the adduct it is anticipated that the cross-linking partner would have a molecular weight between 70-75 kDa. Sec63p and Kar2p are proteins that are essential for the translocation of precursor into the ER (Young *et al.*, 2001). Furthermore they have molecular weights of 73 and 75 kDa respectively making these two proteins prime candidates for the 85 kDa cross-linking partner.

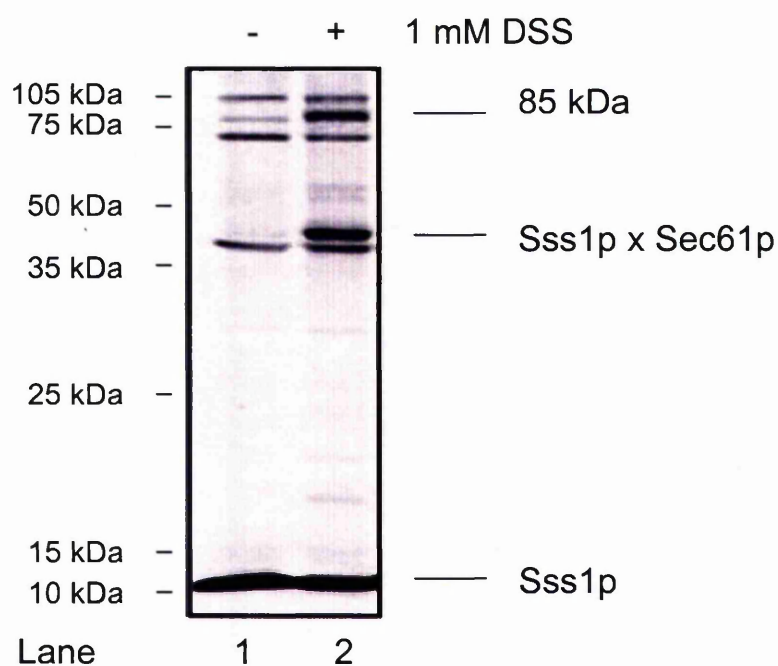


Figure 4.1 Investigating interactions formed by Sss1p by lysine dependent crosslinking. 1 $A_{280\text{nm}}$ of membranes isolated from w303 α cells were either mock treated with DMSO (lane 1) or incubated with 1 mM DSS (lane 2) at 30°C for 30 minutes. After this, crosslinking was quenched by incubating samples with 100 mM lysine and glycine for 5 minutes, incubated with an equal volume of 2X Laemmli sample buffer. Samples were analysed by SDS-PAGE on an 8-15% linear gradient and after western transfer were immunoblotted with anti Sss1p antibodies. Sss1p and Sss1p crosslinked adducts are indicated.

4.1.1 Characterising the 85 kDa crosslinked adduct

To investigate the DSS cross-linking profile of both Sec63p and Kar2p, membranes isolated from wild type cells were treated with 1 mM DSS and then probed with antisera immunoreactive to either Kar2p or Sec63p. Membranes incubated with or without 1 mM DSS probed with anti Kar2p antibodies identified one major immunoreactive species of 75 kDa (figure 4.2 A, lanes 1 and 2) and this band has been reported to represent Kar2p (Tyson and Stirling, 2000). When we probed wild type membranes that had not been treated with DSS with anti Sec63p antibodies one major immunoreactive species of approximately 73 kDa was detected that has previously been shown to be Sec63p (Young *et al.*, 2001) (figure 4.2 B, lane 1). However, when we probed membranes that had been incubated with 1 mM DSS a DSS dependent, Sec63p containing adduct with a rMw of approximately 85 kDa was detected in addition to the non-crosslinked Sec63p (figure 4.2 B, lane 2). This made Sec63p the prime candidate for the 70-75 kDa cross-linking partner for the 85 kDa Sss1p adduct.

4.1.2 Sss1p can be cross-linked to Sec63p

To identify whether the formation of the DSS dependent 85 kDa Sss1p adduct requires the expression of *SEC63*, we investigated the Sss1p cross-linking profile in membranes harvested from cells where the expression of *SEC63* had been regulated by the growth conditions. The BYY5 strain has the *SEC63* gene under the control of the methionine repressible *MET3* promoter. Previous analysis has demonstrated that expression of this *SEC63* allele is repressed by growth for 6 hours in media supplemented with 2 mM methionine or in nutrient rich media as Sec63 protein levels are significantly reduced in microsomes at this

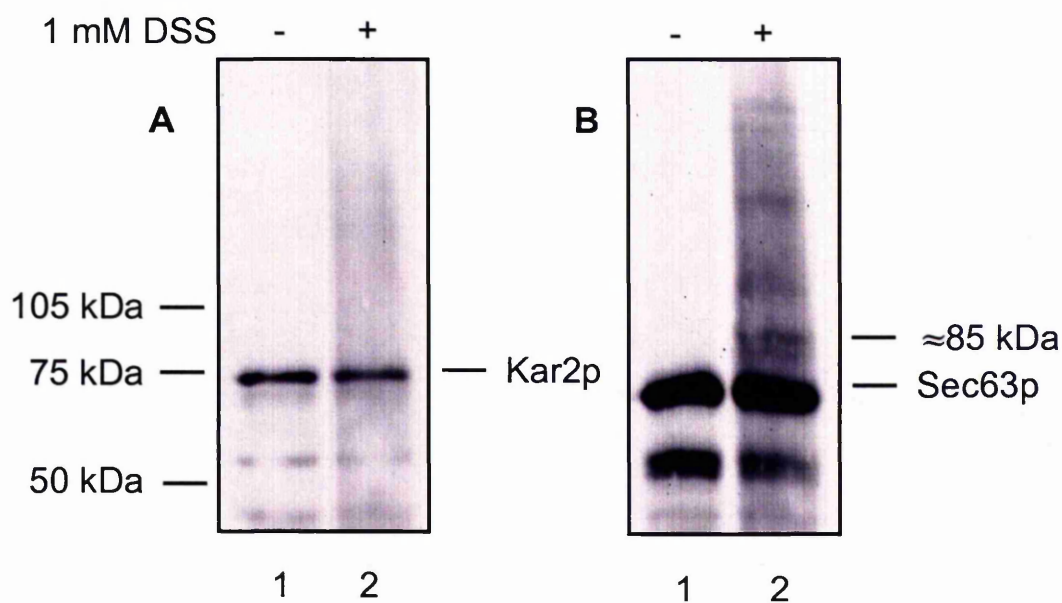


Figure 4.2 Investigating interactions formed by Kar2p and Sec63p by lysine dependent crosslinking. 1 A_{280nm} of membranes isolated from w303α were either mock treated with DMSO (lane 1) or incubated with 1 mM DSS (lane 2) at 30°C for 30 minutes. After this, crosslinking was quenched by incubating samples with 100 mM lysine and glycine for 5 minutes and incubated with an equal volume of 2X Laemmli sample buffer. Samples were analysed by SDS-PAGE on an 8-15% linear gradient and after western transfer were immunoblotted with either anti Kar2p antibodies (**A**) or anti Sec63p antibodies (**B**).

point (Young *et al.*, 2001). Membranes were harvested from BYY5 cells grown in the presence or absence of methionine and then incubated with or without 1 mM DSS. After SDS-PAGE and western transfer, samples were probed with either anti Sec63p or anti Sss1p antibodies. Western blot analysis of membranes isolated from cells, grown in the absence of methionine, with anti Sec63p antibodies again identified the 85 kDa adduct (figure 4.3 A, compare lanes 1 and 2). When membranes isolated from BYY5 cells that had been grown in nutrient rich media were probed with anti Sec63p antibodies it was found that the Sec63 protein had been significantly depleted in microsomes that had been mock treated (figure 4.3 A, lane 3) and no DSS dependent Sec63p adducts were isolated in samples that had been incubated with DSS (figure 4.3A, lane 4). The reduction in Sec63 protein levels in membranes isolated from cells grown in nutrient rich media compared to media depleted of methionine is entirely consistent with the methionine dependent regulation of the *MET3-SEC63* allele. Following this, samples were probed with anti Sss1p antibodies. The 46 and 85 kDa Sss1p crosslinked adducts were again detected following the treatment of membranes that had been isolated from BYY5 cells grown in the absence of methionine with DSS (figure 4.3 B, lane 2). However, when the Sss1p cross-linking profile was examined in membranes extracted from cells grown in the presence of methionine, we found that depletion of Sec63p did not affect the efficiency with which the Sss1p x Sec61p adduct was generated but the 85 kDa adduct could no longer be detected (figure 4.3 B, lane 4). This indicates that the formation of the 85 kDa Sss1p adduct requires the expression of *SEC63* and it may be Sec63p itself, which crosslinks to Sss1p. Conversely, depletion of Sec63p may indirectly perturb the ability of Sss1p to associate with its 70-75 kDa cross-linking partner and the protein that is covalently coupled to Sec63p, to form an 85 kDa Sec63p adduct, is not Sss1p.

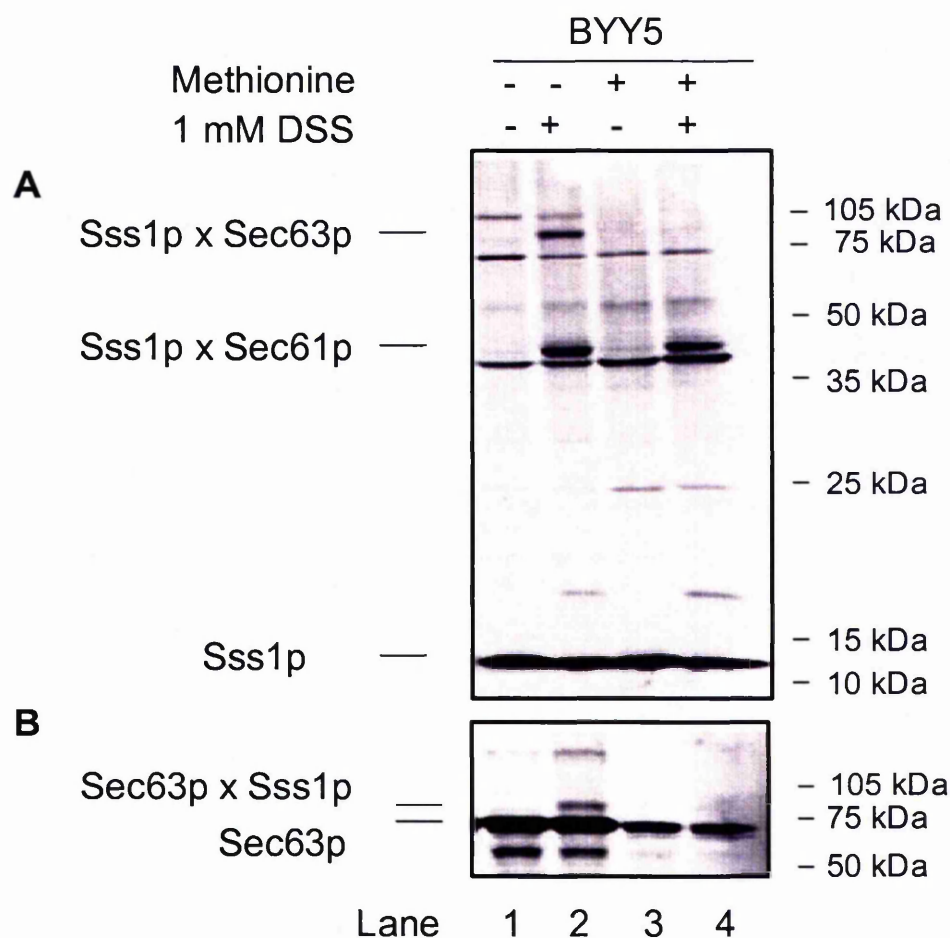


Figure 4.3 DSS dependent crosslinking profile of Sss1p following the depletion of Sec63p. Expression of *SEC63* in BYY5 cells is regulated by the presence of methionine in the growth media. Membranes were isolated from BYY5 cells incubated in the absence (expression) or presence (repression) of methionine for 6 hours to manipulate the expression of *MET3-SEC63*. After this, 1 A_{280nm} of membranes were either mock treated with DMSO or incubated with 1 mM DSS at 30°C for 30 minutes. After this, crosslinking was quenched by incubating samples with 100 mM lysine and glycine for 5 minutes, incubated with an equal volume of 2X Laemmli sample buffer and then analysed by SDS-PAGE on an 8-15% linear gradient. After western transfer samples were either probed with anti Sss1p antibodies (**A**) or anti Sec63p antibodies (**B**). Crosslinked adducts are indicated.

To discriminate between these two possibilities we examined the effect of Sss1p depletion on the DSS dependent cross-linking profile of Sec63p. The FKY198 strain possesses the *SSS1* coding sequence under the control of the regulatable *GAL10-CYC1* hybrid promoter (Esnault *et al.*, 1993; 1994). Thus the expression of *SSS1* in this strain can be regulated by the carbon source present in the growth media. The *GAL10* promoter is transcriptionally activated by the Gal4p transcription factor. This is active in the presence of galactose but inactive in the presence of glucose due to binding of its regulator protein, Gal80p.

The *GAL10-SSS1* allele has previously been shown to be no longer expressed when these cells are grown in the presence of glucose (Esnault *et al.*, 1994). Furthermore, the Sss1 protein has been shown to be efficiently depleted from cells after growth for 6 hours in media containing glucose. Therefore, membranes were isolated from cells that had been grown in either galactose or glucose for 6 hours and treated with or without DSS. Western blot analysis of membranes harvested from cells grown in media containing galactose again identified the 46 and 85 kDa Sss1p adducts following incubation with 1 mM DSS (figure 4.4 A, compare lanes 1 and 2). When membranes were isolated from cells grown in glucose, western analysis indicated that there was a vast reduction of Sss1p in membranes that were mock treated (figure 4.4A, lane 3) and could no longer be detected after treatment with 1 mM DSS (figure 4.4 A, lane 4). The reduction in Sss1 protein levels in membranes isolated from cells grown in glucose compared to galactose as well as the inability of these cells to grow on glucose containing media is entirely consistent with the glucose induced repression of the *GAL10* regulated *SSS1* allele. The DSS cross-linking profile of Sec63p was unperturbed in membranes isolated from cells grown in 2% galactose (figure 4.4B, lane 2). But when the ability of Sec63p to form cross-links in membranes where Sss1p had been depleted was investigated it was found that the 85 kDa Sec63p adduct could no longer be detected (figure 4.4B, lane 3).

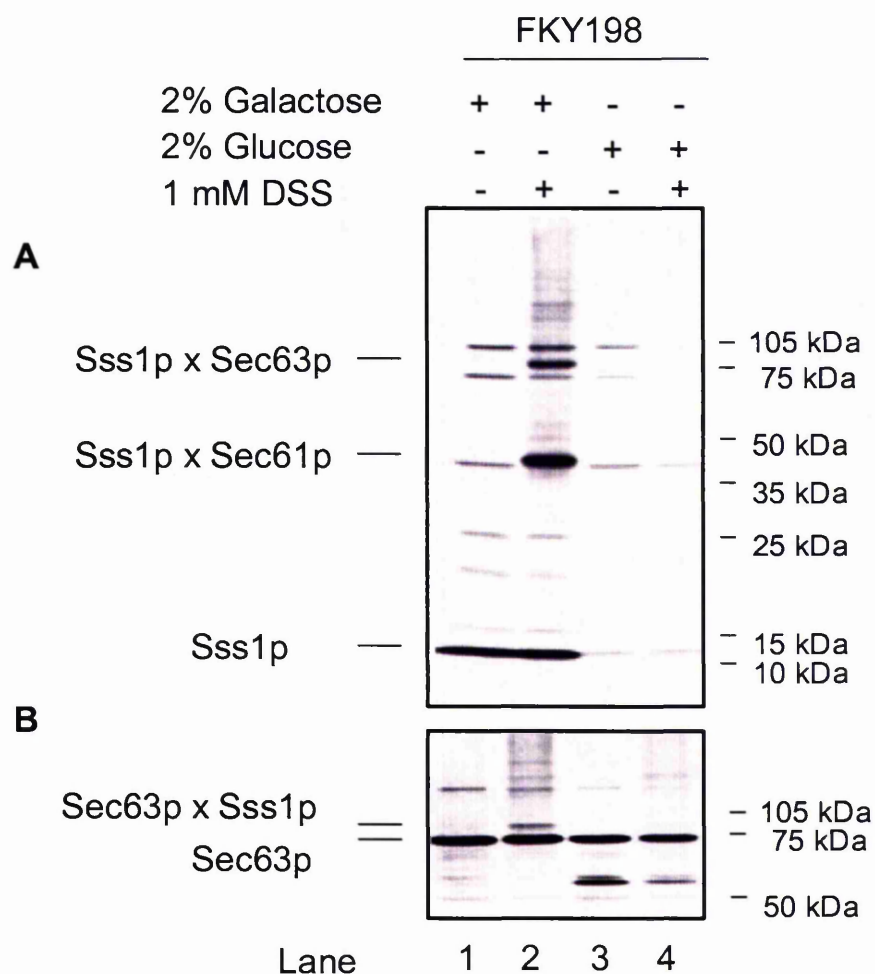


Figure 4.4 DSS dependent crosslinking profile of Sec63p following the depletion of Sss1p. Expression of *SSS1* in FKY198 cells is regulated by the carbon source in the growth media. Membranes were isolated from FKY198 cells grown in nutrient rich media supplemented with either 2% galactose or 2% glucose, in which the *GAL10-SSS1* is either expressed or repressed respectively, for 6 hours to manipulate the expression of *GAL10-SSS1*. After this, 1 A_{280nm} of membranes were either mock treated with DMSO or incubated with 1 mM DSS at 30°C for 30 minutes. After this, crosslinking was quenched by incubating samples with 100 mM lysine and glycine for 5 minutes, incubated with an equal volume of 2X Laemmli sample buffer and samples were analysed by SDS-PAGE on an 8-15% linear gradient. After western transfer samples were either immunoblotted with anti Sss1p antibodies (A) or anti Sec63p antibodies (B). Crosslinked adducts are indicated.

Collectively, these results indicate that the 85 kDa Sss1p adduct and the 85 kDa Sec63p adduct are one and the same and correspond to cross-linking between Sss1p and Sec63p.

As it has been identified that Sss1p can interact with the Sec63 protein as well as Sec61p we wondered if depletion of Sss1p from cells would affect either Sec61p or Sec63p at steady state. To investigate this, both Sec61p and Sec63p were analysed by SDS-PAGE in conditions that would allow a greater resolution of proteins. Microsomes were isolated from cells in conditions that regulate the expression of *GAL10-SSS1* and 0.5 A_{280nm} of membranes, whereby 0.5 A_{280nm} equivalents of membranes is taken from a stock suspension that possesses a tryptophan and tyrosine content that gives an absorbance of 50 A_{280nm}/ml in a cuvette with 1 cm pathlength, were then prepared for SDS-PAGE and immunoblotted with either anti Sss1p, anti Sec61p or anti Sec63p antibodies. Western analysis with anti Sss1p antibodies identified a single major immunoreactive species of approximately 12 kDa in microsomes isolated from cells grown in YP Gal and this has been shown to correspond to Sss1p (Esnault *et al.*, 1993) (figure 4.5 A, lane 1). Conversely, the same analysis indicated that although this band is present in microsomes isolated from cells that had been grown in glucose, the intensity was significantly lower than when FKY198 was grown in YP Gal (figure 4.5 A, compare lanes 1 and 2). Again, this is consistent with the *SSS1* gene being repressed. Immunoblot analysis with anti Sec61p antibodies identified a single major species of approximately 35 kDa, which corresponds to the Sec61 protein, in microsomes where *SSS1* had been expressed (figure 4.5 B, lane 1) or repressed (Figure 4.5B, lane 2). Depletion of Sss1p does not appear to affect the level of Sec61 protein at steady state as there are no apparent differences in the amount of Sec61p in either sample (figure 4.5 B, compare lanes 1 and 2) and is consistent with previous observations (Esnault *et al.*, 1994). However, western analysis with anti Sec63p antibodies identified a 73 kDa immunoreactive species in membranes isolated from cells where *SSS1* had

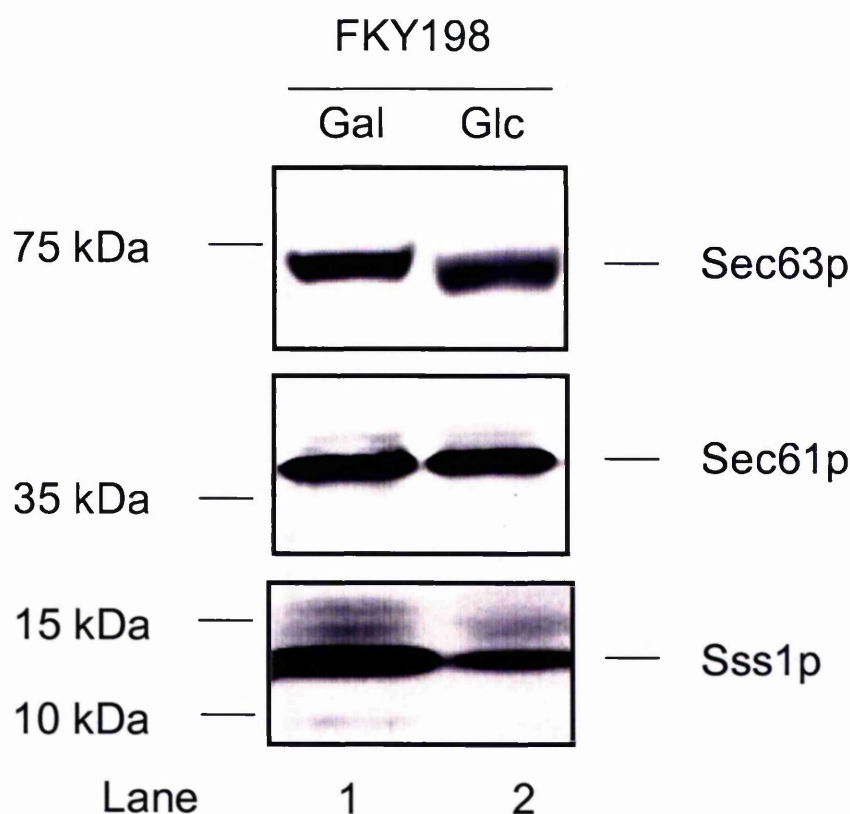


Figure 4.5 Analysis of translocon components following the depletion of Sss1p.

Membranes were isolated from FKY198 cells grown in nutrient rich media supplemented with either 2% galactose (lane 1) or 2% glucose (lane 2), in which the *GAL10-SSS1* is either expressed or repressed respectively, for 6 hours to manipulate the expression of *GAL10-SSS1*. After this, 1 A_{280nm} equivalent of membranes was incubated with an equal volume of 2X Laemmli sample buffer and analysed by SDS-PAGE on an 8% gel containing a 15% step at the bottom of the gel. After this samples were transferred to nitrocellulose membrane by western transfer and were immunoblotted with anti Sec63p antibodies (A), anti Sec61p antibodies (B) or anti Sss1p antibodies (C).

been expressed (figure 4.5 C, lane 1). But, analysis of microsomes isolated after repression of *SSS1*, by SDS-PAGE on an 8% acrylamide gel, identified a form of Sec63p that had a faster electrophoretic mobility than that isolated in cells where *SSS1* had been expressed (figure 4.5 C, compare lanes 1 and 2). Furthermore, the intensity of this band appeared significantly lower in microsomes where Sss1p had been depleted (figure 4.5 C, compare lanes 1 and 2). Therefore depletion of Sss1p has a detrimental affect on the Sec63 protein at steady state.

4.2 Sss1p requires a functional transmembrane domain to interact with Sec63p

Functional analysis of the *E. coli* Sec YEG translocon has revealed that TM3 of SecE is required to stabilise this complex as a SecE mutant where TM3 had been replaced with a transmembrane domain from a protein of unrelated function could no longer instigate the assembly of the translocation channel (Pohlschroder *et al.* 1996). A collaborative study in this laboratory has investigated the function of the Sss1p TMD in a similar manner. Previous analysis has demonstrated that a mutant of Sss1p where the C- terminal 28 residues has been deleted is no longer functional as it can no longer complement the lethal *sss1Δ* null mutation (J. K. Brownsword, 2000). This implies that the N- terminal 52 residues cannot function alone because it could no longer associate with the ER membrane.

4.2.1 The transmembrane domain of Sss1p is essential for function

The function of the Sss1pTM has been investigated by domain substitution analysis where the TM of Sss1p has been replaced with that of Ubc6p, an ER tail anchor protein required for the conjugation of ubiquitin to substrate (Yang *et al.*, 1997). This chimeric protein has been termed SUSp. We postulated that if the function of the Sss1p transmembrane domain is to anchor the protein to the ER membrane the chimeric protein would be functional.

However, if this chimeric protein is not functional it would imply that the transmembrane domain is required for another function other than membrane localisation.

A region of 16 residues corresponding to residues 53-68 in Sss1p was replaced with residues 233-249 of Ubc6p and this construct was created via PCR. pBW236 was constructed by PCR where a 255 bp fragment was generated using the SUS_F and SUS_R primers. The SUS_F primer encodes for the Ubc6p transmembrane domain which is flanked by a 5' *Bam*HI restriction site and a 3' *SSS1* annealing region starting at codon K69. The SUS_R primer encompasses the natural *Ssp*I restriction site in the 3' untranslated region (UTR) of *SSS1*. The PCR product was digested with 10 U of *Bam*HI and 10 U *Ssp*I and this was ligated into the *Bam*HI/*Ssp*I site of pJKB1, a plasmid where a *Bam*HI site had been incorporated immediately after the *SSS1* codon encoding K52. The subsequent *Bam*HI/*Ssp*I restriction digest excises the sequence encoding for the Sss1p TM, the extreme C-terminus and 3' UTR. Following ligation, the SUS encoding vector, pBW236, was verified by DNA sequencing.

The functionality of the SUS fusion protein was assessed by its ability to complement the otherwise lethal *sss1* null mutation by plasmid shuffle. The strain BWY530 was transformed with either pRS313 (vector control), pJKB2 (*SSS1*), or pBW236 and transformants were selected for histidine prototrophy. Transformants were then plated out onto media containing 5-FOA and incubated at 30°C for 2 days (figure 4.6 A). Cells transformed with pRS313 did not grow under these conditions as the plasmid does not encode for a functional copy of *SSS1* that can complement the loss of the *URA3* selective wild type plasmid. Cells transformed with pJKB2 could grow as there is a functional *SSS1* gene encoded by this plasmid. However, cells transformed with pBW236 could not grow on media containing 5-FOA (figure 4.6 A). This demonstrates that the SUS is not a functional mutant of *SSS1*.

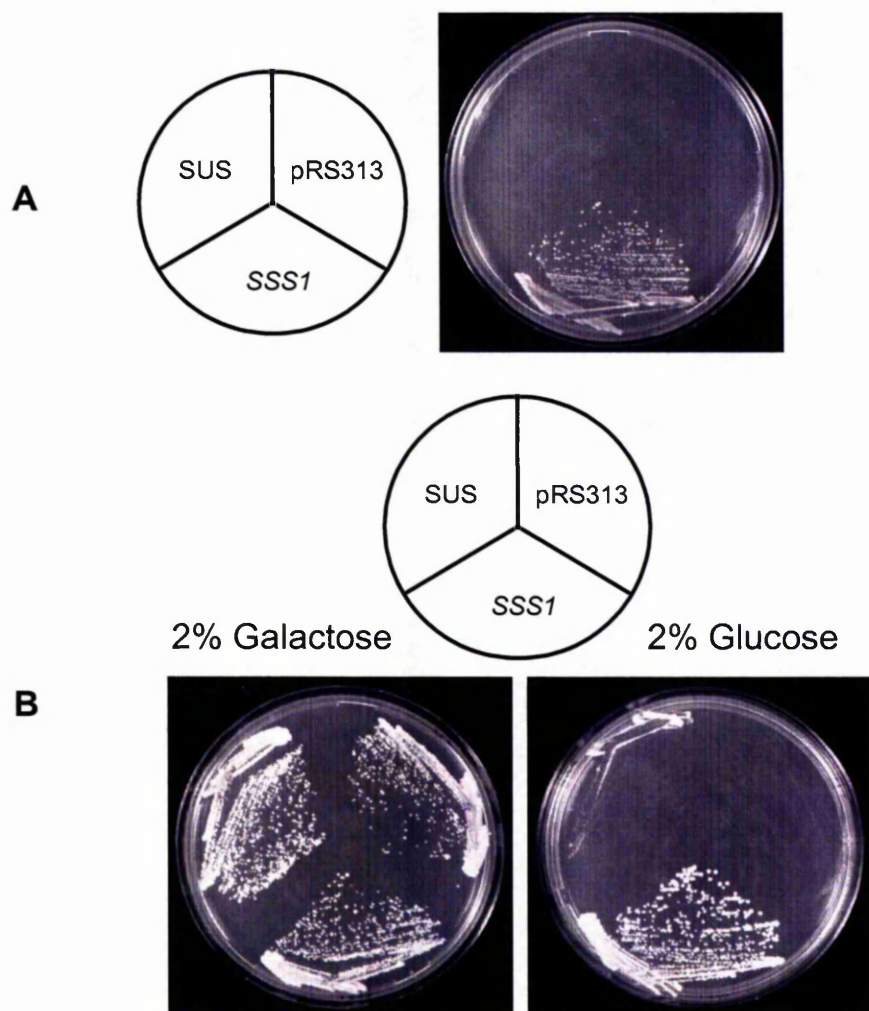


Figure 4.6 Investigating the functionality of the SUS mutant. (A) The functionality of the SUS mutation was assessed by its ability to complement the inviable *sss1Δ* mutation. BWY530 cells were transformed with either pRS313 vector control, pJKB2 (*SSS1*) or pBW236 (SUS) and selected for histidine prototrophy. After this transformants were plated out onto minimal media supplemented with 1 mg/ml 5-FOA to counterselect for the *URA3* selective, *SSS1* encoding vector. Cells were incubated at 30°C for 2 days. (B) The functionality of the SUS mutation was assessed following the repression of a regulatable allele of *SSS1*. FKY198 cells were transformed with either pRS313, pJKB2 or pBW236 and selected for histidine prototrophy. Transformants were then plated out onto YNB supplemented with either 2% galactose, that promotes the expression of *GAL10-SSS1*, or 2% glucose which represses the *GAL10-SSS1* allele. Cells were incubated at 30°C for 2 days

In order to further investigate the SUS mutant it is required that this mutant is expressed in the FKY198 strain containing the *GAL10* regulated *SSS1* allele. FKY198 cells were transformed with pRS313, pJKB2 or pBW236 and transformants were selected for histidine prototrophy on selective media supplemented with 2% galactose to drive the expression of *GAL10-SSS1*. Transformants were subsequently plated out onto selective media that was supplemented with either 2% galactose or 2% glucose. FKY198 transformed with either pRS313, pJKB2 or pBW236 were able to grow on histidine selective media containing galactose as the *GAL10-SSS1* allele is expressed under these conditions (figure 4.6B) but only cells transformed with the pJKB2 plasmid could grow on media containing glucose (figure 4.6 B). This supports the plasmid shuffle analysis as neither pRS313 or pBW236 encode a functional copy of *SSS1* that can complement the otherwise lethal repression of the *GAL10-SSS1* gene.

