Biochemical Analysis of the Yeast SRP Receptor

Allison Robb BSc

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Declaration

This study was carried out under the supervision of Dr Jeremy D. Brown in the Institute of Cell and Molecular Biology, University of Edinburgh between October 1998 and December 2001.

The experimental work carried out in this thesis, unless otherwise stated, is my own and this manuscript has been composed by myself.

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Abstract

The yeast *Saccharomyces cerevisiae* utilises many transport pathways for the efficient and accurate segregation of proteins to the compartments of the cell in which they function. Of these, the secretory pathway is responsible for the localisation of proteins to the endoplasmic reticulum (ER), Golgi apparatus, endocytic and vacuolar compartments and the cell surface. The first step along this pathway is the targeting of nascent polypeptides to the ER membrane. For many proteins, the signal recognition particle (SRP), a cytosolic ribonucleoprotein, and its cognate receptor (SR) on the ER membrane are responsible for this step. SRP and the SR direct proteins to the translocon, which forms an aqueous channel through which the nascent proteins are translocated or from which they are then integrated into the ER membrane. The aim of this study was to dissect incompletely understood interactions that occur at the ER membrane between SRP, SR and the translocon and to reconstitute the SRP-dependent translocation reaction with purified proteins. In particular, through 2-hybrid analysis and pull-down assays a novel interaction was identified between SR and the major translocon component Sec61p. To facilitate the study of this and other interactions, attempts were made to reconstitute the SRP-dependent targeting pathway with SR and translocon purified from yeast. It was demonstrated that SRP-dependent translocation could be reconstituted with solubilised yeast ER membrane proteins. SR was purified and shown to be functional, and the translocation reaction was shown to be stimulated by the presence of the ER-lumenal chaperone Kar2p. Preliminary experiments were also carried out that suggested that the purified translocon was active; indicating that the goal of reconstituting SRP-dependent translocation with purified components is attainable.

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Abbreviations

3-AT	3-aminotriazole
ATP	adenosine triphosphate
ADP	adenosine diphosphate
CPY	carboxypeptidase Y
DEPC	diethyl pyrocarbonate
$D_{HC}\alpha F$	dipeptidylaminopeptidase-B-pro-alpha factor fusion
DNA	deoxyribonucleic acid
DPAP-B	dipeptidylaminopeptidase-B
DSS	disuccinimidyl suberate
DTT	dithiolthreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis(beta-aminoethylether)-N,N'-tetraacetic acid
ER	endoplasmic reticulum
ERAD	ER-associated degradation
Ffh	fifty-four homologue
GAL4DBD	GAL4 DNA binding domain
GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GNRP	guanine nucleotide release protein
Gpp(NH)p	β-γ-imidoguanosine5'-triphosphate
GST	glutathione-S-transferase
GTP	guanosine triphosphate
HMG-CoA reductase	3-hydroxy-3-methylglutaryl coenzyme A reductase
HRP	horseradish peroxidase
HA	haemagglutanin
IgG	immunoglobulin G
KOAc	potassium acetate
KPO_4	potassium phosphate buffer
LB	Luria broth
LiOAc	lithium acetate
$Mg(OAc)_2$	magnesium acetate
mRNA	messenger RNA
OSTase	oligosaccharyltransferase
PAGE	polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PEG	polyethylene glycol
PMSF	phenylmethylsulphonalfluoride
ppαF	pre-pro-alpha factor
PrA	protein A
RAMP	ribosome-associated membrane protein

RNA	ribonucleic acid
RNC	ribosome-nascent chain
RNP	ribonucleoprotein
rRNA	ribosomal RNA
scR1	small cytoplasmic RNA 1
SDS	sodium dodecylsulphate
SPase	signal peptidase
SR	signal recognition particle receptor
SRP	signal recognition particle
SR-ΔTMβ	transmembraneless SRβ within the SR complex
SR(ΔTM)-GST	GST-tagged transmembraneless SRβ within the SR complex
SRβ-ΔTM	transmembraneless SRβ
SRβ-ΔTM-GST	GST-tagged transmembraneless SRβ
SRβ-PrA	"zz" domain-tagged SRβ
TAE	Tris acetate, EDTA
TAP-tag	tandem affinity purification-tag
TAT	twin-arginine translocation
TBS	Tris buffered saline
TCA	trichloroacetic acid
TE	Tris, EDTA
TEV	tobacco etch virus
Tic	translocon inner chloroplast membrane
Tim	translocon inner membrane
Toc	translocon outer chloroplast membrane
Tom	translocon outer membrane
TRAM	translocating chain-associated membrane protein
tRNA	transfer RNA
UPR	unfolded protein response
XTP	xanthosine triphosphate
YNB	yeast nitrogen base
YPD	yeast extract, peptone, dextrose
yRM	yeast rough microsomal membranes
YTE	yeast translation extract
"ZZ" domain	IgG binding domain of protein A

Commonly used buffers

PBS	137 mM NaCl, 2.7 mM KCl, 10 mM NaH_2PO_4, 1.76 mM KH_2PO_4 (adjusted to pH 7.2)
TBS	25 mM Tris.HCl, 137 mM NaCl, 27 mM KCl (adjusted to pH 7.4)
TAE	40 mM Tris-acetate, 1 mM EDTA (pH 8.0)
TE	10 mM Tris.HCl (pH 7.4), 1 mM EDTA (pH 8.0) (pH 7.4 final)

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Chapter 1

Introduction

1.1 Introduction

The yeast *Saccharomyces cerevisiae* utilises many transport pathways in order to efficiently and accurately target proteins to the area of the cell where they carry out their specific functions. One of these is the secretory pathway, responsible for targeting proteins destined for secretion from the cell, integration into membranes and a subset of other subcellular organelles. The first step in the secretory pathway involves translocation of proteins into the lumen of the endoplasmic reticulum (ER) or insertion into the ER membrane. The lumen of the ER contains many chaperone proteins that ensure that all proteins sent further along the secretory pathway are correctly folded, and many modifying enzymes, which add disulphide bonds to, glycosylate and add co-factors to proteins. In order for these proteins to be correctly modified they must first be targeted accurately from the cytosol, where they are synthesised, to the ER membrane. A population of proteins exist, some of which are conserved from bacteria to man, that ensure that this targeting event occurs. *S. cerevisiae* utilises two methods of targeting proteins to the ER. The first involves a soluble cytosolic ribonucleoprotein known as the signal recognition particle (SRP), its cognate receptor, the signal recognition particle receptor (SR), Sec63p and the lumenal chaperone Kar2p. The second involves the proteins Sec62, 63, 71 and 72p and Kar2p. Both of these targeting pathways converge on a group of proteins known collectively as the translocon, consisting in yeast of Sec61p, Sbh1p and Sss1p. The translocon components form a protein channel through which nascent polypeptide chains are translocated into the lumen of the ER. The pathway involving SRP and SR is known as the SRP-dependent targeting pathway and targets proteins with a hydrophobic signal sequence at their N-terminus to the translocon. The Sec62/63/71/72p pathway is known as the post-translational targeting pathway and proteins targeted through this pathway do so independently of SRP and SR. The aim of this study was to dissect incompletely understood interactions at the ER membrane between SRP, SR and the translocon. To achieve this, SRP-dependent protein targeting was reconstituted in an *in vitro* system using components of the pathway purified from yeast. The assay itself is described alongside details of experiments that show that the purified material is active for promoting translocation of both SRP-dependent and –independent substrates. The purification of one of the

components necessary for SRP-dependent translocation, SR, brought to light a previously unidentified interaction between the β subunit of SR and Sec61p, the major component of the translocon. The defining experiment is described, along with experiments carried out to further characterise this novel and unexpected interaction.

1.2 Translocation

There are numerous modes of protein translocation in eukaryotic and prokaryotic cells. Many similarities exist between the different transport mechanisms, however varied the substrates or environments may be (Agarraberes and Dice, 2001). In this section I describe translocation pathways that exist in bacteria and two eukaryotic organelles: mitochondria and chloroplasts.

In addition to the conventional Sec-dependent and SRP-dependent pathways in bacteria (sections 1.5.3 and 1.6.2) there exists the twin-arginine translocation (TAT) system. Proteins exported by the TAT pathway have unusually long signal sequences of up to 48 amino acids. The signal sequence has an invariant twin arginine motif in its amino terminal region followed by a short hydrophobic sequence and one or more basic amino acids. A consensus twin arginine targeting motif is serine-arginine-arginine-X-phenylalanine-leucine-lysine (SRRXFLK) in which X can be any amino acid (Stanley *et al.*, 2000). This motif constitutes a Sec avoidance signal (Bogsch *et al.*, 1998). Any disruption of the motif renders the protein incapable of translocation by the TAT system and transforms it into a substrate for the Sec pathway (Cristobal *et al.*, 1999). Proteins can be translocated across the membrane via the TAT system while folded (Berks, 1996). Many of the substrates for this pathway in bacteria are metalloenzymes that reside in the periplasmic space or are associated with the periplasmic face of the inner membrane (Berks, 1996). It is not completely understood how fully folded proteins are competent translocation substrates, however transport is dependent on the proton-motive force across the membrane (Berks *et al.*, 2000).

Mitochondrial import in eukaryotic cells has been the focus of much investigation. Although mitochondria contain their own genome, most mitochondrial proteins are encoded by nuclear genes and are synthesised in the cytosol as precursors. Mitochondrial biogenesis requires protein targeting to four compartments: the outer membrane, the intermembrane space, the inner membrane and the matrix. Translocation of mitochondrial precursors is an energy-dependent process that is assisted by heteromeric translocation complexes in both membranes. Four translocation complexes have been identified, two translocons in the outer mitochondrial membrane (Tom) and two in the inner mitochondrial membrane (Tim) (Dekker *et al.*, 1996; Dekker *et al.*, 1997; Dekker *et al.*, 1998). Some proteins can be imported into mitochondria independently of Tom and Tim complexes, perhaps due to their abilities to associate spontaneously with lipid bilayers (Rassow and Pfanner, 2000). Precursor proteins destined for the mitochondrial matrix are usually hydrophilic polypeptides with amino-terminal signal peptides that form amphipathic helices in solution (von Heijne, 1986; Roise and Schatz, 1988). Inner membrane proteins, such as the ATP/ADP carrier (AAC) and the phosphate carrier, are hydrophobic and contain internal targeting signals not yet characterised (Truscott and Pfanner, 1999). Translocation across the inner membrane is dependent on the proton-motive force (Schleyer *et al.*, 1982; Martin *et al.*, 1991). The driving force for translocation across the outer membrane has not yet been determined, although ATP is required for the transfer of substrate from a receptor-bound to membrane-inserted state (Pfanner *et al.*, 1987; Pfanner and Neupert, 1987).

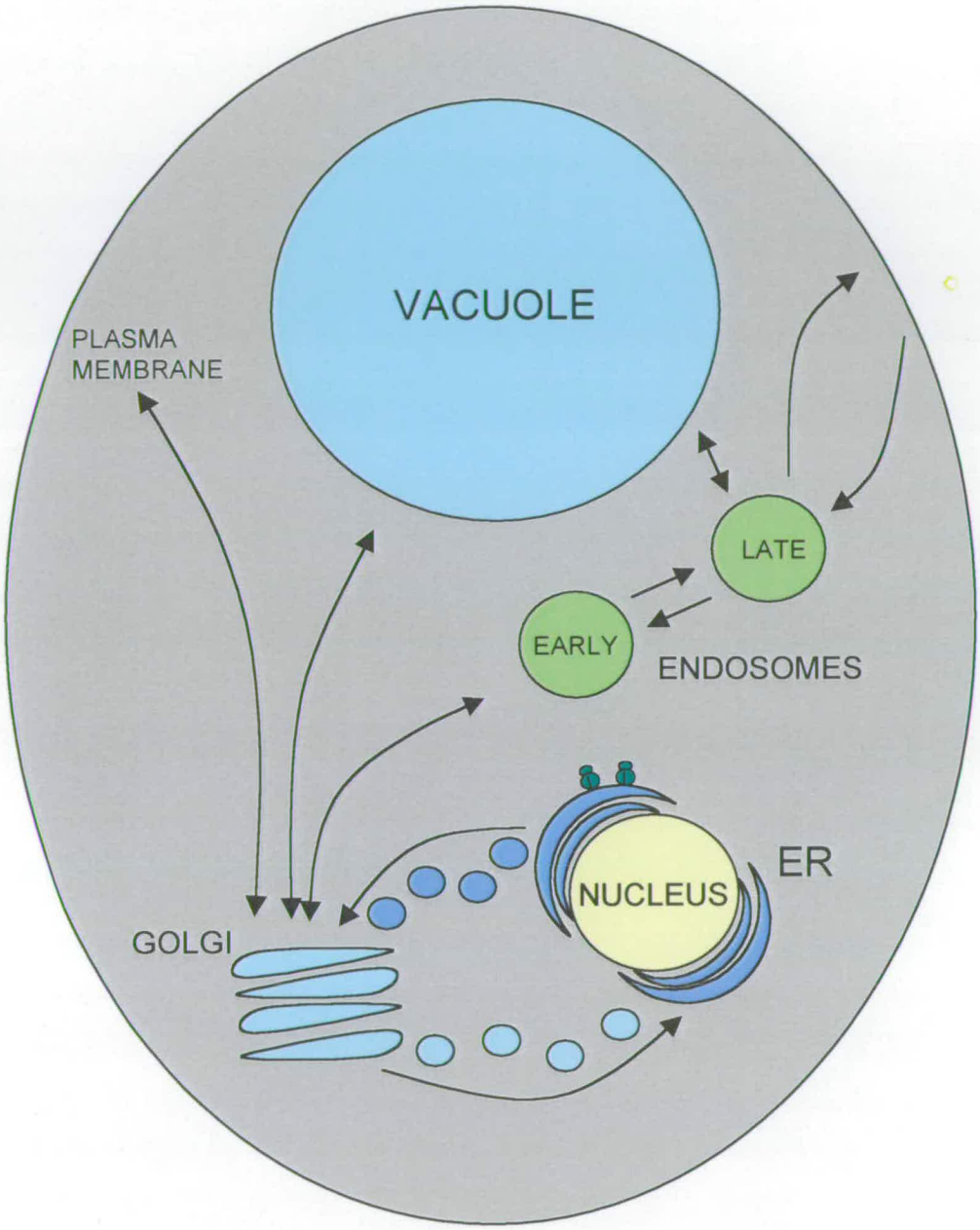
Chloroplasts have six distinct compartments: outer membrane, intermembrane space, inner membrane, stroma, thylakoid membrane, and thylakoid lumen (Agarraberes and Dice, 2001). Each compartment contains specific proteins. Like mitochondria, chloroplasts contain their own genome, but most chloroplast proteins are synthesised in the cytosol and imported post-translationally. Chloroplast targeting sequences are variable in length (from 20 to more than 120 amino acids) and contain basic amino acids with a high content of serine and threonine (Schnell, 1995; Chen and Schnell, 1999). Translocation across the outer and inner membrane occurs simultaneously for most proteins, probably at regions where the outer and inner membranes are in close contact (May and Soll, 1999; Chen and Schnell, 1999). The translocon at the outer

membrane of chloroplasts (Toc) binds to a substrate protein and transfers the protein to the translocon at the inner chloroplast membrane (Tic). This process is stimulated by ATP and in the absence of ATP only weak binding of substrate proteins to Toc subunits occurs. In addition, precursor insertion into the Tic complex requires ATP hydrolysis within the stroma (Chen and Schnell, 1999). Protein translocation into thylakoid membranes is varied similarly to the translocation pathways in bacteria. Many proteins utilise the components homologous to the Sec apparatus while others require a stromal homologue of SRP for import (Robinson *et al.*, 1998; Thompson *et al.*, 1999). Other proteins are transported into the thylakoid lumen by a route similar to the TAT system. The proteins are transported while folded and contain a twin arginine motif in their targeting sequences (Thompson *et al.*, 1999).

Many common features exist between the different translocation pathways. Most involve ATP or the proton-motive force, almost all require cytosolic or membrane associated chaperones and in all cases insertion is through a gated, aqueous channel. In this study, the focus is on how proteins are translocated into the lumen of the ER, as the first step in entering the secretory pathway.

1.3 The secretory pathway

Eukaryotic cells are characterised by the presence of organelles that carry out a variety of highly specialised functions. The structure and function of each organelle is defined by its unique complement of proteins. Since most proteins begin their synthesis in the cytosol, targeting mechanisms and transport pathways exist to efficiently direct proteins to their correct destinations. One of these is the secretory pathway. The endoplasmic reticulum (ER) plays a very important part in intracellular protein trafficking and targeting to the ER is the first step in the secretory pathway. Proteins translocated into the ER can either remain resident there, or may be transported to the Golgi apparatus, the lysosomal network, or the cell surface (Figure 1.1). Protein targeting to the ER is mediated by a variety of proteins, both cytosolic and ER membrane bound, and can occur either co- or post-translationally. Co-translational targeting of polypeptides requires a cytosolic


VACUOLE
PLASMA
MEMBRANE
LATE
EARLY
ENDOSOMES
NUCLEUS
ER
GOLGI

ribonucleoprotein the signal recognition particle (SRP) and its ER bound receptor (SR). This pathway is known as SRP-dependent targeting, and is described below.

1.4 SRP-dependent targeting

In order to explain how proteins in the cell were integrated into or translocated across cellular membranes the signal hypothesis was proposed (Blobel and Sabatini, 1971; Blobel and Dobberstein, 1975; Blobel, 1980). The signal hypothesis proposed that each protein translocated across or integrated into a cellular membrane must contain a signal sequence that is membrane specific. A signal sequence-specific recognition factor and a cognate receptor on target membranes must mediate recognition of the signal sequence and the subsequent protein targeting. Finally, translocation across the membrane would occur *via* a proteinaceous channel. Subsequent experiments confirmed that this hypothesis was essentially correct and defined what is now a well-established protein-targeting pathway to the ER. Thus, the signal recognition particle targets ribosomes synthesising nascent secretory and membrane proteins to the ER. The nascent proteins are, at this point, integrated into or translocated across the membrane into the lumen of the ER (Figure 1.2). During translocation, signal sequence removal and protein glycosylation occur by proteolysis by the signal peptidase complex and through the action of oligosaccharyltransferase (OSTase) respectively (Görlich *et al.*, 1992). Signal peptidase typically cleaves at the sequence AXA where A is any small, aliphatic amino acid (Perlman and Halvorson, 1983). OSTases transfer an oligosaccharyl moiety to asparagine residues in the polypeptide when the glycosylation site (NXS/T) has reached a distance of at least 12 amino acids from the lumenal side of the ER membrane (Nilsson and von Heijne, 1993). Both signal peptidase and oligosaccharyltransferase are multi-subunit, membrane-spanning complexes of which there appears to be one for every translocation site in the ER membrane (Rapoport *et al.*, 1996).