4.2.2 ER translocation is defective in SUS cells

The Sss1 protein has been shown to be ubiquitous for ER translocation (Esnault *et al.*, 1993; 1994). ER translocation activity was therefore analysed in cells, following growth in conditions that have been shown to deplete the endogenous Sss1 protein in FKY198 cells, bearing either pRS313 control plasmid, *SSS1* or SUS to investigate whether loss of function directly correlated to an inability to translocate proteins into the ER. ER translocation was analysed by pulse labelling where cells are incubated for a short period of time with a ³⁵S labelled cocktail of methionine and cysteine. Following this, only newly synthesised material will be radiolabeled and the biogenesis of precursor protein can be followed by the level of processing. After growth for 6 hours in glucose containing media, 5 O.D._{600nm} of cells

containing either vector control, *SSS1* or *SUS* were labelled with ^{35}S methionine/cysteine for 5 minutes. Cell extracts were then prepared and immunoprecipitated with antisera directed against secretory precursor proteins known to be translocated either by Sec62p dependent translocation (CPY) or by SRP dependent protein translocation (DPAP B) (Ng *et al.*, 1996).

CPY is a vacuolar peptidase that is trafficked to this organelle via the secretory pathway (Stevens *et al.*, 1986). Following synthesis, pre pro CPY (ppCPY) is translocated into the ER where its signal sequence is cleaved via the signal peptidase complex and the resulting polypeptide is glycosylated at 4 glycosylation sites, giving the 67 kDa p1CPY form. Following this, p1CPY is then translocated to the Golgi where the glycan chains are further modified giving the 69 kDa p2 form of CPY. Finally, p2CPY is trafficked to the vacuole where the pro-domain is released by the Pep4p protease leaving the 61 kDa mature form of CPY (mCPY) (Stevens *et al.*, 1986).

Like CPY, DPAP B is vacuolar protease that is trafficked to the vacuole via the secretory pathway. DPAP B is a type II integral membrane protein that possesses a non-cleavable signal sequence which functions to tether the protein to the membrane. Once DPAP B has been translocated into the ER it is glycosylated and receives 5-8 N- linked glycan chains (Roberts *et al.*, 1989) before being trafficked to the Golgi where the glycan chains are further modified. Finally it is trafficked to the vacuole where the mature form of DPAP B (mDPAP B) functions as a 120 kDa polypeptide (Roberts *et al.*, 1989).

Following the depletion of *Sss1p* in FKY198 cells that had been transformed with pRS313 vector control, immunoprecipitation isolated only ppCPY (figure 4.7A, lane 3) and pDPAP B (figure 4.7 B, lane 3) again demonstrating that *Sss1p* is required for both ER translocation pathways. Immunoprecipitation of secretory precursor proteins from cells where *SSS1* was expressed from a plasmid following the repression of the *GAL10-SSS1* allele identified the 67 kDa p1 and 69 kDa p2 forms of CPY (figure 4.7 A, lane 1) as well as the 120

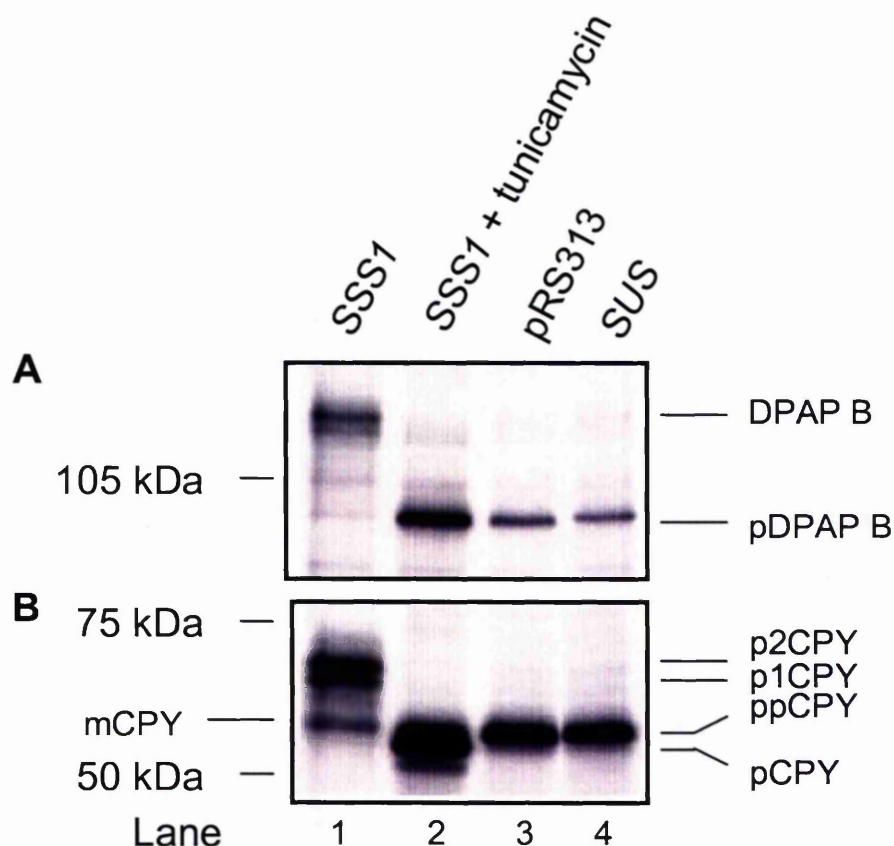


Figure 4.7 Investigating the translocation of secretory precursors into cells expressing SUS. FKY198 cells that had been transformed with either pRS313 vector control (BWY585) or pJKB2 (BWY586) or pBW236 (BWY591), expressing SSS1 or SUS respectively, were grown to early exponential phase in low sulphate YNB containing 2% galactose. At this point, cells were switched to low sulphate YNB supplemented with 2 % glucose and grown for 6 hours to repress the *GAL10-SSS1* allele. One aliquot of FKY198 cells transformed with pJKB2 was incubated with 10 μ g/ml tunicamycin for 1 hour. 5 O.D._{600nm} of cells were isolated, resuspended in 1 ml of pre-warmed low sulphate YNB and incubated with 20 μ Ci/ O.D._{600nm} ³⁵S methionine/cysteine for 5 minutes. After this total cell protein extracts were prepared and then incubated with either 2 μ l/ O.D._{600nm} of anti DPAP B antibodies (A) or 1 μ l/ O.D._{600nm} of anti CPY antibodies (B) after which antibodies were immobilised with Protein A conjugated sepharose beads. Samples were then incubated with 1X Laemmli sample buffer, analysed by SDS-PAGE and visualised by autoradiography (taken from Wilkinson *et al.*, in preparation).

kDa mature form of DPAP B (figure 4.7, lane 1). This indicates that ER translocation proceeds normally under these conditions. However, cells expressing SUS were found to accumulate both ppCPY (figure 4.7 A, lane 4) and pDPAP B (figure 4.7 B, lane 4) following a shift from galactose containing media to media containing glucose. This indicates that the Sss1 protein requires a functional transmembrane domain for ER translocation.

4.2.3 Investigating the ability of SUSp to be crosslinked to both Sec61p and Sec63p

A mutant of *sss1* where the transmembrane domain of this protein has been substituted with that of an unrelated protein is non-functional. This implies that the TM of Sss1p has a function as well as to anchor this protein to the ER membrane. Sss1p is an essential component of the Sec61 complex and we have demonstrated that this protein can also interact with Sec63p. Therefore, we wondered whether this chimera could still form these interactions. As previously reported, association of Sss1p with Sec61p and a newly defined interaction with Sec63p can be assayed by chemical crosslinking with DSS. Therefore, this assay was employed to examine whether the SUS mutant could form the same cross-links as Sss1p in order to investigate its ability to form protein complexes required for ER translocation. Microsomes were harvested from FKY198 that had been transformed with either pJKB2 or pBW236 after growth in glucose containing media for 6 hours and cross-linked as previously described. The samples were then probed for Sss1p containing adducts. DSS cross-linking in microsomes harvested from cells expressing a plasmid borne copy of *SSS1* again isolated the two major DSS specific adducts of approximately 46 kDa and 85 kDa corresponding to an adduct between Sss1p and Sec61p or Sec63p respectively (figure 4.8, compare lanes 1 and 2). Analysis of microsomes isolated from cells expressing SUS indicated that the SUS mutation

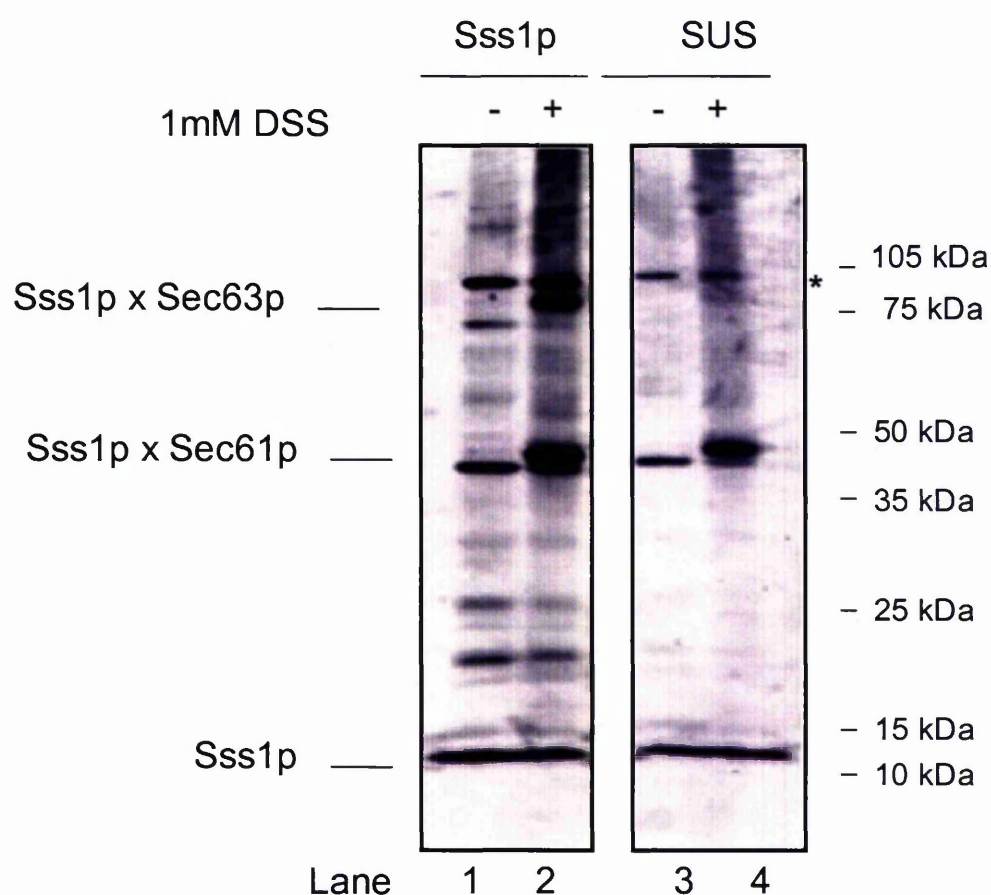


Figure 4.8 DSS crosslinking profile of SUSp. Membranes were isolated from FKY198 cells that had been transformed with either pJKB2 (BWY586) or pBW236 (BWY591) grown in YP media supplemented with 2% glucose for 6 hours to repress the *GAL10-SSS1* allele. After this, 1 A_{280nm} equivalent of membranes was either mock treated with DMSO or incubated with 1 mM DSS at 30°C for 30 minutes. After this, crosslinking was quenched by incubating samples with 100 mM lysine and glycine for 5 minutes, incubated with an equal volume of 2X Laemmli sample buffer and samples were analysed by SDS-PAGE on an 8-15% linear gradient. After this samples, were either probed with anti Sss1p antibodies. Sss1p and Sss1p crosslinked adducts are indicated (taken from Wilkinson *et al.*, in preparation).

did not appear to affect the levels of the SUS protein at steady state as the levels of Sss1p and SUSp are comparable (figure 4.8, compare lanes 1 and 3). Also the efficiency with which the 46 kDa adduct with Sec61p was generated appeared to be comparable between Sss1p and SUSp (figure 4.8, compare lanes 2 and 4). However the efficiency with which the 85 kDa was generated was markedly reduced in microsomes containing SUSp but not Sss1p (figure 4.8, compare lanes 2 and 4). This data indicates that the SUS mutation does not affect the ability of the protein to be cross-linked via DSS to Sec61p but perturbs the ability to be cross-linked to Sec63p. This implies that an interaction between Sss1p and Sec63p requires the transmembrane domain of Sss1p.

4.3 Analysis of the Sec61 complex in membranes isolated from cells expressing SUS

The failure to crosslink SUSp to Sec63p may be direct where the transmembrane domain of Sss1p is required to recruit Sec63p to the translocation channel. Alternatively this may be indirect as the observed DSS dependent interaction between Sss1p and Sec63p may be dependent on the Sec61 complex and although the SUS does not perturb the efficiency with which it can be cross-linked to Sec61p, the interaction may be aberrant and the resulting change in Sec61 complex conformation may affect the cross-linking between Sss1p and Sec63p.

To discriminate between these two possibilities it is necessary to investigate whether the manner which SUS interacts with Sec61p resembles that of Sss1p. Previously, it was identified in chapter 3 that the endogenous cysteine residue in Sss1p, C39, can be covalently coupled by either BMH or o-PDM to a mutant of Sec61p that contains three consecutive missense mutations in TM9 where the mutagenised codons encode cysteine. It has also been

proposed that one face of the Sss1p TMD makes single point contacts with TM 1, 5, 6 and 10 of Sec61p and this may be sufficient to brace the N- and C- terminal halves of Sec61p to form a functional translocation channel (van den Berg *et al.*, 2004). We therefore reasoned that if SUS can interact with Sec61p in an identical manner to that of Sss1p a BMH dependent cross-link between C39, which is also present in SUS, and the functional Sec61p^{TM9CCC} mutant when both are co-expressed would be detected. To do this the methodology used in chapter 3 was employed where a strain that has the genomic copy of both *sec61Δ* and *sss1Δ* deleted where cell viability is sustained by the expression of these genes from a plasmid (CMY5). However, the SUS mutation is not functional so it is required that a plasmid born copy of *SSS1* where its expression can be regulated by the growth conditions.

4.3.1 Creating a *MET3* regulated allele of *SSS1*

Plasmid pCM201 (*TRP1 SSS1*) was constructed as a cloning intermediate for the expression of *sss1* mutants in CMY5. Therefore, a methionine regulated *MET3-SSS1* allele was constructed, based on pCM201, which can be transformed into CMY5. pCM201 was mutagenised with the pJKB18 5'NdeI forward and reverse mutagenic primers (previously described in chapter 3.2) to incorporate an *NdeI* restriction site immediately upstream of the *SSS1* translation initiation codon. Following this, the *SSS1* promoter region was excised out of the *SSS1* gene and replaced with the *MET3* promoter that had previously been subcloned into plasmid pJT31 (J. R. Tyson, 2000). The *SSS1* promoter was excised as a 700 bp *SalI/NdeI* fragment where pCM201 5'NdeI was initially digested with *NdeI* restriction endonuclease and following this the cohesive ends were treated with Klenow enzyme to create a blunt restriction site. The plasmid was then digested with *SalI* and the promoterless; pCM201 5'NdeI fragment was extracted from a 1% agarose gel. The *MET3* promoter was isolated from pJT31 by, firstly, digesting the plasmid with *BamHI* restriction enzyme and the cohesive ends were blunted with

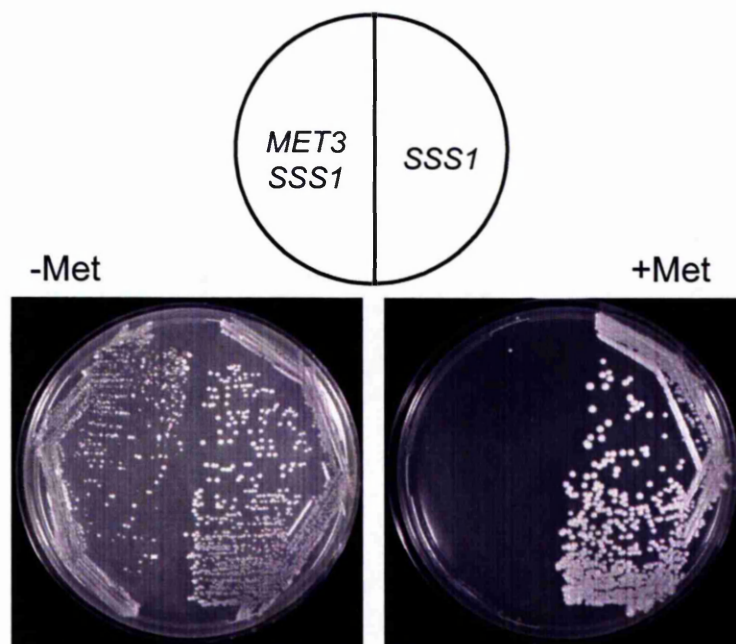


Figure 4.9 Isolating a *MET3* regulated allele of *SSS1*. BWY530 cells were transformed with plasmids expressing either pCM201 (CMY39) or pCM214 (CMY40) and transformants were selected for tryptophan prototrophy. After this, cells plated out onto YNB containing 1 mg/ml 5'-FOA to counterselect for the *URA3* selective, *SSS1* encoding plasmid. pCM201 encodes for *SSS1* whereas pCM214 encodes for a methionine regulatable allele of *SSS1*. CMY39 and CMY40 cells were plated out onto either YNB minus methionine (-Met) or YPD (+Met) and incubated at 30°C for 2 days.

Klenow enzyme. The plasmid was then digested with *Sall* and the 700 bp *MET3* promoter was isolated from a 1.5% agarose gel. These two fragments were then ligated and the ligation reaction was used to transform electrocompetent *E. coli* where the plasmid encoding *MET3*-*SSS1* (pCM214) was subsequently isolated.

BWY530 cells were transformed with pCM214 and subsequently counter selected for the *SSS1 URA3* plasmid. After this, cells were found to grow on media that did not contain methionine but could not grow on nutrient rich media (figure 4.9, panel 1 and 2). This is consistent with the *SSS1* gene being under the control of the methionine regulated *MET3* promoter.

In order to characterise non-functional *sss1* mutants in cells that are co-transformed with this plasmid it is necessary to further characterise the repression profile of this construct. Western blot analysis of cells grown in the absence of methionine showed the expression of *SSS1* at all time points (figure 4.10 C, lanes 1-5). There appeared to be a noticeable defect in the translocation of alpha factor in cells expressing *SSS1* under the control of the *MET3* promoter as sufficient levels of pp α f were observed at all time points where *SSS1* was expressed (figure 4.10 B, lanes 1-5). As the *MET3* promoter has been previously shown to be a low expression promoter (Mao *et al.*, 2002), the observed translocation defect in cells expressing wild type protein under the control of this non-native promoter may be the consequence of the Sss1 protein becoming limiting for ER translocation.

Following growth in nutrient rich media the level of Sss1p was found to be rapidly diminished (figure 4.10 C, lanes 6-10) Indeed, Sss1p was barely detectable after growth in nutrient rich media for between 6-8 hours (figure 4.10 B, lanes 9 and 10). This was also correlated to a concomitant elevation in pp α f accumulation, consistent with the loss of this essential protein (figure 4.10 B, lanes 6-10). The reduction in Sss1 protein levels in cells grown in nutrient rich media compared to when methionine has been omitted, as well as the

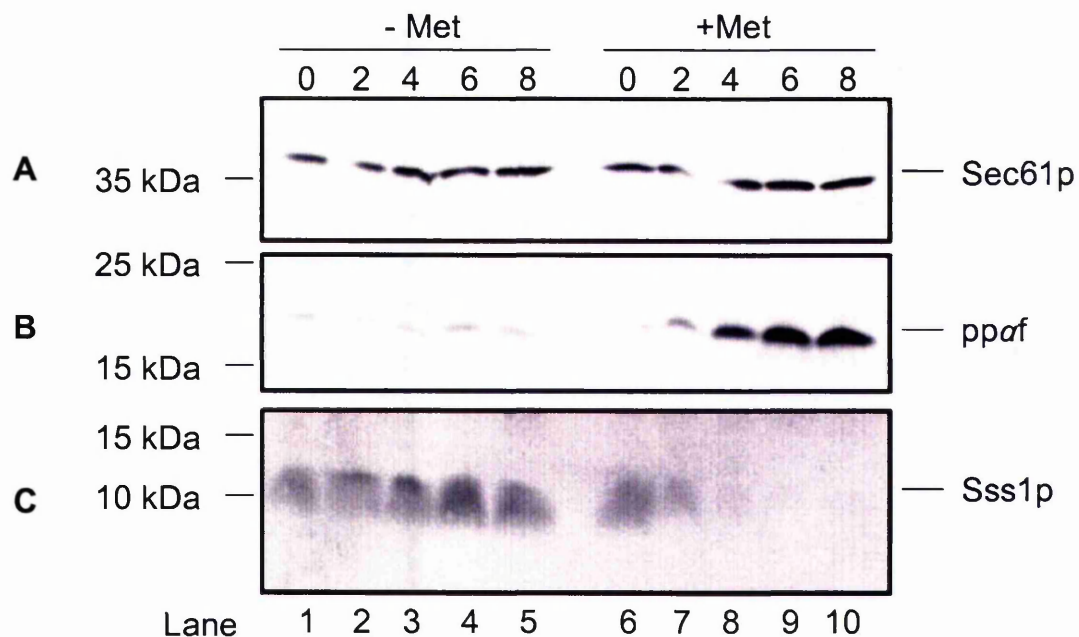


Figure 4.10 Expression profile of *MET3-SSS1*. CMY40 cells expressing a plasmid borne copy of a methionine regulatable allele of *SSS1* were grown in YNB minus methionine to early exponential growth phase and then switched into either fresh selective media minus methionine (-Met) or YPD (+Met) and incubated at 30°C for either 0, 2, 4, 6 or 8 hours. Total cell protein extracts were prepared from 5 O.D._{600nm} of cells from the specified time points and 0.1 O.D._{600nm} equivalents of each sample was analysed by SDS-PAGE. After western transfer samples were incubated with either anti Sec61p antibodies (A), anti alpha factor antibodies (B) or anti Sss1p antibodies (C).

inability of these cells to grow on media containing methionine indicates that the pCM214 plasmid encodes for a regulatable allele of *SSS1* whose expression is repressed by methionine. Therefore, this is a suitable regulatable allele of *SSS1* that allows the expression and analysis of non-functional alleles of *sss1*.

4.3.2 Investigating the interaction of SUSp with Sec61p by site specific crosslinking

CMY5 was then transformed with pCM108 (Sec61pTM9CCC) and pCM214 (*MET3-SSS1*) by electroporation and transformants were selected for leucine and tryptophan prototrophy. Following this, cells were counterselected for the *URA3 SSS1 SEC61* (pCM203) plasmid by selecting for cells that could grow on media supplemented with 5-FOA but not methionine, giving CMY28.

To allow for the selection of an SUS encoding vector in this strain, the SUS coding sequence had to be subcloned into a vector that contained the *URA3* selectable marker. The FKp52 plasmid is a yeast centromeric vector that encodes for *SSS1* and transformation with this plasmid confers uracil prototrophy. The sequence encoding for SUS was subcloned into the multiple cloning site of pRS316 as a 1.1 kb *Sall/SmaI* fragment giving pCM209. Following this, CMY28 was transformed with either pRS316, FKp52 or pCM209 and transformants were selected for uracil prototrophy.

As the protein product of the *MET3-SSS1* allele also contains the cysteine residue that can promote covalent coupling of Sss1p to the Sec61pTM9CCC mutant protein, it is essential to show that this adduct is no longer detected following the methionine dependent depletion of Sss1p from membranes isolated from cells containing the *MET3-SSS1* allele. Previous analysis has indicated that the Sss1 protein can no longer be detected following the growth of cells

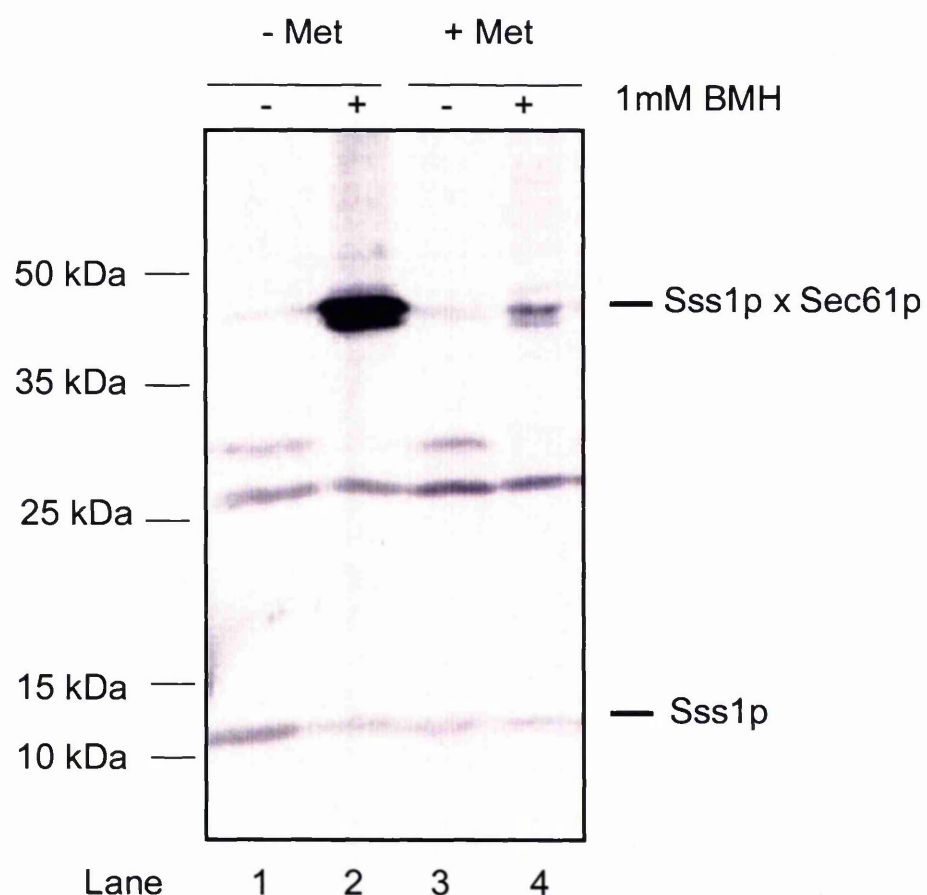


Figure 4.11 BMH dependent crosslinking profile of Sss1p after the repression of *MET3-SSS1*. CMY5 cells were transformed with pCM108 (Sec61p TM9CCC) and pCM214 (*MET3-SSS1*) by electroporation and transformants were selected for tryptophan and leucine prototrophy giving CMY28. CMY28 cells were then transformed with pRS316 by electroporation and transformants were selected for uracil prototrophy. CMY28 cells were grown in YNB minus methionine to early exponential growth phase and then switched into either fresh YNB minus methionine (-Met) or YPD (+Met) and grown at 30°C for 8 hours. After this, membranes were isolated from these cells, mock treated with DMSO or incubated with 1 mM BMH at 30°C for 30 minutes. After this, crosslinking was quenched by incubating samples with 100 mM DTT for 5 minutes, incubated with an equal volume of 2X Laemmli sample buffer and analysed by SDS-PAGE on an 8-15% linear gradient. After western transfer, samples were immunoblotted with anti Sss1p antibodies. Sss1p and Sss1p crosslinked adducts are indicated.

containing pCM215 have grown in methionine rich media ([methionine] ≥ 2 mM) for 6-8 hours (figure 4.10 C, lanes 9 and 10). Membranes were isolated from cells that had been grown in methionine containing media for 8 hours and then incubated with BMH (1 mM final concentration). After this, samples were prepared for SDS-PAGE and immunoblot analysis. Incubation of microsomes isolated from cells where *SSS1* had been expressed with BMH shows one major Sss1p containing adduct of 46 kDa corresponding to a BMH dependent cross-link between Sss1p and Sec61p^{TM9CCC} (figure 4.11 A, lane 2). The same cross-linking was then performed using microsomes where Sss1p had been depleted. As expected, the 46 kDa adduct was absent in these microsomes (figure 4.11 A, lane 4). This indicates that isolation of a 46 kDa adduct in microsomes isolated from cells expressing SUS following the repression of *MET3-SSS1* would indicate that the interaction is SUS dependent.