The SRP-dependent co-translational targeting pathway requires interactions between SRP, its receptor and a group of proteins in the ER membrane, known collectively as

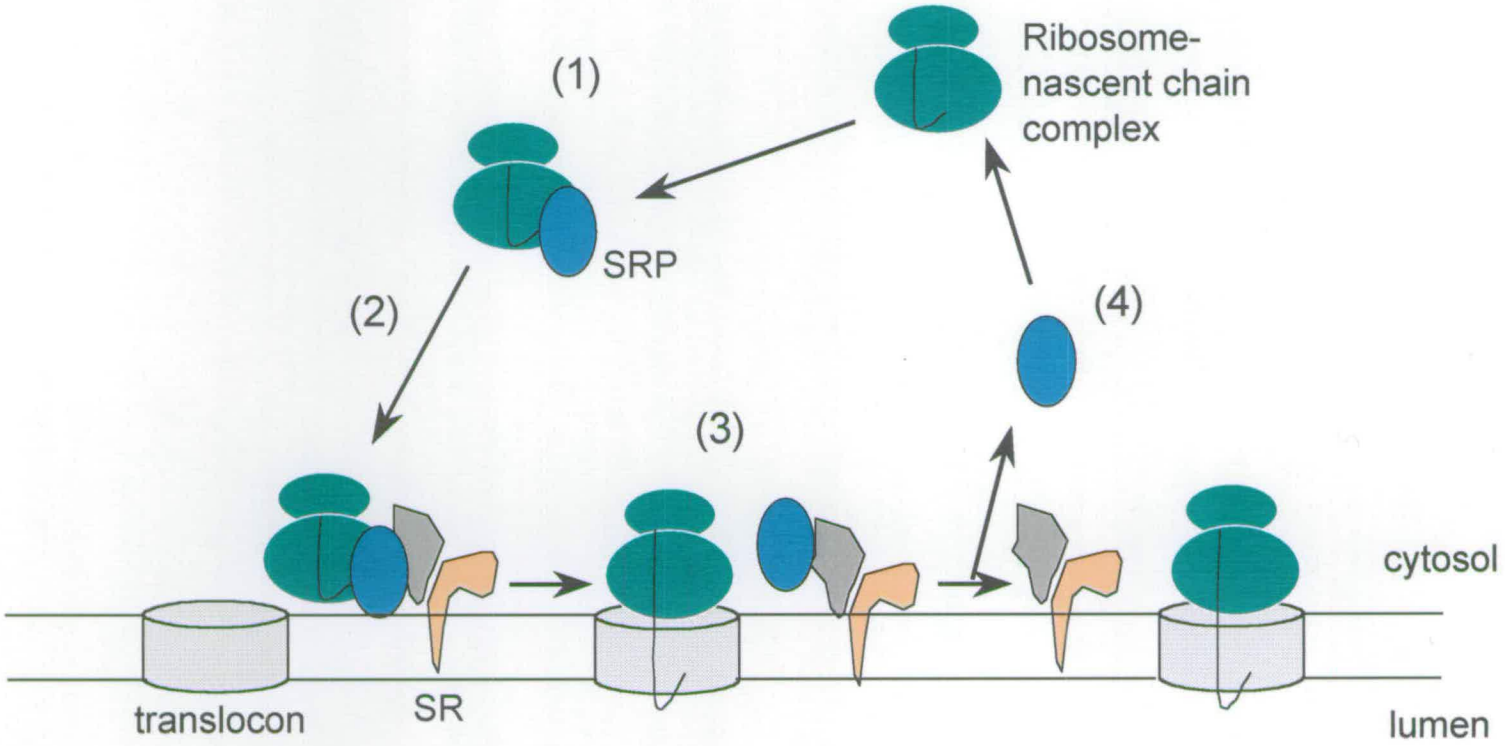
Figure 1.2 SRP-dependent protein targeting pathway

1) SRP binds to emerging signal sequence of nascent chain and the ribosome causing elongation arrest of the nascent polypeptide

2) SRP docks with the SRP receptor (SR) in the ER membrane

3) Ribosome is targeted to the translocon and dissociates from SRP, releasing elongation arrest. Nascent protein is translocated through the translocon to the lumen of the ER

4) SRP releases from SR


Ribosome-
nascent chain
complex
(1)
SRP
(2)
(3)
(4)
cytosol
translocon
SR
lumen

the translocon (Wilkinson *et al.*, 1997). *In vitro* experiments with mammalian components have shown that SR and the translocon are the minimum apparatus required for translocation. These experiments have also shown a requirement of some nascent proteins for the translocating chain-associated membrane (TRAM) protein whose function will be discussed later (Görlich and Rapoport, 1993).

1.5 The signal recognition particle

1.5.1 Components of SRP

Mammalian SRP was first isolated from canine pancreas and was found to be an 11S ribonucleoprotein (Walter and Blobel, 1980). SRP consists of six polypeptides named SRP9, 14, 19, 54, 68 and 72 according to their apparent molecular weight in kilodaltons and the 7SL RNA (Walter and Blobel, 1982). 7SL RNA provides a "backbone" to SRP with the protein subunits binding to it as either heterodimers (SRP9/14 and SRP68/72) or monomers (SRP54 and SRP19) (Walter and Blobel, 1983).

SRP has been identified in all organisms studied to date. It comprises a highly conserved group of proteins and RNA, homologues of which are found from bacteria to mammals. In this study, I have been concerned with SRP-dependent targeting of proteins in the yeast *Saccharomyces cerevisiae*. SRP in yeast, as in mammalian cells, consists of six polypeptides Srp14, 21, 54, 68 and 72p and Sec65p, and one RNA, scR1 (Hann and Walter, 1991; Hann and Walter, 1992; Stirling and Hewitt, 1992; Brown *et al.*, 1994). Srp14, 54, 68 and 72p and Sec65p are homologous to mammalian SRP14, SRP54, SRP68, SRP72 and SRP19 respectively. There are no known homologues of Srp21p, except in other yeast species (J. Brown and R. van Neus, personal communication) and the yeast particle does not contain an SRP9 homologue (Brown *et al.*, 1994). Sec65p is required for the stable association of Srp54p with scR1, however neither Sec65p nor Srp54p are required for the stability of SRP, a core SRP remaining with all other SRP proteins stably associated with scR1 in their absence. A temperature-sensitive *sec65-1* mutant has no Sec65p or SRP54 associated with scR1 at the restrictive temperature. Over-expression of

SRP54 in a *sec65-1* mutant will suppress this phenotype (Hann *et al.*, 1992). Deletion of all but the central domain of Sec65p, which shares the most homology to SRP19, showed that this portion is required for activity of Sec65p *in vivo* (Regnacq *et al.*, 1998). Only in deletions of the four core components of SRP do destabilisation of scR1 and the particle itself occur (Hann and Walter, 1991).

1.5.2 Functions of SRP

Initial experimental evidence showed that nascent chain translocation across the ER membrane was a protein-mediated event. It was shown that protein-protein interactions (signal sequence + ribosome + SRP), at the ER membrane, provided the specificity for translocation and not protein-lipid interactions (signal sequence with lipid bilayer) (Walter *et al.*, 1981). SRP was also shown to induce a site-specific arrest in nascent chain elongation on binding the amino-terminal signal peptide of a nascent chain. This arrest was released upon interactions between the SRP-nascent chain-ribosome complex (SRP-RNC) and the SRP receptor (SR) at the ER membrane, resulting in chain completion and translocation into the microsomal vesicle (Walter and Blobel, 1981).

The functions of each component of SRP were established in experiments in which purified mammalian SRP was dissociated into its four protein subunits (SRP54, 19, 9/14 and 68/72) and RNA and the particle reconstituted in the absence of components or with individual components chemically modified (Siegel and Walter, 1988b). Three activities of SRP were measured: signal sequence recognition, elongation arrest and translocation promotion. Elongation arrest activity is thought to increase the efficiency of protein translocation by lengthening the amount of time available for the nascent chain to be targeted to the ER membrane (Thomas *et al.*, 1997). SRP9/14 are required for elongation arrest and SRP68/72 are required for translocation promotion. SRP54 is required for signal sequence recognition and was later shown to bind signal sequences directly by chemical cross-linking (Bernstein *et al.*, 1989; Mothes *et al.*, 1998). Cloning of SRP54 revealed that it has a GTPase domain. This is essential for its function and the structure of this conserved domain has been elucidated (Freymann *et al.*, 1997). Neither elongation arrest nor

translocation promotion occurs without SRP54 binding to the nascent chain. Therefore, binding of SRP54 to the signal sequence is the first step in SRP-dependent targeting of nascent polypeptides (Walter and Blobel, 1983; Siegel and Walter, 1988).

Functions have been assigned to components of yeast SRP based on their homology to mammalian proteins (Figure 1.3). Srp54p has a GTPase domain and is thought to be responsible for signal sequence binding. Srp68/72p proteins exist as a stable heterodimer and may be important for translocation promotion of the nascent chain, while Srp21p has no known function. It is known that intact Srp14p function is required for elongation arrest (Strub *et al.*, 1999; Mason *et al.*, 2000). A recent, unexpected, discovery indicated that while mammalian SRP14 exists as a dimer with SRP9, in yeast Srp14p exists as a homodimer, occupying an area on scR1 in close proximity to Srp21p (Figure 1.3) (Strub *et al.*, 1999; Mason *et al.*, 2000; J. Brown and L. Ciufo, unpublished observation).

(i) Signal sequence binding

Signal sequences of nascent polypeptides specify which route a protein will travel in targeting to the ER membrane. While they lack primary sequence homology a common feature among ER-targeting signal sequences is a stretch of 10-15 hydrophobic amino acids (von Heijne, 1994). In yeast, the more hydrophobic the signal sequence the more likelihood the protein has of being targeted in an SRP-dependent fashion, while less hydrophobic signal sequences result in targeting through the posttranslational translocation route (Ng *et al.*, 1996). It has been shown with mammalian proteins that the length of the nascent chain is critical for SRP binding. In the case of the secretory protein preprolactin, once the chain length exposed from the ribosome is greater than 70 amino acids SRP is no longer able to sample and bind the polypeptide (Siegel and Walter, 1988a). SRP54 recognises signal sequences regardless of their lack of primary sequence homology. A model has been proposed in which a domain of SRP54 at the C-terminus rich in methionine (M domain) constitutes the critical region for the signal sequence-binding pocket. Binding of signal sequences of variable length and composition to SRP54 would be

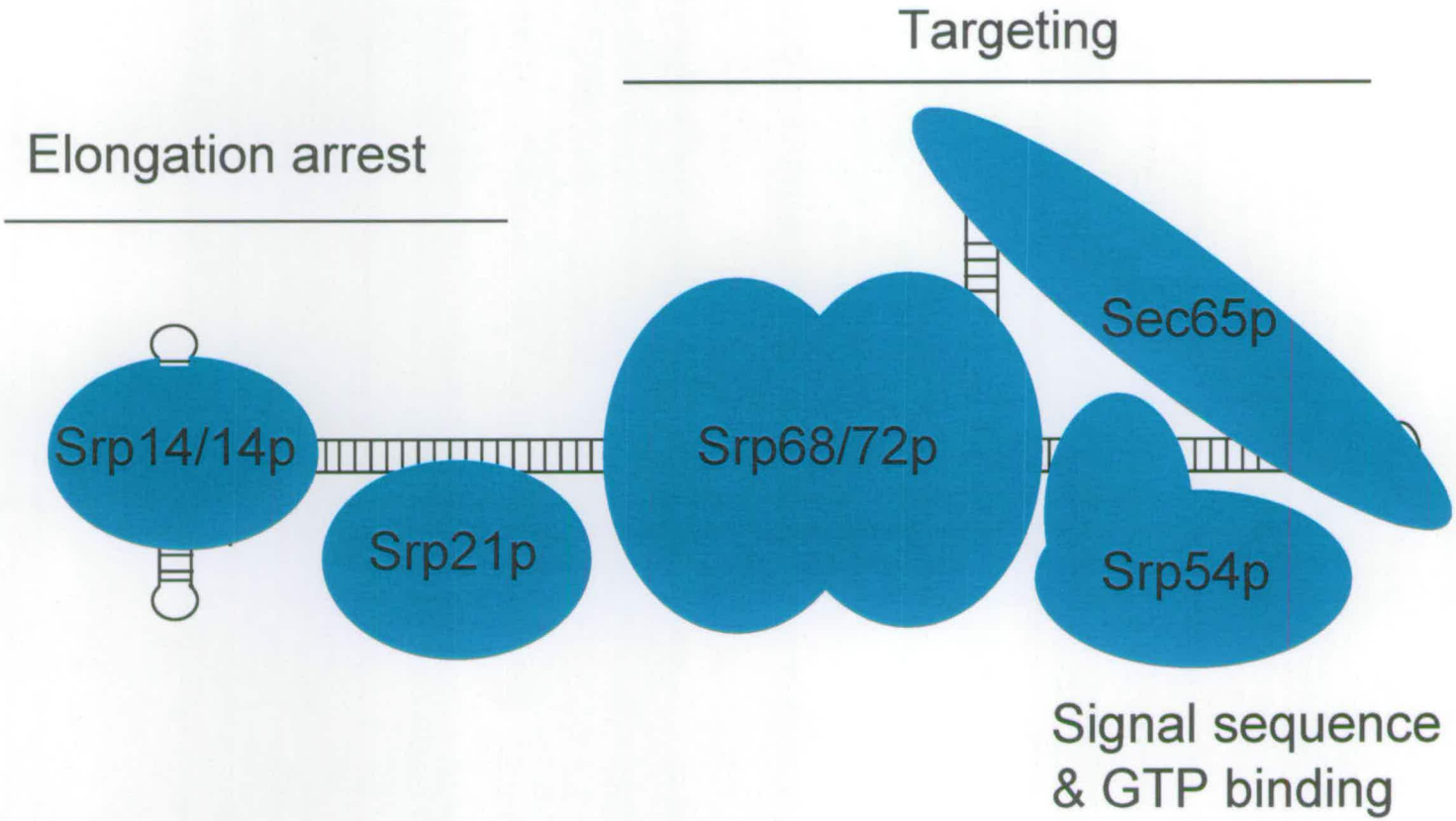
Figure 1.3 Components and functions of yeast SRP

On the basis of structural and biochemical studies of mammalian SRP, and through homology to those proteins, possible functions and potential binding sites on scR1 have been assigned to yeast SRP proteins.

Srp14p is a homodimer and has been shown to be necessary for elongation arrest, potentially with Srp21p, which can be cross-linked to Srp14p and thus is localised close to it in the particle (J. Brown, unpublished observation).

Srp68/72p are thought to be involved in the targeting of SRP to the ER membrane.

Srp54p is predicted to bind signal sequences as they emerge from the ribosome, and is necessary for targeting the ribosome-nascent chain complex to the ER membrane through its interaction with SRα.


Targeting
Elongation arrest
Sec65p
Srp14/14p
Srp68/72p
Srp21p
Srp54p
Signal sequence
& GTP binding

possible through the flexibility of the methionine side chains (Bernstein *et al.*, 1989). An atomic view of the interaction between SRP54 bacterial homologue Ffh and 49 nucleotides of 4.5S RNA (homologous to 7SL RNA, (section 1.5.3)) was achieved through x-ray crystallography. The structure resolved an unusual interaction between the two components. Ffh recognises a distorted RNA minor groove (Batey *et al.*, 2000). Based on this structure and that of the M domain within Ffh (Keenan *et al.*, 1998), it was proposed that the signal sequence recognition surface is composed of both protein and RNA. This surface would interact with signal sequences through a combination of hydrophobic and electrostatic interactions (Bernstein, 2000a). Changes in as little as one or two amino acids in a signal sequence can greatly reduce the efficiency of protein targeting and translocation (Belin *et al.*, 1996) despite signal sequence recognition being independent of primary sequence. It was illustrated that signal sequence structure determined SRP binding affinity and the stability of translocation competent complexes (Belin *et al.*, 1996). Collectively, the data on signal sequence binding suggest that SRP samples signal sequences as they emerge from the ribosome. This sampling appears to occur by sequential binding and release from the ribosome in a cyclic fashion (Walter and Johnson, 1994; Ogg and Walter, 1995). When SRP binds to the signal sequence elongation of the nascent chain arrests. However, if a signal sequence is not present at this point SRP releases and elongation resumes.

(ii) Elongation arrest

Elongation arrest has long been thought an important function of SRP from *in vitro* experiments (Walter and Blobel, 1981; Ibrahimi, 1987; Rapoport *et al.*, 1987; Wolin and Walter, 1989). Recently, the importance of elongation arrest for translocation *in vivo* was shown in yeast (Mason *et al.*, 2000) confirming this as a crucial function of the particle. Elongation arrest activity requires the Alu domain of SRP RNA and the proteins that bind to it (Siegel and Walter, 1985,1986; Strub and Walter, 1990; Bui *et al.*, 1997; Thomas *et al.*, 1997; Chang *et al.*, 1997). Alu-like elements comprise the most abundant family of interspersed repetitive sequences in primates and rodents (Schmid and Jelinek, 1982). The most highly conserved feature in the Alu-domain is a mostly single-stranded motif within the context of two hairpin structures at the 5'

end of 7SL RNA (CCUGUAAYCY; Strub *et al.*, 1991). SRP particles that lack the SRP9/14 heterodimer and subparticles that lack the Alu domain both lack their elongation arrest activity (Siegel and Walter, 1985, 1986). These same particles have been found to be defective in binding directly, independently of signal sequences, to ribosomes (Hauser *et al.*, 1995; Powers and Walter, 1996). This suggests that these particles have a more general defect in the way they interact with the ribosome as opposed to a specific defect in elongation arrest. A truncation of SRP14 (ΔC20) was found to form, with SRP9, a stable complex with SRP RNA. However, SRP containing this truncated SRP14 completely lacked elongation arrest activity. The particle had intact signal recognition, targeting and ribosome binding activities. Therefore, the truncated SRP14 only impaired interactions with the ribosome required to effect elongation arrest (Thomas *et al.*, 1997). This provided evidence that direct interactions between the Alu-domain components of SRP and the ribosome are required for elongation arrest. A recent study identified the Alu-domain present in yeast scR1. This is bound specifically by a homodimer of Srp14p. Mammalian SRP9/14 does not bind scR1 indicating substantial evolutionary changes in RNA-protein recognition between yeast and mammalian SRP Alu domains (Strub *et al.*, 1999). A similar truncation to the mammalian SRP14 (ΔC20) was made in yeast Srp14p (ΔC29). Expression of this truncated protein as the only Srp14p in yeast led to a complete lack of elongation arrest function of yeast SRP, both *in vitro* and *in vivo*. This demonstrated not only similar structural features of mammalian and yeast SRP14 but also provided evidence that elongation arrest is important *in vivo* (Mason *et al.*, 2000). Homologues of SRP9 and 14 have not been identified in organisms other than eukaryotes and the eubacterial SRP RNAs identified so far lack the entire Alu-domain found in eukaryotes. The two exceptions are the SRP RNAs of *Bacillus* and *Clostridium* species with HBsu protein identified in *Bacillus* as interacting with the Alu domain of the SRP RNA (Nakamura *et al.*, 1999). Recent evidence suggests that elongation arrest may have a function in bacterial cells despite the lack of SRP9/14 homologues (Avdeeva *et al.*, 2002). The M domain of Ffh, the bacterial SRP54 homologue, was over-expressed in *E. coli* with a cleavable affinity tag and ribosome-SRP complexes recovered. The M-domain affinity tag fusion

retained its ability to bind signal sequences. However, in the absence of the N or G domains of Ffh, responsible for membrane binding and GTP hydrolysis, SRP could not dissociate from ribosomes. The recovery of stable complexes of SRP and ribosomes suggests that translation was halted by binding of the Ffh M domain to ribosome-nascent chains. In the absence of membrane targeting or GTP hydrolysis translation was unable to proceed. Perhaps in bacterial cells homologues of SRP9 and 14 are not necessary, with Ffh responsible for other functions in protein targeting than signal sequence binding, just as the SRβ subunit appears unnecessary in the bacterial SR homologue (section 1.5.3).