The SUS interaction with Sec61p was then investigated using the same cross-linking procedure. Membranes were isolated from CMY28 cells transformed with either FKp52 (CMY29) or pCM209 (CMY30) after growth in nutrient rich media for 8 hours and incubated with 1 mM BMH. Membranes were then probed with anti Sec61p antibodies to the ability of either Sss1p or SUSp to be chemically coupled to Sec61p^{TM9CCC}. Immunoblot analysis of microsomes isolated from cells expressing a plasmid born copy of *SSS1* again identified the BMH dependent 46 kDa adduct between Sec61p^{TMCCC} and Sss1p (figure 4.12, lane 1). The 46 kDa adduct was also observed in microsomes harvested from cells expressing SUS after treatment with BMH (figure 4.12, lane 2) although the efficiency with which this adduct was generated in these microsomes was lower than that identified in microsomes containing Sss1p (figure 4.12, compare lanes 1 and 2). This data along with the comparable efficiencies with which Sss1p and SUSp can be covalently coupled to Sec61p via DSS dependent cross-linking (figure 4.9, compare lanes 2 and 4) may imply that SUSp interacts with Sec61p in a manner similar to which Sss1p interacts with Sec61p and may therefore support a model where

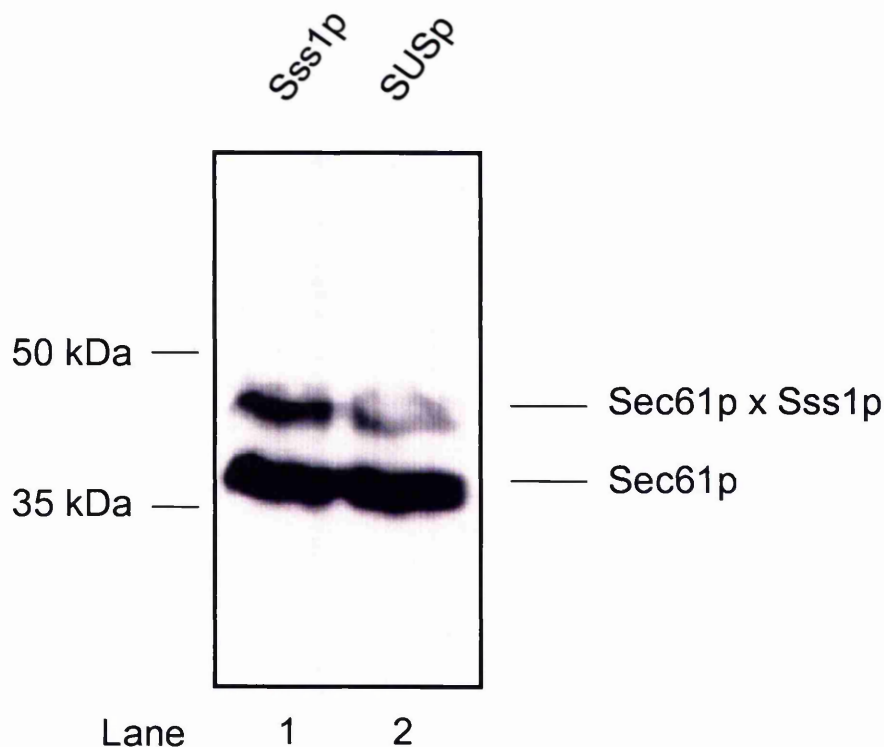


Figure 4.12 Investigating the interaction of SUSp with Sec61p by site specific crosslinking. CMY27 cells were transformed with either FKp52 (*SSS1*) or pCM209 (*SUS*) by electroporation and transformants were selected for uracil prototrophy giving CMY29 and CMY30 respectively. CMY29 and CMY30 were grown to early exponential phase in YNB without methionine and then switched into YPD and incubated at 30°C for 8 hours to repress the *MET3-SSS1* allele. After this, membranes were isolated from these cells, incubated with 1 mM BMH at 30°C for 30 minutes. After this, crosslinking was quenched by incubating samples with 100 mM DTT for 5 minutes, incubated with an equal volume of 2X Laemmli sample buffer and analysed by SDS-PAGE. After western transfer, samples were immunoblotted with anti Sec61p antibodies. Sec61p and Sec61p crosslinked adducts are indicated.

one function of the Sss1p transmembrane domain is to recruit Sec63p to the Sec61 complex to form functional translocons.

4.4 Probing the molecular environment of the Sss1p C- terminus

This study has indicated that chemical cross-linking is an appropriate molecular tool to investigate the interactions formed by Sss1p to instigate the translocation of proteins into the ER. The previous results have investigated such interactions by lysine specific chemical cross-linking. The Sss1 protein contains 11 lysine residues however, 10 of these residues are present in the N- terminal 52 residues. Therefore, probability would predict that the observed cross-linking is likely to indicate the molecular environment of the N- terminal 52 residues. Therefore, chemical cross-linking was used to investigate the molecular environment of the extreme C- terminus of Sss1p. An Sss1p mutant was engineered where a cysteine residue was incorporated at the extreme C- terminus which would ultimately allow the C- terminal environment of Sss1p to be investigated by cysteine dependent chemical cross-linking. pJKB2 was mutagenised by site directed mutagenesis using the *sss1* 81C forward and reverse primers giving pCM207 and this plasmid was then used to transform BWY530. This mutant form of *sss1* was found to be functional as it could complement the otherwise lethal *sss1* null mutation. Following this membranes were harvested from cells expressing the Sss1p 81C mutant, treated with cysteine specific homobifunctional cross-linking reagents and probed with anti Sss1p antibodies to identify Sss1p containing adducts. Analysis of microsomes isolated from a wild type strain of yeast, w303 α , with 1 mM (final concentration) BMH with anti Sss1p antibodies did not identify any cysteine dependent adducts with the non-mutagenised protein (figure 4.13, lane 2). This implies that the isolation of Sss1p containing adducts by cysteine dependent cross-linking in membranes containing Sss1p 81C is the consequence of this Sss1p mutation.

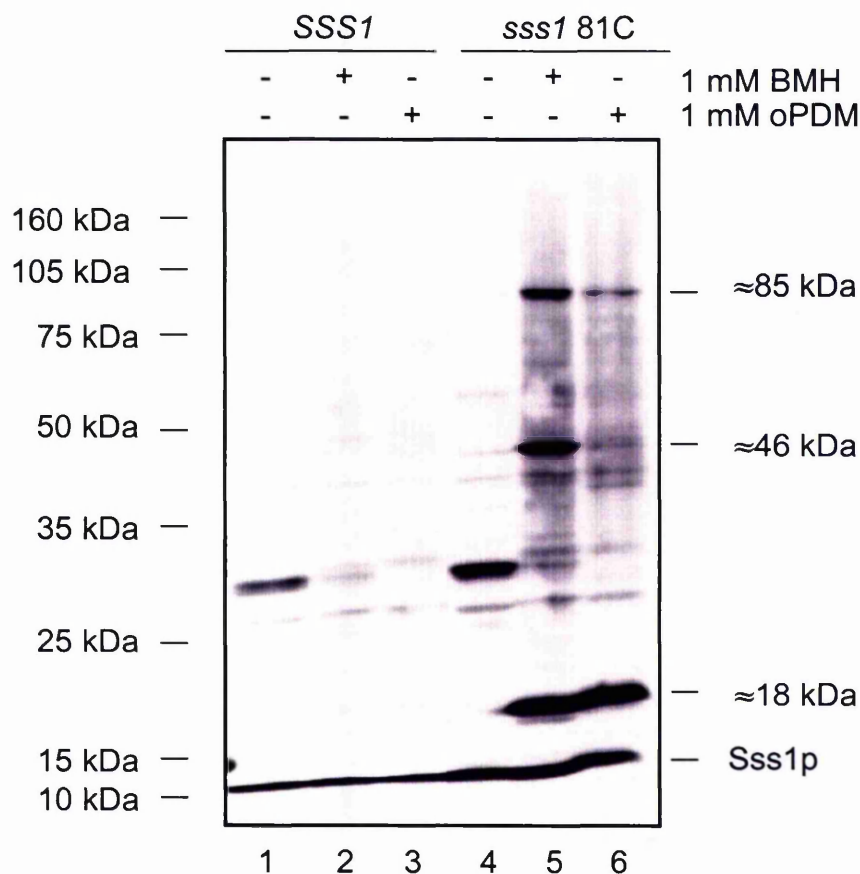


Figure 4.13 Investigating the molecular environment of the extreme C- terminus of Sss1p by cysteine dependent crosslinking. BWY530 cells were transformed with pCM207 by ONE-STEP transformation and transformants were selected for histidine prototrophy. After this, transformants were plated out onto YNB containing 1 mg/ml 5'-FOA to counterselect the *URA3* selective *SSS1* encoding vector giving strain CMY23.1 A_{280nm} equivalent of membranes isolated from either wildtype cells (w303 α) or *sss1 81C* (CMY23) were either mock treated with DMSO or incubated with either 1 mM BMH or 1 mM o-PDM at 30°C for 30 minutes. After this, crosslinking was quenched by incubating samples with 100 mM DTT for 5 minutes, incubated with an equal volume of 2X Laemmli sample buffer and analysed by SDS-PAGE on an 8-15% gradient. After western transfer, samples were immunoblotted with anti Sss1p antibodies. Sss1p and Sss1p crosslinked adducts are indicated.

4.4.1 The C- terminus of Sss1p can be crosslinked to Sec61p and Sec63p

Following the treatment of microsomes harvested from cells expressing Sss1p 81C with 1 mM BMH, western analysis with anti Sss1p antibodies identified three major Sss1p containing adducts of approximately 18, 46 and 85 kDa (figure 4.13, lane 5). The 18 and 85 kDa adducts were also detected following the treatment of membranes with o-PDM (figure 4.13, lane 6). This compound differs to BMH in its cross-linking arm. BMH is a flexible cross-linking reagent that is 15Å whereas o-PDM is a rigid cross-linking reagent that is 6Å. Therefore the 18 and 85 kDa adducts represents the crosslinking of Sss1p to very close interacting partner proteins.

Both the 46 and 85 kDa adducts were identified in wild type membranes by DSS cross-linking (figure 4.1, lane 2) and represent the cross-linking of Sss1p to Sec61p (Esnault *et al.*, 1994) and Sec63p (figure 4.4). Therefore Sec61p and Sec63p were the prime candidates to be the cross-linking partner of Sss1p in the 46 and 85 kDa adduct respectively. To investigate this both the Sec61p and Sec63p BMH dependent cross-linking profiles were investigated in membranes isolated from both w303 α and CMY23 (Sss1p 81C). Analysis of membranes isolated from w303 α that had been treated with BMH with both anti Sec61p and anti Sec63p antibodies did not identify any BMH dependent cross-links as treated microsomes appeared as untreated microsomes (figure 4.14 A and B, compare lanes 1 and 2). Again this indicates that the isolation of BMH dependent adducts in membranes harvested from CMY23 is a consequence of the Sss1p 81C mutation. However, a 46 kDa Sec61p containing adduct (figure 4.14 A, lane 5) and a 85 kDa Sec63p containing adduct (figure 4.13 B, lane 5) was isolated in membranes containing the Sss1p mutant indicating that the C- terminus of Sss1p can be covalently coupled, and is therefore in close proximity to both Sec61p and Sec63p.

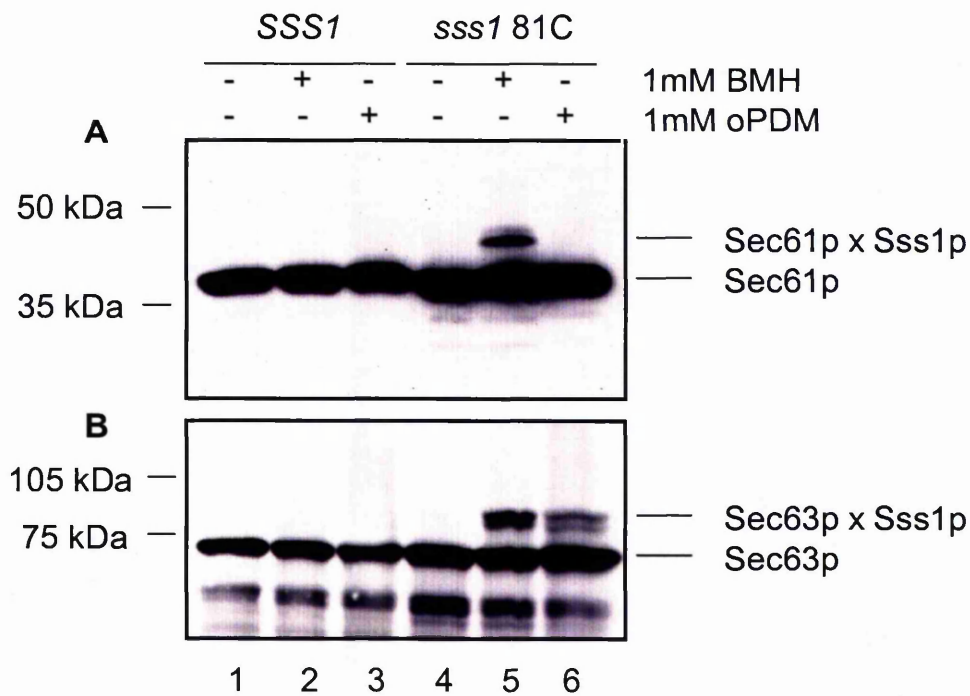


Figure 4.14 The C- terminus of Sss1p can be crosslinked to both Sec61p and Sec63p. 1

A_{280nm} equivalent of membranes isolated from either wildtype cells (w303 α) or cells expressing a plasmid borne copy of *sss1 81C* (CMY23) were either mock treated with DMSO or incubated with either 1 mM BMH or 1 mM o-PDM at 30°C for 30 minutes. After this, crosslinking was quenched by incubating samples with 100 mM DTT for 5 minutes, incubated with an equal volume of 2X Laemmli sample buffer and analysed by SDS-PAGE on an 8-15% gradient. After this, samples were probed with either anti Sec61p antibodies (**A**) or anti Sec63p antibodies (**B**).

Interestingly the 85 kDa cross-linked adduct detected by anti Sec63p antibodies appeared to be comprised of two species.

Sequence and hydropathy analysis of the Sec63 protein indicates that there is a single cysteine residue at the membrane/luminal interface of TM3 that could be covalently coupled to the C- terminus of Sss1p. Therefore, this indicates that the extreme C-terminus of Sss1p is in close proximity to TM3 of Sec63p.

4.4.2 The C- terminus of Sss1p can be cross-linked to TM3 and 4 of Sec61p

As reported in chapter 3, the Sec61 protein contains three cysteine residues located in TM3, 4 and 8. To identify the residue in Sec61p that can be covalently coupled to the C-terminus of Sss1p in a BMH dependent manner, Sss1p 81C was co-expressed with plasmid expressing either Sec61p, Sec61p_{C121A}, Sec61p_{C150A}, Sec61p_{C121, 150AA} or a cysteineless copy of Sec61p, into CMY5. To allow for the selection of CMY5 transformants with the Sss1p 81C mutation, this mutation was incorporated into pCM201. Following co-transformation, transformants were subsequently counter selected for pCM203. Membranes were then isolated from each of these strains and investigated for their ability to form the BMH dependent Sss1p x Sec61p adduct. This adduct was again isolated in membranes co-expressing Sss1p 81C and Sec61p (figure 4.15, lane 2) but was absent in membranes where Sss1p 81C was co-expressed with a cysteineless copy of Sec61p (figure 4.15, lane 4). This again confirms that this is a cross-link between Sss1p and Sec61p. Membranes harvested from cells co-expressing Sss1p 81C with either Sec61p_{C121A} or Sec61p_{C150A} were still competent for the formation of the 46 kDa adduct (figure 4.15, lanes 6 and 8). However, the 46 kDa adduct was not isolated in membranes harvested from cells co-expressing Sss1p 81C with Sec61p_{C121, 150AA}

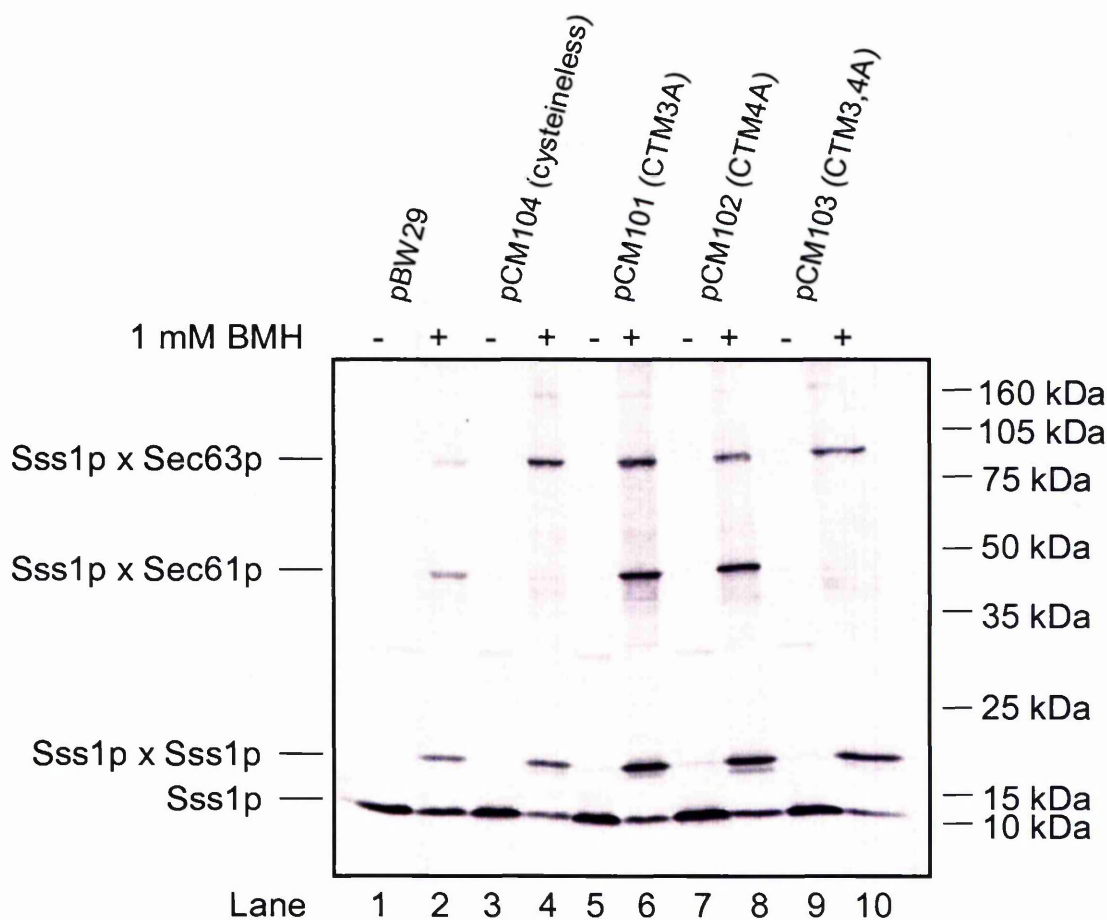


Figure 4.15 The C- terminus of Sss1p is in close proximity to TM3 and TM4 of Sec61p. CMY5 cells were co-transformed with pCM218 (*sss1* 81C) and either pBW29, pCM104 (cysteineless Sec61p) pCM101 (Sec61p_{C121A}, TM3), pCM102 (Sec61p_{C150A}, TM4) or pCM103 (Sec61p_{C121AC150A}, TM3, 4) by electroporation and transformants were selected for tryptophan and leucine prototrophy. After this, transformants were plated out onto YNB containing 1mg/ml 5'-FOA counterselected for the *URA3* selective plasmid encoding *SEC61* and *SSS1*. Membranes were isolated from these cells and 1 A_{280nm} equivalent of membranes were mock treated with DMSO or incubated with 1 mM BMH at 30°C for 30 minutes. After this, crosslinking was quenched by incubating samples with 100 mM DTT for 5 minutes, incubated with an equal volume of 2X Laemmli sample buffer and analysed by SDS-PAGE on an 8-15% gradient. After western transfer samples were immunoblotted with anti Sss1p antibodies. Sss1p and Sss1p crosslinked adducts are indicated.

(figure 4.15, lane 10). This indicates that the extreme C-terminus of Sss1p can be covalently coupled, and is therefore in proximity to either TM3 (C121) or TM4 (C150) of Sec61p.

4.4.3 Sss1p can form dimers

Structural analysis of the mammalian translocon has indicated that three to four copies of the Sec61 complex oligomerise to form the translocon (Hanein *et al.*, 1996). Subsequently, biochemical analysis has identified that Sec E in *E. coli* can dimerise with an adjacent Sec E molecule (Veenendaal *et al.*, 2001). Isolation of an 18 kDa Sss1p containing adduct in Sss1p 81C mutant membranes may indicate that the translocon is indeed oligomeric in yeast. To investigate whether the 18 kDa adduct does represent the cross-linking of two adjacent Sss1p molecules we compared the BMH dependent cross-linking profile of both Sss1p 81C and a polyhistidine tagged derivative of this mutant (10x His Sss1p 81C).

A 10x His Sss1p 81C copy of the Sss1p 81C mutant was created by fusing a 10x His tag and linker region, derived from pET16b, immediate upstream of the Sss1p initiation methionine. Firstly, pCM207 was mutagenised with pJKB18 5'NdeI forward and reverse primers to incorporate an *NdeI* restriction site immediately 5' of the Sss1p start codon. After this, this vector was cut with *NdeI* and *SspI* and a 500bp *SSSI* coding sequence was isolated after agarose gel electrophoresis. This fragment was then ligated into pET16b creating a polyhistidine tagged copy of the Sss1p 81C mutant. Following this, this vector was cut with *XbaI* and *SspI* and ligated into the *XbaI* and *SmaI* restriction sites in pRS313. Finally, the 10X His Sss1p81C coding sequence was fused downstream of the *SSSI* promoter. pCM201 5'NdeI was cut with *NdeI* and then incubated with Klenow enzyme. This was then incubated with *EcoRI* restriction endonuclease and the 5.4 kb pRS314 fragment containing the *SSSI* promoter was isolated after agarose electrophoresis. The cloning intermediate where 10x His Sss1p 81C had been ligated into pRS313 was cut with *NcoI* and then treated with Klenow enzyme and a

565 bp fragment encoding for the 10x His Sss1p 81C was isolated after cutting with *EcoRI* and then ligated into the *EcoRI*, *NdeI* (blunt) restriction sites of the 5.4 kb pRS314 fragment containing the *SSS1* promoter. The 10x His Sss1p 81C mutant was found to be functional as it could complement the lethal *sss1* null mutation (data not shown).

To investigate the nature of the 18 kDa Sss1p containing adduct identified in membranes harvested from cells expressing the Sss1p 81C mutation we compared the BMH dependent cross-linking profile of Sss1p81C and a polyhistidine tagged derivative of this mutant. Sequence analysis predicts that the non-crosslinked tagged derivative of Sss1p 81C has a rMw of approximately 11 kDa and if this species was to crosslink to itself it would form an adduct of approximately 22 kDa. An 18 kDa Sss1p adduct and the 10 kDa non-crosslinked material was again identified in membranes containing the Sss1p 81C mutant (figure 4.16, lanes 1 and 2). The non-crosslinked polyhistidine tagged derivative of Sss1p 81C was found to migrate as expected (figure 4.16, lane 3). However, an Sss1p containing adduct of approximately 21 kDa was identified upon incubation of membranes with BMH (figure 4.16, lane 4). The size difference between the non-crosslinked Sss1p 81C and 10X His Sss1p 81C is indicative of one polyhistidine tag of approximately 2 kDa (figure 4.16, compare lanes 1 and 3). However, the adduct is approximately 4 kDa heavier in membranes containing the 10X His Sss1p than those containing Sss1p 81C and this difference is consistent with two polyhistidine tags. This data is consistent with the Sss1p containing adduct of approximately 18 kDa identified in membranes containing the functional Sss1p 81C mutant representing the covalent coupling, and potential dimerisation, of two adjacent Sss1p molecules.

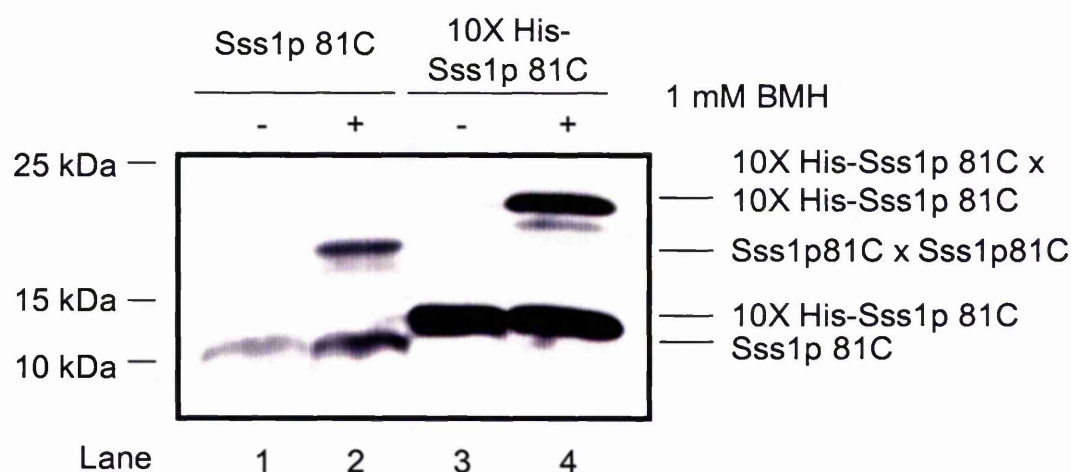


Figure 4.16 Characterising the 18 kDa Sss1p containing crosslinked adduct. BWY530 cells were transformed with either pCM207 (Sss1p81C) or pCM217 (10X His-Sss1p81C) by ONE-STEP transformation and transformants were selected for histidine prototrophy. After this, transformants were plated out onto YNB containing 1mg/ml 5'-FOA counterselected for the *URA3* selective plasmid encoding *SSS1* giving strains CMY23 and CMY31 respectively. Membranes were isolated from either CMY23 or CMY31 and 1 A_{280nm} equivalent of membranes were either mock treated with DMSO or incubated with 1 mM BMH at 30°C for 30 minutes. After this, crosslinking was quenched by incubating samples with 100 mM DTT for 5 minutes, incubated with an equal volume of 2X Laemmli sample buffer and analysed by SDS-PAGE. After western transfer samples were immunoblotted with anti Sss1p antibodies. Sss1p and Sss1p crosslinked adducts are indicated.

4.5 C- terminal interactions of Sss1p are ribosome independent

The ER translocon has been shown to form a regulated channel in the ER membrane (reviewed in Johnson and van Waes, 1999). Structural studies have predicted the size of the translocon pore to be approximately 40-60 Å in diameter (Hamman *et al.*, 1997) although this analysis appeared to contradict an earlier study that predicted the protein conducting channel to be approximately 20 Å in diameter (Hanein *et al.*, 1996). After this, this discrepancy was later proposed to be the functional consequence of the translocon existing in two states, open and closed. A translocon pore diameter of 40-60 Å was proposed to represent the active translocon a pore width of 20 Å the closed translocon and this switch has been proposed to induce a significant change in translocon conformation (Hamman *et al.*, 1997).

The co-translational translocon has been shown to be gated at the cytosolic face through the interaction of a translating ribosome (Crowley *et al.*, 1993; 1994). However, release of the ribosome at the cytosolic face of the pore has been shown to induce the switch from the open to the closed state resulting in the subsequent recruitment of BiP to seal the luminal face of the translocation channel (Hamman *et al.*, 1998). This process can be simulated *in vitro* through the incubation of membranes with both puromycin and high salt (Hamman *et al.*, 1998). Puromycin is an inhibitor of translation as its structural similarity to tRNA molecules that stop translation means that this drug prematurely terminates translation. After this, the ribosomes interaction with the translocon then becomes susceptible to high salt. With this in mind, we investigated the molecular environment of the extreme C- terminus of Sss1p by chemical crosslinking before and after the treatment of membranes with puromycin and high salt. We reasoned that a difference in the Sss1p crosslinking profile before and after treatment with puromycin and high salt would be indicative of a conformational change in the translocon.

Firstly it was paramount to demonstrate that incubation of membranes with puromycin and high salt does result in the release of ribosomes. The strain BWY436 expresses a HA-tagged derivative of the Rpl25 protein, a component of the large ribosomal subunit. Therefore, this provides a method of monitoring the levels of membrane associated ribosomes by blotting for membrane associated HA-Rpl25p with anti HA antibodies. Immunoblot analysis of membranes harvested from BWY436 that had been mock treated with anti HA antibodies identifies a 20 kDa protein that corresponds to HA- Rpl25p (figure 4.17 A, lane 1). Significantly, the level of this protein is considerably reduced following incubation of these membranes with puromycin and high salt (figure 4.17 A, lane 2) indicating that HA- Rpl25p, and therefore ribosomes, are released from membranes following incubation with puromycin and high salt.

Membranes containing the Sss1 81C mutant protein were treated and mock treated in parallel to membranes isolated from strain BWY436. After this, they were then incubated with increasing concentrations of BMH, specifically 0.5, 1 and 2 mM, and probed for Sss1p containing adducts. The major Sss1p adducts were again observed in membranes that had been mock treated in membrane storage buffer and the adducts were generated with comparable intensities despite elevating the concentration of crosslinking reagent (figure 4.17 B, lanes 2-4). Following the release of ribosomes by puromycin and high salt we again found that each of the Sss1p containing adducts previously described were observed (figure 4.17 B, lanes 6-8), the efficiencies with which they were formed is comparable to that identified in mock treated membranes (figure 4.17 B, compare lanes 2-4 with 5-8). Furthermore, no additional cross-links were identified. This indicates that chemical crosslinking does not identify a change in the molecular environment of the extreme C- terminus of Sss1p following the release of ribosomes from membranes and may imply that a large conformational change is not induced following a switch from an open to a closed translocon.