Yeast mutants with reduced levels of expression of functional SRP have reduced growth rate and accumulate untranslocated polypeptides in the cytosol. This phenotype is alleviated by the addition of cycloheximide or sordarin antibiotics to the growth media (Ogg and Walter, 1995; J. Brown, unpublished observation). Cycloheximide inhibits the transfer of peptidyl-tRNA from the aminoacyl (A) site to the peptidyl (P) site during the synthesis of nascent polypeptides (McKeehan and Hardesty, 1969). Sordarin antibiotics specifically stall the ribosome-eEF-2 complex (Justice *et al.*, 1998; Dominguez *et al.*, 1999). Thus, both these antibiotics inhibit the translocation step in the translation elongation cycle. If SRP binds the ribosome, to cause elongation arrest, at the same position in the elongation cycle the drugs will provide an increased window of opportunity for low levels of SRP to bind to and target nascent chains to the ER. Alternatively, the drugs could provide more time for the nascent chain to be targeted to the ER independently of SRP. Importantly, blocks at other stages of translation (initiation and peptidyl-transferase) do not alleviate these types of mutants indicating that the effect is specific to a particular step in the elongation cycle (Ogg and Walter, 1995). The similar effect of sordarin, as cycloheximide, on SRP-deficient cells suggests the possibility that SRP interacts with the ribosome while eEF-2 is still bound to the ribosome (Justice *et al.*, 1998; Shastry *et al.*, 2001).

(iii) Translocation promotion

Alkylation of SRP68/72 subunits results in a decreased affinity of SRP for SR (Siegel and Walter, 1988b). This observation suggests a function for SRP68/72 either targeting of SRP-RNCs to the ER membrane or in the promotion of translocation at the membrane. Further evidence, from manipulation of Srp72p in yeast, suggests that Srp72p may interact with the SRP receptor (J. McDonald and J. Brown, unpublished observations). Mutants of Srp72p have been generated that display translocation defects for SRP-dependent substrates *in vivo*, but which do not compromise overall SRP stability. SRP containing such mutants are unable to associate stably with immobilised SR *in vitro*. This suggests that Srp72p may have a function required for the association of SRP-RNCs with the ER membrane *via* SR.

1.5.3 SRP is evolutionarily conserved

Since homologues of SRP components have been found across all three kingdoms, it has been suggested that SRP-dependent protein targeting is a process of ancient evolutionary origin (Althoff *et al.*, 1994). While the study of related SRP proteins has been concerned mostly with homologues in *E. coli* and budding yeast a number of other species are studied, if to a lesser extent. Homologues of SRP54 have been identified in chloroplasts (Franklin and Hoffman, 1993; Schuenemann *et al.*, 1999). 11S and 10S SRP has been identified in *Schizosaccharomyces pombe* and *Yarrowia lipolytica* respectively (Poritz *et al.*, 1988). A homologue of SRP has been found in *Bacillus subtilis* that, unlike other bacterial homologues, contains an Alu domain in its RNA component. The protein HBsu binds specifically to this domain of the RNA (Kohler and Marahiel, 1998; Nakamura *et al.*, 1999; Yamazaki *et al.*, 1999).

The *E. coli* SRP homologue consists of 4.5S RNA, homologous to 7SL RNA SRP54 binding domain (domain IV), and Ffh a homologue of SRP54 (fifty-four homologue) (Bernstein *et al.*, 1989; Romisch *et al.*, 1989). The Ffh/4.5S RNA complex is essential in bacteria and, like the eukaryotic SRP interacts specifically with the signal sequence of nascent secretory proteins and is important for protein translocation (Brown, 1987; Luirink *et al.*, 1992; Phillips and Silhavy, 1992). The Ffh/4.5S RNA complex binds to a bacterial homologue of SRα, FtsY. This interaction is entirely

GTP-dependent and results in the stimulation of GTP hydrolysis, in the same way as eukaryotic SRP and SR (section 1.6.3) (Miller *et al.*, 1994). This GTP hydrolysis is essential for Ffh function *in vivo* (Samuelsson *et al.*, 1995). Homologues of the *E. coli* Ffh exist in other bacteria. The many bacterial Ffh proteins exhibit marked homology throughout the entire sequence both amongst themselves and with the mammalian SRP54 (Zwieb and Samuelsson, 2000). Ffh-4.5S RNP and FtsY can replace mammalian SRP and SRα in targeting nascent secretory proteins to microsomal membranes *in vitro*. This requires a hydrophobic signal sequence, GTP hydrolysis and occurs co-translationally. However, bacterial SRP was unable to cause elongation arrest, presumably due to the considerably shorter RNA molecule and the lack of homologues of SRP9/14 (Powers and Walter, 1997). Ffh protein has since been found to function in co-translational signal sequence recognition *in vivo* (Valent *et al.*, 1998). That bacterial proteins can replace their eukaryotic homologues is remarkable and supports the belief that SRP-dependent targeting must have a role in bacterial protein transport (Herskovits *et al.*, 2000). Bacterial SRP and SR have been found necessary for the targeting of α-helical membrane proteins to the inner membrane (MacFarlane and Muller, 1995; de Gier *et al.*, 1996; Ulbrandt *et al.*, 1997; Koch *et al.*, 1999). As in yeast, the specificity of bacterial SRP for nascent chains increases with hydrophobicity of the signal sequence (Ulbrandt *et al.*, 1997; Lee and Bernstein, 2001).

Soluble periplasmic and β-pleated outer membrane proteins of *E. coli* are targeted to the inner membrane *via* SecB and translocated across the inner membrane *via* the motor protein SecA, in a process that is completely independent of SRP and SR (Behrmann *et al.*, 1998; Koch *et al.*, 1999). Despite the existence of two separate targeting pathways, both of these appear to converge, as in eukaryotic cells (section 1.7.1), on the same translocation apparatus in the inner membrane. This translocon is composed of SecY, SecG and SecE (Valent *et al.*, 1998). This is consistent with the finding that SecA is necessary for the translocation of SRP-dependent substrates in *E. coli* (Qi and Bernstein, 1999).

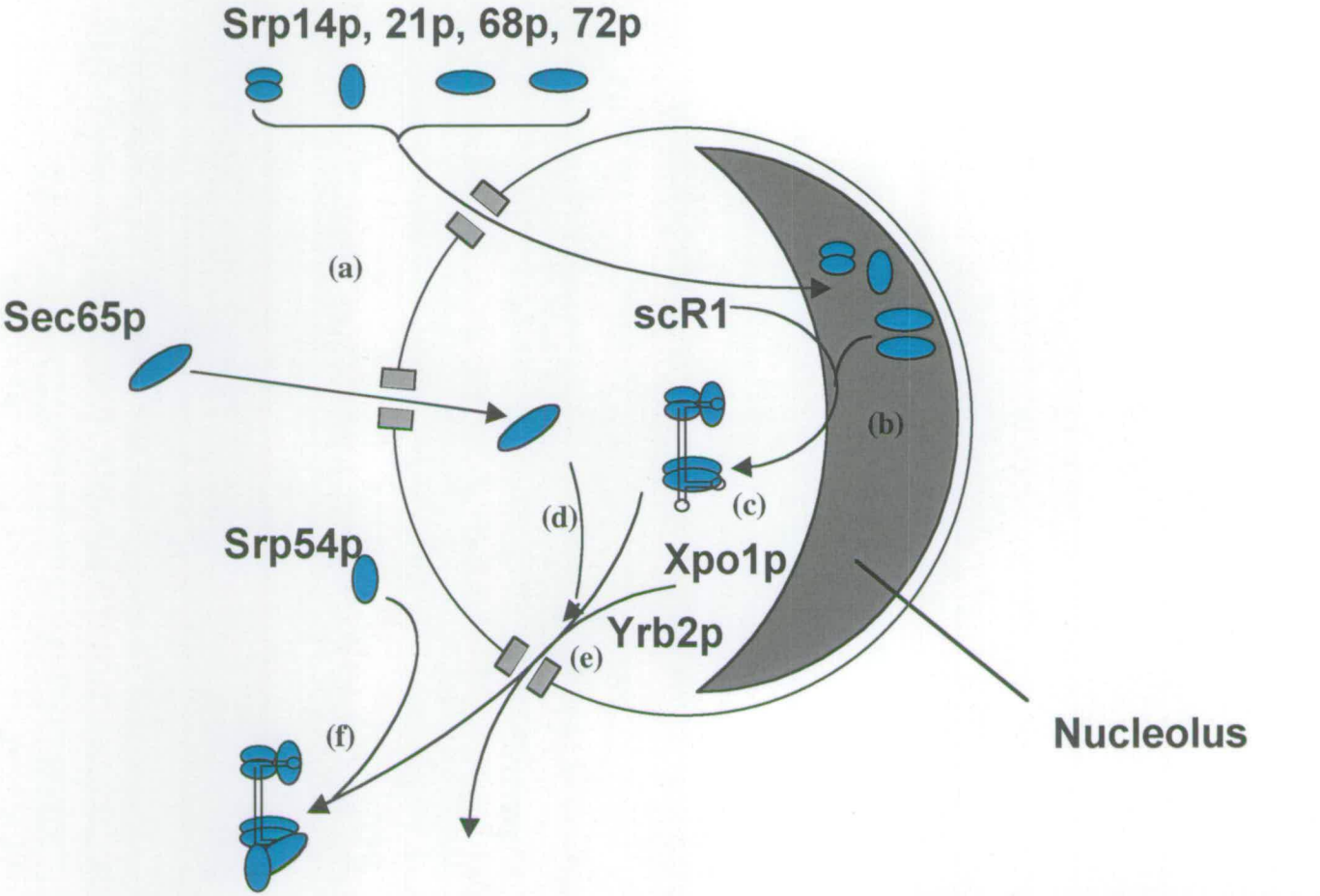
1.5.4 Biogenesis of SRP

Whilst the activities of SRP have been examined extensively, both *in vivo* and *in vitro*, until recently very little work had been carried out to determine how and where the proteins in the particle are assembled with SRP RNA. SRP is a relatively simple ribonucleoprotein (RNP) and is thus a good model for the study of RNP assembly. Recently, SRP RNA was found to localise to the nucleolus of mammalian cells, suggesting that perhaps the processing of SRP RNA or its assembly into SRP involved a nucleolar phase (Jacobson and Pederson, 1998). Ciufo and Brown (2000) defined the assembly of SRP. They found that most of the assembly of the particle takes place in the nucleus. Four of the SRP proteins (Srp14, 21, 68 and 72p) had nucleolar pools and these same proteins are all required to form stable "core" SRP complexes with scR1 RNA. The core SRP is also a competent nuclear export substrate. Of the remaining SRP components, Sec65p also enters the nucleus and assembles onto the core particle there, whereas Srp54p is solely cytoplasmic. The export of SRP from the nucleus required the transport receptor Xpo1p/Crm1p and Yrb2p, both components of the pathway that exports leucine-rich nuclear export signal (NES)-containing proteins from the nucleus (Figure 1.4).

Further work determined that the import of the core SRP components into the nucleolus requires the ribosomal protein import receptors Pse1p and Kap123p/Yrb4p (Grosshans *et al.*, 2001). These receptors have been speculated to constitute a nucleolar import pathway. It has also been shown that ribosomal subunit export may be linked to the same pathway as that used by SRP. Export of 60S subunits of the ribosome requires the nuclear export sequence-containing non-ribosomal protein Nmd3p. This protein binds directly to the large ribosomal subunit protein Rpl10p. Nmd3p shuttles between the nucleus and the cytoplasm and this shuttling requires the nuclear export factor Xpo1p. Therefore, the export of 60S ribosomal subunits, like the export of SRP, is Xpo1p dependent (Gadal *et al.*, 2001). Nmd3p is not, however, required for SRP export (Ciufo and Brown, 2000) and it is currently unclear if Xpo1p binds to a nuclear export signal in SRP or whether an adapter protein is required.

Figure 1.4 Model for yeast SRP assembly

All SRP proteins except Srp54p are imported into the nucleus. Core subunits are directed to the nucleolus, whereas Sec65p is not (a). Core SRP proteins are assembled with nascent scR1 RNA. This step may take place in the nucleolus (b). The assembled core SRP is released from the nucleolus to the nucleoplasm (c) where Sec65p binds to it (d). The particle is exported to the cytoplasm bound to the importin homologue Xpo1p/Crm1p (e), where Srp54p binds, completing the particle (f). Circle, the nucleus; grey crescent, the nucleolus; grey rectangles, nuclear-pore complexes (from Ciufo and Brown, 2000).



Reproduced from Ciufo and Brown, 2000

1.5.5 Cellular response and structural consequences to loss of SRP and mutations in SR

As previously discussed, both the components and the mechanism of SRP-dependent targeting are conserved in every organism studied to date from bacteria to eukaryotic cells. SRP-dependent targeting is essential in all organisms that have been examined except *S. cerevisiae*. Deletion of the genes encoding any of the components necessary for SRP-dependent targeting in yeast result in the disruption of the entire pathway. Yeast strains lacking SRP are petite (they lack mitochondrial function), grow three to six-fold slower than isogenic wild type strains and accumulate SRP-dependent substrates in the cytosol (Hann and Walter, 1991; Ogg *et al.*, 1992; Hann *et al.*, 1992; Stirling *et al.*, 1992; Brown *et al.*, 1994; Ogg *et al.*, 1998). Therefore, while SRP may not be essential in *S. cerevisiae* the loss of SRP-dependent targeting of proteins has severe consequences for the cell (Mutka and Walter, 2001). By depleting SRP or SR subunits from a population of yeast cells, the adaptation of yeast to the loss of SRP can be examined in more detail. Two to four hours after depletion of SRP the accumulation of SRP-dependent precursor proteins can be seen that disappear by six hours after depletion (Mutka and Walter, 2001). Previous studies indicate that the adaptation to the loss of SRP-dependent targeting is not due to the accumulation of a suppressor mutation. It is a physiological adaptation to a situation requiring an immediate stress response from the cell (Ogg *et al.*, 1992). It would be logical to assume that the adaptation to the accumulation of precursor proteins in the cytosol is simply due to the up-regulation of expression of translocon components. However, this is not the case. While an increase in available translocation sites in the ER membrane would alleviate the precursor accumulation problem this is not the adaptive route taken by the cell. Instead, the cell responds immediately by repressing the synthesis of ribosomal proteins, inducing the expression of a large number of heat shock factors and chaperone proteins and repressing the synthesis of mRNAs encoding mitochondrial and energy generating proteins. In all, 11% of the total yeast genome is affected by this adaptation with one-third of these genes being induced while the other two-thirds are repressed. The major response of the cell appears to be the repression of synthesis of ribosomal proteins. This results in not only a reduced growth rate of the cell but also a decrease

in the level of untranslocated protein in the cytosol. Less ribosomal protein results in less protein synthesis therefore the cell trades speed of growth for fidelity in protein synthesis, and survives the stress of losing the SRP-dependent protein targeting pathway (Mutka and Walter, 2001). The loss of SRP has many effects on the yeast cell, from carbon utilisation and growth rate to protein synthesis rates. However, these drastic adaptive measures allow the cell to survive a defect that is lethal to all other organisms studied.

The peripheral ER membrane in yeast forms a dynamic network of interconnecting membrane tubules, similar to the ER in higher eukaryotes (Prinz *et al.*, 2000b). Maintenance of this network does not require microtubule or actin filaments, but its dynamic behaviour is largely dependent on the actin cytoskeleton. Mutants in SR have been isolated that disrupt peripheral ER structure (Prinz *et al.*, 2000b). In both wild type and mutant cells, the ER and mitochondria have been found to partially co-align. Mutations that cause defects in peripheral ER structure also cause defects in mitochondrial structure. It has been suggested that both trafficking between the ER and Golgi complex and ribosome targeting are important for the maintenance of ER structure (Prinz *et al.*, 2000b). Perhaps the maintenance of proper ER structure may be required to maintain mitochondrial structure. The defect in ER, Golgi and mitochondrial structure was a result of mutations in SR which decreased its affinity for ribosomes. The decrease in ribosome binding to the ER membrane, due to the mutations in SR, led to the abnormal ER structure. In cells lacking SR abnormal ER structure is not observed. Also, in mutant cells grown in the presence of sublethal doses of the translational inhibitor cycloheximide, the defect in ER structure is suppressed. This, presumably, is due to cycloheximide slowing the rate of translation, giving the SRP-bound ribosomes more time to reach the ER in SR defective strains. As cells lacking the SRP-dependent targeting pathway have reduced global synthesis of protein, perhaps the general slowing of protein targeting allows ribosome binding to the ER not possible in a SR deletion mutant during rapid growth. This, albeit reduced, ribosome binding to the ER membrane would result in normal ER structure (Prinz *et al.*, 2000b). It can only be speculated why reduction in ribosomal binding to the ER membrane should cause such a drastic structural phenotype in otherwise healthy cells. Recently, a Sec63p-GFP fusion was used to

investigate ER cortical and nuclear structure and inheritance. Cortical ER morphology was found to be sensitive to mutations in the actin-encoding gene (*ACT1*) and to the actin-destabilising drug latrunculin-A (Fehrenbacher *et al.*, 2002). Cortical ER inheritance appears to be cytoskeleton dependent, relying on anchorage as opposed to directed movement (Fehrenbacher *et al.*, 2002). Perhaps ribosome binding to the ER affects its anchorage to the plasma membrane leading to a disruption of ER structure in the absence of ribosome binding.

1.6 The signal recognition particle receptor

1.6.1 Components of SR

The SRP receptor (SR) was first thought to be a single protein whose function was mediating both the binding of SRP-RNCs to the ER membrane and release of SRP from the ribosome (Gilmore and Blobel, 1983). However, it was quickly established that SR was a complex consisting of two distinct polypeptide chains that tightly associate with one another on the ER membrane.

Mammalian SR is composed of two subunits, α and β. The α subunit is a 69 kDa protein bound to the ER membrane through stable association with the 30 kDa transmembrane anchored β subunit. Both SRα and β are GTP binding proteins and the GTPase activity of both subunits of SR are essential for targeting of SRP-dependent substrates to the ER membrane. SR was first purified through the affinity of SRα for SRP54 using SRP immobilised on a chromatography column. This allowed identification of two polypeptides that associated on the column with SRα, mp30 and SRβ. mp30 binds to SRP-sepharose directly and is present in the ER membrane in several-fold molar excess of SRα and SRβ. The affinity of mp30 for SRP suggests that it may serve a yet unknown function in protein translocation. mp30 was found in mammalian cells, however no homologue of this protein has been identified or investigated in yeast (Lauffer *et al.*, 1985; Tajima *et al.*, 1986).

Homologues of mammalian SR components were identified in *S. cerevisiae.* The yeast SR is also composed of two GTPases, α and β. As with mammalian SR, the α subunit is a peripheral membrane protein associated with the transmembrane anchored β subunit (Ogg *et al.*, 1992; Ogg *et al.*, 1998). Depletion of SR from yeast leads to impaired translocation of soluble and membrane proteins across the ER membrane. This is substrate dependent with Kar2p and dipeptidyl aminopeptidase-B (DPAP-B), a soluble ER lumenal protein and a vacuolar integral membrane protein, showing the worst defects. Less affected are invertase and pre-pro-α-factor, and no defect at all is seen for carboxypeptidase Y (CPY) (Ogg *et al.*, 1992). These defects are similar to those found in SRP deficient cells. Deletion of the genes encoding SRα or β leads to a six-fold reduction in growth rate, deletion of both scR1 and SRα results in indistinguishable phenotypes confirming that SRP and SR function in the same pathway (Ogg *et al.*, 1992; Ogg *et al.*, 1998).

During its synthesis a 140 residue N-terminal domain of SRα targets and anchors the polypeptide to the ER membrane through its interaction with SRβ by a mechanism independent of the pathway involving SRP. It has been shown *in vitro* that the mammalian SRα assembles co-translationally on the ER membrane during an mRNA-encoded translation pause. The interaction between SRα and SRβ is sufficient for this to occur. This has not been shown using yeast components, however, it has obvious implications for how or if the subunits of SR may be expressed independently (Young and Andrews, 1996).

1.6.2 Functional domains within SR

Both subunits of yeast SR are GTPases and the GTPase domain of SRβ has been shown to be essential for its function (Ogg *et al.*, 1998). During a round of targeting SRP interacts with SR at the ER membrane. This interaction leads to the GTP-dependent transfer of the nascent chain from SRP to the translocation apparatus in the ER membrane. Displacement of SRP from the signal sequence of a nascent

polypeptide is GTP dependent and mediated by SRα. The GTPase domain of SRβ, although essential for SRP-dependent translocation, has no assigned function in the pathway. The GTPase domains of SRP54 and SRα are related, defining their own GTPase subfamily. The GTPase domain of SRβ, however, is structurally distinct, falling into its own subfamily of GTPases with its closest relative being Sar1p, an ARF-like GTPase involved in ER to Golgi trafficking (Nakano and Muramatsu, 1989; Miller *et al.*, 1995). The discovery of a GTP/GDP binding domain makes it unlikely that SRβ is simply a membrane anchor for SRα. It suggests that a network of three directly interacting GTPases function during protein targeting to the ER membrane. The role of GTP hydrolysis in SR function was examined using the non-hydrolysable analogue β-γ-imidoguanosine 5'-triphosphate (Gpp(NH)p) during the targeting and insertion steps of a protein translocation reaction. It was found that in the presence of Gpp(NH)p SRP co-sedimented with ER membranes, presumably unable to dissociate from SR (Rapiejko and Gilmore, 1997). Previous work had established that SRP54 and SRα are reciprocally activating GTPase activating proteins (GAPs). The interaction between SRP54 and SRα on the ER membrane activates their GTPase function, both proteins hydrolyse the GTP bound to them, this allowing the dissociation of SRP from SR for another round of targeting (Connolly *et al.*, 1991; Bacher *et al.*, 1996). Mutants in the GTPase domain of SRβ were generated (Ogg *et al.*, 1998). Many of these were predicted to have phenotypes such as reduced nucleotide affinity and reduced GTP hydrolysis. However, though many of the mutants are temperature and/or cold sensitive most have not been characterised further. It has also been established that the transmembrane domain of SRβ is not required for its function (Ogg *et al.*, 1998). It should be noted however, that a proportion of this SRβ-ΔTM remains associated with the ER membrane (approximately 25% of the total protein), suggesting that an ER membrane protein interacts with SRβ and that this interaction is sufficient to allow the partial localisation of SRβ-ΔTM to the ER.

1.6.3 GTPase involvement in SRP-dependent targeting

All GTPases bind and hydrolyse GTP and thus can exist in any one of three states: GTP-bound, GDP-bound, and empty. Typically the active state of a GTPase is in the GTP-bound form. Hydrolysis of GTP to GDP turns the protein "off" while the empty state of the GTPase is considered a transient intermediate in the exchange of GDP for GTP. Both the rate of hydrolysis of GTP and the release of GDP are usually slow. Regulation of these steps is by GTPase activating proteins (GAPs) and guanine nucleotide release proteins (GNRPs) (Bourne *et al*, 1991). Because the hydrolysis of GTP is effectively irreversible, the cycle is unidirectional. Targeting of proteins to the ER has been modelled as two intersecting conventional GTPase cycles. Cycle-1 begins with SRP54 binding to the ribosome nascent chain complex (RNC) promoting the exchange of bound GDP for GTP. In cycle-2 the GTP-bound form of SRα binds SRP-RNCs at the ER membrane. The two cycles intersect as the GTP-bound SRP and SR not only bind directly to one another but also function as reciprocal GAPs (Powers and Walter, 1995; Millman and Andrews, 1997). Hydrolysis of GTP results in the release of the nascent secretory protein to the translocon. The GDP-bound forms of SRα and SRP54 dissociate and are recycled.

The observation that binding of SRP54 to the ribosome increased the affinity of SRP54 for GTP 10-fold, suggested that the ribosome acts as a GNRP in the targeting reaction (Bacher *et al.*, 1996). In recent years, evidence has been provided that this conventional model of GTPase action is not appropriate to describe the interactions involved in the targeting of SRP-bound RNCs to the ER membrane.
Rapiejko and Gilmore (1997) examined the GTPase activity of SR in the following way. They utilised a mutant of SRα with altered nucleotide binding specificity such that XTP replaces GTP as the preferred substrate. SRα mutants were incorporated functionally into microsomes using an established assay (Andrews *et al.*, 1989). Using different combinations of XTP, GTP and nonhydrolysable analogues of the two, they were able to analyse the nucleotide bound states of each protein during a functional targeting reaction. GTP binding to both proteins was shown to be reversible, and the nucleotide-binding status of SRP54 and SRα remained unfixed until the SRP-RNC interacted with SR. Furthermore, GTP hydrolysis was not

required for either the stable docking of the RNC or the signal sequence transfer to the translocon, but was necessary for dissociation of SRP54 and SRα.

Further study of the GTPase cycle in SRP-dependent protein targeting by Bacher *et al.*, 1999 led to the proposal that the third GTPase involved, SRβ, regulates the interaction of SR with ribosomes, allowing SRα to "scan" ribosomes for the presence of SRP. The interaction between SRP and SRα would lead to signal sequence release from SRP and insertion into the translocon and GTP hydrolysis would dissociate SR from the ribosome and SRP from SR. To test GTP binding to SR subunits they used UV light-mediated cross-linking of α[^{32}P]GTP to purified SR reconstituted into liposomes with RNCs and/or translocon components. They found that GTP binding to SRβ was drastically reduced in the presence of RNCs and that an increase in GTP hydrolysis was observed (Bacher *et al.*, 1999). Legate *et al.*, 2000 showed that GTP binding by SRβ was necessary for dimerisation of SRα and SRβ. They also proposed that dissociation of SRα and SRβ could play an important role in SRP-dependent targeting. GTP hydrolysis by SRβ would release the RNC-SRP-nascent chain complex for transfer to the translocon, with SRα still bound to SRP54. Transfer of the nascent chain to the translocon would trigger hydrolysis of GTP by SRP54 and SRα, allowing SRP to return to the cytoplasm and SRα to rebind to SRβ. Fulga *et al.*, 2001 extended this model by showing that a 21 kDa ribosomal protein could be cross-linked to SRβ only in the absence of bound nucleotide or in the presence of GDP. Binding of SRβ to this 21 kDa ribosomal protein was lost upon transfer of the RNC to the translocon and appeared to stabilise the nucleotide-free form of SRβ. The use of the D181N mutant of SRβ (Legate *et al.*, 2000), which has a higher affinity for XTP than GTP, demonstrated that GTP binding by SRβ is essential for translocation (Fulga *et al.*, 2001). GTP bound SRβ is essential for the release of the nascent chain from SRP and binding of SRβ to the 21 kDa ribosomal protein stabilises its nucleotide-free state. These two observations are compatible as

binding of SRβ to the 21 kDa ribosomal protein is lost when the translocon is present. Therefore, GTP binding to SRβ ensures that SRP releases the nascent chain only in the presence of an available translocon. A model, integrating all of these observations, and data described in this study (chapter 5), is described in Figure 1.5. SRP binding to the signal sequence of a target nascent chain and the ribosome promotes GTP binding by SRP (1). Strong interaction between SRP54 and SRα targets the SRP-RNC complex to the ER membrane, stimulating GTP binding by SRα (2). GDP-bound SRβ interacts with the ribosome (GNRP) and loses the bound GDP, causing SRα and SRβ to dissociate (3). Tight binding between SRα and Srp54p is sufficient to maintain the RNC-SRP-SR complex on the ER membrane. Ribosome binding to the translocon, and possibly SRβ binding to the translocon, stimulates GTP binding and hydrolysis by SRβ and release of the RNC from SRP (4). Translocation of the nascent chain proceeds, causing structural changes in the ribosome that mediate GTP hydrolysis by SRα and SRP54. This leads to recycling of SRP and binding of SRα to GDP bound SRβ in the ER membrane (5). In this way, the ribosome acts as a GNRP for SRβ, while the translocon, or the translocon and the ribosome together, act as a GAP for SRβ.

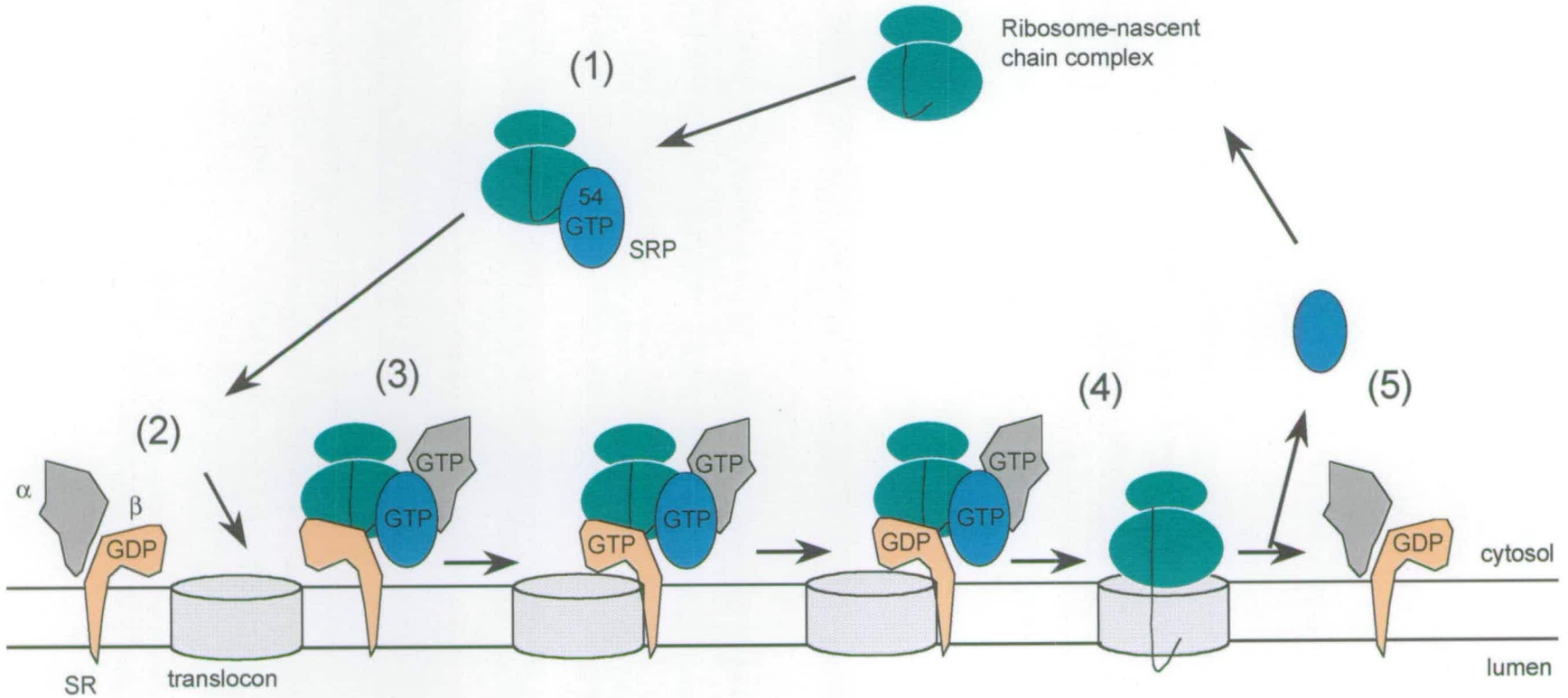
1.6.4 SRα is evolutionarily conserved

Homologues of SRα have been found in eukaryotic, bacterial, and archaeal species. The existence of SRP and SR in all organisms examined indicates a universal conservation of SRP-dependent targeting. While homologues of SRα have been found, homologues of SRβ appear only to exist in eukaryotic cells. The existence of this third GTPase reveals an apparent need for another layer of regulation in SRP-dependent targeting in eukaryotes not required in bacterial cells.

Figure 1.5 Model of GTPase action in SRP-dependent targeting

SRP binding to the signal sequence of a target nascent chain and the ribosome promotes GTP binding by SRP (1). Strong interaction between Srp54p and SRα targets the SRP-RNC complex to the ER membrane, stimulating GTP binding by SRα (2). GDP-bound SRβ interacts with the ribosome (GNRP) and loses the bound GDP, causing SRα and SRβ to dissociate (3). Tight binding of SRα to Srp54p is sufficient to maintain the SRP-RNC-SR complex on the membrane. Ribosome binding to the translocon, and possibly SRβ binding to the translocon, stimulates GTP binding and hydrolysis by SRβ and release of the RNC from SRP (4). Translocation of the nascent chain proceeds causing structural rearrangements in the ribosome that mediate GTP hydrolysis by SRα and Srp54p, leading to recycling of SRP and re-binding of SRα to GDP-bound SRβ in the ER membrane (5).

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Ribosome-nascent
chain complex
(1)
54
GTP
SRP
(2)
(3)
(4)
(5)
α
β
GDP
GTP
GTP
GTP
GTP
GTP
GTP
GDP
GDP
cytosol
lumen
SR
translocon

The bacterial SRα homologue is FtsY. FtsY is essential in *E. coli* for the biogenesis of integral membrane proteins, indicating that FtsY and eukaryotic SRα have similar functions (Seluanov and Bibi, 1997). The binding of Ffh to FtsY requires 4.5S RNA, and FtsY alone does not interact with 4.5S RNA. The interaction between Ffh and FtsY is strongly influenced by the structure of a region of 4.5S RNA known as the tetraloop region. There is strong evidence that both 4.5S RNA and Ffh undergo structural changes to form a functional interaction with FtsY and that 4.5S RNA may regulate the GTPase cycle of the Ffh-FtsY complex (Peluso *et al.*, 2000; Jagath *et al.*, 2001; Peluso *et al.*, 2001). Ribosome binding to the inner membrane in *E. coli* requires FtsY but is not dependent on the SRP54 homologue Ffh, suggesting that perhaps Ffh operates downstream of FtsY (Herskovits and Bibi, 2000).

Sequence alignments of all SRP-type GTPases, except SRβ, identified a domain conserved throughout called the NG domain (Romisch *et al.*, 1989; Bernstein *et al.*, 1989). In FtsY and SRα, the NG domain is linked to a highly charged domain at the N-terminus, which, in SRα is involved in membrane attachment through its interaction with SRβ (Young *et al.*, 1995). This N-terminal, 198-residue long, segment of FtsY constitutes an independent domain, required only for the targeting of the C-terminal NG domain of FtsY to the membrane (Zelazny *et al.*, 1997). Herskovits *et al* ., 2001 showed *in vivo* evidence that the catalytic NG domain of FtsY could be separated from the N-terminal targeting domain by proteolytic cleavage with no loss of FtsY function. When these domains were separated by a polypeptide spacer function was lost suggesting that FtsY is targeted to the inner membrane and assembled co-translationally. Targeting of FtsY to the inner membrane was described previously. It was shown that FtsY binds to the inner membrane through interactions with phosphatidylethanolamine (PE) and an unknown membrane protein (Millman *et al.*, 2001). In the absence of PE, FtsY is still targeted to the membrane and assembled through an interaction sensitive to the addition of trypsin. The crystal structure of the NG domain of FtsY (Montoya *et al.*, 1997) revealed 3 regions: the N region, a GTPase region and an α-β-α insertion

called the "I box". The I box was suggested to be a built-in factor that stabilises the nucleotide-free form of FtsY. To bind GTP, a conformational change is needed which might be induced by the interaction of the I box with a regulatory factor, such as Ffh/4.5S RNA (Montoya *et al.*, 1997). Lu *et al.*, 2001 provided evidence that Ffh and FtsY do not bind GTP unless previously "primed" by binding to an external "substrate". In the case of Ffh this would be a hydrophobic signal sequence and for FtsY the translocation apparatus. The existence of a priming step in SRP-dependent targeting in bacteria suggests that targeting is not dependent on the completion of one cycle of GTP binding and hydrolysis but on two intersecting cycles which cannot interact productively unless both are bound by appropriate substrate molecules. These findings correlate with those of Legate *et al.*, 2000 (section 1.6.3) and strengthen the evidence for a possible role in targeting and/or translocation for dissociation or significant rearrangement of the interactions between eukaryotic SRα and SRβ. Re-association of these proteins could constitute one "priming" step in eukaryotic SRP-dependent targeting with signal sequence binding providing the other. The requirement for priming steps in targeting of proteins might be a way of ensuring the faithful delivery of nascent chains to available translocons and prevents non-productive association of SRP54 and SRα at the membrane that would effectively short-circuit the targeting pathway.

1.7 Co-translational translocation

Protein insertion through the ER membrane is not by diffusion through the lipid-bilayer. Polypeptide insertion is an active process requiring energy and proteins. Protein transport across the membrane of the ER can occur either co- or post-translationally. Events at the membrane during translocation can be dissected into individual steps. The first involves the interaction of signal sequences with membrane proteins. The second involves the formation of the ribosome-membrane junction (in co-translational translocation only) and the third is translocation. Translocation can be detected experimentally by modification of the translocated

protein by enzymes within the lumen of the ER (OSTase and the signal peptidase complex) and by its protection from exogenously added proteases.

Nascent chains translated in the presence of rough microsomes (RMs) are relatively resistant to protease treatment. This suggests that an interaction with an integral or peripheral membrane protein is responsible for the protection of the nascent protein (Sabatini and Blobel, 1970; Connolly *et al.*, 1989). Early evidence for an aqueous translocation channel (translocon) in the ER membrane was demonstrated by electrophysiological techniques, Simon and Blobel (1991) finding that attached ribosomes kept the translocon in an open conformation and that they closed when ribosomes detached. Fluorescence collisional quenching has also been used to detect the open or closed conformation of the translocon and what proteins or complexes are necessary for closing or blocking the pore (Liao *et al.*, 1997; Hamman *et al.*, 1998). The functions of the translocon complex will be discussed in relation to each step of translocation as it occurs at the ER membrane.

1.7.1 The Translocon

Genetic studies in *S. cerevisiae* led to the isolation of a variety of mutants defective in the secretory pathway, the *sec* mutants (Schekman *et al.*, 1983). One such mutant identified Sec61p, a multi-spanning integral membrane protein of the ER required for the translocation of both secretory and membrane proteins. This protein can be isolated from yeast ER membranes in a tight complex with two other proteins, Sbh1p and Sss1p and these heterotrimers form the translocon. The analogous complex in mammalian ER consists of Sec61α, β and γ these being homologous to Sec61p, Sbh1p and Sss1p respectively (Deshaies *et al.*, 1991; Görlich *et al.*, 1992; Stirling *et al.*, 1992; Sanders *et al.*, 1992; High *et al.*, 1993). The evolutionary conservation of the translocon extends to the bacterial translocon SecYEG, in which two components, SecY and SecE are homologous to Sec61α and γ (Meyer *et al.*, 1999).