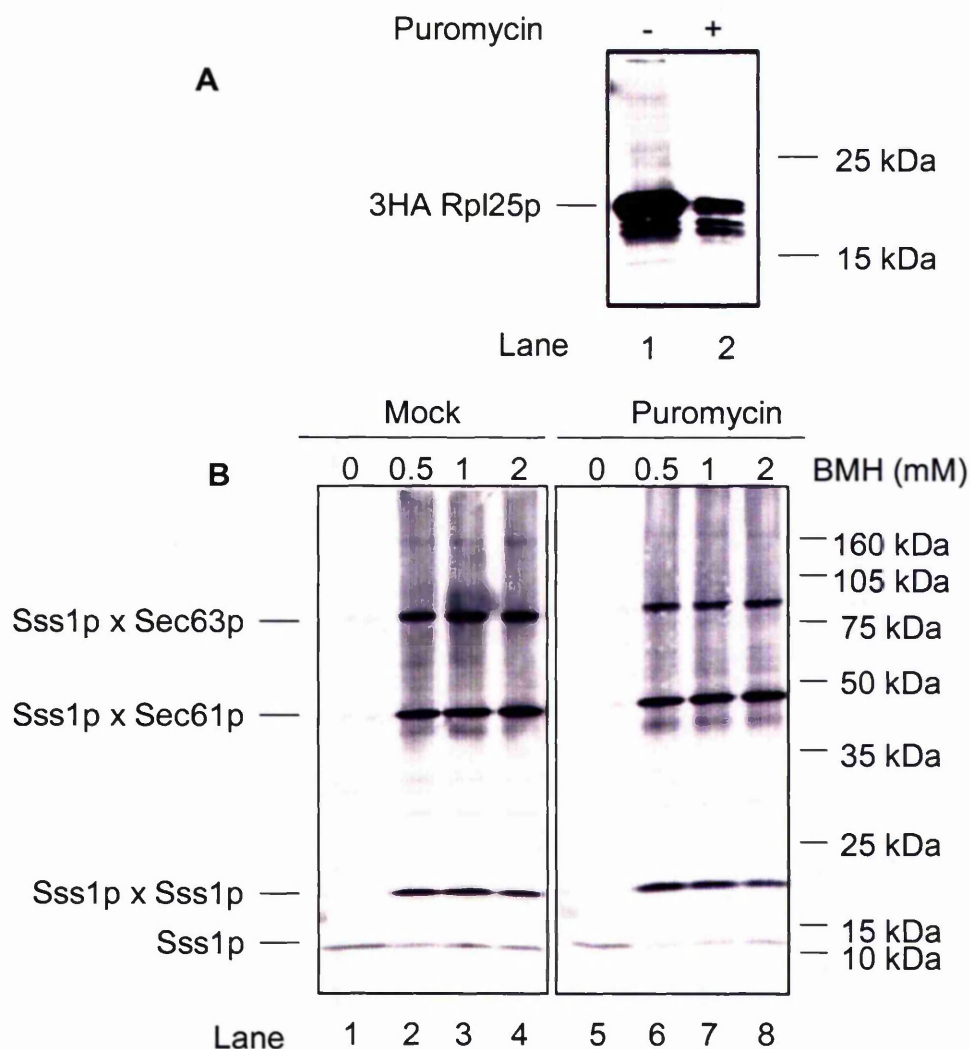


Figure 4.17 Investigating the affect of puromycin on the BMH dependent crosslinking profile of *sss1 81C*. (A) Membranes were isolated from BWY436 cells, which express a 3HA tagged copy of Rpl25p. After this, membranes were either mock treated with membrane storage buffer alone or incubated with membrane storage buffer supplemented with 2 mM GTP and 1 mM puromycin at 30°C for 30 minutes to release ribosomes from membranes. Membranes were recovered at this treatment was repeated twice. After this, membranes were analysed by SDS-PAGE and probed with anti HA antibodies. (B) Membranes isolated from cells expressing *sss1 81C* (CMY23) were treated as above either mock treated with membrane storage buffer alone or incubated with membrane storage buffer supplemented with 2 mM GTP and 1 mM puromycin at 30°C for 30 minutes to release ribosomes from membranes. After this membranes were either mock treated with DMSO or incubated with 1 mM BMH at 30°C for 30 minutes. After this, crosslinking was quenched by incubating samples with 100 mM DTT for 5 minutes, incubated with an equal volume of 2X Laemmli sample buffer and analysed by SDS-PAGE. After western transfer samples were immunoblotted with anti Sss1p antibodies. Sss1p and Sss1p crosslinked adducts are indicated.

4.6 Discussion

The translocation of secretory proteins into the ER is mediated by a multimeric ER membrane complex. The Sec61 complex alone is insufficient to drive the translocation of secretory precursors (Young *et al.*, 2001). However, *in vitro* reconstitution of post-translational translocation has indicated that the heptameric SEC complex is the minimum ER membrane protein requirement (Panzner *et al.*, 1995). Genetic analysis in *Saccharomyces cerevisiae* has indicated that the product of the *SEC63* gene is essential for both Sec62p dependent and SRP dependent protein translocation. As Sec63p is a component of the SEC complex this data would imply that for SRP dependent translocation to proceed, Sec63p must be recruited to the Sec61 complex in order to form a functional translocation channel.

In this chapter the molecular architecture of the translocon has been investigated by chemical cross-linking using the lysine specific homobifunctional cross-linking reagent DSS. Previous analysis has demonstrated that Sss1p is a component of the Sec61 complex (Panzner *et al.*, 1995). Treatment of wild type membranes with DSS results in the formation of two main Sss1p containing adducts of 46 kDa and 85 kDa. The 46 kDa adduct has previously been reported to represent the covalent coupling of Sss1p to Sec61p (Esnault *et al.*, 1994) but the cross-linking partner of the 85 kDa adduct has not been reported. This study has identified the 85 kDa adduct to represent a DSS dependent interaction between Sss1p and Sec63p and this has been confirmed using yeast strains where either Sss1p or Sec63p has been specifically depleted from membranes.

In a related project in the laboratory the nature of the Sss1p-Sec63p interaction has been investigated in more detail. Early topological analysis predicted the Sss1 protein to be tripartite in structure where it was proposed to possess a 40-50 amino acid cytosolic domain, a short transmembrane domain proceeded by a 12 amino acid luminal projection. The contribution of these three domains to Sss1p function was investigated by domain substitution

analysis where the sequence was substituted with that of an alternative ER resident C- terminal anchor protein of non-related function, in this case Ubc6p. Each mutant was found to be functionally defective as they could not complement the otherwise lethal *sss1* null mutation and each mutation was found to induce a total block in ER translocation (J. K. Brownsword, 2000). The loss of function following the substitution of either the cytosolic or luminal domain was attributed to the inability of the *sss1* mutant to interact with the Sec61 protein (personal communication, B. M. Wilkinson). However, substitution of the Sss1p TMD, SUS, was particularly informative. Incubation of membranes harvested from cells expressing SUS with DSS identified the 46 kDa adduct. This implies that the substitution of the Sss1p TMD with that of an unrelated protein does not perturb the ability of the protein to interact with Sec61p. Furthermore, the efficiency with which the adduct was generated was equivalent to that of Sss1p. However, an 85 kDa Sss1p containing adduct was not obtained following the treatment of these membranes with DSS. This would imply that the transmembrane domain of Sss1p is required to recruit Sec63p to the translocation channel, resulting in the formation of an active translocon. Recruitment of Sec63p to the Sec61 complex via a hydrophobic interaction with the TMD of Sss1p may explain the observation that extraction of translocation complexes with detergents such as Triton X-100 results in the destabilisation of the SEC complex as it would disrupt the Sss1p-Sec63p interaction.

The absence of a DSS dependent interaction between SUS and Sec63p may imply that a direct interaction proceeds between the TMD of Sss1p and Sec63p. However, this interaction may be indirect as the SUS mutation may induce a conformational change in the translocon that prevents the recruitment of Sec63p. To investigate this we tested the ability of SUSp to interact with Sec61p via site specific chemical cross-linking. In chapter 3 it was found that the endogenous cysteine residue of Sss1p, C39, can be covalently coupled by BMH to a mutant of Sec61p where 3 consecutive cysteine residues had been incorporated into TM9

of this protein. The SUS protein also contains this residue so it was postulated that if a consequence of the SUS mutation is the formation of an aberrant Sec61 complex then this may manifest itself in the inability of the SUS C39 residue to be covalently coupled to the Sec61p^{TM9CCC} mutant protein. Both *SSS1* and SUS were co-expressed with the *sec61* mutant in the presence of a methionine regulatable allele of *SSS1*. Treatment of membranes isolated from either strain following the repression of the *MET3-SSS1* with BMH indicated that the SUS protein can be covalently coupled to Sec61p^{TM9CCC}. Importantly the efficiency with which SUSp could be coupled to the *sec61* mutant appears to be comparable to Sss1p. This as well as the comparable efficiency to Sss1p with which SUSp can be cross-linked to Sec61p in a DSS dependent manner would imply that the SUS mutation does not affect the conformation of the Sec61 complex indicating that a function of the Sss1p TMD is to recruit Sec63p to the translocation channel. However to be certain of this we require a more robust assay that identifies a direct interaction between the TMD of Sss1p and those of Sec61p.

A study in *E. coli* investigating the assembly of the homologous SecYEG complex reported that the transplacement of TMD3 in SecE, believed to be homologous to the TMD of Sss1p, with that of a non-related TMD resulted in the destabilisation of the SecYEG heterotrimer (Pohlschroder *et al.*, 1996). Structural analysis of an archaeobacterial SecYE β complex has indicated that a function of the SecE TMD is to brace the N- and C- terminal halves of SecY in order to form the translocation channel (van den Berg *et al.*, 2004). The data reported in this study may indicate that the region of the Sss1p TMD that interacts with Sec61p is more tolerant to changes and that this may represent a difference in function of these homologues in their respective organism. Significantly, eubacterial and archaeobacterial post-translational translocation is mechanistically distinct to that in *Saccharomyces cerevisiae*. Furthermore, prokaryotes do not contain homologues of the Sec63 protein. Therefore the

observed recruitment of Sec63p to the Sec61 complex via the TMD of Sss1p would provide a molecular explanation for the differences observed.

The translocation defect observed in the SUS mutant is consistent with the failure of this mutant protein to recruit Sec63p to the Sec61 complex as Sec63p is essential for translocation into the ER. The recruitment of Sec63p to the translocon also has important ramifications regarding the function of Kar2p in this process. The genetic analysis that identified *SEC63* to be essential for ER translocation also identified that the product of the *KAR2* gene is also required for SRP dependent translocation as well as Sec62p dependent translocation. The ATPase activity of Kar2p has been shown to be regulated by the J-domain of Sec63p (Corsi and Schekman, 1997) and this has been shown to be important for ER translocation. Furthermore, Kar2p can also bind to the J-domain of Sec63p. Therefore, Kar2p can be indirectly recruited to the translocon, via the recruitment of Sec63p to the Sec61 complex via the TMD of Sss1p, to drive ER translocation.

Analysis of a functional *sss1* mutant where a cysteine residue has been inserted at the extreme C- terminus, Sss1p 81C, by chemical cross-linking with cysteine specific homobifunctional cross-linking reagents has identified that the extreme C-terminus of Sss1p can also be covalently coupled to Sec63p. Using a variety of cross-linking reagents Sec63p was found to be within 6Å, approximately the distance of two disulphide bonds (Wang and Kaback, 1999; Kwaw *et al.*, 2000), away from the C- terminus of Sss1p. The only available cysteine residue in Sec63p that could form this cross-link resides, as determined by hydropathy analysis, at the lumen/membrane interface of Sec63p TMD3. Significantly TMD3 of Sec63p is immediately after the J-domain of Sec63p. The crystal structure of the *M. jannaschii* SecYE β complex predicts that the C- terminus of SecE, and its homologues, resides at the luminal face of the pore. The observed chemical cross-linking between the C- terminus of Sss1p to TMD3 of Sec63p and to TM 3 and 4 of Sec61p may imply that the J-domain of

Sec63p is immediately adjacent to the translocation channel. The significance of this in post-translational translocation is that the J-domain would position Kar2p at the translocation channel, in the immediate vicinity of substrate once it has been incorporated into the pore. Interestingly, the 85 kDa crosslinked adduct detected by anti Sec63p antibodies identified this adduct to contain two crosslinked species. As this crosslink is dependent on the mutation incorporated into Sss1p it is a consequence of the crosslinking between Sec63p and Sss1p. One possibility could be that this represents second site crosslinking. However, as there is only a single cysteine residue in Sec63p that can be crosslinked to the extreme C- terminus of Sss1p this would indicate that this cannot be the case. It is therefore more likely that one or both of these proteins are alternatively modified in these adducts.

There is a significant body of evidence suggesting that a functional translocon is oligomeric and this has been demonstrated biochemically as two adjacent Sec E molecules in *E. coli* have been shown to interact (Veenendaal *et al.*, 2001). We have shown that the extreme C- terminus of Sss1p can be cross-linked to an adjacent Sss1p molecule and this would indicate that the Sec61 complex can oligomerise in yeast. But the functionality of this interaction is not known as it does not indicate whether this represents an interaction between two independent Sec61 complexes or whether multiple complexes have merged to form the pore.

To date, conflicting models exist regarding the conformation of the translocon and the process with which it cycles between an active and inactive state. One model proposes that this switch is accompanied by a significant structural re-modelling of the translocon (Hamman *et al.*, 1997) whereas the second postulates that the structure is similar in both states (Menetret *et al.*, 2000). The latter has been refined to propose a switch in active state is coupled to the relocalisation of a single transmembrane domain that does not significantly affect the conformation of the complex (van den Berg *et al.*, 2004). To investigate these models in yeast

we have probed the molecular environment of the extreme C- terminus of Sss1p by chemical cross-linking. As previously reported this region can be coupled to Sec61p and Sec63p as well as being able to dimerise. Significantly these interactions are not perturbed following the release of ribosomes from membranes by treatment with puromycin and high salt. As this treatment has been previously shown to induce a switch from an active to an inactive translocon (Hamman *et al.*, 1998) we must conclude that this event does not change the molecular environment of the Sss1p C- terminus. As the ability of crosslinking Sss1p to Sec61p is retained it is apparent that the Sec61 heterotrimer does not dissociate following the completion of a round of translocation. Also, recent studies have indicated that the Sec61 translocon is oligomeric (reviewed in Johnson and van Waes, 1999). Identification that Sss1p can still dimerise following the release of ribosomes by puromycin and high salt implies that the Sec61 complex is still oligomeric in an inactive state and agrees with previous observations (Potter and Nicchitta, 2000). Although this data does not disprove the model implying that a switch from an open to a closed state requires a significant conformational change, it does indicate that the environment of the extreme C-terminus appears to remain constant.

Strikingly, cross-linking analysis has indicated that the Sec63p is an integral component of the ER translocon. Although it is known that Sec63p is absolutely required for translocation, previous models proposing Sec63p function appear to imply that Sec63p is recruited to the Sec61 complex during each round of translocation (Young *et al.*, 2001). This study has indicated that the Sec63 protein remains in association with the complex following inactivation of the translocation machinery. This interaction may have a functional significance regarding gating the ER translocon. It has been demonstrated in mammalian microsomes that the luminal face of the translocon is sealed by a mechanism that is BiP dependent (reviewed in Johnson and van Waes, 1999). However, to date there is no direct

evidence that BiP can directly bind the translocon. As *SEC63* is conserved in eukaryotes the observed chemical cross-linking between the C-terminus of Sss1p and Sec63p may indicate that BiP/Kar2p can gate regulate the closed state through its association with the J-domain of Sec63p.

Chapter 5

Phenotypic analysis of *sss1* mutants

5.1 The conserved C- terminus of Sss1p is required for function

The components of the core ER translocation machinery are highly conserved through evolution. Specifically, the Sec61 protein is 56% identical to its mammalian homologue Sec61 α and Sss1p is 45% identical to its mammalian counterpart Sec61 γ (Hartmann *et al.*, 1994). Furthermore, the extent of conservation is such that Sec61 γ can complement the otherwise lethal *sss1* mutation.

Sequence alignment of Sss1p and its homologues identifies two regions of sequence similarity (figure 5.1). Firstly, there is a region in the cytosolic domain, residues F₂₈-I₅₆ that encompasses an amphipathic helix that is conserved (Sato *et al.*, 2003). A second region of strong sequence similarity exists in the C- terminal 12 residues, K₆₉LIHIPIRYVIV₈₀, that contains 83% sequence identity throughout evolution. Furthermore, this sequence is absolutely required for function as deletion of these residues or substitution with a scrambled sequence results in an *sss1* mutant that cannot complement the lethal *sss1* null mutation (J. K. Brownsword, 2000). Early topological analysis predicted these residues to extend into the lumen (Esnault *et al.*, 1994). However, a more recent analysis has indicating that only the extreme four residues may be luminal and K₆₉-R₇₆ are positioned at the luminal interface of the transmembrane domain (van den Berg *et al.* 2004).

Previous analysis has shown that substitution of either residue L₇₀, I₇₁ and H₇₂ with alanine does not affect the function of *SSS1* (J. K. Brownsword, 2000). However replacement of I₇₃ with two alanine residues resulted in the partial disruption of Sss1p function and the joint replacement of both P₇₄ and I₇₅ with alanine residues (*sss1-6*) (figure 5.1 B) resulted in a more severe phenotype (J. K. Brownsword, 2000).

A

S.c Marasekgeekqsn NQVEKLVEAPVEFVREGTQFLAKCKKPDLETKYTKIVKAVGI**GF**IAVGII**GYA**IKLIHPIRYVIV
A.t M----- EAIDSAIDPLRDFAKSSVRLVQRCHKPDRKEFTKVAVRTAI**GF**VVMGFV**GF**FFV**KL**VF**IP**INNII**VG** ss
S.p Ma----- DNADDLFQIPKNFYKEGSHFIKRCVKPDRKEFLSISKAVAT**GF**VL**MGL**I**GY**II**KL**IHIPINKVL**VG** ga
H.s M----- DQVMQFVEPSRQFVKDSIRLVKRC**TKP**DRKEFQKIAMATAI**GF**AIM**GF**I**GF**FFV**KL**IHIPINNII**VG** g
C.e M----- DQFQALIEPARQFSKDSYRLVRC**TKP**DRKEFYQKIAMATAI**GF**AIM**GF**I**GF**FFV**KL**IHIPINNII**VG** a
P.f Mnvkipefltden-- HPVGYCVNGIQTFVEDSVRLIRK**CTKPN**K**KE**YTNIVYACSF**GL**IM**GF**I**GY**II**KL**VF**IP**INNIF**VG** sy
O.s M----- DAVDSVVDPLREFAKDSVRLVRC**CHKP**DRKEFTKVAARTAI**GF**VVM**GF**V**GF**FFV**KL**IF**IP**INNII**VG** sg
* * ** *** ***** ** * *** ***** ** * * * * * ** ****

B

Sss1p MARASEKGEEKKQSNQVEKLVEAPVEFVREGTQFLAKCKKPDLETKYTKIVKAVGIGFIAVGII**GYA**IKLIHPIRYVIV
Sss1-6p MARASEKGEEKKQSNQVEKLVEAPVEFVREGTQFLAKCKKPDLETKYTKIVKAVGIGFIAVGII**GYA**IKLIHIA**AA**RYVIV
Sss1-7p MARASEKGEEKKQSNQVEKLVEAPVEFVREGTQFLAKCKKPDLETKYTKIVKAVGIGFIAVGII**GYA**IKLI**KI**PIRYVIV

Figure 5.1 Sequence alignment of Sss1p and its homologues. (A) Sss1p and homologues from *Arabidopsis thaliana* (A.t), *Schizosaccharomyces pombe* (S.p), *Homo sapiens* (H.s), *Caenorhabditis elegans* (C.e), *Plasmodium falciparum* (P.f) and *Oryza sativa* (O.s) were aligned using Clustal W sequence alignment. Absolutely conserved residues are coloured red and sequence similarity in a minimum of 5 out of 7 species is denoted by *. (B) Sequence comparison of Sss1p, Sss1-6p and Sss1-7p. The residues mutated in Sss1-6p and Sss1-7p are coloured red.

Further characterisation of this mutant identified that although it is viable at 30°C it could not grow at 37°C. Also, there is a significant accumulation of secretory precursor at the permissive temperature (J. K. Brownsword, 2000) again confirming that this region of Sss1p is required for function.

Subsequently, a second conditional mutant allele of *sss1* has been isolated where the mutation is mapped to the C-terminal 12 amino acids of Sss1p. Although substitution of residue H₇₂ with alanine does not affect *SSS1* function, replacement of the same residue with lysine does (figure 5.1 B). This mutant was obtained by SDM where pJKB2 was mutagenised with *sss1* H72K forward and reverse primers and the H₇₂K substitution was confirmed by DNA sequencing, giving plasmid pCM205. After this, pCM205 was transformed into BWY530 cells and transformants were selected for histidine prototrophy and the wild type plasmid was subsequently counterselected by growth on media containing 5-FOA giving CMY21. BWY530 cells that had been previously transformed with pJKB2 (*SSS1*), *sss1-6* and CMY21 cells were plated out onto YPD and grown at 30°C and 37°C for 2 days. As expected, cells transformed with *SSS1* were viable at both temperatures (figure 5.2). Cells expressing the *sss1-6* mutation were found to be viable at 30°C but did not appear to grow as well as wild type cells and this mutation was again found to be non-functional at 37°C (figure 5.2). CMY21 cells like *sss1-6*, were viable at 30°C (figure 5.2). However, there was a profound growth defect at the permissive temperature which was more severe than that identified with *sss1-6*. CMY21 cells were also found to be temperature sensitive as this mutant cannot grow at 37°C unlike cells expressing *SSS1* (figure 5.2). This demonstrates that H₇₂ and P₇₄ I₇₅ residues in the C- terminal 12 residues are important for *SSS1* function.

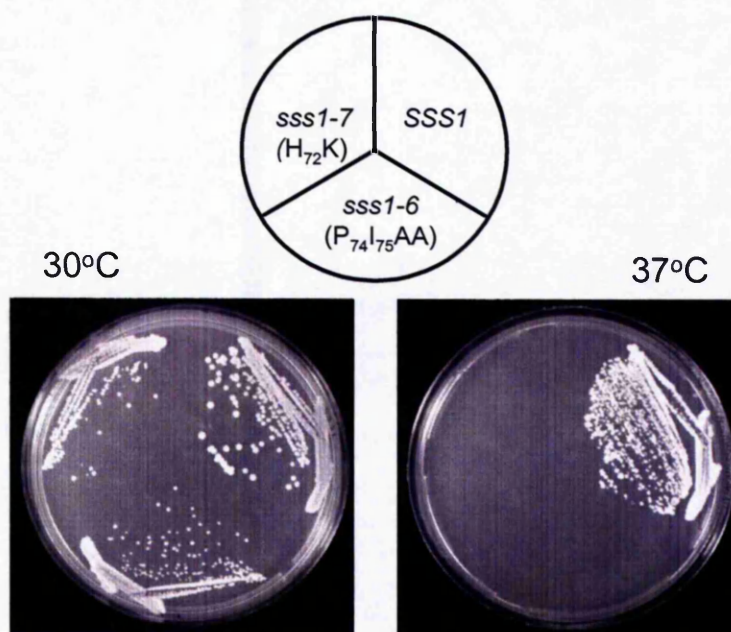


Figure 5.2 *sss1-6* and *sss1-7* are temperature sensitive alleles of *SSS1*. BWY530 cells were transformed with plasmid encoding either *SSS1*(pJKB2), *sss1-6* (pJKB16) and *sss1-7* (pCM205) and selected for histidine prototrophy. After this, cells were grown on YNB media containing 1 mg/ml 5'FOA to counterselect the wildtype plasmid giving BWY538, BWY544 and CMY21 respectively. BWY538, BWY544 and CMY21 cells were then plated out onto YPD and incubated at 30°C or 37°C for 2 days.

As *sss1-6* cells have previously been shown to exhibit an ER translocation defect at the permissive temperature ER translocation activity was investigated in *sss1-7* cells at the permissive temperature. ER translocation activity was investigated by pulse labelling, as previously described in chapter 4. Wild type, *sss1-6* and *sss1-7* cells were grown to early/mid log phase in sulphate free media and then labelled for 5 minutes with ^{35}S labelled methionine and cysteine. After this, total cell protein extracts were prepared from each culture and subjected to immunoprecipitation with antisera against CPY or DPAP B. A total cell protein extract was also prepared from wild type cells that had been treated with the drug tunicamycin, a reagent that inhibits N- linked glycosylation.

. Immunoprecipitation of either CPY and DPAP B from a total cell protein extract isolated from wild type cells that had been treated in tunicamycin isolated a 59 kDa species (figure 5.3 A, lane 1) and a 90 kDa species (figure 5.3 B, lane 1) respectively. The form of CPY isolated corresponds to proCPY as CPY possesses a cleavable signal sequence that is removed upon translocation into the ER, however, this protein is not glycosylated after the treatment of cells with tunicamycin. The 90 kDa form of DPAP B observed co-migrates with and can function as a marker for pDPAP B. Immunoprecipitation of either CPY or DPAP B in cells expressing *SSS1* identified the 67 kDa p1 and 69 kDa p2 forms of CPY (figure 5.3 A, lane 2) as well as the 120 kDa mature form of DPAP B (figure 5.3 B, lane 2). This shows the processing of both the CPY and DPAP B proteins under wild type conditions

Immunoprecipitation of CPY and DPAP B from cells expressing either *sss1-6* or *sss1-7* identified the p1 form and p2 form of CPY (figure 5.3 A, lanes 3 and 4) and mDPAP B (figure 5.3 B, lanes 3 and 4) but were also found to accumulate significant levels of ppCPY (figure 5.3 A, lanes 3 and 4) and pDPAP B (figure 5.3 B, lanes 3 and 4). However, the extent with which ppCPY and pDPAP B are accumulated in *sss1-6*

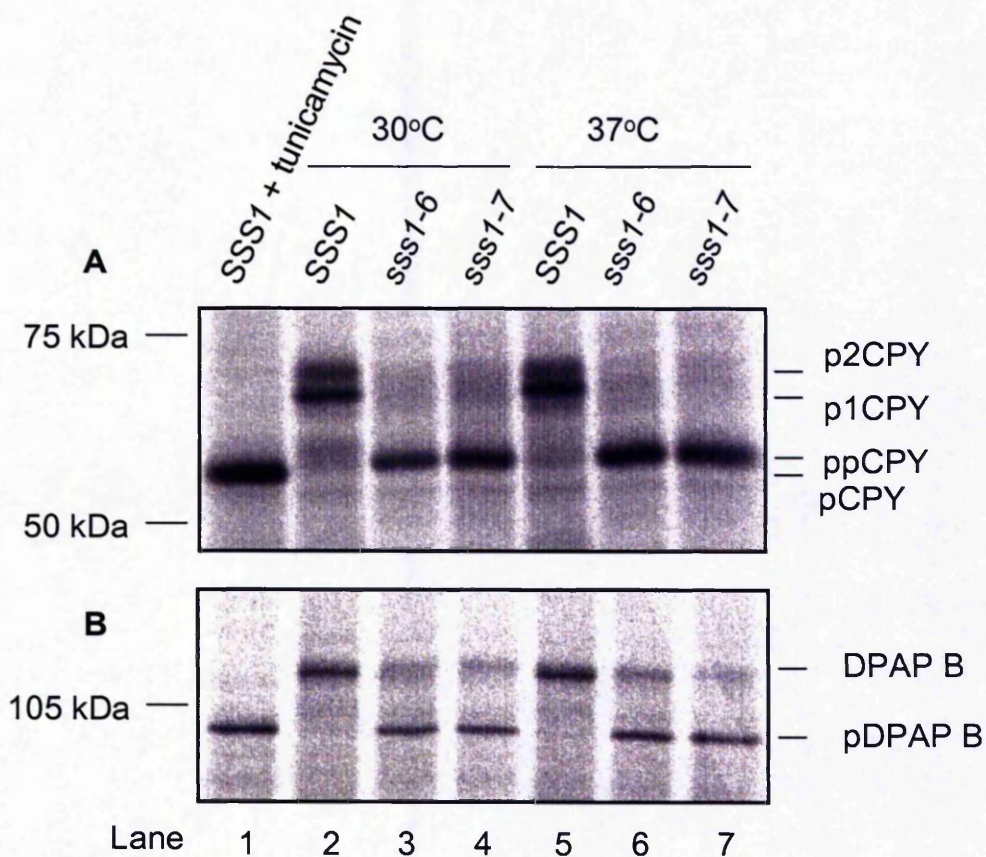


Figure 5.3 Investigating the translocation of secretory precursors in *sss1-6* and *sss1-7* cells. BWY538, BWY544 and CMY21 cells expressing plasmid born copies of *SSS1*, *sss1-6* or *sss1-7* respectively were grown to early exponential phase in low sulphate selective media containing 2% glucose. At this point, cells were grown at either 30°C or 37°C for 3 hours. One aliquot of wild type cells was incubated with 10 µg/ml tunicamycin. 5 O.D._{600nm} of cells were then isolated and incubated with ³⁵S methionine/cysteine for 5 minutes after which, total cell protein extracts were prepared. Cell extracts were then incubated with either 1 µl/ O.D._{600nm} of anti CPY antibodies (**A**) or 2 µl/ O.D._{600nm} of anti DPAP B antibodies (**B**) after which antibodies were immobilised with Protein A conjugated sepharose beads. Associated material was analysed by SDS-PAGE and visualised by phosphorimaging.

and *sss1-7* is not exacerbated when cells were incubated at the non-permissive temperature (figure 5.3 A and B, compare lanes 3 and 4 with lanes 6 and 7). Significantly, the levels of p1 and p2 CPY as well as mDPAP B isolated from both *sss1-6* and *sss1-7* were lower than those isolated from wild type cells but the species isolated from *sss1-6* and *sss1-7* appeared to be more heterogeneous as the protein bands were more diffuse (figure 5.3 A and B, compare lane 1 with lanes 3 and 4). This phenomenon will be discussed later in this chapter. Intriguingly the translocation defect observed in *sss1-7* does not appear to be more significant than the translocation defect observed in *sss1-6* (figure 5.3 A and B, compare lane 3 and 4) and may imply that the growth defect at 30°C does not correlate to the observed translocation defect. Accumulation of both ppCPY and pDPAP B in *sss1-6* and *sss1-7* implies that the residues mutagenised in these mutants are required for the translocation of both Sec62p dependent and SRP dependent secretory precursor proteins into the ER. The *sss1-6* and *sss1-7* mutant phenotypes will be further characterised herein.

5.2 Investigating the affect of Sss1-6p and Sss1-7p on the assembly of protein complexes required for ER translocation

The ER translocation defect detected in both *sss1-6* and *sss1-7* cells at the permissive temperature would be consistent with these mutants having a partial loss of Sss1p function. In chapter 4.1.3 it was shown that Sss1p can be crosslinked with DSS to Sec61p and Sec63p and the latter requires a functional Sss1p transmembrane domain. As recent structural analysis of an archaeobacterial SecYE β translocon would predict that the residues mutated in *sss1-6* and *sss1-7* are positioned towards the luminal face of the Sss1p transmembrane domain we wondered whether these mutants are defective in protein complex assembly. We therefore investigated the ability of both the Sss1-6p and Sss1-7p to form protein complexes required

for ER translocation by investigating the DSS crosslinking profile of these two mutant proteins. Membranes were harvested from cells expressing either *SSS1*, *sss1-6* or *sss1-7* and were then incubated with DSS or mock treated and probed with anti Sss1p antibodies. Again, the 46 kDa and 85 kDa adducts, corresponding to the covalent coupling of Sss1p to Sec61p and Sec63p respectively, were detected in membranes isolated from cells expressing *SSS1* (figure 5.4, lane 2). Analysis of membranes isolated from both *sss1-6* and *sss1-7* indicated that the efficiency with which either Sss1-6p or Sss1-7p was covalently coupled to Sec61p was not perturbed (figure 5.4, lanes 4 and 6) as the efficiency with which this adduct was detected was comparable to wild type (figure 5.4, compare lane 2 with lane 4 and 6). In contrast, analysis of the 85 kDa adduct in membranes isolated from *sss1-6*, the 85 kDa crosslink between Sss1-6p and Sec63p was detected but the intensity of this adduct was lesser than that in membranes isolated from cells expressing *SSS1* (figure 5.4, compare lanes 2 and 4) and the 85 kDa adduct was barely detectable in membranes isolated from cells expressing *sss1-7* (figure 5.4, compare lanes 2 and 6). Together this indicates that both the *sss1-6* and *sss1-7* mutations do not affect the efficiency with which Sss1p and Sec61p can be crosslinked but would appear to affect the efficiency with which they can be crosslinked to Sec63p. This may imply that an interaction between Sec63p and either mutant is perturbed and this would account for the translocation defect detected at steady state.