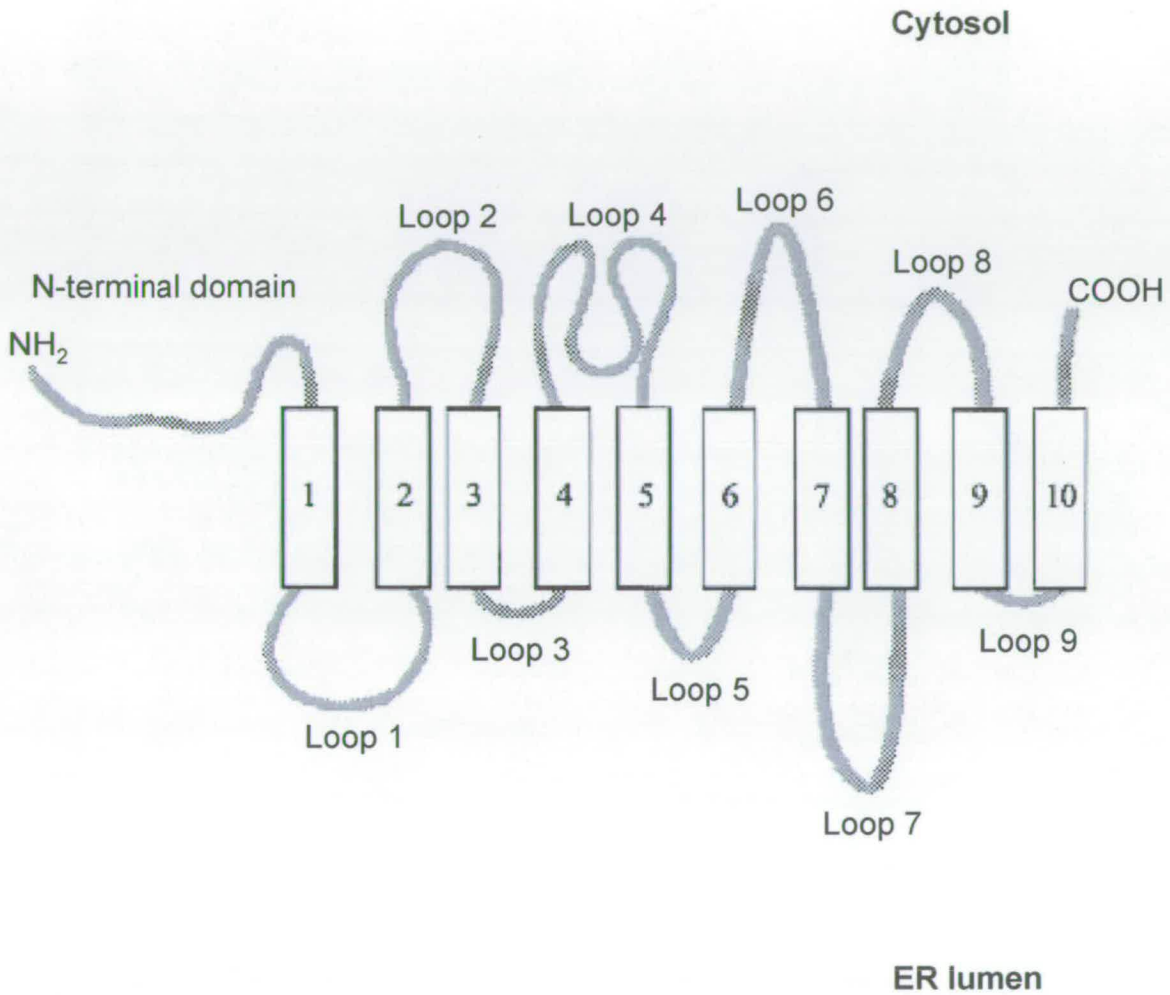
Genetic screens and biochemical reconstitution experiments have indicated that the Sec61p complex is essential for translocation and forms the core of the translocon (Deshaies and Schekman, 1987; Rothblatt *et al.*, 1989; Görlich and Rapoport, 1993; Panzner *et al.*, 1995). Cross-linking experiments have demonstrated that Sec61p,

with ten putative membrane-spanning domains, is in close proximity to polypeptide chains as they pass through the membrane. Indeed, a translocating nascent chain can be cross-linked to Sec61α at every amino acid within the plane of the lipid bilayer, implying that Sec61α surrounds the nascent chain in a ring (Görlich *et al.*, 1992; Müsch *et al.*, 1992; High *et al.*, 1993a+b; Mothes *et al.*, 1994). The characterisation of cold sensitive mutants in *sec61* helped to define two early stages in translocation for which the function of Sec61p is necessary. The first class of mutants is defective in the docking of nascent chains onto the cytosolic face of the Sec complex, with Sec61p required for this interaction. The second class of mutants allow the interaction of precursor proteins with the docking site but are defective in the ATP-mediated release from this site, which in wild type membranes leads to translocation. Sec61p may have to undergo a conformational change for release of the polypeptide to occur (Pilon *et al.*, 1998). While factors such as the ribosome and Kar2p are known to regulate the state of the translocon, these *sec61* mutants indicate that Sec61p itself has a role in the regulation of the transition from the closed to the open conformation of the pore, and therefore a regulatory role in translocation itself.

Sss1p is an 8.9 kDa, C-terminally anchored essential component of the Sec61 complex. Purified Sss1p, in SDS micelles, is very stable and adopts a helical secondary structure (Beswick *et al.*, 1998). In order to gain some insight into the architecture of the Sec61 complex, complementary N- and C-terminal fragments of Sec61p were expressed in yeast (Figure 1.6; Wilkinson *et al.*, 1997). Chemical cross-linking using membranes derived from these strains revealed that Sss1p interacts with transmembrane domains 6, 7 and 8 (Wilkinson *et al.*, 1997). Suppression of normally lethal *sec61* mutants by over-expression of Sss1p provided supporting evidence of this interaction (Wilkinson *et al.*, 1997). It has been speculated that Sss1p acts as a surrogate signal sequence in the empty translocon and is a blocking protein for the pore, moving out of the translocon during translocation. The small, helical structure of Sss1p would fit inside the translocon until a suitable substrate was available, blocking any non-specific translocation of small cytosolic proteins.

Figure 1.6 Membrane topology of Sec61p

The 10 transmembrane domains of Sec61p are depicted (rectangles 1-10) with the relative lengths of each cytoplasmic and lumenal loop (1-9). The orientation of the N-terminal domain of Sec61p along the plane of the lipid bilayer is based on the data of Wilkinson *et al.*, 2000.



Reproduced from Wilkinson *et al.*, 2000.

It is apparent that the Sec61 complex is not only involved in translocation into the ER but also dislocation from it. When protein folding or processing within the lumen of the ER is prevented the protein is handled in one of three ways. First, the protein may aggregate and elicit the unfolded protein response (UPR) (McMillan *et al.*, 1994). The UPR is mediated through an ER transmembrane protein, Ire1p, which along with tRNA ligase removes a non-canonical intron from the mRNA encoding a transcription factor (Hac1p) responsible for induction of expression of many chaperones and translocon components (Shamu and Walter, 1996; Shamu, 1998). Second, the protein may be transported to the vacuole for degradation (Hong *et al.*, 1996). Third, the ER quality control machinery may recognise the misfolded protein and target it for immediate degradation and removal from the secretory pathway by a process known as the ER-associated degradation process (ERAD) (McCracken and Brodsky, 1996). ERAD is carried out by the 26S proteasome, the multicatalytic protease complex that mediates the majority of protein degradation in the cytosol and nucleus (Plemper and Wolf, 1999). It has been demonstrated that ERAD substrates (with possible exceptions; Walter *et al.*, 2001) gain access to the proteasome by being extracted from the ER via the Sec61p translocon in a process referred to as dislocation (Wiertz *et al.*, 1996b; Pilon *et al.*, 1997; Plemper *et al.*, 1997; Zhou and Schekman, 1999). Major substrates for ERAD include proteins that are conformationally abnormal as a result of mutation, lack of assembly partners, or imperfect folding.

1.7.2 Ribosome binding to the Sec61p complex

In co-translational translocation, the Sec61p complex associates with the translating ribosome at the ER membrane (Görlich and Rapoport, 1993; Kalies *et al.*, 1994; Hanein *et al.*, 1996; Beckmann *et al.*, 1997). Electron micrograph images of purified Sec61 complex from yeast confirmed that it forms an oligomeric ring structure comprising 3-4 Sec61 trimers (consisting of Sec61p, Sbh1p and Sss1p). The overall diameter of the ring is 85-95Å with the internal pore having a mean diameter of 20-35Å. This diameter is greater or lesser depending on the open or closed state of the pore and if ribosomes are bound (Hanein *et al.*, 1996; Beckmann *et al.*, 1997). Recently it has been established that the tunnel in the large ribosomal subunit

through which the nascent polypeptide exits (Lake, 1985; Yonath *et al.*, 1987) is aligned with the protein-conducting channel of the translocon (Beckmann *et al.*, 1997; Beckmann *et al.*, 2001). Fluorescence collisional quenching data showed that the ribosome, while bound to the translocon, regulates the gating of the pore during membrane protein integration (Do *et al.*, 1996; Liao *et al.*, 1997). Regulation of the pore by the ribosome includes recognition of membrane proteins requiring insertion into the lipid bilayer, leading to structural changes in both the ribosome and the translocon required to effect insertion. One striking observation is that the lumenal and cytosolic sides of the translocon are never open simultaneously. Significant structural rearrangement would be required to close a pore with a mean diameter of 40-60Å which suggests that a lumenal protein acts as a "gate". Further fluorescence collisional quenching experiments identified this "gate" protein as the ER lumenal chaperone BiP (Kar2p in yeast) (Hamman *et al.*, 1998). BiP, only in the nucleotide-bound state, not only acts as a lumenal seal during translocation but also seals the pore when no ribosomes are present (Hamman *et al.*, 1998; Section 1.9).

Cryo EM reconstruction of the yeast ribosome-nascent chain translocon complex has recently been extended to 15.4 Å. The reconstruction revealed four connection points between the ribosome and the Sec61p complex across a gap of approximately 10-20 Å and this "gap" was also seen with mammalian ribosome-translocon complexes (Ménétret *et al.*, 2000; Beckmann *et al.*, 2001) (Figure 1.7a). The compact appearance of the channel indicated that gating of the translocon by a signal sequence could lead to an opening just large enough to be completely occupied by an inserted nascent polypeptide chain, without allowing ion conductance (Beckmann *et al.*, 2001). These findings led to the proposal of a model of ribosome-protein-conducting channel function in co-translational translocation and membrane protein insertion (Figure 1.7b). In this model the exit tunnel of the large ribosomal subunit constitutes a functional domain of the ribosome allowing folding of secondary helical structure. Any hydrophobic signal sequence would be probed by the channel and lead to insertion and capture with the C-terminal side of the signal sequence either exposed to the cytosol or the lumen depending on its orientation (Figure 1.7b). The channel would open wide enough to only allow insertion of the signal sequence, as a result, the nascent chain following the signal sequence will either accumulate on the

Figure 1.7 Alignment of translocon and ribosome during translocation

A.

(a) Reconstruction of the translating and translocating ribosome-nascent chain complex (RNC)-Sec61 complex in 0.2% (w/v) deoxyBigCHAP at 15.4 Å resolution. Note the presence of tRNA density in the P site and the Sec61p channel with the shape of a compact disc in the active channel. Colour coding: yellow, small ribosomal subunit (40S); blue, large ribosomal subunit (60S); red, Sec61p complex; green, P site tRNA.

(b) Same as (a) but rotated upward by 90°.

(c) Reconstruction of the empty complex in 0.2% (w/v) deoxyBigCHAP at 18.9 Å resolution. Note the absence of tRNA density and the elongated shape of the channel. Colour coding as described for (a).

(d) Same as (c) but rotated upward by 90°. Bar: 100 Å (Beckmann *et al.*, 2001).

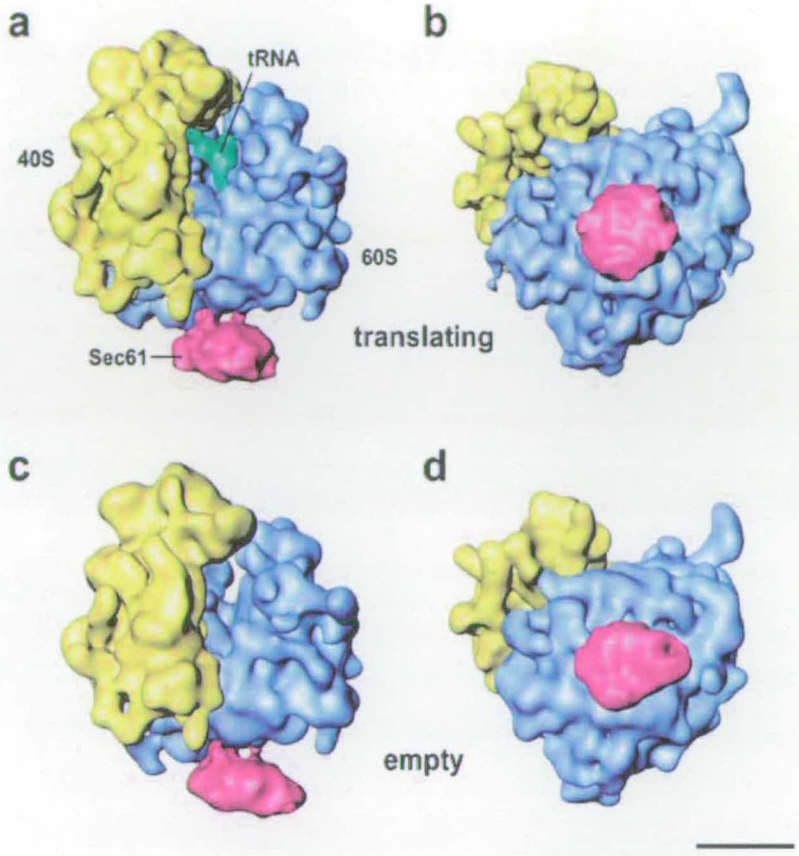
B.

Binary Model of Cotranslational Protein Translocation

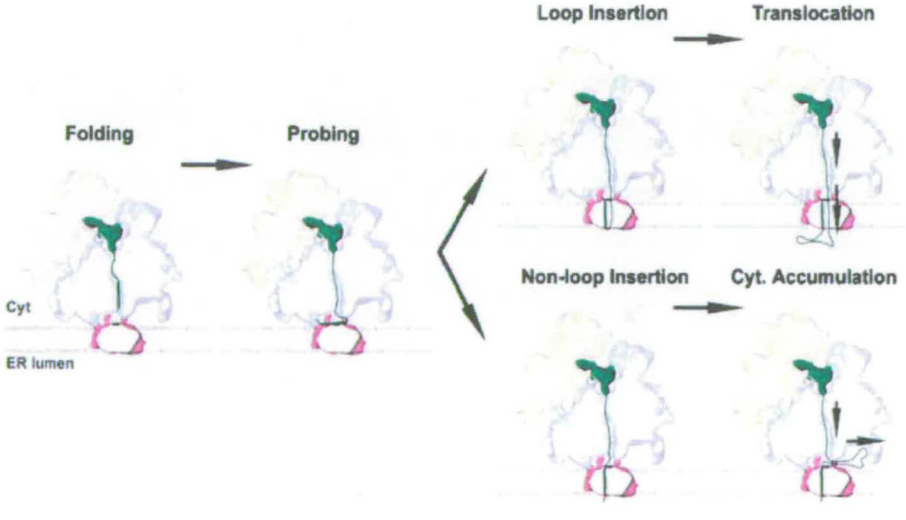
The model is based on the finding that a gap exists between the RNC and the protein conducting channel (PCC), and that the translating PCC has a compact conformation. The PCC can provide a seal to maintain the ion permeability barrier of the ER membrane: (1) The tunnel in the large ribosomal subunit facilitates folding of α-helical segments. (2) The emerging segment is probed by the PCC before insertion. Hydrophobicity, helicity, and the nature of the flanking regions (i.e., positive charges) determine if and in what orientation the segment is inserted. (3) Insertion can occur in two different orientations with the channel opening just wide enough to accommodate the inserted nascent polypeptide. (4) In case of loop insertion, the following nascent chain is guided through the membrane, and translocation is possible. In case of non-loop insertion, the following nascent chain cannot translocate, accumulating on the cytosolic side of the membrane. Because of the sealed channel and the existence of the gap between ribosome and PCC, such cytosolic domains can easily exit into the cytosol at any time without compromising the ion permeability barrier.

Nascent chain translocation after loop insertion and cytosolic accumulation after non-loop insertion are the only two principally different functional states of the RNC-PCC complex. A simple secretory protein would experience only one loop insertion of the signal sequence after targeting by SRP and translocate. For a polytopic membrane protein, the states would alternate with every new hydrophobic transmembrane domain. This model may be expanded by including additional regulation (i.e., pause transfer sequences (Chuck and Lingappa, 1992)) and exceptions (Beckmann *et al.*, 2001).

A.



B.



Reproduced from Beckmann *et al*., 2001

cytosolic side or, if it has been co-inserted in a loop, translocated across the membrane (Beckmann *et al.*, 2001). This provides a model of insertion and translocation of secretory proteins, signal anchor proteins and polytopic membrane proteins.

Assays in which the binding of radiolabelled ribosomal components to purified ER membranes were analysed revealing that the ribosome-Sec61p interaction is mediated by the 28S rRNA of the eukaryotic large ribosomal subunit. Bacterial ribosomes bound via their 23S rRNA to the bacterial homologue of the Sec61p complex, SecYEG. Interestingly, eukaryotic ribosomes bind SecYEG while bacterial ribosomes bind the Sec61p complex. This indicates that rRNA-mediated ribosome binding to the translocation channel is evolutionarily conserved (Prinz *et al.*, 2000a).

As mentioned earlier, Sec61p is an integral membrane protein with 10 transmembrane domains. The topology of the protein was elucidated through the use of C-terminal reporter-domain fusions and *in situ* digestion of specifically inserted factor Xa cleavage sites (Wilkinson *et al.*, 1996). A representation of the topology of Sec61p is shown in Figure 1.6. As Sec61p has been highly conserved throughout evolution this data can be applied to homologues of Sec61p in other organisms. Cytoplasmic loops of Sec61α have been found to be important in ribosome binding and translocation promotion by the Sec61 complex. Mild protease treatment of microsomal membranes results in the removal of SRα and, depending on the conditions and protease used, cytoplasmic loops of Sec61α. It was found that membrane binding of non-translating ribosomes decreased upon removal of cytoplasmic loop 8 and the carboxy-terminal cytoplasmic tail of Sec61α. In contrast, removal of cytoplasmic loop 6 (but not loop 8 or the carboxy-terminal tail) severely inhibited translocation (Raden *et al.*, 2000) (Figure 1.6). Thus, it appears there is differential regulation of ribosome binding and translocation within Sec61α itself. This may allow for more flexibility within the protein targeting and translocation pathways and separate regulation of SRP-dependent and –independent translocation. Wilkinson *et al.*, 2000 extended these findings by describing a role for

transmembrane domain 2 of Sec61p in yeast in post-translational translocation. They also found this domain to be dispensable for co-translational translocation and protein dislocation.

Sec61α is not the only protein within the translocon implicated in ribosome binding. Levy (2001) identified an interaction between non-translating ribosomes and Sec61β. It was shown that the cytoplasmic domain of Sec61β could inhibit the binding of ribosomes to ribosome-stripped ER membranes and that this interaction was sensitive to high salt concentrations. The conclusion that this represents an important Sec61β-ribosome interaction is strengthened by the observation that the absence of Sec61β results in impaired translocation (Kalies *et al.*, 1998). It has also been suggested that one function of Sec61β may be to stabilise the ribosome-Sec61α interaction (Raden *et al.*, 2000). It has not been investigated whether Sec61β binding to the ribosome is protein- or rRNA-mediated.

Puromycin is an aminoacyl-tRNA analogue that it is covalently incorporated into a nascent chain, and terminates protein synthesis. The reaction is catalysed by the peptidyl transferase activity of the ribosome, and the resulting peptidyl-puromycin is released (Blobel and Sabatini, 1971). Membranes stripped of ribosomes by puromycin and high salt treatment, as well as proteoliposomes reconstituted from microsomal proteins after detergent solubilisation, still contain large numbers of Sec61p oligomers (Kalies *et al.*, 1994). The survival of these complexes after puromycin and high salt treatment suggests that translocons are stable Sec61 complex oligomers. These results are supported by recent work that showed that non-translating ribosomes remain ER-bound following translocation until engaged by mRNA encoding a cytosolic protein (Potter and Nicchitta, 2002). Ribosomes bound to the ER membrane could support the translation and translocation of secretory protein independent of the SRP receptor. However, for a translationally active membrane-bound ribosome to maintain its association with the membrane, the emergence of a signal sequence must occur. The potential physiological importance

of this data is that it suggests a mechanism of regulating the equilibrium of membrane-bound and free ribosomal pools. Should the demand for the synthesis of cytoplasmic proteins exceed the capacity of free ribosomes, membrane-bound ribosomes could initiate protein synthesis and detach from the membrane. Conversely, should the demand for secretory proteins rapidly increase, an efficient mechanism may exist whereby membrane-bound ribosomes can undergo repetitive cycles of translation while remaining on the membrane, bypassing the need for detachment and re-targeting to the ER (Potter and Nicchitta, 2000; Potter and Nicchitta, 2002).

1.7.3 Mechanisms of co-translational translocation

In co-translational translocation nascent chain-ribosome complexes first interact with SRP in the cytosol. Upon interaction of the ribosome-nascent chain with the ER-localised SR, the nascent-chain ribosome complex dissociates from SRP and is targeted to the Sec61p complex. This targeting is determined by the affinity of the ribosome for the Sec61p complex and by signal sequence-Sec61p complex interactions (Walter and Johnson, 1994; Jungnickel and Rapoport, 1995). During co-translational translocation the ribosome forms a tight seal with the Sec61p complex (Liao *et al.*, 1997). The driving force for translocation of a co-translational precursor is thought to be provided by the elongation of the nascent chain on the translating ribosome. Kar2p, a lumenal Hsp70, is thought to assist in folding of emerging polypeptides and to provide additional gating of the pore (Hamman *et al.*, 1998). It has been shown by chemical cross-linking that Sec61β (the mammalian homologue of Sbh1p in yeast) interacts with Sec61α and the 25 kDa subunit of the signal peptidase complex (SPC25) (Kalies *et al.*, 1998). Cross-linking of Sec61β to SPC25 demonstrated for the first time a tight interaction between the Sec61 complex and the SPC. The cross-linking was dependent on the presence of membrane bound ribosomes, suggesting that these interactions only occur during translocation or upon translocation initiation (Kalies *et al.*, 1998).

During co-translational integration of a type I membrane protein into the ER membrane, the opening or closing of each end of the aqueous translocon pore is tightly controlled. Type I membrane proteins have an N-terminal transmembrane domain and a large C-terminal cytosolic domain. Each event in the insertion of these proteins occurs in a fashion that does not compromise the integrity of the membrane's permeability barrier. The ribosome first recognises the transmembrane segment and triggers long-range structural changes that may be involved with shifting the function of the translocon from translocation to integration (Liao *et al.*, 1997). Closing of the translocon pore on the lumenal side to maintain the permeability barrier precedes exposure of the pore to the cytosol during integration. This result is remarkable in that the ribosome, not the translocon, triggers the structural changes. It can be supposed that the translocon would be insufficient for the recognition of the transmembrane domain as there would be too little time to effect the structural changes needed for integration.

Further investigation has revealed how the transmembrane domain of a membrane protein is co-translationally integrated into the ER membrane. It was demonstrated that the Sec61p channel allows the transmembrane domain of the protein to bypass the barrier posed by the polar head groups of the lipid bilayer and come into contact with the hydrophobic interior of the membrane. Together with the TRAM protein, Sec61p provides a site in the membrane, at the interface of translocon and lipid, through which a transmembrane domain can partition into the lipid bilayer (Mothes *et al.*, 1997; Heinrich *et al.*, 2000).

1.8 Post-translational translocation

Post-translational translocation, identified in yeast, occurs after the polypeptide has been fully synthesised in the cytosol and released from the ribosome and involves interactions between substrate and cytosolic chaperones, the translocon and the Sec62/63p complex (Figure 1.8). Post-translational translocation occurs in distinct phases. In an initial binding reaction, a translocation substrate interacts with the cytosolic face of the Sec complex. This interaction is ATP- and Kar2p-independent. Subsequently, Kar2p interacts with the J domain of Sec63p to move the substrate

through the translocation channel. This interaction and translocation is ATP-dependent (Matlack *et al.*, 1997). Post-translational targeting in yeast can be reconstituted using proteoliposomes containing a heptameric membrane protein complex consisting of trimeric Sec61p and the tetrameric Sec62/63p complex (Panzner *et al.*, 1995). Neither of the sub-complexes can support post-translational translocation alone but wild type translocation levels can be restored if both complexes are reconstituted together. In yeast, cytosolic Hsp70 proteins, the products of the *SSA* gene family, and Ydj1p, a cytosolic DnaJ homologue, are required for translocation of a few post-translational substrates. Among these are pre-pro-α factor and proteinase A (Chirico *et al.*, 1988; Deshaies *et al.*, 1988; Caplan *et al.*, 1992; Brodsky *et al.*, 1995; Becker *et al.*, 1996). *KAR2*, the gene encoding Kar2p in yeast, shows synthetic lethality with *sec63-1* mutants confirming its essential role in translocation (Deshaies *et al.*, 1991).

1.8.1 The Sec complex

Sec62, 63, 71 and 72p form the Sec62/63 or Sec complex in yeast (Panzner *et al.*, 1995). Selection of yeast cells defective in translocation of a signal peptide-cytosolic enzyme hybrid protein led to the identification of the *SEC62, SEC63* genes. The observation that haploid yeast with mutations in any pair of the genes were inviable at 24°C and showed a marked increase in translocation defects led to the assumption that the products of these genes interacted with Sec61p and each other (Rothblatt *et al.*, 1989). Sec62p is a 30 kDa protein with two membrane spanning domains. The protein is believed to be oriented such that the amino and carboxy termini are exposed to the cytosol, with the N-terminal domain of Sec62p binding the C-terminal 14 amino acid residues of Sec63p (Deshaies and Schekman, 1990; Wittke *et al.*, 2000).

Sec63p is a 73 kDa protein that spans the bilayer three times and has an ER lumenal domain which is 42% identical to the *E. coli* DnaJ protein (Feldheim *et al.*, 1992; Sadler *et al.*, 1989). The ER lumenal chaperone Kar2p interacts with the Sec complex via the lumenal J domain of Sec63p. The J domain is a 70 amino acid segment that defines the Hsp-70 interacting J protein family (Sanders *et al.*, 1992;

Brodsky and Schekman, 1993; Scidmore *et al.*, 1993; Lyman and Schekman, 1995; Corsi and Schekman, 1997; Matlack *et al.*, 1997). Peptide binding of Kar2p is activated by the J domain of Sec63p and is ATP-dependent (Corsi and Schekman, 1997; Misselwitz *et al.*, 1998).

When membrane targeting of proteins was reconstituted *in vitro* in the absence of Sec62p, two of the substrates affected were what are now thought of as classic post-translational translocation substrates, carboxypeptidase Y (CPY) and pre-pro-α factor (ppαF) (Deshaies and Schekman, 1989). While in mammalian cells co-translational translocation is favoured, the Sec61 complex has been found to associate with mammalian homologues of Sec62 and 63p, despite the lack of observed post-translational translocation (Meyer *et al.*, 2000).

SEC72 encodes the 23 kDa subunit of the Sec complex. The DNA sequence of *SEC72* predicts a 21.6 kDa protein with neither a signal peptide nor any transmembrane domains. Antibodies directed against a carboxyl-terminal peptide of Sec72p confirmed the membrane location of the protein. *SEC72* is not essential for yeast cell growth, although a *sec72* null mutant accumulates a subset of post-translationally translocated secretory precursors *in vivo* (Green *et al.*, 1992; Fang and Green, 1994). *SEC71* encodes a 31.5 kDa transmembrane protein of the Sec complex that is also non-essential in yeast and a null mutant of which accumulates the same subset of secretory precursors *in vivo* (Green *et al.*, 1992; Fang and Green, 1994).

The heptameric Sec complex, consisting of Sec61p, Sbh1p, Sss1p, Sec62/63/71 and 72p may constitute a stable closed pore; it can be purified as a complete unit. The suggestion that the Sec62/63p complex may induce oligomerisation of Sec61p predicts that this heptameric Sec complex would open only in the presence of a suitable signal sequence or substrate. These assembly and gating processes are thought to occur independently and consecutively (Simon and Blobel, 1991; Hanein *et al.*, 1996).

Mutants of *sec63, 71* and *72* have defects in karyogamy. From this observation it has been proposed that Sec63, 71 and 72p have auxiliary roles in the nuclear envelope fusion complex, with the ER lumenal chaperone Kar2p required for the process (Brizzio *et al.*, 1999; Ng *et al.*, 1996).

1.8.2 Signal sequence detection by the Sec62/63p complex

A post-translationally translocated substrate, used often in the analysis of this pathway, is pre-pro-alpha factor (ppαF). Cross-linking studies have examined how the signal sequence of ppαF is recognised in the first step of translocation, signal sequence recognition. The signal sequence interacts in a Kar2p- and ATP-independent reaction with Sec61p and is specifically cross-linked to transmembrane domains 2 and 7 (Figure 1.6). While bound to Sec61p, the signal sequence (in a helical structure formed inside the ribosome) is contacted on one side by Sec62p and Sec71p. This binding site is located at the interface of the protein channel and the lipid bilayer (Plath *et al.*, 1998). Signal sequence recognition in co-translational translocation in mammals appears to occur in a similar fashion (Mothes *et al.*, 1998). This suggests a general mechanism by which the signal sequence opens the translocation channel for polypeptide transport (Plath *et al.*, 1998). Previous cross-linking experiments suggested that the initial contact of the signal sequence occurs with Sec62p, Sec71p and Sec72p, and that contact with Sec61p requires a subsequent ATP- and Kar2p-dependent step (Müsch *et al.*, 1992; Sanders *et al.*, 1992; Lyman and Schekman, 1997). However, these experiments used wild type ppαF, in which all the cross-linkable lysine residues were in the C-terminus of the protein. Therefore, the data from these experiments can be explained by the movement of the C-terminal portion of ppαF into the translocon during translocation, and not by ATP- and Kar2p-dependent signal sequence recognition by Sec61p (Plath *et al.*, 1998).

Experiments using chimeras of signal sequences of one protein and the mature polypeptide of another demonstrated that a translocation defect seen in mutants of *sec72* is associated with the signal peptide rather than with the mature region of the secretory precursor. These mutants failed to translocate an arginine permease-

invertase-HIS4C fusion protein and a Sec63-invertase chimera, both of which use internal hydrophobic segments as signal peptides. The phenotype of the *sec72* mutants is consistent with at least two potentially overlapping roles in translocation. Sec72p may be involved in recognition of "difficult" or charged signal peptides or Sec72p may increase the efficiency of transfer of these "difficult" secretory precursors to the translocation pore (Feldheim and Schekman, 1994).

1.9 Kar2p

Kar2p is essential for the post-translational translocation pathway in yeast where it is required at two stages. The first is in the initiation of translocation prior to the stable interaction of the precursor protein with the translocon. The second is in the promotion of vectorial transport of the polypeptide chain through the translocon to the ER lumen (Sanders *et al.*, 1992; Lyman and Schekman, 1995, 1997). It has been shown *in vitro* that Kar2p stimulates the level of post-translational translocation three-fold (Brodsky and Schekman, 1993). Kar2p binds to nascent chains during translocation. ATP is required for the repeated binding and release of the nascent chain from Kar2p (Matlack *et al.*, 1997). However, there is much speculation over whether Kar2p acts as a ratchet during translocation, binding to nascent chains to prevent them slipping back through to the cytosol, or whether it actively pulls the chain through. Recent evidence supports the ratchet model but this is unconvincing as only one substrate was analysed and a pared-down minimal reconstituted system was used (Matlack *et al.*, 1999). Perhaps a combination of both effects is required to explain the translocation of all substrates into the ER lumen.

It has been shown in co-translational translocation that BiP, the mammalian homologue of Kar2p in yeast, maintains the lumenal seal of the translocon, and is displaced when the translocating chain reaches greater than 70 amino acids in length (Hamman *et al.*, 1998). In this way, BiP maintains the integrity of the ER lumen by effectively closing the translocon channel until the ribosome interacts with Sec61p and seals the pore from the surrounding cytosol (Crowley *et al.*, 1994). More recently, it has been shown that in the absence of Kar2p or Sec63p (but not Sec62p)

co-translational translocation was compromised *in vivo*. This suggests a role for Kar2p in translocation itself as well as providing a lumenal seal (Young *et al.*, 2001). This is supported by previous data gathered through an *in vitro* assay involving co-translational translocation into microsomes defective in Kar2p and/or Sec63p activity. Data from these experiments showed an absolute requirement for Sec63p and Kar2p in co-translational targeting *in vitro* (Brodsky *et al.*, 1995).

1.10 Lhs1p

A second ER-resident Hsp70-related protein, encoded by the *LHS1* gene, has been characterised in yeast (Baxter *et al.*, 1996; Craven *et al.*, 1996; Hamilton and Flynn, 1996). The Lhs1 protein (Lhs1p) represents a novel branch of the Hsp70 superfamily (Saris *et al.*, 1997) and appears ubiquitous amongst eukaryotes. Unlike Kar2p, Lhs1p is not essential for cell viability, but *lhs1* null mutant cells display a partial defect in post-translational translocation (Baxter *et al.*, 1996; Craven *et al.*, 1996; Hamilton and Flynn, 1996) and are also defective in the repair of misfolded proteins in the ER (Saris *et al.*, 1997). However, interpretation of the *Δlhs1* phenotype is complicated by the fact that these cells exhibit induction of the unfolded protein response (UPR). The *LHS1* gene itself is UPR regulated, suggesting that Lhs1p plays a role in the normal cellular response to folding stress (Baxter *et al.*, 1996; Craven *et al.*, 1996). A functional UPR is required for the near normal growth rate observed in *Δlhs1* cells when compared with the parental strain. *SIL1* is an UPR-regulated gene that is essential for the survival of *Δlhs1* cells. Sil1p interacts with the ATPase domain of Kar2p. Over-expression of Sil1p is sufficient to suppress the phenotypes associated with an *lhs1* deletion. However, a *Δlhs1Δsil1* double mutation results in a lethal phenotype with a complete defect in protein translocation into the ER (Tyson and Stirling, 2000).

Whatever the actual functions and interactions of Lhs1p and Sil1p, the translocation defect seen in *Δlhs1Δsil1* double mutant cells demonstrate that Kar2p is not sufficient to drive co- or post-translational translocation into the ER *in vivo*.

1.11 TRAM

Photocross-linking has been used extensively to analyse interactions of nascent chains during their insertion into the ER membrane (Wiedmann *et al.*, 1987; Krieg *et al.*, 1989; High *et al.*, 1991). One glycoprotein that is cross-linked to nascent chains is the 36 kDa translocating chain-associated membrane protein (TRAMp) (Görlich *et al.*, 1992a). TRAM protein contacts the amino-terminal region of the signal sequence of translocating proteins in mammalian cells (High *et al.*, 1993) and has been found to have a stimulatory role in the translocation of SRP-dependent substrates *in vitro* (Görlich and Rapoport, 1993).

TRAM protein also regulates which domains of polytopic membrane proteins are exposed to the cytosol during translocation pauses. This process appears to be highly specific with the result of regulating the conformational state of the translocation apparatus, the rate of translocation and the environment of the nascent chain (Hegde *et al.*, 1998). *S. cerevisiae* has no identified homologue of TRAM protein; therefore it is still unclear how this process is accomplished in yeast.

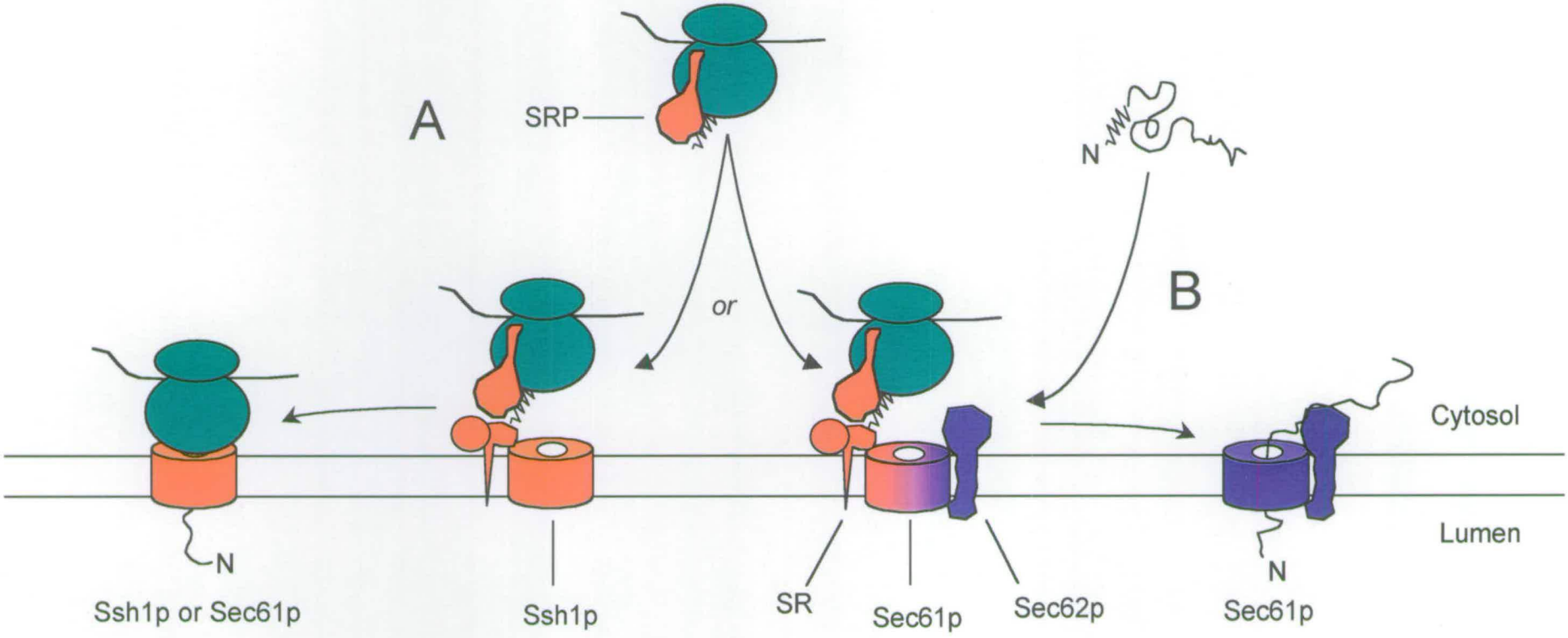
1.12 The Ssh1p complex

The Sec61p translocon accepts proteins through both the co-translational and post-translational translocation pathways and allows dislocation from the ER. The yeast gene *SSH1* (Sec sixty-one homologue), encodes a protein 30% identical to Sec61p that is ER localized and associated with a homologue of Sbh1p, termed Sbh2p, and Sss1p (Finke *et al.*, 1996).