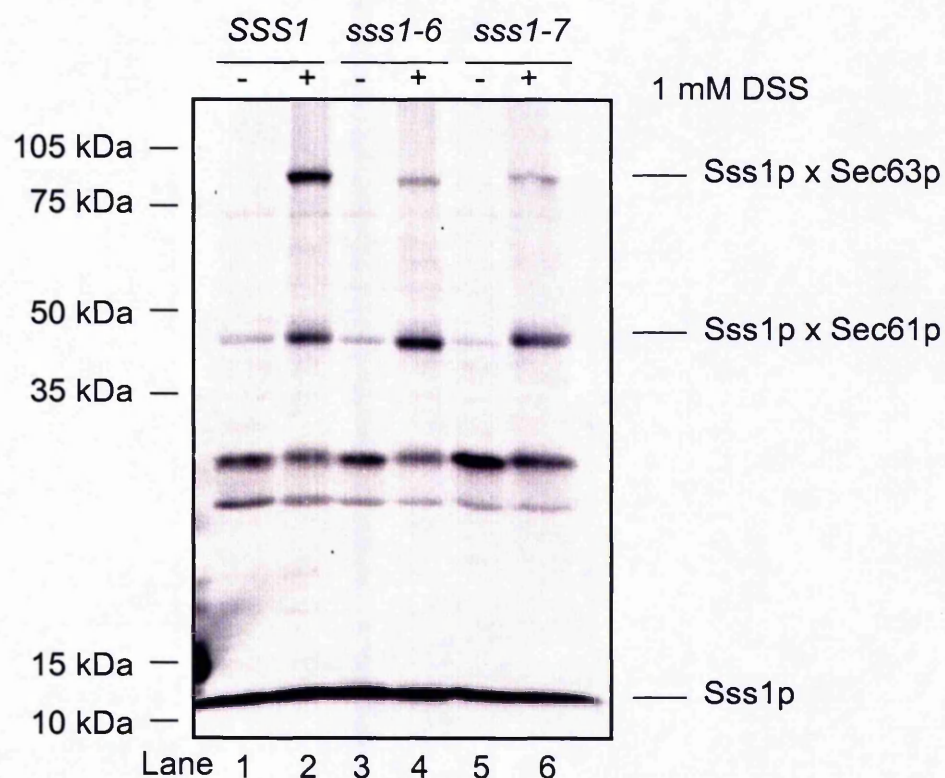


Figure 5.4 Investigating interactions formed by Sss1-6p and Sss1-7p by lysine dependent crosslinking. 1 A_{280nm} of membranes isolated from cells expressing either *SSS1* (BWY538), *sss1-6* (BWY544) or *sss1-7* (CMY21) were either mock treated with DMSO or incubated with 1 mM DSS at 30°C for 30 minutes. Crosslinking reactions were then terminated by the addition of 100 mM lysine and glycine. After this samples were incubated with 1X Laemmli sample buffer and analysed by SDS-PAGE on an 8-15% linear gradient. After western transfer samples were immunoblotted with anti Sss1p antibodies. Sss1p crosslinked adducts are indicated.

5.3 Investigating translocon and ER luminal components at steady state in *sss1-6* and *sss1-7*

Depletion of Sss1p from cells has previously been shown in chapter 4 to affect the electrophoretic mobility of the Sec63 protein at steady state. Furthermore, the transmembrane domain of Sss1p is required to interact with Sec63p to recruit it to the Sec61 complex in order to form the functional translocon. As the efficiency with which Sss1p can be covalently coupled to Sec63p in membranes isolated from cells expressing either *sss1-6* or *sss1-7* is significantly reduced we wondered whether the Sec63 protein would again be affected at steady state. The opportunity was also taken to investigate the biogenesis and levels of ER luminal components, specifically Kar2p and Sil1p, to see if the *sss1-6* and *sss1-7* mutations compromise the ER lumen.

Cells expressing either *SSS1*, *sss1-6* or *sss1-7* were grown to early/mid log phase and total cell protein extracts were prepared from 5 O.D._{600nm} of cells. A total cell protein extract was also prepared from 5 O.D._{600nm} of cells expressing *SSS1* that had been treated with tunicamycin. Proteins of interest were detected by western blot analysis. Analysis of Sss1 protein levels in these cells identified that the mutations did not appear to destabilise the protein at steady state as the level of Sss1p detected in either *sss1-6* and *sss1-7* was comparable to that detected in a haploid wild type strain, w303 α (figure 5.5 A, compare lane 2 with lanes 3 and 4). But this analysis is limited as the Sss1 protein does not adhere to nitrocellulose/PVDF membrane efficiently. However, the *sss1-7* mutation did appear to affect the electrophoretic mobility of the Sss1 protein as it appeared as a slower migrating species when analysed by SDS-PAGE (figure 5.5 A, compare lanes 2 and 3).

The levels of Sec61p in cells has been previously shown to be unaffected after the depletion of Sss1p (figure 4.5, lane 2; Esnault *et al.*, 1993; 1994) and neither the *sss1-6* nor the

sss1-7 mutation appeared to affect the level of Sec61p at steady state (figure 5.5 B, compare lane 2 with lanes 3 and 4).

As previously described, the aberrant Sec63p gel mobility upon depletion of Sss1p from cells is consistent with the Sec63 protein being covalently modified. Therefore depletion of Sss1p would appear to affect the covalent modification of Sec63p. Analysis of Sec63p in cells expressing either *SSS1*, *sss1-6* or *sss1-7* indicated that this aberrancy could not be detected in these mutants as the form of Sec63p detected in both *sss1-6* and *sss1-7* co-migrates with the species of Sec63p identified in cells expressing *SSS1* (figure 5.5 C, compare lane 2 with lanes 3 and 4). However, the level of Sec63p appears to be reduced in both *sss1-6* and *sss1-7* (figure 5.5 C, compare lane 2 with lanes 3 and 4) implying that Sec63p is potentially destabilised in these cells. Consistent with this, there is a concomitant increase in a lower Mw species that is detected by anti Sec63p antibodies in cells expressing either *sss1-6* or *sss1-7* compared to *SSS1* (figure 5.5 C, compare lane 2 with lanes 3 and 4). This immunoreactive species has also been observed in a study investigating *sss1* mutants and has been postulated to represent a breakdown product of Sec63p (J. K. Brownsword, 2000).

Analysis of ER luminal components has been particularly informative. The *KAR2* gene encodes for the major ER luminal Hsp70 chaperone protein and genetic analysis has determined this protein to be essential for all ER translocation (Young *et al.*, 2001) as well as being required to contribute to the folding environment of the ER lumen and to function in the early stages of ER associated degradation (ERAD) (discussed later). The Sil1 protein was identified by genetic analysis where the overexpression of the *SIL1* gene was found to suppress the synthetic lethality of an *ire1*, *lhs1* double deletion (Tyson and Stirling, 2000). Sil1p was later found to function as a nucleotide exchange factor for Kar2p (Kabani *et al.*,

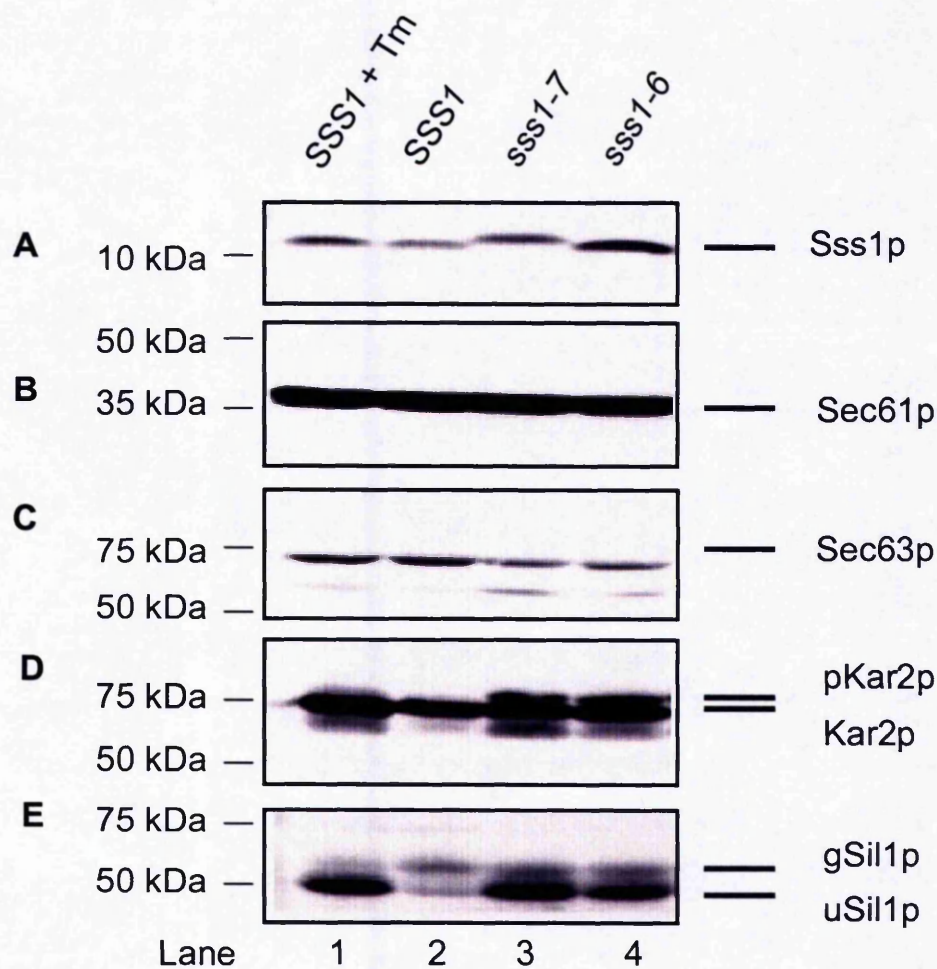


Figure 5.5 Analysis of translocon and ER luminal components in *sss1-6* and *sss1-7*. Total protein extracts were prepared from 5 O.D._{600nm} of either BWY538 cells treated with 10 µg/ml tunicamycin, BWY538 (*SSS1*), BWY544 (*sss1-6*) and CMY21 (*sss1-7*) that were growing logarithmically. After this, extracts were analysed by SDS-PAGE and after western transfer were immunoblotted with either anti Sss1p antibodies (A), anti Sec61p antibodies (B), anti Sec63p antibodies (C), anti Kar2p antibodies (D) or anti Sil1p antibodies (E).

2000), as does Lhs1p (Steel *et al.*, 2004), and the overexpression of *SIL1* was found to compensate the loss of Lhs1p nucleotide exchange activity (Steel *et al.*, 2004).

In wild type cells, Kar2p is detected as a 75 kDa protein by immunoblot analysis (figure 5.5 D, lane 2). This form of Kar2p is also observed in both *sss1-6* and *sss1-7* cells (figure 5.5 D, lanes 4 and 3 respectively). But a second immunoreactive species with a higher Mw is detected in cells expressing either *sss1-6* or *sss1-7* (figure 5.5 D, lanes 4 and 3). This represents non-translocated pre Kar2p and its detection is consistent with the ER translocation defect detected in these cells. However, the level of ER luminal Kar2p in these cells does not appear to be perturbed in comparison to cells expressing *SSS1* (figure 5.5 D, compare lane 2 with lanes 3 and 4) and may appear to be higher. In fact, the level of Kar2p in *sss1-6* cells appears to be approximately equal to the level of Kar2p in wild type cells that have been treated with tunicamycin (figure 5.5 D, compare lane 1 and 4).

Certain similarities between wild type cells treated with tunicamycin and *sss1-6* and *sss1-7* cells are more apparent when the levels of the Sil1 protein were investigated. Sil1p biogenesis is poorly defined. In wild type cells two forms are detected by anti Sil1p antibodies (figure 5.5 E, lane 2). The faster migrating species, unlike the slower, is also present in wild type cells that have been treated with tunicamycin (figure 5.5 E, compare lane 1 and 2). This would be consistent with this being a signal peptide cleaved, unglycosylated form of Sil1p (uSil1p). The significant reduction of the slower migrating species in wild type cells treated with tunicamycin compared to untreated cells (figure 5.5 E, compare lane 1 and 2) is consistent with this being a glycosylated form of Sil1p (gSil1p). Treatment of wild type cells with tunicamycin leads to a significant increase in the amount of total Sil1p synthesised in these cells compared to those that have been untreated (figure 5.5 E, compare lane 1 and 2). Significantly, the amount of total Sil1p at steady state in either *sss1-6* or *sss1-7* is comparable to that in wild type cells that have been treated with tunicamycin (figure 5.5 E, compare lane 1

with lanes 3 and 4). Also, the major species of Sil1p in both *sss1-6* and *sss1-7* co-migrates with the form identified in wild-type cells that have been treated with tunicamycin (figure 5.5 E, lanes 3 and 4). Therefore, treatment of wild type cells with tunicamycin affects the ER lumen and results in the increased levels of luminal Kar2p and Sil1p and this phenotype is prevalent in *sss1-6* and *sss1-7* regardless of incubation of these cells with tunicamycin.

As previously discussed, tunicamycin inhibits N-linked glycosylation in the ER. However, many proteins are glycosylated and require this in order to adopt their final native conformation as inhibition of glycosylation results in the failure of glycoproteins to fold, leading to the accumulation of mis-folded protein in the ER lumen. Accumulation of misfolded protein in the ER results in the induction of the unfolded protein response (UPR) in order to alleviate the ER stress. Therefore the comparative analysis of Kar2p and Sil1p in *sss1-6* and *sss1-7* cells with wild type cells treated with tunicamycin might indicate that the UPR is induced in both *sss1-6* and *sss1-7* cells.

5.4 The unfolded protein response is induced in *sss1-6* and *sss1-7*

Conditions that alter ER homeostasis can lead to the accumulation of misfolded proteins, a condition that can lead to cell death. To cope with this stress, cells activate an intracellular signalling pathway, the unfolded protein response, which transmits information about the protein folding environment of the ER lumen to the cytoplasm and the nucleus. The UPR is a highly conserved signalling pathway observed in all eukaryotes studied to date (Patil and Walter, 2001) and it is required to upregulate the expression of a set of effector genes to alleviate the stress.

The effector molecules of the UPR were initially identified by genetic analysis in *Saccharomyces cerevisiae*. Changes in the ER environment are sensed by an ER/nuclear membrane localised serine/threonine kinase, Ire1p (Cox *et al.*, 1993) where the amino

terminus of Ire1p indirectly senses an elevation in misfolded proteins. In the absence of ER stress, Ire1p is maintained in a monomeric state through the interaction of its amino terminus with the ER resident chaperone Kar2p (Okamura *et al.*, 2000; Bertolotti *et al.*, 2000). Upon the accumulation of unfolded protein, Kar2p dissociates from Ire1p to bind to and promote the folding of misfolded protein allowing the dimerisation of Ire1p via its amino terminus (Shamu *et al.*, 1994). Oligomerisation of Ire1p leads to *trans*-autophosphorylation through its cytosolic kinase domain which in turn stimulates the activity of its endoribonuclease activity towards its extreme carboxyl terminus (Shamu and Walter, 1996; Welihinda and Kaufman, 1996; Patil and Walter, 2001).

The substrate for the Ire1p endonuclease activity is the pre-mRNA of Hac1p (Cox and Walter, 1996; Kawahara *et al.*, 1997; Sidrauski and Walter, 1997), a transcription factor required for the transcription of UPR target genes (Cox and Walter, 1996). Under normal growth conditions *HAC1*^u (uninduced) mRNA translation is attenuated as it contains an intron towards the 3' end of the open reading frame that prevents its translation (Chapman and Walter, 1997). Upon activation of the UPR, the intron has been identified, *in vitro*, to be removed by the endonuclease activity of Ire1p (Sidrauski and Walter, 1997). *HAC1*ⁱ (induced) mRNA is generated by the ligation of the 5' and 3' fragments by the tRNA ligase, Rlg1p, and this can be translated to give functional Hac1p transcription factor (Sidrauski *et al.*, 1996).

Following synthesis, Hac1p translocates to the nucleus and activates the transcription of approximately 381 genes in yeast (208 of which have known function or are homologous to genes of known function) (Travers *et al.*, 2000). It is believed that one function of the UPR is to modify the secretory pathway to eradicate the ER stress (Travers *et al.*, 2000). These genes share a common element within their promoter region, the

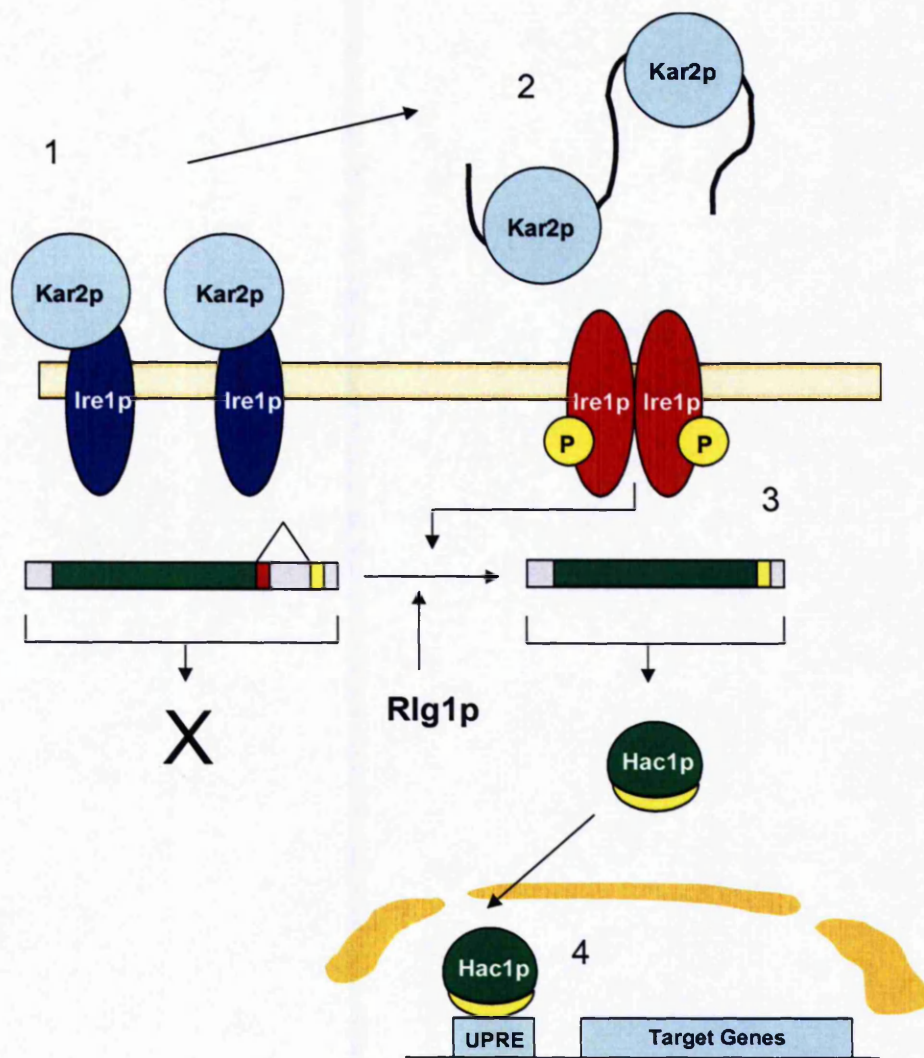


Figure 5.6 The unfolded response in *Saccharomyces cerevisiae*. In the absence of ER stress Ire1p is maintained in a monomeric state via an interaction with Kar2p (1). Following stress, Kar2p dissociates and binds to unfolded protein allowing Ire1p to dimerise (2). Ire1p dimerisation results in autophosphorylation which in turn stimulates its endoribonuclease activity and this removes an intron that otherwise inhibits translation, from the mRNA of the HAC1 transcription factor (3). The 5' and 3' fragments of HAC1 mRNA are ligated by Rlg1p, the transcript is then translated, giving Hac1p, which is then translocated to the nucleus where it activates the transcription of UPR target genes (4).

unfolded protein response element (UPRE), that is bound by Hac1p and this has been shown to be both necessary and sufficient for the UPR dependent transcription of a gene (Kohno *et al.*, 1993; Mori *et al.*, 1992).

We therefore proceeded to investigate whether the UPR is induced in both *sss1-6* and *sss1-7* cells. The yeast centromeric pJT30 plasmid contains the β -galactosidase reporter gene whose expression is controlled by the UPR (Wilkinson *et al.*, 2000). This is so because, as previously described, genes that are induced by the UPR contain a UPRE within their promoter region. Therefore following the activation of the Hac1p transcription factor upon ER stress, Hac1p binds and activates the transcription of genes containing this *cis*- acting element for transcription. The *KAR2* gene is transcriptionally activated upon induction of the UPR (Travers *et al.*, 2000). In order to place the β -galactosidase gene under the control of the UPR the UPRE and flanking sequence from the *KAR2* promoter was fused upstream of the β -galactosidase gene. Therefore this construct is only transcribed upon activation of the Hac1p transcription factor allowing UPR induction to be quantified by determining the β -galactosidase activity in cells that have been transformed with this construct.

Wild type, *sss1-6* and *sss1-7* cells were transformed with pJT30 and transformants were selected for uracil prototrophy. For a positive control of UPR induction β -galactosidase activity was investigated in cells containing a genomic deletion of the *lhs1* gene that had been transformed with pJT30. This strain is a suitable positive control as the UPR is constitutively induced in these cells (Tyson and Stirling, 2000).

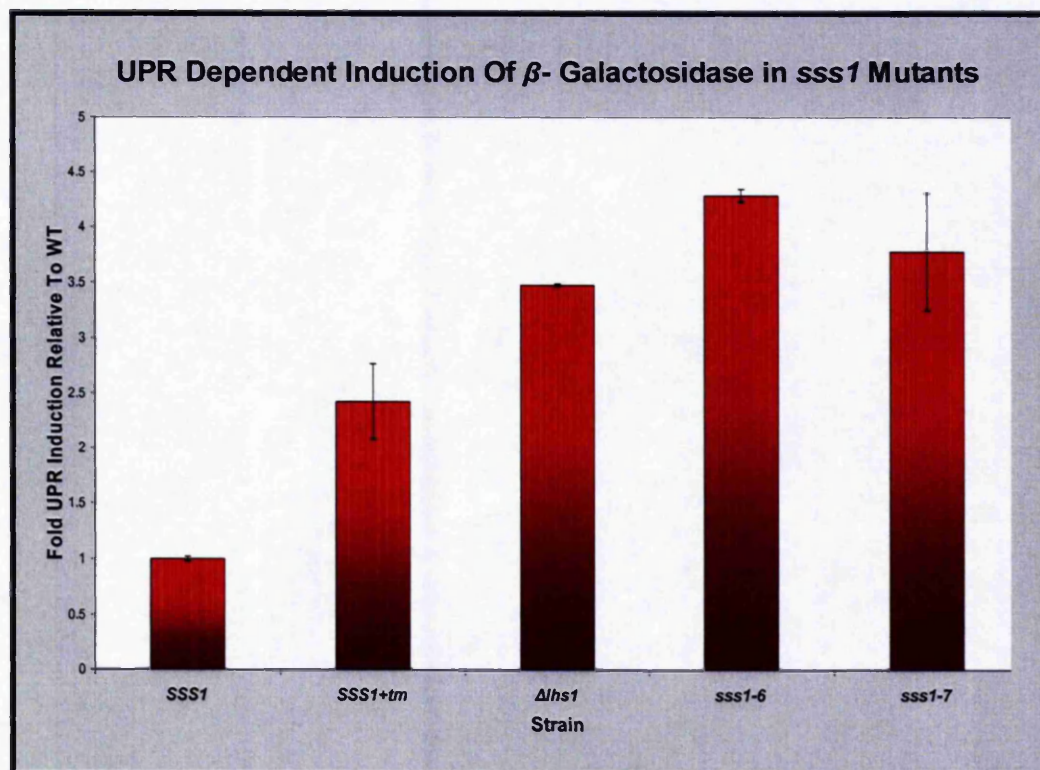


Figure 5.7 Investigating the UPR dependent induction of β -galactosidase in *sss1-6* and *sss1-7*. BWY530 (*SSS1*), JTY33 (*Δ hs1*), BWY544 (*sss1-6*) and CMY21 (*sss1-7*) cells were transformed with the pJT30 plasmid, encoding for UPRE LacZ, were grown in YNB minus uracil to early/mid log phase. Also, an aliquot of BWY530 cells were incubated with 10 μ g/ml tunicamycin (*SSS1* + tm) for 30 minutes to induce the UPR. Approximately 5 O.D._{600nm} of cells were isolated and resuspended in 5 ml of Z-buffer. After this, 800 μ l aliquots were solubilised and the β -galactosidase activity was assessed, according to materials and methods, by the hydrolysis of ONPG, which was detected by a concomitant increase in A_{440nm}.

Cells were grown to early/mid phase in selective media and then approximately 5 O.D._{600nm} of cells were harvested and the cells disrupted. β -galactosidase activity was then measured spectrophotometrically by monitoring the hydrolysis of o-nitrophenyl β -D- galactopyranoside (ONPG), which can be detected by a concomitant increase in absorbance at A_{420nm}.

There is a basal level of UPR induction in wild type cells at steady state and consistent with this β -galactosidase activity was detected in these cells (figure 5.7, column 1). As these cells share the same genetic background as the other strains of interest, w303, we normalised the β -galactosidase activity in these cells to 1. Subsequently, by doing this we can measure the induction of β -galactosidase activity, which correlates to UPR induction, relative to wild type. As previously discussed, incubation of cells with tunicamycin artificially induces the UPR through the inhibition of N-linked glycosylation. We therefore wanted to show that we could induce the UPR in cells expressing *SSSI* and demonstrate that the reporter construct is sensitive to UPR induction. Cells expressing *SSSI* were incubated with tunicamycin for 30 minutes and then the specific β -galactosidase activity was measured in these cells. Analysis indicated that following this incubation there was a 2.5 fold induction of β -galactosidase activity relative to wild type (figure 5.7, column 2). This demonstrates that the pJT30 plasmid is suitable for sensing the induction of the UPR in cells.

When we analysed β -galactosidase activity in cells containing the *lhs1* null mutation we found that it was approximately 3.5 times higher than that in wild type cells (figure 5.7, column 3). As induction of the UPR in *lhs1* cells is vital to sustain cell viability, a 3.5 fold induction of β -galactosidase activity would serve as a suitable marker to predict a strong induction of the UPR in cells. However, the level of UPR induction in *lhs1* cells compared to wild type cells that have been treated with tunicamycin in this study is greater than in results that have been published (Tyson and Stirling, 2000), this is as wild type cells were only treated for 30 minutes with tunicamycin in this study rather than for 60 minutes.

Analysis of β -Galactosidase activity in both *sss1-6* and *sss1-7* indicated that the UPR is induced in both of these strains, as the measured β -galactosidase activity was approximately 4.5 times higher in *sss1-6* (figure 5.7, column 4) and approximately 4 times higher in *sss1-7* (figure 5.7, column 5) compared to wild type. Significantly, the observed induction of β -galactosidase activity appears to be greater in both *sss1-6* and *sss1-7* than that observed in *lhs1* null cells (figure 5.7, compare column 3 with columns 4 and 5). This would be consistent with UPR induction in these cells being required to maintain cell viability. Together this data implies that the UPR is induced in both *sss1-6* and *sss1-7* and would suggest that the residues mutagenised in these strains are required to control the environment of the ER lumen and this function is perturbed in these mutants.

5.5 Investigating ER Associated Degradation in *sss1-6* and *sss1-7*

Proteins recognised as misfolded are targeted for proteolysis by a process termed ER associated degradation (ERAD) (Brodsky and McCracken, 1997). As this does not occur in the ER lumen it requires that proteins are retrotranslocated from the ER into the cytosol where the misfolded protein is degraded by the ubiquitin-proteasomal pathway. Interestingly, this pathway is utilised by some pathogens to enter the host's cytoplasm or to destroy the host's immune defence, for example the US2 and US11 proteins of the human cytomegalovirus target the destruction of the major histocompatibility complex (MHC) class I for degradation via ERAD.

The ERAD process can be broken down into three stages. Firstly, the misfolded protein is recognised as a substrate for ERAD and targeted to the "dislocon". It is then transported across the membrane and released into the cytosol where it is, finally, degraded.

During the folding of a polypeptide chain hydrophobic domains can be transiently exposed to the ER lumen and ERAD substrates may be recognised as misfolded due to the prolonged exposure of these surfaces. This timing mechanism has been proposed for misfolded glycoproteins where the terminal glucose residue of the glycan chain (GlcNAc₂Man₉Glc₃) is constantly removed and added back by the action of glucosidase II and UDP-glucose glycosyl transferase (UGGT) respectively (reviewed in Helenius and Aebi, 2004), and the monoglucosylated forms are recognised by two ER lectin like chaperones, calnexin and calreticulin, that facilitate the folding of these precursors (Helenius and Aebi, 2004). Although yeast possesses a protein homologous to calnexin, the UGGT pathway does not appear to be conserved due to a lack of a functional UGGT homologue (Kostova and Wolf, 2003). However, the signal for the degradation of misfolded glycoproteins appears to be conserved as the removal of the terminal mannose residue by the action of ER mannosidase I, leaving the GlcNAc₂Man₈ sugar moiety, targets proteins for degradation (Knop *et al.*, 1996; Jakob *et al.*, 1998b). As this process is slow, only acutely misfolded proteins are targeted for degradation (Jakob *et al.*, 1998b). Genetic analysis in *S. cerevisiae* has indicated that the ER chaperones Pdi1p and Kar2p are required to target misfolded proteins for degradation (Gillece *et al.*, 1999; Plemper *et al.*, 1997; Brodsky *et al.*, 1999). It is believed that they maintain misfolded proteins in solution to retain them as substrates for ERAD (Nishikawa *et al.*, 2001; Braakman *et al.*, 1992).