In contrast to *SEC61*, *SSH1* is not essential, and initial analysis of the Ssh1p complex by Finke *et al.* (1996) showed that though in its absence cells grew slower than wild-type, they had no detectable translocation defects. However, the Ssh1p complex binds ribosomes with similar affinity to Sec61p (Prinz *et al.*, 2000c), but does not bind Sec62p (Finke *et al.*, 1996), suggesting that it may form translocons active only in co-translational translocation. Recent evidence supports the existence of two functionally nonequivalent translocons in the yeast ER, containing Sec61p and Ssh1p, respectively, both of which mediate translocation and dislocation (Figure 1.8). First, it was shown that deletion of *SSH1* was strongly synthetic negative with a

Figure 1.8 Two translocons in the ER membrane

The two pathways are depicted. In (A), the SRP-dependent co-translational pathway (orange), a ribosome translating a protein with a hydrophobic signal sequence, is bound by SRP and becomes localized to the ER membrane through interaction with the SRP receptor (SR). The ribosome may be docked to either the Sec61p or Ssh1p translocon. In (B), the post-translational pathway (blue), accepting proteins with less hydrophobic signal sequences (Ng et al., 1996), functions solely through Sec61p as only this translocon interacts with Sec62p. Recent evidence (Young et al., 2001) indicates that both pathways additionally require Sec63p and the ER lumenal chaperone Kar2p (not depicted). The data of Wilkinson et al. (2001) suggest that the Sec61p translocon does not provide sufficient capacity to accommodate all proteins under maximum growth rates. Thus in the absence of Ssh1p, all SRP-targeted proteins are forced through Sec61p, leading to translocation defects for proteins that use either pathway. Both translocons function in dislocation (not shown) (Robb and Brown, 2001).



(Adapted from Robb and Brown, 2001)

mutation in SRP (*sec65-1*). This synthetic negative interaction was not seen with a *sec62* mutation. Also, it was found that *ssh1*Δ cells had similar slow growth and respiration deficient phenotypes to SRP deficient cells. Cells deficient in Ssh1p or SRP quickly lose mitochondrial function, they become ρ^-. These cells can be forced to retain their mitochondria if grown on non-fermentable carbon sources, such as ethanol and glycerol. These ρ^+ *ssh1*Δ cells have a severe defect in the translocation of dipeptidylaminopeptidase-B (DPAP-B), an entirely SRP-dependent protein. These cells also show defects in the translocation of CPY. CPY is targeted independently of SRP and since Ssh1p does not interact with Sec62p this suggests that lack of Ssh1p may place a general stress on the translocation apparatus (Wilkinson *et al.*, 2001). The identification of a synthetic lethal interaction of Ssh1p with Sec65p, the revelation that Ssh1p, in a complex with Sbh2p and Sss1p binds ribosomes with the same affinity as the Sec61p complex and the identification of translocation defects in cells lacking Ssh1p provides evidence that Ssh1p does indeed form a complex in the ER membrane, sufficient to promote translocation of SRP-dependent substrates. Since *ssh1*Δ cells are slow growing the Sec61p complex is insufficient for translocation on its own during rapid growth, where the burden on the secretory pathway is highest. However, Ssh1p cannot transport a sufficiently broad spectrum of proteins to compensate for a lack of Sec61p, as *sec61*Δ cells are inviable. Therefore, it is a possibility that Ssh1p exists as a "back-up" translocon providing extra translocation capacity and physiological flexibility to the cell under rapid growth conditions (Figure 1.8) (Robb and Brown, 2001).

1.13 Reconstitution of protein targeting *in vitro*

Reconstitution of the SRP-dependent targeting pathway determined the minimum complement of membrane proteins necessary for translocation. Görlich and Rapoport (1993) reconstituted the co-translational translocation apparatus of the mammalian ER membrane into proteoliposomes from pure phospholipids and purified membrane proteins. The results from this indicated that the minimum

translocation apparatus was very simple, comprising only the Sec61 complex and SR. These proteins were all that were required for translocation of preprolactin (Hegde *et al.*, 1998; Voigt *et al.* 1996). However, translocation of pre-pro-α-factor required the addition of TRAM protein. Post-translational translocation in yeast was reconstituted by Panzner *et al.* (1995) using reconstituted proteoliposomes containing purified Sec complex. The Sec complex included the heterotrimeric Sec61p complex and the Sec62/63p complex. Efficient post-translational translocation required the addition of Kar2p and ATP. The trimeric Sec61 complex was found to exist as a separate entity that, in contrast with the larger Sec complex, was associated with membrane-bound ribosomes. From this it was hypothesised that distinct membrane protein complexes function in co- and post-translational translocation pathways (Panzner *et al.*, 1995). Reconstitution of protein targeting is an established method of elucidating the components and interactions necessary for different modes of translocation. These previously established assays provided a starting point in the reconstitution of SRP-dependent targeting from entirely yeast components described in this study.

1.14 Yeast as a model organism

The study of SRP-dependent targeting in yeast is simpler than corresponding study in mammalian cells due to the amenability of yeast to genetic and biochemical manipulation. The growth rate of yeast cells coupled with their ability to be transformed with heterologous DNA make the organism an excellent tool for the investigation of such a conserved pathway. The publication of the complete yeast genome also makes this organism an excellent model for the study of the first step of the secretory pathway. An obvious advantage to studying SRP and SR components in yeast is the fact that SRP and SR encoding genes are non-essential. The ability to delete SRP components from the yeast genome without inducing apoptosis allows the study of SRP-dependent targeting by a variety of other methods not open to the study of essential genes.

1.15 Aims

1.15.1 Purification of SR from yeast

While the functions of many of the proteins involved in SRP-dependent targeting have been elucidated, as described in the introduction, the function of SRβ has remained elusive. While an active GTPase domain is required for SRβ function and its transmembrane domain is not essential (Ogg *et al.*, 1998), little else is known about this protein and no function has been assigned to it. In order to identify the role of SRβ in SRP-dependent targeting in yeast, other than as a membrane anchor for SRα, *in vitro* reconstitution of the targeting pathway was to be attempted. In order to carry out this reconstitution SR had to be purified from yeast along with other membrane proteins required for translocation (Sec61p and the Sec62/63p complex) and the lumenal chaperone Kar2p (required for co- and post-translational translocation). Purification of these components, for the eventual reconstitution of protein targeting, is described.

1.15.2 Reconstitution of SRP-dependent targeting *in vitro*

In order to investigate the sequence of interactions that occur in the delivery of a ribosome-nascent chain complex to the translocon in the ER membrane, reconstitution of the targeting pathway was attempted. Optimisation of reconstitution was carried out at every stage. Reconstitution of SRP-dependent targeting was successfully carried out with solubilised membrane proteins recovered from appropriate yeast strains and additional purified components.

1.15.3 Identification of interactions of SR

To aid in elucidation of the sequence of events that occur at the ER membrane and in determining a function for SRβ experiments to identify proteins that interact with SRβ were carried out. Pull-down assays, two-hybrid analysis, chemical cross-linking and immunoprecipitation were attempted to this end. Pull-down assays successfully identified a salt-concentration sensitive interaction between SRβ and Sec61p. Two-hybrid analysis localised this interaction to the N-terminus of Sec61p.

Chapter 2

Materials and Methods

Materials

2.1 Chemicals and Biochemicals

General chemicals were obtained from Sigma Chemical Co., BDH Chemicals, Fisher Ltd, Calbiochem or Boehringer Mannheim. Media components were from Difco Laboratories. IgG, Q, SP, CM and DEAE Sepharoses (fast flow) and ^{35}S-methionine were from Amersham Pharmacia. Ni-NTA agarose and kits for plasmid DNA preparation were from Qiagen Ltd. 0.02 μm filters used to filter sterilise liquids and centricon centrifugal filter devices were obtained from Millipore. Spectra/Por molecularporous regenerated cellulose dialysis membrane and clips were from Spectrum Laboratories Inc. Prosieve 50 modified acrylamide gel solution was obtained from Flowgen.

2.2 Enzymes, proteins and antibodies

Lysozyme, trypsin and micrococcal nuclease were all obtained from Sigma Chemical Co.. All DNA restriction and modification enzymes were from Promega or NEB and used as recommended by the manufacturer. *Taq* polymerase was from Promega. Standard PCR reactions contained 0.2 μM each dNTP, 100 pmol each oligonucleotide in 10 mM Tris.HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.01% (w/v) gelatin. Antibodies not raised in this study were from Sigma Chemical Co., Diagnostics Scotland or BABCO (California) and are listed in table 3. *In vitro* mRNA transcription kit and the $m^7G(5')ppp(5')G$ Cap analogue were obtained from Ambion.

2.3 Bacterial strains and plasmids

The strains of *E. coli* used in this study are listed in table 1. Transformants are denoted by listing the strain, followed by the plasmid with which it has been transformed. All plasmids used in this study are described in table 2.

2.4 Yeast strains and plasmids

The strains of *S. cerevisiae* used in this study are listed in table 1. Transformants are denoted by listing the strain, followed by the plasmid with which it has been transformed.

2.5 Media

Yeast cultures were grown in complete medium (YPD) (1% (w/v) Bacto yeast extract, 2% (w/v) Bacto peptone and 2% (w/v) glucose (dextrose)). Bacto agar was added to the above to 2% (w/v) when solid medium was required. Maintenance of transformed plasmids and/or verification of auxotrophic markers inserted into the genome was achieved by growth in media comprising 0.67% yeast nitrogen base (minus amino acids), 2% glucose and 10% (v/v) 10x Drop-out media. Drop-out media was made from powder from Bio101 Inc. dissolved in dH_2O producing a complete synthetic defined drop-out medium lacking the desired component(s). Bacto agar was added to 2% (w/v) when solid medium was required.

Bacterial cultures were grown in Luria broth (LB) (1% (w/v) Bacto tryptone, 0.5% (w/v) Bacto yeast extract and 0.5% (w/v) NaCl). Bacto agar was added to the above to 1.5% (w/v) when solid medium was required. Liquid and solid media were supplemented where necessary with 100 μg/ml ampicillin and/or 50 μg/ml kanamycin.

Methods

2.6 DNA manipulations

Standard DNA manipulation techniques including PCR amplification, ligation, extraction with phenol, precipitation with ethanol and restriction endonuclease cleavage were performed as described in Sambrook and Russell (2001), or according to the enzyme manufacturers instructions.

Plasmid DNA was isolated from bacterial cells using QAIprep spin miniprep purification kits supplied by Qiagen using the manufacturers instructions. A miniprep routinely yielded approximately 20 μg of plasmid DNA that was stored at –20°C.

Initial cloning of PCR products to be included in pAR143 (section 3.2.1) was by "TA-cloning" using the commercial vector pCR™ (Invitrogen). Gel electrophoresis, for the separation and visualisation of DNA fragments, was routinely carried out using 1% (w/v) agarose gels in TAE containing 1 μg/ml ethidium bromide. The size of the fragments of DNA was estimated by comparing their mobility with that of fragments of known size (1kB ladder markers, NEB).

2.7 Transformation of bacterial cells

Bacterial cells were made chemically competent by treatment with $CaCl_2$ and were transformed with plasmid DNA as described (Sambrook and Russell, 2001).

2.8 Transformation of yeast cells

Yeast cells were transformed with circular plasmid or linear integrative DNA by a lithium acetate method adapted from Gietz *et al.*, (1992). 100 ml of yeast cells were

grown to logarithmic phase and harvested when the OD_{600} reached 0.5. The cells were pelleted by centrifugation at 1500 *g* for 5 min in a benchtop centrifuge (HERAEUS Labofuge 410). The cells were washed with 20 ml of sterile lithium acetate mix (LiOAc mix – 0.1M LiOAc, 1xTE), by resuspension and centrifugation and were resuspended finally in 1 ml of LiOAc mix. 100 µl of cells were added to 0.1-0.5 µg of transforming DNA and 10 µl of 10 mg/ml freshly denatured and chilled carrier DNA (sonicated salmon sperm). After mixing these thoroughly, 700 µl of PEG mix (40% (w/v) PEG 2000, 0.1M LiOAc, 1xTE) was added and the cells incubated at 30°C for 30 min (or 24°C if transforming a temperature-sensitive strain). The cells were then transferred to 42°C for 15-20 min, pelleted by centrifugation at 3000 rpm for 1 min in a microcentrifuge and the supernate removed. The cells were washed once with sterile water by resuspension and centrifugation and resuspended in 200 µl of residual liquid from the wash step. Cells were spread onto solid YPDA medium for overnight incubation at 30°C before replica plating onto selective media (integrative transformation) or were spread directly onto solid selective medium (plasmid transformation) and incubated at 30°C.

2.9 Yeast and bacterial stock preservation

Glycerol was added to freshly grown saturated yeast culture to a final concentration of 20% (v/v), which was then stored at –80°C. Glycerol was added to freshly grown saturated bacterial culture to a final concentration of 30% (v/v), which was then stored at –80°C.

2.10 DNA sequence analysis

Plasmid DNA was isolated as described in section 2.6. 1 µg of plasmid DNA was precipitated with $1/10^{th}$ volume of 3M sodium acetate (pH5.3) and an equal volume

of 95% (v/v) ethanol and pelleted by centrifugation at 13000 rpm for 15 min in a microcentrifuge. The DNA pellet was washed once with 95% (v/v) ice cold ethanol and resuspended in 20 µl of dH_2O. 5 µl (250 ng) of the resuspended DNA were added to 4 µl of the Perkin Elmer BigDye sequencing kit sequencing mix along with 1.6 pmol of oligonucleotide to a final volume of 10 µl with dH_20. The PCR sequencing reaction and preparation of DNA for running on the sequencing gel was carried out according to the manufacturers instructions. Sequencing reactions were run by the ICMB sequencing facility.

2.11 Purification of GST-fusion proteins from *E. coli* for antibody production

2.11a Induction of expression from the *lac* promoter

A 100 ml culture of DH5α cells, harbouring either pSOY88 or pSOY90, was grown overnight at 37°C to saturation in LB medium containing ampicillin. The cells were diluted back to an OD_{600} of 0.1 in 6 l of LB medium and allowed to grow to an OD_{600} of 0.5. At that time expression was induced by addition of IPTG to 0.1 mM and was allowed to proceed for 5-6 hr at 37°C. After this time the cells were harvested by centrifugation at 8000 rpm for 10 min in a Beckman JLA10.500 rotor, washed in dH_2O and stored at -80°C.

2.11b Cell lysis and recovery of fusion protein

Cells were thawed on ice and resuspended in 40 ml buffer A (50 mM Tris.HCl pH 8.0, 200 mM NaCl, 3 mM EDTA, 2 mM β-mercaptoethanol, 1 mM PMSF, 1 µM pepstatin and leupeptin) containing 1 mg/ml lysozyme and incubated at 24°C for 30 min. TritonX-100 was then added to a final concentration of 1% (v/v) and

incubation continued at 24°C for a further 15 min. The cells were sonicated on ice (four bursts of 15 s with 1 min cooling intervals) and insoluble material pelleted by centrifugation at 15000 rpm for 30 min in a Beckman JA25.5 rotor.

2.11c Solubilisation and purification of fusion protein

As both the SRα-GST and SRβΔTM-GST fusion proteins were insoluble they were recovered in the pellet of the initial lysate clarification step. The pellet was washed by resuspending in 20 ml of PBS and resedimenting. The pellet was then resuspended in 3 ml of PBS and 30 ml of urea solution (8 M urea, 500 mM Tris.HCl pH 8.0, 500 mM NaCl, 1 mM EDTA, 30 mM β-mercaptoethanol) added. Any insoluble material remaining was then pelleted by centrifugation at 15000 rpm for 30 min in a Beckman JA25.5 rotor. The supernate of this centrifugation step was dialysed against 1 l of dialysis buffer (50 mM HEPES.KOH pH 7.6, 100 mM KCl, 30% (v/v) glycerol) for 8 hr with the buffer replaced after 3 and 6 hr. Insoluble material present in the dialysate was pelleted by centrifugation for 30 min at 15000 rpm in a Beckman JA25.5 rotor. The supernate of this step was incubated with approximately 200 mg (dry weight) of pre-swelled glutathione agarose beads on a rotating platform for 4 hr. The mixture was then poured into a 20 ml Poly Prep (BIORAD) chromatography column and the flow through collected. The beads were washed with 50 ml of PBS containing 1% (v/v) tritonX-100, 50 ml of PBS containing 1% (v/v) tritonX-100 and 0.5 M NaCl and 50 ml of PBS. Finally, the fusion protein was eluted from the agarose beads by competition with free glutathione using 10 x 2 ml washes of 50 mM Tris.HCl (pH 8.0) containing 5 mM reduced glutathione. The eluted protein was dialysed against 1 l of dialysis buffer for 8 hr with the buffer replaced after 3 hr.

2.12 Over-expression and preparation of lyticase

This lyticase preparation is based on an osmotic shock method of lysing the outer cell wall of bacterial cells to release periplasmic components. The major constituent of the recovered periplasm is lyticase due to its over-expression in the cells. Strain DH5α(pUV5-G1S) (tables 1 and 2) was grown, induced and harvested as in section 2.11a. The culture was diluted 100-fold into 1 l of pre-warmed (37°C) LB medium containing ampicillin. The cells were then grown at 37°C with vigorous shaking until they reached an OD_{600} of 0.5. Over-expression of lyticase was induced by addition of IPTG to 1 mM and allowed to proceed for 5 hr. The cells were harvested by centrifugation at 8000 rpm for 10 min in a Beckman JLA10.500 rotor. The cells were washed in 200 ml of 25 mM Tris (pH 7.4) by resuspension and centrifugation as before. The cells were then resuspended in 1/50th of the original culture volume of 25 mM Tris (pH 7.4) with EDTA added to a final concentration of 2 mM. An equal volume of 40% (w/v) sucrose and 25 mM Tris (pH 7.4) was added and the cells mixed with gentle swirling for 20 min. The cells were pelleted by centrifugation at 8000 rpm for 10 min in a Beckman JLA10500 rotor and the supernate removed. The cell pellet was then resuspended in 1/50th of the original culture volume of ice cold 0.5 mM $MgSO_4$ and the cells mixed gently on ice. The cells were pelleted as before and the lyticase-containing supernate reserved. The supernate was immediately frozen in liquid nitrogen in 10 ml aliquots and stored at –80°C.