A body of evidence suggests that substrates of ERAD are retrotranslocated from the ER into the cytosol via the Sec61 complex. This was initially proposed as a substrate targeted for ERAD could be co-immunoprecipitated with Sec61 α (Wiertz *et al.*, 1996). This was later supported genetically as a number of mutations in the yeast *sec61* gene were found to delay or prevent degradation (Plemper *et al.*, 1997; Pilon *et al.*, 1997; Gillece *et al.*, 1999) and these mutations were later found to be mapped to the luminal domains of Sec61p (Zhou and

Schekman, 1999). However, the data to date is not entirely conclusive as there are difficulties in repeating the findings reported in Zhou *et al.*, 1999 preventing further Characterisation of these mutants (personal communication C. J. Stirling). Also, in order to determine a function for Sec61p in the dislocation of mis-folded protein it is necessary to identify *sec61* mutants that are defective in the dislocation of misfolded protein but do not compromise the translocation activity. Such mutants are proving particularly problematic to isolate.

DER1 is an evolutionary conserved polytopic membrane protein that spans the ER membrane four times (Hitt and Wolf, 2004a) that is required for the degradation of numerous misfolded proteins in yeast (Knop *et al.*, 1996; Vashist and Ng, 2004) and is upregulated by the UPR (Travers *et al.*, 2000). The mammalian homologue of Der1p, Derlin, has been recently identified to associate with substrates of ERAD (Ye *et al.*, 2004) and associated, via an interaction with VIMP, with a complex that provides the energy for the movement (Cdc48p/p97 complex, discussed later) of proteins from the ER into the cytosol (Ye *et al.*, 2004). Therefore, a model has been proposed which postulates that Der1p/Derlin forms the channel through which misfolded proteins are retrotranslocated (Ye *et al.*, 2004).

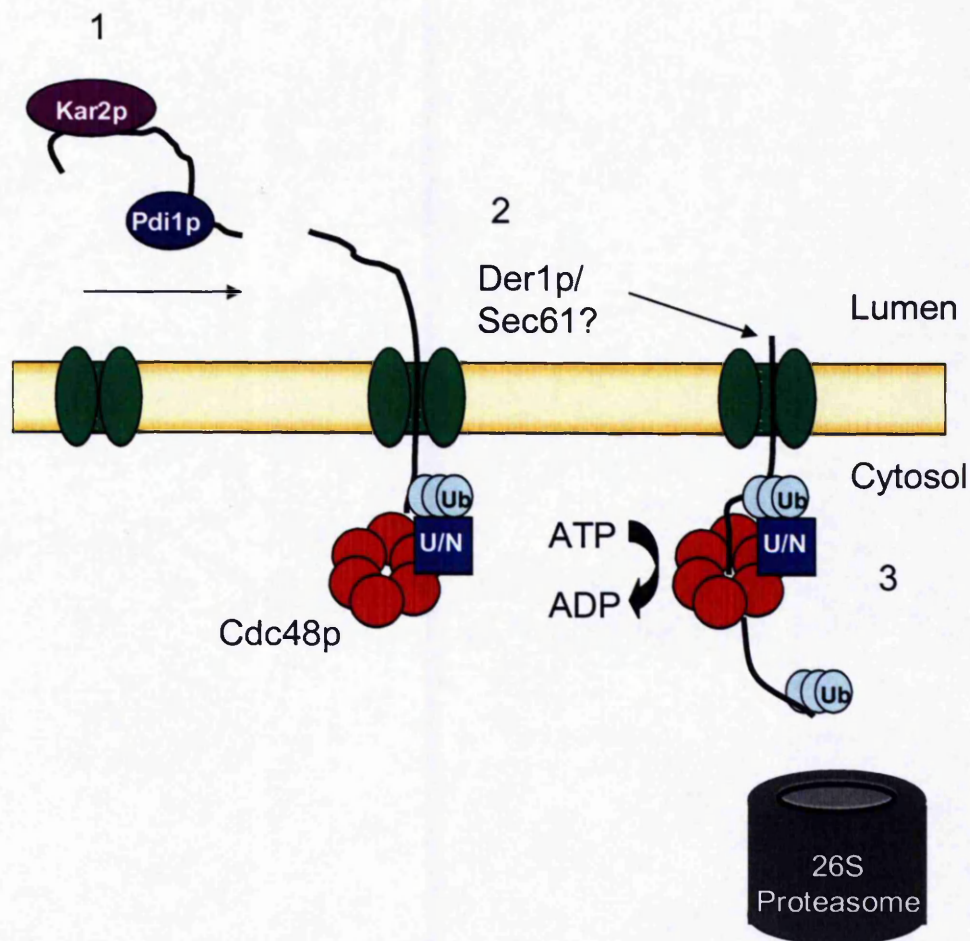


Figure 5.8 ER associated degradation in *Saccharomyces cerevisiae*. Misfolded proteins are prevented from aggregating by Kar2p and Pdi1p (1). Misfolded proteins are then targeted to the ER membrane where they are retrotranslocated into the cytosol by a mechanism that requires Der1p and potentially the Sec61p complex (2). During retrotranslocation, ERAD substrate is polyubiquitinated (3). This is then bound by Cdc48p, a member of the AAA ATPase family and two co-factors Ufd1p (U) and Npl4p (N) and is retrotranslocated into the cytosol in an ATP dependent manner (3). The polyubiquitinated protein is then degraded by the proteasome.

Substrates for ERAD are polyubiquitinated at the ER membrane prior to their export and this has been shown to be required for ERAD as mutations in the components of the ubiquitinylation complex have been shown to stabilise misfolded protein (Hiller *et al.*, 1996; Shamu *et al.*, 1999; 2001). Furthermore, polyubiquitination of the ERAD substrate may serve as the signal for binding by the Cdc48p/Ufd1p/Npl4p complex that has been implicated in the movement of proteins from the ER into the cytosol (Flierman *et al.*, 2003).

Recently, Cdc48p in yeast (p97 in mammals), a member of the AAA ATPase family, has been shown to be required for the extraction of ERAD substrate from the ER into the cytosol (Jarosch *et al.*, 2002). Cdc48p is found in a stable complex with two co-factors, Ufd1p and Npl4p, and mutations in *cdc48*, *ufd1* and *npl4* results in the delayed degradation of misfolded proteins (Ye *et al.*, 2001; Tsai *et al.*, 2002). Cdc48p forms hexameric structures. It has been proposed that these structures bind to misfolded protein, presented at the cytosolic face of the ER membrane via an interaction with VIMP, and pull the polypeptide chain in an ATP dependent manner (Tsai *et al.*, 2002). There is now experimental evidence to support this model (Elkabetz *et al.*, 2004). Following the extraction of polyubiquitinated misfolded protein from the ER by Cdc48p/Ufd1p/Npl4p the protein is delivered to the 26S proteasome for degradation in a process that may depend on Cdc48p, Dsk2p and Rad23p (Medicherla *et al.*, 2004).

As there is a sufficient body of evidence to suggest that the Sec61 complex is required for the retrotranslocation of misfolded proteins from the ER lumen into the cytosol we investigated whether *sss1-6* and *sss1-7* are defective in ERAD. This is a very attractive hypothesis considering the substantial induction of the UPR in these cells, as the mutations are positioned towards the ER membrane/lumen interface and also that Sss1p is a component of

the Sec61 complex and the homologous Ssh1 complex. Intriguingly, *ssh1Δ* cells have been shown to be defective in ERAD (Wilkinson *et al.*, 2001).

In yeast, ERAD has been investigated by examining the stability of a misfolded protein by pulse chase analysis. The protein encoded by the *PRC1* gene, CPY, functions in the yeast vacuole (Stevens *et al.*, 1986). Cells expressing the *prc1-1* mutation, CPY*, were found to be phenotypically similar to a *prc1* null mutant as no CPY was found to be trafficked to the vacuole. Further analysis indicated that p1 CPY* accumulated in the ER, as this mutant cannot fold, and is targeted for degradation by the cytosolic proteasome (Finger *et al.*, 1993). Importantly, mutants that were found to be defective in ERAD were shown to increase the half life, and therefore the stability, of CPY*.

sss1-6 or *sss1-7* cells were examined for defects in ERAD by investigating the half life of CPY* in these cells by pulse chase analysis. As wildtype, *sss1-6* and *sss1-7* cells express a functional copy of *PRC1* it was necessary to introduce the *prc1-1* mutation into these cells. The plasmid pCT43 encodes *prc1-1*. Furthermore, this derivative contains a 3-HA tag allowing it to be distinguished from both p1 and p2 CPY. Wildtype, *sss1-6* and *sss1-7* cells were transformed with plasmid and grown under selective conditions. To control for defects in ERAD, HA-CPY* biogenesis was investigated in *der3Δ* cells, that have previously been shown to be defective in CPY* degradation (Knop *et al.*, 1996). Cells transformed with HA-CPY* were grown to early/mid log phase in low sulphate media and were labeled with ³⁵S methionine/cysteine for 20 minutes (pulse). A chase was initiated by incubating cells with unlabeled methionine/cysteine and CPY* was immunoprecipitated from total cell protein extracts prepared at 0, 30, 60 or 120 minutes of chase. CPY* was then visualised by phosphorimaging.

In wild type cells the amount of immunoprecipitable radiolabeled CPY* was found to decrease over the time course of the chase and was barely detectable following a 2 hour chase

(figure 5.9 A, lane 4). This is consistent with CPY* being degraded over the time course and implies that ERAD is functional in these cells. When *der3Δ* cells were analysed it was found that although the amount of radiolabeled CPY* immunoprecipitated from these cells decreases over time there is a substantial stabilisation of CPY* in these cells (figure 5.9 B). This is confirmed as radiolabeled CPY* is immunoprecipitated from *der3Δ* cells, unlike in wild type cells, after a 2 hour chase with non-radiolabeled methionine/cysteine (compare figure 5.9 A, lane 4 with figure 5.9 B, lane 4). Stabilisation of radiolabeled CPY* in *der3Δ* cells is consistent with ERAD being perturbed in these cells. When we investigated CPY* in *sss1-6* and *sss1-7* cells we found that radiolabeled CPY* had been stabilised in these cells (compare figure 5.9 A, lane 4 with figure 5.9 C and D, lane 4). Furthermore, the amount of material immunoprecipitated from these cells following chase for 2 hours was far greater than that immunoprecipitated from *der3Δ* cells (compare figure 5.9 B, lane 4 with figure 5.9 C and D, lane 4). This indicates that ERAD is defective in *sss1-6* and *sss1-7* and that this defect is more substantial than that identified in *der3Δ* cells. Interestingly, p1 HA-CPY* immunoprecipitated from *sss1-6* and *sss1-7* appeared to be larger and more heterogeneous than that immunoprecipitated from wild type cells (compare figure 5.9 A, lane 1 with figure 5.9 C and D, lane 1) and is consistent with the difference in p1 CPY observed between wild type and *sss1-6* and *sss1-7* observed in figure 5.3. Together this indicates that induction of the UPR in *sss1-6* and *sss1-7* cells may be the consequence of the accumulation of misfolded protein in the ER lumen due to ERAD being defective in these cells. This also suggests that Sss1p may be directly required for the degradation of misfolded ER proteins.

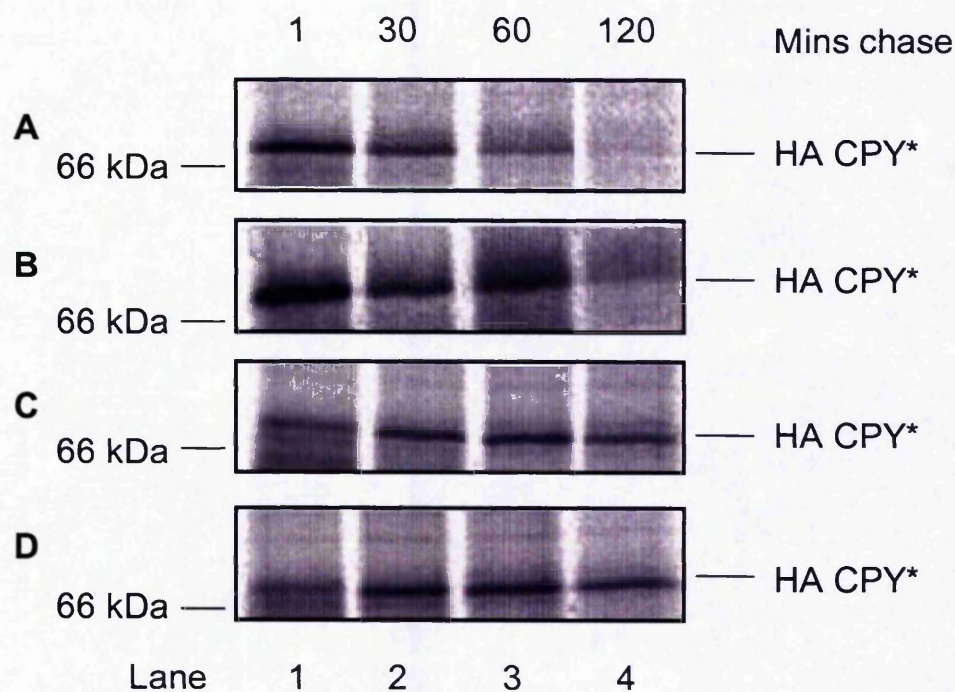


Figure 5.9 Investigating the degradation of misfolded proteins in *sss1-6* and *sss1-7*. Cells expressing plasmid borne copies of either *SSS1* (BWY538) (A), *sss1-6* (BWY544) (C) or *sss1-7* (CMY21) (D) as well as cells containing the *der3Δ* (B) mutation were transformed with pCT43 plasmid expressing a 3 HA tagged copy of the extensively characterised ERAD substrate CPY* (Prc1-1p). After this, cells were grown in sulphate free selective media minus uracil to early log phase and then 5 O.D._{600nm} of cells were isolated and labeled with ³⁵S methionine/cysteine for 20 minutes (pulse). Next, cells were incubated with 2 mM unlabelled methionine/cysteine for either 0, 30, 60 or 120 minutes (chase) after which total cell protein extracts were prepared and incubated with 1 μl CPY antiserum/O.D._{600nm}. Then, antibodies were immobilised with Protein A conjugated sepharose beads and the associated material was analysed by SDS-PAGE and visualised by phosphorimaging.

5.6 *In vitro* translocation of pp α f into *sss1-6* and *sss1-7* membranes

Immunoprecipitation of p1 CPY and p1 HA-CPY* from both *sss1-6* and *sss1-7* cells showed that these were aberrant when compared to those isolated from wild type (figure 5.3 A, lane 1 with lanes 3 and 4) as they appeared to be both larger and heterogeneous in all cases indicating a defect in biogenesis. The size difference is too slight for it to imply that signal peptide processing has been perturbed. Therefore, as the polypeptide chain is constant throughout comparison it was reasoned that the anomaly observed may be the consequence of the processing of the glycan chains that are added to the protein in the ER lumen.

To investigate whether the glycan chains of glycoproteins are aberrantly processed in *sss1-6* and *sss1-7*, we investigated the *in vitro* translocation of pp α f into membranes that have been isolated from these cells. The biogenesis of this secretory precursor has been extensively characterised. It is predominantly translocated via Sec62p dependent translocation but can also be translocated via SRP dependent translocation. Following translocation, the signal peptide is cleaved and the pro-protein is glycosylated three times via N- linked glycosylation.

As pp α f is translocated post-translationally *in vitro*, the precursor is first translated in a rabbit reticulocyte lysate and translation is then inhibited via the addition of cycloheximide and ribosomes and ribosome associated protein are isolated and removed via centrifugation. The *in vitro* translated material is then incubated with a crude membrane preparation that has been isolated from strains of interest. The reaction is then prepared for SDS-PAGE and the reaction visualised by autoradiography.

Incubation of the *in vitro* translated pp α f with membrane storage buffer alone identified the 18 kDa pp α f (figure 5.10, lane 1). There are also 2 other higher molecular weight products of unknown origin. However, these are present in the absence of membranes and do not represent translocated forms of pp α f. Following the incubation of *in vitro* translated pp α f with membranes isolated from cells expressing *SSS1*, three other proteinaceous species

are isolated along with the non-translocated pp α f (figure 5.10, lane 2). These represent the glycosylated intermediates of p α f, specifically g1 α f, g2 α f and g3 α f, of which g2 α f and g3 α f are the major species after translocation (figure 5.10, lane 2). Interestingly the two higher molecular weight proteins identified in the translation reaction that had been incubated with membranes storage buffer were no longer detected following the incubation with membranes (figure 5.10, lane 2). Surprisingly, there was no reduction in the efficiency with which pp α f was translocated into membranes isolated from cells expressing either *sss1-6* or *sss1-7* (figure 5.10, compare lane 2 with lanes 3 and 4). However, unlike in membranes isolated from cells expressing *SSS1*, eight translocated forms of alpha factor were identified following the incubation of pp α f with membranes isolated from either *sss1-6* or *sss1-7* (figure 5.10, lanes 3 and 4). Three of these co-migrated with those identified in wild type membranes, specifically g1 α f, g2 α f and g3 α f (figure 5.10, compare lane 2 with lanes 3 and 4). The additional forms of alpha factor appeared to be intermediate forms of g1 α f, g2 α f and g3 α f. One migrated between g1 α f and g2 α f, two between g2 α f and g3 α f and two had a greater molecular weight than g3 α f (figure 5.10, lanes 3 and 4). These additional species of alpha factor have been identified to co-migrate with alpha factor that has been translocated into membranes isolated from either ER glucosidase 1 or ER glucosidase 2 deficient cells (personal communication, B. M. Wilkinson). Specifically, the higher molecular weight intermediates were found to co-migrate with those identified in glucosidase 1 deleted membranes and the lower molecular weight intermediates co-migrate with species identified in glucosidase 2 deleted membranes.

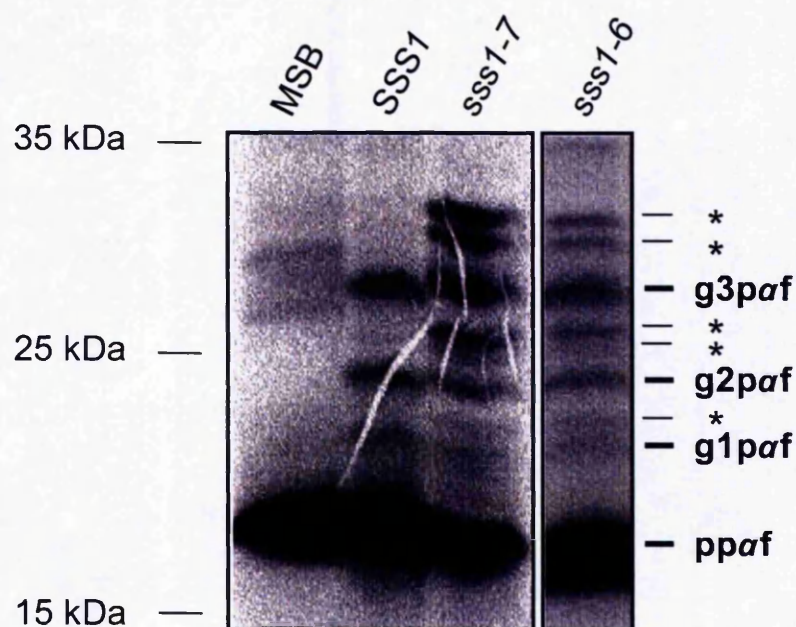


Figure 5.10 *In vitro* translocation of prepro alpha factor into *sss1-6* and *sss1-7* membranes. Prepro alpha factor was translated *in vitro* using rabbit reticulocyte lysate. Ppaf was then incubated with either membrane storage buffer (MSB) alone as a control or incubated with membranes isolated from either w303 α (*SSS1*), CMY21 (*sss1-7*) or BWY544 (*sss1-6*) at 30°C for 20 minutes. After incubation, samples were incubated with an equal volume of 2X Laemmli sample buffer, analysed by SDS-PAGE and detected by phosphorimaging. The bands labeled g1paf g2paf and g3paf are the mono, doubly and triply glycosylated alpha factor respectively and are denoted in bold. * denotes the additional translocated forms of alpha factor identified in membranes isolated from *sss1-6* and *sss1-7*.

ER glucosidase 1 and 2 function during the biogenesis of the N- linked glycan chain following its addition to the acceptor site of a glycoprotein. ER glucosidase 1 removes the terminal α 1, 2- glucose residue and glucosidase 2 cleaves the inner two α 1, 3- glucose residues (figure 5.11). This data suggests that the biogenesis of glycoproteins is perturbed in *sss1-6* and *sss1-7* and perturbation may be the consequence of aberrant trimming of glycan chains.

5.7 Glycosylation in *sss1-6* and *sss1-7* is aberrant *in vivo*

Previous studies have identified that yeast cells which exhibit defective glycosylation and glycan processing are hypersensitive to the aminoglycoside hygromycin B. Initially, hygromycin B sensitivity was contributed to defects in glycoprotein processing in the Golgi (Dean, 1995). However, further characterisation indicated that hygromycin B hypersensitivity is common to all aspects of aberrant glycosylation as mutants that were defective in the assembly of the glycan chain in the cytosol (*alg4*), in N-linked glycosylation (*wbp1*) and N-linked glycan processing in the ER (*gls1*) were all susceptible to this reagent (Dean, 1995 and references there in). Furthermore, the mutants affected at the earliest steps of glycosylation, glycan assembly and in N- linked glycosylation, were more susceptible to hygromycin B than mutants that function late on, e.g. outer chain elongation in the Golgi (Dean, 1995). As many of the cell wall and plasma membrane proteins in yeast are extensively glycosylated it was concluded that hygromycin B hypersensitivity is the result of aberrant glycosylation that affects the permeability of the cell wall and/or plasma membrane to this reagent.

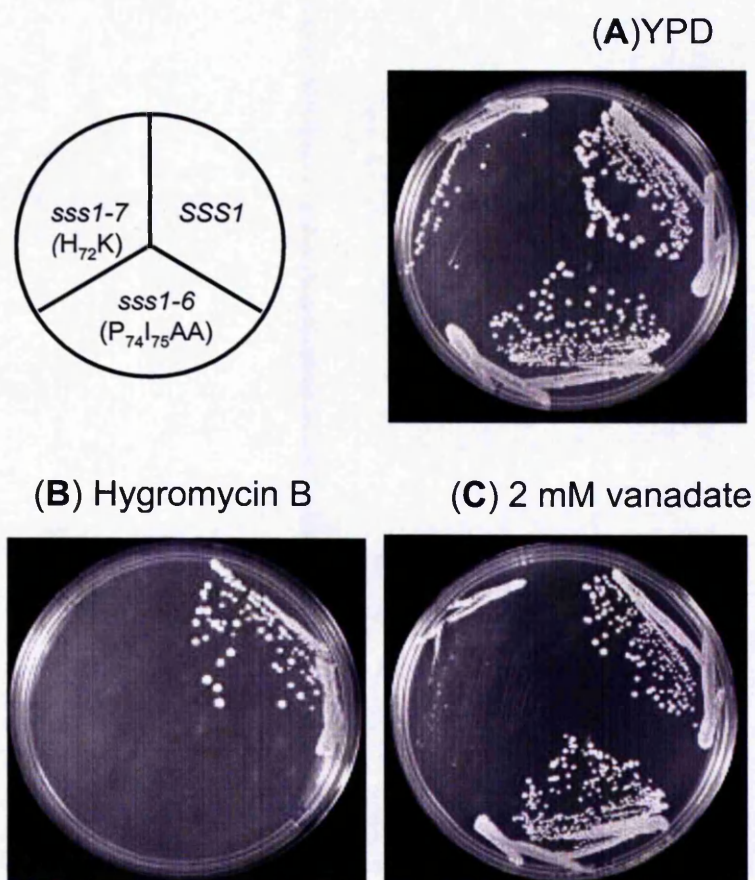


Figure 5.11 *sss1-6* and *sss1-7* are sensitive to hygromycin B and vanadate. BWY538, BWY544 and CMY21 cells expressing plasmid born copies of *SSS1*, *sss1-6* or *sss1-7* respectively were plated out onto either YPD (A), YPD supplemented with 12.5 $\mu\text{g/ml}$ hygromycin B (B) or 2 mM sodium metavanadate (C). Cells were incubated at 30°C for 2 days

As the gene encoding glucosidase 1, *GLS1*, was found to define a complementation group for hygromycin B hypersensitivity and that N-linked glycan processing was aberrant, *in vitro*, in *sss1-6* and *sss1-7* membranes we postulated that these cells would be hypersensitive to hygromycin B. Cells expressing either *SSS1*, *sss1-6* or *sss1-7* were plated out onto either nutrient rich media or nutrient rich media supplemented with 12.5 $\mu\text{g/ml}$ hygromycin B and grown at 30°C for 2 days. As expected, all of the strains were able to grow on nutrient rich media (figure 5.11 A). However, only cells expressing *SSS1* were able to grow on media supplemented with 12.5 $\mu\text{g/ml}$ hygromycin B (figure 5.11 B). Hygromycin B hypersensitivity in *sss1-6* and *sss1-7* would imply that glycosylation is aberrant in these cells. Furthermore, the inability to grow on media supplemented with only 12.5 $\mu\text{g/ml}$ hygromycin B may suggest that glycosylation is aberrant during the early stages of glycan processing.

As mutants that were found to be hypersensitive to hygromycin B were isolated following as a consequence of their increased resistance to vanadate (Dean, 1995) we wondered whether *sss1-6* and *sss1-7* cells would be resistant to this reagent. Cells expressing either *SSS1*, *sss1-6* or *sss1-7* were plated out on to nutrient rich media supplemented with 2 mM vanadate. Cells expressing *SSS1* did not appear to be sensitive to 2 mM vanadate (figure 5.11 C). However, *sss1-7* cells appeared to be hypersensitive to 2 mM vanadate (figure 5.11 C). *sss1-6* cells appeared to be mildly sensitive to 2 mM vanadate, although the effect was not as severe as that identified with *sss1-7* (figure 5.11 C). Therefore hypersensitivity hygromycin B does not correlate to an increase in resistance to vanadate in *sss1-6* and *sss1-7*.

5.8 Discussion

Sss1p is conserved through evolution in both sequence and function and is essential for the translocation of proteins into the ER in eukaryotes or into the periplasm of prokaryotes. Sequence alignment analysis identifies the C- terminal 12 residues of Sss1p to be particularly highly conserved. Furthermore, these residues are required for Sss1p function as mutants of *sss1* where this region has been deleted or replaced with that of an unrelated sequence can no longer function (J. K. Brownsword, 2000). We have identified two mutants of *sss1* where by mutations in this region of the protein disrupt function. These mutants, *sss1-6* and *sss1-7*, containing the P₇₄A I₇₅A and H₇₂K mutations respectively, are temperature sensitive and accumulate secretory precursors. Accumulation of both SRP- dependent and Sec62p dependent precursor proteins in *sss1-6* and *sss1-7* again reflects that this protein is essential for ER translocation. The ability of these mutant proteins to form protein complexes required for the translocation of proteins into the ER was investigated by cross-linking with DSS. The efficiency with which Sec61p could be crosslinked to either mutant protein was unperturbed. However, in both cases the efficiency with which either Sss1-6p or Sss1-7p could be covalently coupled to Sec63p was greatly reduced in comparison to the efficiency with which Sss1p can be cross-linked to Sec63p. Again, failure to recruit Sec63p to the translocon would be detrimental to ER translocation as this protein, like Sss1p, is absolutely required for this function. But, ER translocation is not completely defective. ER translocation activity indicates that a significant amount of protein is still translocated into the ER. Therefore the observed translocation defect and decrease in efficiency with which Sec63p could be covalently coupled to Sss1-6p and particularly Sss1-7p may indicate that the interaction of Sec63p with these mutants is much more transient and/or labile than that identified between Sec63p and Sss1p.

Early topological analysis of Sss1p indicated that the C- terminal 12 residues extend into the ER lumen and follow a short transmembrane domain of relatively weak

hydrophobicity (Esnault *et al.*, 1994). More recent structural analysis predicts that only the C-terminal 4 residues extend into the lumen and the preceding 8 residues are part of the Sss1p transmembrane domain. In chapter 4 we report that recruitment of Sec63p to the Sec61 complex requires the TMD of Sss1p. Therefore the apparent decrease in efficiency with which both Sss1-6p and Sss1-7p can be covalently coupled to Sec63p would support this model as these mutations are predicted to be in the transmembrane domain of Sss1p.

Although it would appear that *sss1-6* and *sss1-7* may affect the stability of the Sec63 protein they did not affect its gel mobility, unlike the depletion of Sss1p from cells. However, analysis of the Kar2p and Sil1p levels were significantly elevated in *sss1-6* and *sss1-7*. As these proteins are required to function in protein folding as well as in ER translocation, their elevation is consistent with the ER lumen being compromised in these cells and the UPR being induced. Genomic analysis has indicated that the UPR induces the transcription of 381 target genes in yeast (Travers *et al.*, 2000), of which *KAR2* and *SIL1* are two of these genes. Consequently, the levels of Kar2p have been found to be elevated upon UPR induction (Dürr *et al.*, 1998), however, the affect of UPR induction on Sil1p has not been previously characterised. A direct comparison between wild type cells that have either been mock treated or treated with tunicamycin would indicate that under normal conditions the major Sil1 protein would appear to be glycosylated. Based on size, it would be predicted that this would be glycosylated either once or twice. However, upon UPR induction the major Sil1 protein would appear to be unglycosylated. The significance of this is unknown. But, the Sil1 protein has previously been shown to have a very short half life (personal communication, B. M. Wilkinson). One possibility is that the substantial increase in unglycosylated Sil1p is that this is more stable than its glycosylated derivative. Alternatively, glycosylation of Sil1p may affect the activity of the protein. All of this remains to be resolved.

The detected increase in Kar2p and Sil1p levels in *sss1-6* and *sss1-7* indicated that the UPR may be induced in these cells. This possibility was investigated by detecting the expression and activity of the β -galactosidase gene whose expression is induced by the UPR. Analysis indicated that β -galactosidase activity was induced 4.5 fold in *sss1-6* and 4 fold in *sss1-7* relative to the activity identified in wild type cells. Significantly, the β -galactosidase activity was higher than that observed in *lhs1* null cells (3.5 fold induction of β -galactosidase activity) whose viability requires the UPR to be induced. Therefore, this would imply that UPR induction is significant in these cells and that constitutive UPR induction maybe required to maintain cell viability, although the latter needs to be confirmed.

UPR induction in yeast is the consequence of the accumulation of misfolded protein in the ER. This occurs as Kar2p is titrated away from the UPR signal transduction molecule, which in turn activates this molecule, in order to facilitate the folding of misfolded protein. In order to investigate whether induction of the UPR could be the consequence of the accumulation of misfolded protein in the ER lumen we investigated ERAD in *sss1-6* and *sss1-7*. We identified that ERAD of the model substrate, CPY*, was severely impaired in *sss1-6* and *sss1-7* cells and this would correlate with the induction of the UPR. As there is a large body of evidence suggesting that the Sec61 translocon also functions as the ER dislocon, our data indicating that ERAD is severely impaired in these *sss1* mutants would provide substantial evidence to support this. However, we cannot rule out the possibility that the UPR is an indirect consequence of the observed translocation defect associated with both the *sss1-6* and *sss1-7* mutation. But, analysis of the translocated forms of CPY, p1 and p2 CPY, proved particularly informative. Upon translocation the signal peptide is removed from CPY and then this is glycosylated four times. Therefore, as the CPY protein backbone is the same in wild type and both *sss1-6* and *sss1-7* the observed p1 and p2 heterogeneity in *sss1-6* and *sss1-7* compared to wild type must be a consequence of the glycan chains added to the protein.

In order to resolve this we investigated the early stages of the *in vitro* biogenesis of alpha factor. Upon translocation into the ER alpha factor is glycosylated three times and these forms as well as non-translocated alpha factor were observed with wild type membranes. These forms were also detected in membranes isolated from *sss1-6* and *sss1-7* however, five other derivatives were also detected in these membranes and these appeared to represent higher molecular weight intermediates of the $g1\alpha f$, $g2\alpha f$ and $g3\alpha f$ forms. Infact, these species were found to co-migrate with $g1\alpha f$, $g2\alpha f$ and $g3\alpha f$ that were detected following incubation of $pp\alpha f$ with membranes that were defective in either glucosidase 1 or glucosidase 2 activity (personal communication, B. M. Wilkinson). Glucosidase 1 and glucosidase 2 are required to process the glycan chain once it has been linked to the polypeptide chain. Glucosidase 1 cleaves the outer most $\alpha 1, 2$ - linked glucose residue of the $GlcNAc_2Man_9Glc_3$ core glycosylation unit whereas glucosidase 2 cleaves the two inner most $\alpha 1, 3$ glucose residues. Therefore loss of glucosidase 1 activity would result in the accumulation of N- linked glycoproteins with $GlcNAc_2Man_9Glc_3$ moieties whereas perturbation of glucosidase 2 activity would result in the accumulation of N- linked glycoproteins with $GlcNAc_2Man_9Glc_2$ or $GlcNAc_2Man_9Glc_1$ moieties. As $g1\alpha f$, $g2\alpha f$ and $g3\alpha f$ can all be detected following the translocation of $pp\alpha f$ into *sss1-6* and *sss1-7* membranes it would appear that the catalytic efficiency of glucosidase 1 and 2 is reduced rather than lost. However it remains to be confirmed that it is indeed glucosidase 1 and 2 activity that is compromised in *sss1-6* and *sss1-7* membranes and not other glycan modifying proteins and it would prove particularly informative to analyse the global modification of N- linked glycan chains from *sss1-6* and *sss1-7* cells.

In agreement with the defective glycan processing detected *in vitro* in both *sss1-6* and *sss1-7* were found to be hygromycin B. Hygromycin B hypersensitivity was characterised to be the result of defective glycan chain processing in the Golgi. However, more severe

phenotypes were identified in mutant cells that were deficient in early glycosylation processes. Identification that 12.5 $\mu\text{g/ml}$ of hygromycin B is toxic to *sss1-6* and *sss1-7* cells would be consistent with early stages of glycosylation being defective. As a similar phenotype is identified in mutants that are either defective in glycosylation or in trimming the N- linked glycan in the ER this is consistent with the ER lumen being perturbed in *sss1-6* and *sss1-7*.

We do not know how mutations in the C- terminal 12 residues of Sss1p could be so detrimental to the ER lumen. We cannot rule out the possibility that the translocation defect associated with these mutations depletes the ER of enzymes that are required for efficient glycosylation. Alternatively Sss1p may be directly required to regulate the activity of enzymes that are required for these processes. Interestingly, it has been reported that Sss1p is required to co-ordinate N- linked glycosylation and directly interacts with a component of the oligosaccharyl transferase complex (OST), Wbp1p an essential component of the OST complex, and this interaction has been proposed to regulate function (Scheper *et al.*, 2003). Although there is evidence that glycoproteins maybe underglycosylated at the restrictive temperature in *sss1-6* and *sss1-7* this does not appear to be so at the permissive temperature. These mutations appear to affect the processing of glycan chains rather than affect the actual glycosylation of the protein. We cannot dismiss the possibility that Sss1p may directly regulate glycan processing enzymes. However, this would appear to be unlikely as the *sss1-6* and *sss1-7* mutations appear to be in the TMD of Sss1p and many glycan processing enzymes are soluble luminal factors. It would therefore appear that aberrant glycan processing is indirect.

The correlation between hygromycin B hypersensitivity and aberrant glycosylation was detected following the characterisation of mutants that were resistant to vanadate, a potent inhibitor of tyrosine phosphatases and adenosine triphosphatases. As mutants that were found to be resistant to vanadate were identified to be sensitive to hygromycin B we wondered

whether the converse would be true for *sss1-6* and *sss1-7*. Surprisingly we found that *sss1-6* and *sss1-7* cells in particular were also sensitive to vanadate.

Vanadate has been shown to be a potent inhibitor of tyrosine phosphatases and adenosine triphosphatases, in particular P- Type ATPases (Strayle *et al.*, 1999). Three P- type ATPases in yeast have been shown to be required to maintain organellar cation homeostasis, specifically Cod1p, Pmc1p and Pmr1p. These have been shown to be localised to the ER, Vacuole and Golgi specifically (Dürr *et al.*, 1998; Cronin *et al.*, 2002 and results therein). However, these proteins appear to functionally overlap as Pmr1p is also required for ER cation homeostasis (Strayle *et al.*, 1999) and the overexpression of *PMCI* has been shown to suppress specific phenotypes associated with the *pmr1Δ* mutation (Dürr *et al.*, 1998). Incubation of wildtype cells with 1 mM vanadate has been shown to deplete the total free ER luminal calcium by half as does the *pmr1* null mutation (Strayle *et al.*, 1999).

Interestingly, a number of mutants that were found to be hypersensitive to hygromycin B encode for proteins that require cations as co-factors for function. In particular, ER mannosidase I requires calcium for function (Vashist *et al.*, 2002) and a number of enzymes that are required for N- linked glycan processing in the Golgi require manganese to function (Dürr *et al.*, 1998) and this raises the possibility of whether aberrant glycan processing in *sss1-6* and *sss1-7* cells correlates with aberrant cation homeostasis in the ER and Golgi?

We report in this study that the ERAD of CPY* is severely impaired in *sss1-6* and *sss1-7* cells. Although we cannot rule out the possibility that this defect is directly the consequence of Sss1p being required for the dislocation of proteins from the ER lumen into the cytosol the defect may be the consequence of aberrant glycan processing. We have identified that *sss1-6* and *sss1-7* may possess phenotypes that are common to *cod1Δ*, *pmr1Δ* and *cod1Δ pmr1Δ* mutants, specifically sensitivity to vanadate. Intriguingly these mutants are also defective in ERAD (Dürr *et al.*, 1998; Vashist *et al.*, 2002). However, these mutants are

defective in the ERAD of misfolded glycoproteins, specifically CPY*, as the dislocation of a misfolded non-glycosylated protein, Ste6-166p, was not impaired (Vashist *et al.*, 2002). ERAD of misfolded glycoproteins was found to be defective in these mutants due to defective glycan trimming by ER mannosidase I (Vashist *et al.*, 2002) as *cod1Δ*, *pmr1Δ* cells were found to accumulate glycoproteins with the GlcNAc₂Man₉ structure, a structure that does not support the degradation of CPY* (Knop *et al.*, 1996; Jakob *et al.*, 1998b). To investigate whether ERAD in general or whether the degradation of only a subset of misfolded proteins is impaired in *sss1-6* and *sss1-7* cells it is necessary to investigate the degradation of other misfolded proteins. This would be of particular interest considering that glycan trimming appears to be aberrant in these *sss1* mutants and would reveal if there are more phenotypic similarities between these *sss1* mutants and *cod1Δ*, *pmr1Δ* and *cod1Δ pmr1Δ* mutants.

To date, the observed phenotypic similarities between *sss1-6* and *sss1-7* and mutants that are required to maintain organellar cation homeostasis leads to the postulation of a hypothetical model that implicates *sss1-6* and *sss1-7* to be mutants that are defective in gating the ER translocon. This model would predict that the translocon, and therefore the ER membrane, would become more porous allowing the cross contamination of cytosol with ER lumenal contents. However these phenotypic similarities may be the consequence of the levels of these ion transporters becoming limiting in the cells due to the severity of the translocation defect associated with these mutations.

In order to prove or disprove this model a number of suppressers of *sss1-7* temperature sensitivity have been isolated. It is anticipated through the characterisation of these suppressers of *sss1-7* temperature sensitivity we will gain an insight into why mutations within the conserved C- terminal 12 residues of Sss1p are so detrimental to the lumen of the ER.

Chapter 6

Sec63p is phosphorylated at steady state

6.1 Sec63p is phosphorylated at steady state

In chapter 4 it was discovered that the Sec63 protein migrates aberrantly following the repression of *SSS1*. This may be the consequence of covalent modification or through the protein becoming more prone to proteolysis during the isolation of microsomes from these cells. Analysis of the Sec63p sequence by both prosite and NetPhos sequence analysis tools (www.expasy.ch) predicts the cytosolic C- terminus to contain numerous consensus sites for phosphorylation (figure 6.1 A), specifically 29 serine, 12 threonine and 9 tyrosine. To test whether the increase in Sec63p electrophoretic mobility following the depletion of Sss1p could be the consequence of aberrant phosphorylation the electrophoretic mobility of Sec63p was investigated following the treatment of wild type microsomes with calf intestinal alkaline phosphatase (CIP). 0.5 A_{280nm} of membranes was either mock-treated in dephosphorylation buffer and glycerol or incubated with 10U of CIP for 30 minutes at 30°C. After SDS-PAGE and western transfer samples were incubated with anti Sec63p antibodies. When wild type microsomes that had been mock treated were investigated we identified an anti Sec63p reactive species of approximately 73 kDa (figure 6.1 B, lane 1). However, following the treatment with CIP it was found that there was a subtle increase in the electrophoretic mobility of Sec63p when compared to microsomes that had been mock treated (figure 6.1 B, compare lanes 1 and 2). Furthermore, this band shift was related to the Sec63p aberration observed in microsomes that had been isolated from cells where *SSS1* had been repressed. These data imply that Sec63p is phosphorylated at steady state and that phosphorylation may require expression of *SSS1*.

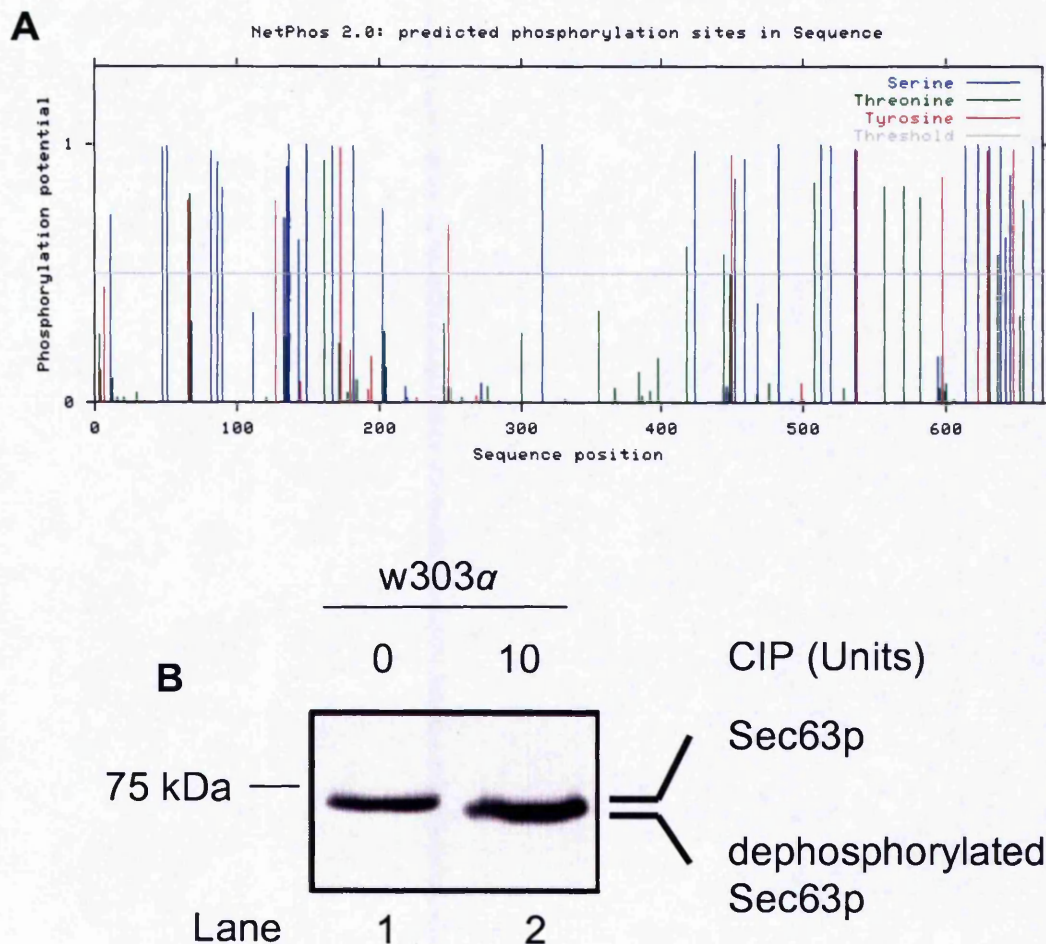


Figure 6.1 Sec63p is potentially phosphorylated. (A) Potential phosphorylation sites in Sec63p were predicted using the NetPhos sequence analysis program (www.expasy.ch). (B) 1 A_{280nm} equivalents of membranes isolated from the wildtype strain w303 α were either mock treated with membrane storage buffer (lane 1) or incubated with 10 U calf intestinal alkaline phosphatase (lane 2) at 30°C for 30 minutes. Membranes were then isolated and denatured in 1X Laemmli and after SDS-PAGE and western transfer samples were probed with anti Sec63p antibodies.

The reduction in the relative molecular weight of the Sec63 protein following incubation of wild type microsomes with CIP is consistent with Sec63p being phosphorylated under normal growth conditions. To confirm this cells were labeled with 32 orthophosphate to observe the *in vivo* phosphorylation of Sec63p. Wild type cells (w303d) were grown in low phosphate medium (LP-YPD) to exponential growth phase and 5 OD_{600nm} units of cells were isolated, resuspended in fresh, pre-warmed LP-YPD and radiolabeled with 25 μ Ci/OD_{600nm} of 32 orthophosphate for 60 minutes. Following this, cell extracts were isolated in the presence of phosphatase inhibitors, sodium vanadate and sodium fluoride, and immunoprecipitated with either a non-specific antiserum or with anti Sec63p antibodies. As expected, no radiolabeled proteinaceous species was immunoprecipitated with non-specific antiserum (figure 6.2, lane 1). However, immunoprecipitation with anti Sec63p antibodies isolated a single 32 P labelled species of approximately 73 kDa (figure 6.2, lane 2). These data are consistent with Sec63p being phosphorylated at steady state.

6.2 Sec63p phosphorylation requires the expression of Sss1p

As an increase in Sec63p electrophoretic mobility after depletion of Sss1p from cells correlated with the retardation in Sec63p electrophoretic mobility after treatment with CIP we hypothesised that phosphorylation of Sec63p requires the expression of *SSS1*. Therefore, Sec63p phosphorylation was examined following the repression of *SSS1* in FKY198 cells. 5 OD_{600nm} of cells in logarithmic growth phase were subcultured into LP-YP supplemented with either 2% glucose or 2% galactose and grown for 6 hours to regulate the expression of the *SSS1* gene.

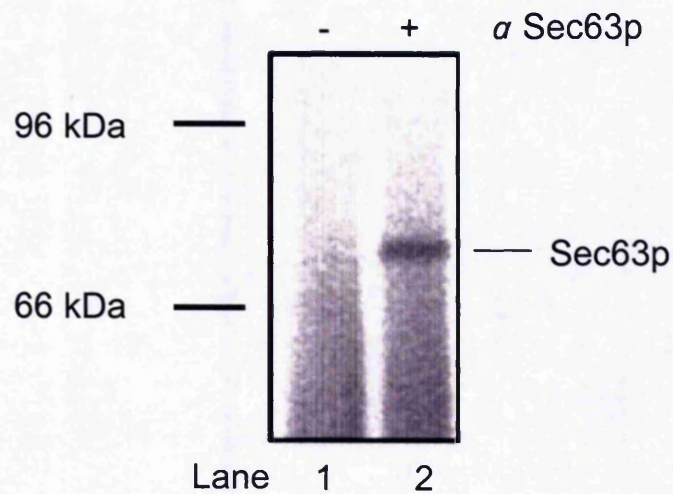


Figure 6.2 Sec63p is phosphorylated at steady state. w303d cells were grown in low phosphate media supplemented with 2% glucose (LP-YPD) to early exponential growth phase. At this point 5 O.D._{600nm} of cells were isolated and resuspended in 5 ml of fresh LP-YPD and labeled with 25 $\mu\text{Ci}/\text{O.D.}_{600\text{nm}}$ ^{32}P orthophosphate for 60 minutes. After this, total cell protein extracts were prepared in the presence of 1 M urea and phosphatase inhibitors and incubated with 2 $\mu\text{l}/\text{O.D.}_{600\text{nm}}$ of non-immunised antisera (lane 1) or with anti Sec63p antibodies (lane 2) Samples were denatured with 1X Laemmli sample buffer, analysed by SDS-PAGE and visualised by phosphorimaging.

After this, cells were labelled for either 0, 10, 20, 30 or 60 minutes with 25 $\mu\text{Ci}/\text{OD}_{600\text{nm}}$ $^{32}\text{orthophosphate}$ and Sec63p was immunoprecipitated from cell extracts. As expected, a 73 kDa phosphate labelled protein was isolated from cells where *SSS1* had been expressed (figure 6.3 A, lanes 1-5). Phosphorylated Sec63p was isolated after cells were incubated with $^{32}\text{orthophosphate}$ for 10 minutes (figure 6.3 A, lane 2) and phosphorylation appeared to be optimal between 30 and 60 minutes (figure 6.3 A, lanes 4 and 5). Following *SSS1* repression, trace amounts of phosphorylated Sec63p were isolated after cells were labeled with $^{32}\text{orthophosphate}$ for 20 minutes (figure 6.3 A, lane 8) and phosphorylated Sec63p was immunoprecipitated from these cells following labelling for 30 and 60 minutes (figure 6.3 A, lanes 9 and 10). Western analysis of total protein extracts isolated from cells that had been grown with or without glucose indicated that although the *SSS1* gene had been repressed the Sss1 protein had not been entirely depleted (figure 6.3 B, compare lanes 1 and 2). However, following 60 minutes of labelling, the level of phosphorylated Sec63p isolated from cells following *SSS1* repression appeared to be less than that incorporated into Sec63p following labelling cells for 10 minutes with $^{32}\text{orthophosphate}$ under normal growth conditions (figure 6.3 A, compare lanes 2 and 10). These results may imply that the phosphorylation of Sec63p requires the expression of *SSS1* and that the level of Sec63p phosphorylation in cells after repression of *SSS1* would be consistent with the trace levels of Sss1 protein remaining in the cells. To verify these findings it is necessary to confirm that Sec63p is phosphorylated when a gene that is essential for cell viability whose function is independent of ER translocation has been repressed.

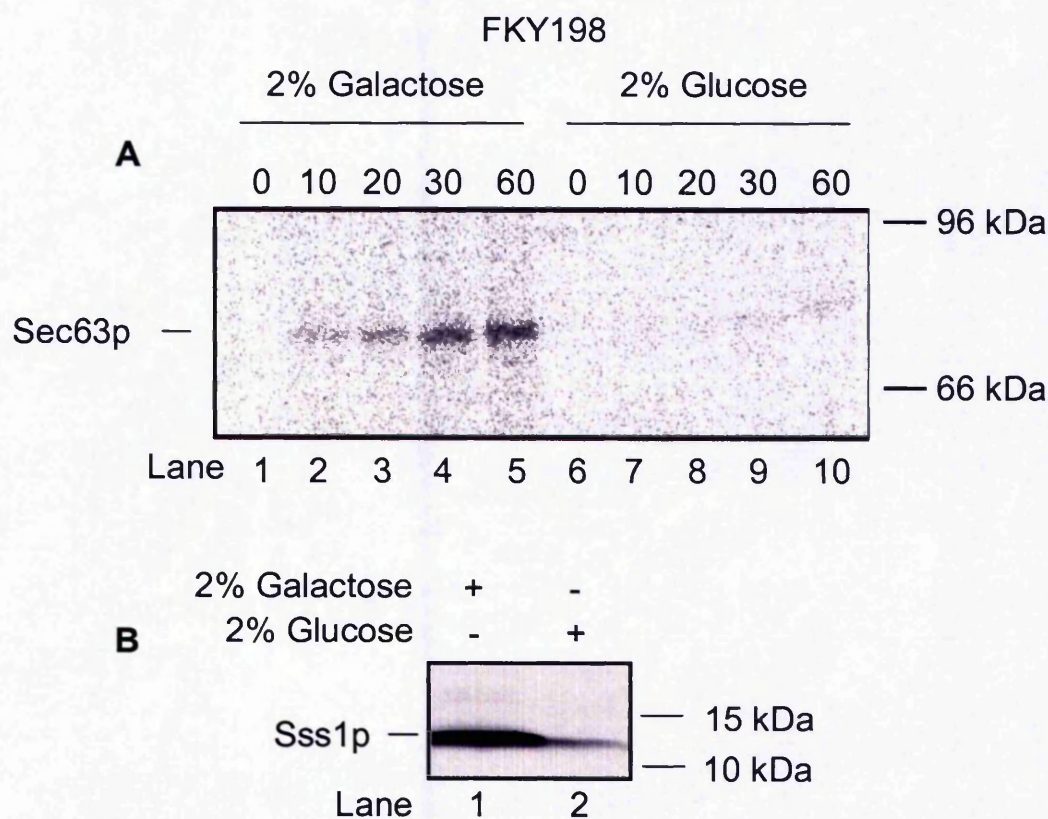


Figure 6.3 Analysis of Sec63p phosphorylation after the depletion of Sss1p. (A) FKY198 cells were grown to early exponential growth phase in low phosphate media containing 2% galactose (LP-YPGal). After this, cells were switched to fresh low phosphate media and grown in the presence of either 2% galactose to promote *SSS1* expression (lanes 1-5) or in 2% glucose to repress *SSS1* in these cells (lanes 6-10) for 6 hours at 30°C. After this, cell cultures were incubated with 25 $\mu\text{Ci}/\text{O.D.}_{600\text{nm}}$ ^{32}P orthophosphate for either 0, 10, 20, 30 or 60 minutes after which, total cell protein extracts were prepared from 5 $\text{O.D.}_{600\text{nm}}$ of cells, in the presence of 1 M urea and phosphatase inhibitors and incubated with 2 $\mu\text{l}/\text{O.D.}_{600\text{nm}}$ of anti Sec63p antibodies. Antibodies were then immobilised by Protein A coupled to sepharose beads, samples were eluted in 1X Laemmli sample buffer, analysed by SDS-PAGE and visualised by autoradiography. (B) Total cell protein extracts were prepared from 5 $\text{O.D.}_{600\text{nm}}$ of FKY198 cells grown in liquid YP supplemented with either 2% galactose (lane 1) or 2% glucose (lane 2), in which the *GAL10-SSS1* is either expressed or repressed respectively, for 6 hours to manipulate the expression of *GAL10-SSS1*. After this, 0.1 $\text{O.D.}_{600\text{nm}}$ equivalents of each extract was analysed by SDS-PAGE and after western transfer immunoblotted with anti Sss1p antibodies.

6.3 Sec63p is phosphorylated within its C- terminal 52 residues

Sequence analysis with the NetPhos protein phosphorylation prediction programme predicts that Sec63p could be phosphorylated in as many as 50 sites. Significantly, there appears to be a clustering of potential phosphorylation sites towards the extreme C- terminus of Sec63p. A number of laboratory strains exist that allow the expression of numerous *sec63* alleles that are truncated at the extreme C- terminus, specifically deletion of the C- terminal 14 (BYY7), 28 (BYY8) and 52 (AJY1) residues. We have used these strains to investigate whether these amino acids are important for Sec63p phosphorylation. The functionality of these truncations has previously been characterised. Although deletion of the C- terminal 14 residues of Sec63p is still viable, Sec62p dependent translocation is partially compromised (Young *et al.*, 2001). However, deletion of either the C- terminal 28 residues (Young *et al.*, 2001) or 52 residues (E. Davies, 2001; personal communication, A. J. Jermy) perturbs Sec63p function as it has been reported that this mutation compromise the ability of Sec63p to interact with Sec62p (Wittke *et al.*, 2000; Young *et al.*, 2001). Therefore, these truncations were expressed in cells containing a *MET3* regulated allele of *SEC63*. Cells were grown overnight in selective media minus methionine to early log phase ($\text{O.D.}_{600\text{nm}} \leq 0.5$) and cells were harvested and resuspended in pre-warmed LP-YPD and incubated for 6 hours at 30°C to repress *MET3-SEC63* (as reported in Young *et al.*, 2001). After this, 5 $\text{O.D.}_{600\text{nm}}$ units of cells were harvested and resuspended in 10ml of LP-YPD and labelled for 1 hour with $25\mu\text{Ci}/\text{O.D.}_{600\text{nm}}$ 32 orthophosphate. Sec63p truncations were then immunoprecipitated from total cell protein extracts prepared from 5 $\text{O.D.}_{600\text{nm}}$ of 32 orthophosphate labeled cells in the presence of phosphatase inhibitors. Again, phosphorylated Sec63p was immunoprecipitated from cells expressing *SEC63* (figure 6.4 A, lane 1). A 73 kDa species was immunoprecipitated from each of the truncations following the repression of *MET3-SEC63* (figure 6.4 A, lanes 2-4). However the intensity of the band is vastly reduced compared to cells expressing *SEC63*

(figure 6.4 A, compare lane 1 with lanes 2-4) and this is consistent with the trace levels of Sec63p remaining after repression of *MET3-SEC63* being immunoprecipitated and further supports the observation that Sec63p is phosphorylated.

Immunoprecipitation with anti Sec63p antibodies from total cell protein extract prepared from cells expressing either Sec63p Δ 14 (figure 6.4 A, lane 2) or Sec63p Δ 28 (figure 6.4 A, lane 3) identified a second ^{32}P labeled species that co-migrates with the predicted size of the truncation (compare figure 6.4 A, lanes 2 and 3 with figure 6.4 B, lanes 2 and 3). However the intensity of the labeled Sec63 Δ 28 protein appeared significantly lower than that of labeled Sec63p Δ 14 (figure 6.4 A, compare lanes 2 and 3). However, the amount of protein identified by western analysis appears comparable (figure 6.4 B, compare lanes 2 and 3). This is consistent with both Sec63p Δ 14 and Sec63p Δ 28 being phosphorylated and the reduction in the level of ^{32}P that is incorporated maybe the consequence of at least one phosphorylation site being present in the sequence between the two different truncations.

Analysis of cells expressing the Sec63p Δ 52 truncation was particularly informative. No other ^{32}P labeled species other than the trace amount of Sec63p present after repression of *MET3-SEC63* was immunoprecipitated from a total cell protein extract expressing this truncation (figure 6.4 A, lane 4). Furthermore, western analysis of an unlabeled extract with anti Sec63p antibodies identifies a protein of predicted molecular weight (figure 6.4 B, lane 4) that is comparable in intensity to both Sec63p Δ 14 and Sec63p Δ 28 (figure 6.4 B, compare lanes 2-4) indicating that the epitope identified by the anti Sec63p antibodies had not been deleted. This data indicates that the C- terminal 52 residues of Sec63p are required for phosphorylation. However, this may imply that phosphorylation proceeds in the C- terminal 52 residues of Sec63p with a minimum of two phosphorylation sites, one between residues D611-T635 and a second between residues T635-D649.

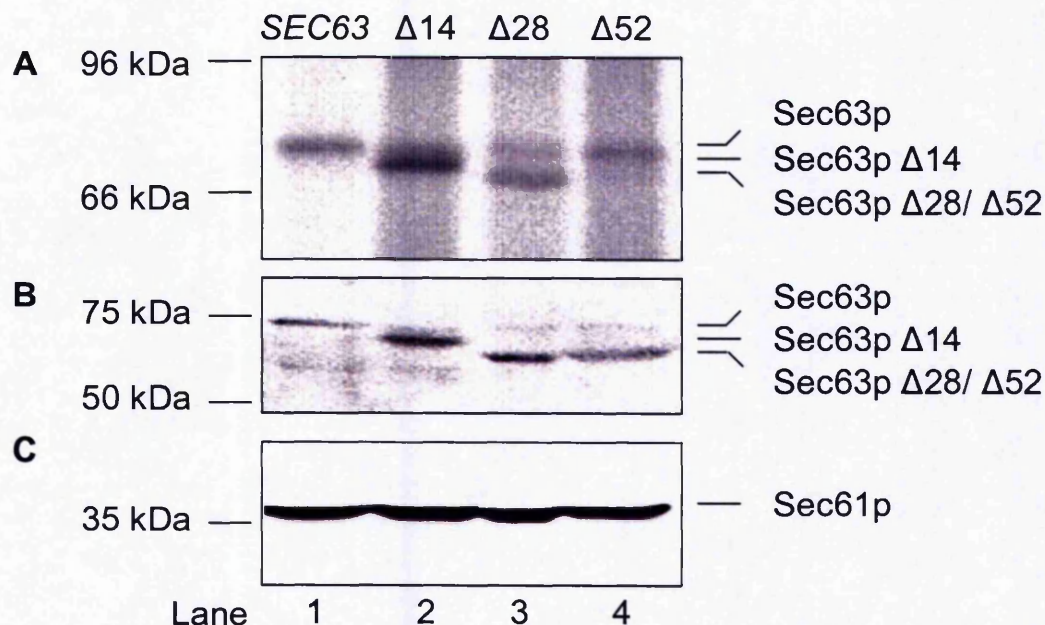


Figure 6.4 Analysis of Sec63p phosphorylation in Sec63p truncations. (A) w303 α , BYY7 (Sec63p Δ 14), BYY8 (Sec63p Δ 28) or AJY1 (Sec63p Δ 52) cells were grown to early exponential phase in YNB minus histidine and then switched to low phosphate media containing 2% glucose and grown for 6 hours at 30°C to repress *MET3-SEC63* expression. After this, 5 O.D._{600nm} cells were isolated and added to fresh LP-YPD and incubated with 25 μ Ci/O.D._{600nm} ³²orthophosphate for 30 minutes. Then total cell protein extracts were prepared in the presence of 1 M urea and phosphatase inhibitors and incubated with 2 μ l/O.D._{600nm} of anti Sec63p antibodies. Antibodies were then immobilised by Protein A coupled to sepharose beads, incubated with 1X Laemmli buffer and then analysed by SDS-PAGE and visualised by phosphorimaging. (B and C) w303 α , BYY7 (Sec63p Δ 14), BYY8 (Sec63p Δ 28) or AJY1 (Sec63p Δ 52) cells were grown to early exponential phase in selective media and then switched to low phosphate media containing 2% glucose, to repress *MET3-SEC63* expression, and grown for 6 hours at 30°C. Then, total cell protein extracts were prepared from 5 O.D._{600nm} of cells and 0.1 O.D._{600nm} equivalents were analysed by SDS-PAGE. After western transfer samples were immunoblotted with anti Sec63p antibodies (B) or anti Sec61p antibodies (C).

6.4 Discussion

Analysis of translocon components following the repression of *SSS1* indicated that the Sec63 protein synthesised in these cells appeared aberrant when compared to wild type as Sec63p had a faster electrophoretic mobility following the repression of *SSS1*. In chapter 4 we report that one function of Sss1p is to recruit Sec63p to the Sec61 complex in order to form functional ER translocons. As Sec63p can no longer interact with the translocon following the depletion of Sss1p from cells the Sec63 protein may be more prone to proteolysis during the extraction of membranes. However, microsomes are isolated from yeast cells in the presence of a large excess of protease inhibitors, making this unlikely, although not impossible. Secondly, it is unlikely that the Sec63p aberration is a consequence of proteolysis as the increase in electrophoretic mobility is very subtle. We therefore reasoned that this anomaly maybe the consequence of covalent modification.

Analysis of the Sec63p sequence with bioinformatics programs that predict potential protein modification indicated that Sec63p contains numerous potential phosphorylation sites. As translocon components have previously been identified to be phosphorylated and this modification was shown to be functionally significant (Gruss *et al.*, 1999) we entertained the possibility that Sec63p is phosphorylated and this is aberrant in the absence of Sss1p.

A Sec63p mobility shift was observed following the incubation of microsomes with calf intestinal alkaline phosphatase. Furthermore the mobility shift was comparable to that observed following the repression of *SSS1* implying that Sec63p may no longer phosphorylated in the absence of Sss1p. Confirmation that Sec63p is a phosphoprotein at steady state was obtained following the immunoprecipitation of a ^{32}P labeled protein with anti Sec63p antibodies from a ^{32}P labeled total cell protein extract, this was not isolated by an antiserum that had not been immunised with Sec63p antigen, and was depleted following the repression of a *MET3* regulated allele of *SEC63* in cells expressing truncated forms of Sec63p.

As this phenotype was originally identified following the repression of *SSS1* it was important to confirm that Sec63p is no longer phosphorylated after depletion of Sss1p. Sec63p phosphorylation was found to be optimal following labelling with ^{32}P for 30-60 minutes although a sufficient amount of Sec63p was labeled after a 10 minute pulse with 32 orthophosphate. However, only trace amounts of Sec63p was phosphorylated after 60 minutes of labelling following the repression of *SSS1*. Furthermore the amount of Sec63p phosphorylation after 60 minutes in *SSS1* repression conditions was lower than the amount observed after 10 minutes in the presence of Sss1p. This would appear to indicate that Sec63p phosphorylation requires the expression of *SSS1* however, we need to verify this with appropriate controls.

To date, it is not known which protein kinase is required to phosphorylate Sec63p. Sec63p phosphorylation would appear to require the expression of *SSS1* and this may indicate that Sss1p is itself a protein kinase that is directly required to phosphorylate Sec63p. However, this would appear to be unlikely as the Sss1p sequence appears to show no sequence similarity to known kinases. It is more likely that Sec63p phosphorylation is linked to the Sss1p dependent recruitment of Sec63p to the Sec61 complex. This model would predict that an unknown protein kinase is recruited to the site of ER translocation in order to phosphorylate Sec63p once it interacts with Sss1p. This remains to be confirmed.

The region of Sec63p that is phosphorylated has been investigated using *sec63* mutants with varying degrees of C- terminal truncation. Upon the repression of a *MET3* regulated allele of *SEC63* it was identified that a mutant of *sec63* where the C- terminal 52 residues had been deleted could no longer become phosphorylated. Expression and immunodetection of this mutant was confirmed by western blotting. However, analysis of *sec63* mutants where either the C- terminal 28 or 14 residues had been deleted may indicate that Sec63p is phosphorylated at least twice, at least once between residues D611-T635 and at least once between residues

T635-D649. This was concluded from the observation that although both Sec63p truncations were phosphorylated, significantly more label had been incorporated into Sec63p Δ 14.

Sequence analysis of the C-terminal 52 residues of Sec63p using the Prosite motifs prediction program has indicated that this region contains 8 strong candidate phosphorylation sites by CK2 (Casein Kinase 2) protein kinase. There are also three potential tyrosine phosphorylation sites. Intriguingly, it has been reported that CK2 has dual specificity as although it is predominantly a serine/threonine kinase it has also been shown to phosphorylate tyrosine residues (reviewed in Litchfield, 2003). Therefore, if Sec63p is phosphorylated in its C-terminal 52 residues it may imply that CK2 is the protein kinase that phosphorylates Sec63p.

The functional significance of Sec63p phosphorylation is not known. However, if Sec63p is phosphorylated within the C-terminal 52 residues it may have interesting implications regarding functionality. This region of Sec63p is extremely acidic, therefore addition of phosphates in this region would further contribute to the negative charge of this domain. Phosphorylation of acidic domains by CK2 has been reported to instigate protein-protein interaction, particularly in signal transduction and protein translation (reviewed in Meggio and Pinna, 2003). However, the region of Sec63p that maybe phosphorylated has been identified to be required to interact with Sec62p (Wittke *et al*, 2000; Young *et al.*, 2001). The nature of this interaction appears to be electrostatic as the N-terminus of Sec62p, required to interact with the acidic domain C-terminus of Sec63p, is positively charged. It is therefore attractive to speculate that Sec63p phosphorylation is required for its efficient interaction with Sec62p. As Sec62p is required exclusively for post-translational translocation this model would predict that phosphorylated Sec63p defines the pool of Sec63p that is required to function in Sec62p dependent translocation. Alternatively, Sec63p phosphorylation may provide the signal that switches the translocon from an inactive to an active state.

In order to ascertain the contribution of phosphorylation to Sec63p function it is paramount that both the kinase that phosphorylates Sec63p is isolated and the residues in Sec63p that are phosphorylated determined. Together these will indicate whether Sec63p phosphorylation is a post-translational phenomenon as, if this is the case, inhibition of Sec63p phosphorylation would be detrimental to the translocation of precursors that are translocated via Sec62p dependent translocation and not via SRP dependent translocation. If this is true the door will be opened to explore the possibility that Sec63p phosphorylation promotes the recruitment of Sec62p via direct interaction studies. However, if this is not the case it may be required to investigate Sec63p phosphorylation in a number of translocation mutants to ascertain the point with which it is phosphorylated.

Chapter 7

Discussion

Sss1p is an essential component of the Sec61 complex that is conserved throughout evolution, however its function in ER translocation is not known. In this study we have utilised numerous biochemical and genetic analyses to investigate the function of Sss1p in ER translocation.

The crystal structure of the homologous SecYE β complex from the archaeobacteria *Methanococcus jannaschii* has recently been solved (van den Berg *et al.*, 2004). The homologue of Sec61p, SecY, has been postulated to be comprised of two domains, TM1-5 and TM6-10 that are related to each other by two-fold symmetry. In order to form the translocation channel it has been proposed that these two domains are clamped together by the transmembrane domain of the Sss1p homologue, SecE (van den Berg *et al.*, 2004). The transmembrane domain of SecE has been suggested to wrap around the two domains of SecY, making single points of contact with TM6, 10, 5 and 1 and the C- terminus of SecE is predicted to be in close proximity to the β subunit of the complex. Also, the N- terminal amphipathic helix of SecE, and its homologues, has been proposed to lie on the plane of the membrane, towards the cytosolic face, and interact with the C- terminal half of SecY (Sato *et al.*, 2003; van den Berg *et al.*, 2004).

This data represents the X-ray structure of protein crystals isolated from an integral membrane complex of an inactive translocon in detergent rather than in lipid and it remains to be seen what rearrangements occur in the active SecY complex in a lipid bilayer. The precise molecular architecture of the yeast Sec61 complex remains unknown. Sequence homology between the components of the Sec61 complex and the SecY complex in archaeobacteria are weakly conserved. Also the mechanisms by which secretory precursors are translocated in these species significantly differ as in order to function the eukaryotic and prokaryotic translocons interact with entirely different sets of essential translocation co-factors, e.g. Sec63p (Young *et al.*, 2001) and SecA (Oliver and Beckwith, 1982) respectively. Therefore it

may be likely that the architecture of the eukaryotic translocation channel significantly differs to that identified in prokaryotes. But, data obtained in this study provides some evidence that the yeast Sec61 complex in microsomes shares some structural similarity to the prokaryotic translocation channel.

Cysteine scanning mutagenesis has been used to investigate the molecular architecture of the yeast Sec61 complex in an attempt to identify transmembrane domains of Sec61p that interact with that of Sss1p. This study did not identify any crosslink dependent interactions between the putative transmembrane domains of Sec61p and Sss1p. This may indicate that such interactions do not exist however there is a significant body of evidence that suggests otherwise. Therefore it is likely that the cysteine residues incorporated into these transmembrane domains are not in the correct orientation or proximity to detect such interactions. In contrast, the endogenous cysteine residue of Sss1p, located in the N- terminal amphipathic helix, was found to interact, by crosslinking, to *sec61* mutants where cysteine residues had been incorporated into either TM9 or TM10. This residue was found to be, by crosslinking, at most 6 Å away from TM9 and 6-15 Å from TM10 and this is consistent with the SecYEβ crystal structure (van den Berg *et al.*, 2004). Secondly, the extreme C- terminus of SecE has been postulated to be in close proximity to the β subunit (van den Berg *et al.*, 2004) and Secβ has been predicted to be in close proximity to TM4 of SecY (van den Berg *et al.*, 2004). Consistent with this, crosslinking analysis in a mutant of Sss1p where a cysteine residue has been inserted at the extreme C- terminus of this protein has indicated that this region of Sss1p is in close proximity to both TM3 and TM4 of Sec61p. Together, these data may suggest that there is a certain degree of structural conservation between the yeast Sec61 complex and the archaeobacterial SecY complex.

It has been demonstrated using a mutant of Sss1p where a cysteine residue has been inserted at its extreme C- terminus that this region can be crosslinked to an adjacent Sss1p molecule. Furthermore, these molecules have been identified to be no more than 6 Å apart indicating that they are in close proximity and may imply that Sss1p can form dimers in the ER membrane. Early structural analysis has indicated that the translocon forms ring like particles of approximately 85-100 Å in diameter (Hanein *et al.*, 1996). Following this it was postulated from the diameter and symmetry of the structure that 3-4 copies of the Sec61p complex associate to form the channel and the assembly may be regulated by interacting factors such as ribosomes and the Sec62/63 complex (Hanein *et al.*, 1996). Therefore, our data would be consistent with the oligomerisation of Sec61 complexes in the ER membrane.

Data isolated from prokaryotes would also suggest that the homologous SecY complex oligomerises to form the translocon as analysis of the SecY complex isolated from *Bacillus subtilis* by cryoelectron microscopy demonstrated that, like the eukaryotic Sec61 complex, the SecY complex forms ring like structures approximately 85 Å in diameter (Meyer *et al.*, 1999). Also, TM3 of SecE can be crosslinked to TM3 of SecE in an adjacent SecY complex (Kaufmann *et al.*, 1999; Veenendaal *et al.*, 2001). Subsequently, it has been postulated that two copies of the SecY complex can associate to form dimers in a back to back orientation where the interacting face of each Sec61 complex is formed by TM6, 10, 5 and 1, as well as the TM of SecE (van den Berg *et al.*, 2004). In this model, the extreme C- terminus of SecE/Sss1p in an adjacent complex would be approximately 40 Å apart. As the crosslinking analysis reported in this study has identified an interaction within 6 Å, this does not support dimerisation in this model. It has been reported that two such dimers may associate to form a tetrameric SecY complex (Clemons *et al.*, 2004). Upon inspection, our crosslinking data may support the formation of a tetramer of Sec61/SecY heterotrimers. Also, in agreement with this, the major species that is detected with anti Sec61p antibodies following BN-PAGE analysis of

microsomes isolated from wild type cells possesses a molecular weight of approximately 280 kDa and this is consistent with this species representing four copies of the Sec61 complex.

Although our data supports previous observations that the translocon is oligomeric, the significance of this remains unknown. It has been proposed that a single copy of the Sec61/SecY complex may form the pore and molecular dynamics modelling has indicated that the pore is 12 Å in diameter (van den Berg *et al.*, 2004; Clemons *et al.*, 2004). This data is consistent with electrophysiological studies that indicate that the eukaryotic translocon is 10-15 Å in diameter in a closed conformation (Hanein *et al.*, 1996; Hamman *et al.*, 1998). However, fluorescence quenching experiments have suggested that the functional channel has a diameter of approximately 40-60 Å and it has been proposed that following oligomerisation a single channel is formed (Hamman *et al.*, 1997). This issue remains to be resolved.

It has been suggested that the translocon is a regulated conduit where activation is coupled with a change in pore size from 15-20 Å to 40-60 Å (Hamman *et al.*, 1997; 1998). To accommodate this change it is predicted that the translocon undergoes significant structural re-modelling. Fluorescence quenching studies in mammalian microsomes have indicated that ribosomes provide the cytosolic seal that prevents the cross-contamination of the cytosol with ER lumenal contents during co-translational translocation. Following the complete translocation of substrate the ribosome is released and this has been postulated to induce a change in pore diameter from 40-60 Å to 15-20 Å and this has been proposed to correlate with a switch from an open to a closed conformation (Hamman *et al.*, 1998). A comparative analysis of the molecular environment of the extreme C- terminus of Sss1p following the release of ribosomes from membranes, indicated that this environment would appear to remain constant in the presence or absence of ribosomes. This would imply that the translocon and

translocation structures remain assembled whether in an open or closed conformation. Interestingly, the extreme C-terminus of Sss1p remains in close proximity to both TM3 and TM4 regardless of whether ribosomes are associated. This data would indicate that the position of the Sss1p C- terminus remains constant prior to or after the treatment of membranes with puromycin. Such treatment has been shown to decrease the pore diameter of the translocon from 40-60 Å to 15-20 Å and this has been proposed to require significant structural remodelling (Hamman *et al.*, 1998). Our data would indicate that if this true then the position of the C- terminus of Sss1p is not affected or that TM3 and TM4 of Sec61p and the C- terminus of Sss1p are in close proximity after a conformational change in the translocon.

Genetic and biochemical analysis in yeast indicates that the Sec61 complex alone is insufficient for ER translocation to proceed. Post-translational translocation requires the heptameric SEC complex (Panzner *et al.*, 1995), formed via an interaction between the heterotrimeric Sec61 complex and the heterotetrameric Sec62/63 complex. Although Sec62p functions solely in post-translational translocation, Sec63p is ubiquitously required for ER translocation (Young *et al.*, 2001). Therefore the co-translational translocon requires the recruitment of other factors in order to be functional. Using chemical crosslinking we have identified that Sec63p and Sss1p are in close proximity and this spatial alignment would appear to be sensitive to mutations in the transmembrane domain of Sss1p. Therefore, we can postulate a model in which one function of Sss1p is to recruit Sec63p to the translocation channel in order to form functional translocons.

Surprisingly, we found that depletion of Sss1p from cells affected the electrophoretic mobility of Sec63p when analysed by SDS-PAGE. Following this, it was discovered that Sec63p is phosphorylated at steady state and the Sec63p mobility shift, after the depletion of

Sss1p, appeared to correlate with Sec63p being no longer phosphorylated. This implies that phosphorylation of Sec63p requires the expression of *SSS1*. However, Sss1p shares no homology to known kinases, therefore it is highly unlikely that Sss1p directly phosphorylates Sec63p. It is more likely that Sec63p is phosphorylated following its Sss1p dependent recruitment to the translocon. How Sec63p is phosphorylated is unknown. It may be that association of Sec63p with the translocon induces a conformational change in the Sec63 protein which reveals sites for phosphorylation. Alternatively, Sec63p maybe readily phosphorylated at steady state but is rapidly dephosphorylated unless it is associated with the translocon. This remains to be seen.

It is not known which kinase is required to phosphorylate Sec63p however, previously analysis has demonstrated that Sec61 β is phosphorylated by protein kinase C in mammalian microsomes (Gruss *et al.*, 1999). Together this may suggest an intriguing possibility that ER translocation may be regulated by phosphorylation. Sequence analysis predicts the extreme C-terminus of Sec63p to contain several consensus sites for phosphorylation by casein kinase 2. Interestingly, deletion analysis has demonstrated that the C- terminal 52 residues of Sec63p, encompassing these sites, are required for Sec63p to be phosphorylated. However, it is not known whether it is this region of Sec63p that is directly phosphorylated. The extreme C-terminus of Sec63p has been demonstrated to interact with Sec62p (Wittke *et al.*, 2000; Young *et al.*, 2001). Furthermore, this region of Sec63p is extremely acidic and interacts with the basic N- terminus of Sec62p, therefore it is likely that this interaction is ionic in nature. The functionality of Sec63p phosphorylation is not clear but following the finding that the C-terminal 52 residues of Sec63p are required for phosphorylation it is interesting to speculate that phosphorylation may be required for Sec63p to interact with Sec62p. Interestingly, BN-PAGE analysis has identified a protein complex of a molecular weight that is consistent with that of the SEC complex without Sec62p (personal communication B. M. Wilkinson and A. J.

Jermy). To test this model it would be of particular interest to detect whether Sec63p is phosphorylated or differentially phosphorylated in this complex compared to that in the SEC complex to determine whether Sec63p phosphorylation is important for its interaction with Sec62p.

Sec63p is essential component of the translocation machinery and crosslinking analysis has indicated that Sss1p and Sec63p are in close proximity following the release of ribosomes from membranes by treatment with puromycin. Therefore, in contrast to previous observations (Panzner *et al.*, 1995; Finke *et al.*, 1996), this would imply that Sec63p is an integral component of the translocon. As a result, Sec63p could recruit factors, such as Sec62p, to form active protein complexes required for ER translocation. Kar2p, like Sec63p, is ubiquitously required for ER translocation (Young *et al.*, 2001) and the J-domain of Sec63p directly binds to and regulates the ATPase activity of Kar2p (Brodsky and Schekman, 1993; Corsi and Schekman, 1997). Through recruiting Sec63p to the translocon Sss1p may indirectly recruit and position Kar2p adjacent to the translocation channel. Consequently, Kar2p would be in an optimal environment to promote the stable interaction between precursor and the translocon and then bind to precursor in a nucleotide dependent manner once they emerge from the translocation channel. Following the stimulation of ATP hydrolysis by the J- domain of Sec63p, Kar2p would translocate precursor into the ER lumen.

It has been previously demonstrated that the ER membrane is permeable to small molecules both *in vitro* and *de novo* (Le Gall *et al.*, 2004 and references there in). However, in mammalian microsomes the homologue of Kar2p, BiP, is required to seal the luminal face of the translocon, however direct binding to the Sec61 complex has never been detected. As Sec63p is conserved in eukaryotes and the J-domain of Sec63p may be in close proximity to

the channel it is attractive to speculate that Kar2p may seal the translocon via a direct interaction with Sec63p. An alternative model for translocon gating has been postulated whereby the SecY/Sec61 complex can gate itself (Beckman *et al.*, 2002; van den Berg *et al.*, 2004). This model proposes that a switch from an open to a closed state is coupled to a relocalisation of a single transmembrane domain that in turn “plugs” the translocation channel (van den Berg *et al.*, 2004). This was postulated following the observation that incorporation of a cysteine residue into this region of the *E. coli* SecY subunit can form a disulphide bond *in vivo* with a cysteine residue introduced toward the C- terminus of SecE. Disulphide bonding results in a dominant negative phenotype and it was proposed that this phenotype is the consequence of the translocon being locked in an open conformation.

How the luminal face of the eukaryotic translocon is sealed is not known. It may be that the closed translocon shares distinct structural similarities to its prokaryotic counterpart. However, whereas translocon gating is BiP dependent in eukaryotes prokaryotes do not possess periplasmic Hsp70 proteins nor do they encode for a *SEC63* homologue. Therefore, the manner with which the mechanism of translocon gating is regulated in prokaryotes and eukaryotes may substantially differ.

The extreme C- terminus of Sss1p and its homologues are highly conserved. Two conditional mutant alleles of *sss1* have been isolated where mutations in this region disrupt Sss1p function. These mutants, *sss1-6* and *sss1-7*, containing the P₇₄A I₇₅A and H₇₂K mutations respectively, are temperature sensitive and are defective in ER translocation. However, the translocation defect observed at the permissive temperature does not appear to be exacerbated when these cells are switched to the restrictive temperature and may indicate that the translocation defect can be tolerated at 30°C but not at higher temperatures. Although

the observed translocation defect may be a consequence of these mutations disrupting the ability of the Sec61 complex to promote ER translocation it is more likely that this defect is linked to the interaction between Sss1p and Sec63p being more transient in these mutants. Consistent with this, the residues mutated in both *sss1-6* and *sss1-7* are predicted to be in the transmembrane domain of Sss1p (van den Berg *et al.*, 2004) which has been shown to be required to recruit Sec63p to the translocation channel. In fact, the extent with which SRP dependent and Sec62p dependent precursors are accumulated in *sss1-6* and *sss1-7* is reminiscent of the *sec63-301* mutation (Young *et al.*, 2001). In this mutant the level of the Sec63 protein synthesised was found to be reduced compared to wild-type (Young *et al.*, 2001).

The UPR was found to be induced in both *sss1-6* and *sss1-7* cells indicating that misfolded proteins accumulate in the ER lumen of these cells. Also, there is a concomitant defect in the degradation of the ERAD substrate CPY* in these cells. There is significant evidence suggesting that the Sec61 complex is required to dislocate mis-folded protein into the cytosol as well as function in the translocation of secretory precursors into the ER lumen. The observation that Sss1p mutants are defective in ERAD would appear to support this hypothesis as well as implicate a novel function for Sss1p in this process. Furthermore, mutations in the region of Sec61p that have been identified in this study to be in close proximity to the C-terminus of Sss1p have previously been shown to affect ERAD. However, it has been discovered that glycosylation is aberrant in both *sss1-6* and *sss1-7* cells. An investigation of the early stages of the biogenesis of alpha factor, *in vitro*, has indicated that translocated forms of alpha factor are detected in addition to the g1 α f, g2 α f and g3 α f. These additional forms were found to co-migrate with translocated forms of alpha factor detected in strains where the glycan processing enzymes ER glucosidase 1 and ER glucosidase 2 were depleted. Also, the translocated forms of CPY detected in these cells appear to be abnormally processed

compared to those detected in wild type cells. The extent of which glycan chains are mis-processed in these cells is not known. Intriguingly it has been found that cells that accumulate glycan structures consistent with mis-processing by ER glucosidase 2 can induce the UPR (Jakob *et al.*, 1998a) and ER glucosidase 1 mutants in yeast are defective in ERAD and are allelic with *DER7* (Hitt and Wolf, 2004b). Whether these defects are an indirect consequence of the profound translocation defect remains to be seen. Importantly, a consequence of defective ER translocation in these mutants may be that ER lumenal factors that are required for the folding and processing of polypeptides may become limiting. It is also possible that if Kar2p or ER factors that function in protein folding become limiting in the ER lumen of *sss1-6* and *sss1-7* that misfolded proteins may form aggregates. As aggregated proteins are no longer substrates for ERAD this may explain the significant stabilisation of CPY* in these cells.

Previous studies have identified that yeast cells which exhibit defective glycosylation and glycan processing are hypersensitive to the aminoglycoside hygromycin B. Initially, hygromycin B sensitivity was contributed to defects in glycoprotein processing in the Golgi (Dean, 1995) although further characterisation indicated that hygromycin B hypersensitivity is common to all aspects of aberrant glycosylation. Such a correlation between hygromycin B hypersensitivity and aberrant glycosylation was detected following the characterisation of mutants that were resistant to vanadate (Dean, 1995), a potent inhibitor of tyrosine phosphatases and adenosine triphosphatases. However, in contradiction to this, *sss1-6* and *sss1-7* cells in particular are, if anything, sensitive to vanadate which has been found to be toxic to cells where either the ER calcium pump, Cod1p, or the Golgi localised ER/Golgi calcium and manganese pump, Pmr1p, have been mutagenised. As these proteins are required to maintain ER cation homeostasis this may imply that ER gating is defective in *sss1-6* and *sss1-7* whereby the ER membrane has become more porous to ER lumenal factors allowing

the cross contamination of cytosol with ER lumen. Using fluorescence collisional quenching it has been demonstrated that the luminal face of the mammalian translocon is sealed by a mechanism that is BiP dependent (Hamman *et al.*, 1998). Also, translocon gating is nucleotide dependent and requires ATP hydrolysis as the luminal face is no longer sealed when BiP is bound to AMP-PNP, a non-hydrolysable analogue of ATP (Hamman *et al.*, 1998). The intrinsic ATPase activity of BiP/Kar2p is stimulated by the J-domain of Sec63p and puromycin release of ribosomes has indicated that Sec63p is a component of the ER translocon. Therefore, if the yeast translocon is gated by a similar mechanism as its mammalian counterpart this may imply that Sec63p is required to gate the translocon. Significantly, the efficiency with which Sec63p can be crosslinked to Sss1p in membranes isolated from either *sss1-6* or *sss1-7* is significantly reduced. Therefore, the efficiency with which the luminal face is sealed in these mutants maybe compromised as insufficient Kar2p may be recruited to the translocon in order to seal the luminal face of the pore. Alternatively, there maybe insufficient Sec63p present to stimulate the ATPase activity of Kar2p that would appear to be required to seal the translocon.

Although it is attractive to speculate that *sss1-6* and *sss1-7* are defective in the gating of the luminal face of the translocation channel we cannot rule out the possibility that these defects are an indirect consequence of the translocation defect associated with these mutations. The Cod1p and Pmr1p cation transporters are integrated into the ER membrane via the Sec61 complex prior to their trafficking to the organelle in which they function. Therefore, the translocation defect observed in *sss1-6* and *sss1-7* may substantially decrease the levels of these proteins that are integrated into membranes. Under normal growth conditions, the levels of divalent cations in the growth media may be sufficient for these mutants to compensate for the decrease in functional ion transporters. However, following the addition of EGTA to the

growth media there may be insufficient quantities of divalent cations that can be scavenged to compensate for this.

The residues of Sss1p that are mutagenised in both *sss1-6* and *sss1-7* are predicted to be in the transmembrane domain of this protein. Therefore it is possible that these mutations may affect the efficiency with which the Sss1-6 and Sss1-7 proteins are integrated into the ER membrane as well as the rate with which they are turned over. Analysis of total cell protein extracts from cells expressing either *SSS1*, *sss1-6* or *sss1-7* would suggest that the more mutant proteins are not turned over more rapidly than Sss1p. Also, analysis of membranes isolated from these cells would suggest that these mutations do not appear to compromise the efficiency with which these proteins associate with membranes.

The region of SecE in *E. coli* that when mutagenised, was found to give a rise to a dominant negative phenotype when co-expressed with certain SecY mutants was found to align with the region of *SSS1* harbouring the *sss1-6* and *sss1-7* mutations. As the dominant lethal phenotype was found to be the consequence of a disulphide bridge being formed between SecY and SecE it was postulated that this stabilises the open conformation of the SecY complex, allowing the cross contamination of the cytosol with periplasmic factors. The *sss1-7* mutation increases the basic nature of the Sss1p C-terminus. This region has been proposed to interact with the acidic TM1-TM2 loop of Sec61p (van den Berg *et al.*, 2004). Therefore it is possible that the *sss1-7* mutation may partially stabilise the open conformation of the Sec61 complex via an ionic interaction. In *sss1-6* it may be that substitution of a proline residue to alanine grossly perturbs the structure of this region which may compromise function.

It remains to be seen whether all of the phenotypes associated with the *sss1-6* and *sss1-7* are a direct or indirect consequence of the mutations compromising the function of Sss1p in these processes. Interestingly, although *sec61* mutants do not appear to possess many of these

phenotypes *sec63* and *kar2* mutants do (personal communication B. M. Wilkinson). Therefore, it may be that a number of these phenotypes are a consequence of the interaction between Sss1p and Sec63p being more transient in these mutants. A number of intergenic suppressors of *sss1-7* temperature sensitivity have been isolated and it is anticipated that following the identification of genes, when mutagenised, that suppress this phenotype that we will gain an insight into the function of the Sss1p.

This study has indicated that Sss1p plays a pivotal role in both the formation of the translocation channel and the recruitment of other factors, specifically Sec63p, in order to form functional ER translocons. Furthermore, it is also apparent that the extreme C- terminus of this protein is required to function in a process that would appear to regulate ER luminal homeostasis. It is anticipated that further investigation into how Sss1p form these interactions and the characterisation of suppresser mutations of conditional *sss1* alleles may further contribute to the understanding of how this protein functions in these processes.

Chapter 8

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