2.13 Purification of Kar2p

The method of purification of hexahistidine-tagged Kar2p was adapted from McClellan et al. (1998). pMR2623 was a kind gift of Dr J Brodsky (University of Pittsburgh). Strain DH5α(pMR2623) was grown, induced and harvested as in section 2.11a except induction was at 24°C for 4 hr. The cells were washed once in water by resuspension and centrifugation as before. The cell pellet was resuspended in 20 ml of sonication buffer (50 mM HEPES.KOH (pH 7.4), 300 mM NaCl, 10 mM

imidazole, 5 mM β-mercaptoethanol, 1 mM PMSF, 1 μM pepstatin) and sonicated for 3 x 60 s with 2 min cooling on ice between bursts. Insoluble material was pelleted by centrifugation at 10,000 rpm for 10 min in a Beckman JA25.5 rotor. The supernate was loaded onto a 5 ml column of Ni-NTA agarose beads equilibrated with sonication buffer. The column was washed sequentially with 20 ml of sonication buffer, 30 ml of wash buffer 2 (50 mM HEPES.KOH (pH 7.4), 300 mM NaCl, 1% (v/v) tritonX-100, 10 mM imidazole, 5% (v/v) glycerol, 5 mM β-mercaptoethanol, 1 mM PMSF, 1 μM pepstatin) and 30 ml of wash buffer 3 (50 mM HEPES.KOH (pH 7.4), 1 M NaCl, 10 mM imidazole, 5% (v/v) glycerol, 5 mM β-mercaptoethanol, 1 mM PMSF, 1 μM pepstatin). Protein was eluted with 30 ml of elution buffer (50 mM HEPES.KOH (pH 7.4), 300 mM NaCl, 250 mM imidazole, 5% (v/v) glycerol, 5 mM β-mercaptoethanol, 1 mM PMSF, 1 μM pepstatin) collected in 30 x 1 ml fractions. Peak fractions, determined by Bradford assay (section 2.23) were pooled, diluted four-fold in buffer 88 (20 mM HEPES.KOH (pH 6.8), 150 mM KOAc, 250 mM sorbitol, 5 mM $Mg(OAc)_2$) and loaded onto a 10 ml Q-sepharose column. The column was washed with 25 ml of buffer 88 and 30 ml of buffer 88 containing 0.2 M KOAc. The protein was eluted from the sepharose with a 30 ml linear gradient of buffer 88 from 0.2 M KOAc to 2 M KOAc. 30 x 1 ml fractions were collected. The peak elution fractions were pooled and dialysed against 4 l of dialysis buffer (50 mM Tris.HCl (pH 7.4), 50 mM NaCl, 0.8 mM DTT, 2 mM $Mg(OAc)_2$, 5% (v/v) glycerol) overnight at 4°C. The protein concentration of the dialysate was determined and 200 μl aliquots frozen in liquid nitrogen and stored at –80°C.

2.14 Purification of bacterially expressed SR-GST

M15pREP4 cells transformed with pJE97 were grown, induced and harvested as in section 2.11a, except that kanamycin was added to maintain pREP4. All growth of cells, except initial overnight culture, was at 24°C and induction was for 6 hr. The

cells were resuspended in 80 ml lysis buffer (50 mM Tris.HCl (pH 7.5), 1 M NaCl, 0.2% (v/v) tritonX-100, 1 μM leupeptin and pepstatin, 1 mM PMSF) and sonicated 4 x 15 s with 2 min on ice between bursts. Insoluble material was pelleted by centrifugation at 15000 rpm for 40 min in a Beckman JA25.5 rotor. The lysate was diluted 3-fold in equilibration buffer (50 mM Tris.HCl (pH 7.5), 150 mM NaCl) and loaded onto a 2 ml column of pre-swelled glutathione agarose, equilibrated in equilibration buffer. The column was washed with 50 ml of wash buffer (50 mM Tris.HCl (pH 7.5), 0.25 M NaCl) and eluted with 10 x 4 ml of wash buffer containing 10 mM reduced glutathione. Peak fractions were pooled and dialysed against 1 l of dialysis buffer (0.15 M KOAc, 20 mM HEPES.KOH (pH 7.5), 15% (v/v) glycerol) at 4°C for 8 hr with 2 changes of buffer.

2.15 Electrophoretic separation and detection of proteins

Electrophoretic separation of proteins was performed using SDS polyacrylamide gels following the method of Laemmli (1970), or with Prosieve 50 acrylamide mix (Flowgen), using the solutions detailed below.

Separating gel buffer	375 mM Tris.HCl pH 8.8, 0.1% SDS
Stacking gel buffer	125 mM Tris.HCl pH 6.8, 0.1% SDS
Acrylamide stock solution	30% (w/v) acrylamide, 0.8% (w/v) N,N' methylene bisacrylamide
Electrophoresis buffer	0.125 M Tris, 0.2 M glycine, 0.1% (w/v) SDS (gives pH 8.3 without adjustment)
Prosieve electrophoresis buffer	0.1 M Tris, 0.1 M tricine, 0.1% (w/v) SDS (gives pH 8.3 without adjustment)

SDS sample buffer 50 mM Tris.HCl pH 6.5 (or pH 11 when protein samples had been precipitated with TCA), 5% (w/v) SDS, 10% (v/v) glycerol, 10 mM DTT, 50 μg/ml bromophenol blue

Routinely, separating gels of 15% (w/v) and 18% (w/v) (10% (w/v) if using Prosieve 50 acrylamide mix) with a 5% (w/v) stacking gel were used for the separation of proteins.

2.15a Coomassie Blue staining

Following electrophoretic separation, protein bands were visualised by staining with Coomassie Blue. The gel was covered with a solution of 0.25% (w/v) Coomassie Brilliant Blue dissolved in 50% (v/v) methanol, 7% (v/v) acetic acid for 30 min. The stain was then decanted and destaining of the gel achieved by gently agitating in 45% (v/v) methanol, 10% (v/v) acetic acid.

2.15b Silver staining

Silver staining was carried out as follows using 100 ml of each solution per gel with gentle swirling. Gels were soaked sequentially in 10% methanol for 30 min, 50% (v/v) methanol, 12% (v/v) TCA, 2% (w/v) $CuCl_2$ for 20 min, solution A (10% (v/v) ethanol, 5% (v/v) acetic acid) for 10 min, 0.01% (w/v) $KMnO_4$ for 20 min, solution A for 10 min, 10% (v/v) ethanol for 10 min, 10 min in water, 10 min with a 0.1% (w/v) solution of $AgNO_3$, water for 20 s and 10% (w/v) K_2CO_3 for 1 min. Finally the gels were developed with 2% (w/v) K_2CO_3 and 0.03% (v/v) formaldehyde until bands appeared. The developing reaction was stopped by transferring the gel to solution A before drying.

2.16 Transfer of proteins on to nitrocellulose

Proteins were transferred from acrylamide gels to Protran nitrocellulose (Schleicher and Schuell) by either of the following two methods.

2.16a Semi-dry blotting

Proteins were transferred from gels, after electrophoresis, onto nitrocellulose filter by a semi-dry blotting procedure using a BIORAD semi-dry blotting apparatus according to the manufacturers instructions.

2.16b Wet blotting

The gel was placed next to a sheet of nitrocellulose which had been soaked in Towbin transfer buffer (50 mM Tris, 380 mM glycine, 0.1% (w/v) SDS, 20% (v/v) methanol) and sandwiched between three sheets of 3MM and two pads of sponge also soaked in transfer buffer. The assembly was then placed in a cassette and slotted into a tank so that it was completely submerged in Towbin transfer buffer with the nitrocellulose closest to the anode. A current of 15 mA was then applied for 16 hr.

2.16c Ponceau S staining

The presence of proteins on nitrocellulose filters was detected by Ponceau S staining. The nitrocellulose was immersed in Ponceau S solution diluted from concentrate (Sigma) as per manufacturers instructions. The stain was decanted and destaining achieved by washing the nitrocellulose repeatedly with distilled water to reveal the stained proteins.

2.17 Immunoblot analysis

Proteins on nitrocellulose membranes were detected as follows using specific (primary) antibodies against the desired protein (table 3). All steps were carried out

on a shaking platform/rocker. Membranes were incubated sequentially for 20 min in blotto (5% (w/v) non-fat dried milk, 0.5% (v/v) Tween 20 in TBS) to block non-specific binding of the antibody, for 1 hr with primary antibody in blotto (diluted as in table 3), twice for 10 min in blotto to remove unbound antibody, for 1 hr with HRP-conjugated anti-rabbit IgG or HRP-conjugated anti-rat IgG (detailed in table 3), twice for 10 min with blotto before a final rinse with TBS. The bound antibodies were visualised by incubation with equal volumes of chemiluminescent detection solution (Solution 1: 2.5 mM 3-aminophthalhydrazide, 0.1 mM Tris.HCl (pH 8.5), 0.396 mM cumaric acid; Solution 2: 0.0192% (v/v) H_2O_2, 0.1 mM Tris.HCl (pH 8.5)) and exposure to film.

2.18 Production of polyclonal antibodies from rabbits

2.18a Immunisation and serum collection

Immunisation of and serum collection from rabbits for production of antibodies against SRα and SRβ was carried out by Diagnostics Scotland. 100 μg of immunogen (in 250 μl of PBS buffer) was mixed with an equal volume of Freund's Adjuvant and injected subcutaneously. Three booster injections of identical antigen/adjuvant mix were applied at one monthly intervals.

2.18b Affinity purification of antibodies using purified protein

Antibodies directed against SRα or SRβ were purified from polyclonal serum by binding and elution from the fusion proteins against which they had been raised, immobilised on affigel-10. Removal of antibodies raised against the GST moiety was by prior incubation with GST immobilised on affigel-10. 10 ml of resin was prepared containing 3 mg of fusion protein or GST coupled as detailed in Harlow and Lane, (1988). The coupled resin was washed twice with 10 ml of 0.1 M

Hepes.KOH (pH 7.4) and incubated overnight with 30 ml immune serum (raised against the appropriate protein) at 4°C on a roller before being packed into a 20 ml Poly Prep (BIORAD) chromatography column. The column was washed with 100 ml of 0.1 M Hepes.KOH (pH 7.4) and the antibodies eluted with 10 x 1 ml washes of 0.2 M glycine (pH 2.5). Fractions were collected into tubes containing 75 µl of 1 M Tris.HCl (pH 8.0) (yielding a final pH of 7.4). Peak fractions (determined by Bradford analysis 2.20) were pooled and dialysed against 2 l of dialysis buffer (1x PBS, 20% (v/v) glycerol, 1 mM PMSF). After dialysis the antibodies were frozen in 100 µl aliquots and stored at –80°C.

2.19 Protein purification

2.19a Preparation of microsomal membranes

To prepare ER-derived microsomes for use in *in vitro* translocation assays, to provide material for solubilisation and reconstitution of proteoliposomes and for purification of SR, the following protocol (adapted from McClellan et al., 1998) was followed. The appropriate yeast strain was grown to an OD_{600} of 2 (or OD_{600} 0.5 for galactose induction of SR-PrA) in 2 l of YPDA medium at 30°C (or 32 l for induction of SR-PrA). The cells were harvested by centrifugation at 5,000 rpm for 5 min in a Beckman JLA10,500 rotor and washed with water by resuspension and centrifugation as before. The cells were resuspended to 50 OD units/ml in 100 mM Tris.HCl (pH 9.4), 10 mM DTT and incubated at 24°C for 10 min with gentle shaking. The cells were then harvested as before and resuspended to 50 OD units/ml in lyticase buffer (0.7 M sorbitol, 1% (w/v) yeast extract and 2% (w/v) peptone, 0.5% (w/v) glucose, 10 mM Tris.HCl (pH 7.4)). 5 µl of cell suspension was sampled, diluted into 995 µl of distilled water and the OD_{600} measured. 1 ml of lyticase (section 2.12) per 1500 OD units of cells was added and the cell suspension incubated with gentle swirling (1 hr) at 30°C. A time course to monitor sphaeroplasting was carried out where 5 µl of cells were sampled and diluted into

995 µl of distilled water and the OD_{600} measured. Sphaeroplasting was considered complete when the final OD_{600} was less than 10% of the initial value. 15 ml aliquots of the sphaeroplasts were overlayed onto 15 ml of ice cold cushion 1 (0.8 M sucrose, 1.5% (w/v) Ficoll 400, 20 mM Hepes.KOH (pH 7.4)) in 50 ml polycarbonate tubes, and the sphaeroplasts collected by centrifugation at 6,000 rpm for 10 min in a Beckman JS13.1 rotor. The sphaeroplasts were resuspended in 35 ml of ice cold lysis buffer (0.1 M sorbitol, 50 mM KOAc, 2 mM EDTA, 20 mM Hepes.KOH (pH 7.4), 1 mM DTT, 1 mM PMSF) and homogenised with 20 strokes in a motor-driven Status R50 homogeniser at setting 16. 15 ml aliquots of homogenate were overlayed onto 15 ml of ice cold cushion 2 (1 M sucrose, 50 mM KOAc, 20 mM Hepes.KOH (pH 7.4), 1 mM DTT) and unbroken cells pelleted by centrifugation at 6,000 rpm for 10 min in a Beckman JS13.1 rotor. The top 20 ml of the supernate of this step was transferred into fresh 50 ml polycarbonate tubes and microsomes pelleted by centrifugation at 17,000 rpm for 20 min in a Beckman JA25.5 rotor. The microsomes were washed in 20 ml of ice cold buffer 88 (20 mM Hepes.KOH (pH 6.8), 150 mM KOAc, 250 mM sorbitol, 5 mM $Mg(OAc)_2$) by resuspension and centrifugation as before. The microsomes were finally resuspended in 500 µl of ice cold buffer 88. 5 µl of this suspension was diluted into 995 µl of 2% SDS and the A_{280} measured. A volume of buffer 88 was then added to the microsome solution to adjust the OD_{280} to 40 (corresponding to a protein concentration of approximately 10-12 mg/ml). The microsomes were frozen by dropping into liquid nitrogen in 60 µl aliquots and stored at –80°C.

When microsomes were to be used in a translocation assay they were washed to remove ribosomes and mRNA from their surface. 60 µl of EDTA solution (20 mM Hepes.KOH (pH 7.5), 250 mM sucrose, 50 mM EDTA (pH 8.0), 1 mM DTT) was added per 60 µl of microsomes and the mixture incubated on ice for 15 min before pelleting by centrifugation at 13000 rpm for 15 min at 4°C in a microcentrifuge. The microsome pellet was then resuspended in 200 µl of salt solution (20 mM Hepes.KOH (pH 7.5), 250 mM sucrose, 500 mM KOAc, 1 mM DTT) and incubated

on ice for 1 hr. The microsomes were pelleted by centrifugation as before and washed twice in 200 µl of membrane storage buffer (20 mM Hepes.KOH (pH 7.5), 250 mM sorbitol, 50 mM KOAc, 1 mM DTT) by resuspension and centrifugation. After the final wash the microsomes were resuspended in membrane storage buffer to the starting volume and frozen by dropping into liquid nitrogen in 60 µl aliquots and stored at –80°C.

2.19b Ion-exchange chromatography

Yeast strain ARY2 was grown harvested and microsomes prepared as described in section 2.19a. Deviations from this protocol are indicated, where appropriate, in Chapter 3. Typically, 8,000 OD_{280} units of microsomal membranes were solubilised in digitonin buffer (3% (w/v) digitonin, 50 mM KOAc, 20 mM Hepes.KOH, 1 mM DTT). Insoluble material was pelleted by centrifugation at 17,000 rpm for 30 min in a Beckman JA25.5 rotor. The supernate containing solubilised membrane proteins was incubated overnight at 4°C on a roller with 5 ml of pre-equilibrated ion-exchange resin (CM, Q, SP or S-sepharose). The resin was packed into a 20 ml Poly Prep (BIORAD) chromatography column. The column was washed with 100 ml of wash buffer (1% (w/v) digitonin, 50 mM KOAc, 20 mM Hepes.KOH, 1 mM DTT) and eluted with 10 x 1 ml washes of elution buffer (1% (w/v) digitonin, 20 mM Hepes.KOH, 1 mM DTT) containing 0.1-1 M KOAc.

2.19c Purification of oligohistidine-tagged proteins

Yeast strains ARY2 or ARY3 were grown harvested and microsomes prepared as described in section 2.19a. Deviations from this protocol are indicated, where appropriate, in Chapter 3. Solubilised membrane proteins were prepared as in 2.19b except buffer contained 0.8 mM imidazole. The supernate was incubated overnight at 4°C on a roller with 0.3 ml of pre-equilibrated Qaigen Ni-NTA agarose. The

protein bound agarose was packed into a 20 ml Poly Prep (BIORAD) chromatography column. The column was washed with 100 ml of wash buffer (1% (w/v) digitonin, 50 mM KOAc, 20 mM Hepes.KOH, 8 mM imidazole, 1 mM PMSF) and eluted with 10 x 1 ml washes of elution buffer (1% (w/v) digitonin, 50 mM KOAc, 20 mM Hepes.KOH, 50 mM imidazole, 1 mM PMSF).

2.20 Purification of SR from yeast

2.20a Over-expression of SR in yeast by induction of the galactose promoter

Typically 32 l of culture containing yeast strain JDY225 (harbouring pAR143) was grown to OD_{600} 0.5 in YPD at which point over-expression of SR was induced by the addition of galactose to a final concentration of 1% (w/v). Induction proceeded until the OD_{600} reached 2.0 at which time the cells were harvested and washed as described in section 2.19a and stored at –80°C.

2.20b Purification of SR

The cells were thawed and microsomes prepared as described in section 2.19a. Microsomes were resuspended in 15 ml of solubilisation buffer (100 mM KPO_4 (pH 7), 20% (v/v) glycerol, 10 mM DTT, 500 mM KOAc) with sufficient tritonX-100 layered on top of the suspension to yield a final concentration of 3% (v/v). The suspension was then vortexed vigorously for 10 s to solubilise the microsomes. Insoluble material was pelleted by centrifugation at 17,000 rpm for 30 min in a Beckman JA25.5 rotor. The soluble protein fraction was subjected to centrifugation at 60,000 rpm for 90 min to pellet ribosomes and ribosome associated membrane proteins. The supernate from this step was incubated overnight at 4°C on a roller with 1 ml of pre-equilibrated IgG sepharose. The sepharose was then packed into a 20 ml Poly Prep chromatography column (BIO-RAD) and washed with 100 ml of

wash buffer A (500 mM KOAc, 100 mM KPO_4 (pH 7), 20% (v/v) glycerol, 10 mM DTT) and 100 ml wash buffer B (100 mM KPO_4 (pH 7), 20% (v/v) glycerol, 10 mM DTT, 150 mM KOAc). Protein was eluted from the sepharose by incubation with 2 ml elution buffer (wash buffer B + 50 U TEV protease (Gibco BRL)) for 1 hr at 24°C. After incubation with TEV protease the protein was collected by washing the sepharose with 5 x 1 ml washes of buffer B containing alternately150 mM KOAc and 500 mM KOAc. Peak fractions (determined by Bradford analysis) were pooled and bound to 0.3 ml pre-equilibrated Qaigen Ni-NTA agarose overnight on a roller at 4°C to remove the hexa-histidine tagged TEV protease. Silver or coomassie staining indicated that this step effectively removed the TEV protease from the protein mixture.

2.21 Purification of Sec61/63p from yeast

Purification of Sec complex through protein A-tagged Sec63p was essentially as described by Beckman *et al.*, (1997). Yeast strain JDY516 (table 1) expressing Sec63-PrA was created by homologous integration of a PCR amplified cassette using oligos 5SEC63PRA and 3SEC63PRA (table 4) consisting of a TEV cleavage site, 2x protein A IgG binding domain and the *S. pombe* $his5^+$ gene from plasmid pJE39 (table 2, gift of Sean Munro, MRC-LMB, Cambridge). Upon purification digitonin in the elution buffer was exchanged for deoxyBigCHAP to facilitate reconstitution as described in section 2.24.

2.22 Concentration of protein

Centricon columns (Amicon) with a cut-off of 30,000 Da were used for the concentration of small volumes of protein following the manufacturer's instructions.

2.23 Estimation of protein concentration

Protein concentration was routinely determined by Bradford assay. 10 µl of the sample to be analysed was added to 740 µl of distilled water and mixed. 250 µl of BIORAD Bradford reagent was then added and mixed. The absorbance of the sample was then measured at OD_{595} at 24°C. The concentration of protein in the sample was estimated by comparing the absorbance of the sample with that of a control with a known concentration of protein (routinely a BSA standard curve from 0.5 µg/ml to 1 mg/ml).

2.24 Detergent exchange

Proteins purified and eluted into buffer containing either digitonin or tritonX-100 had this detergent exchanged for another to facilitate reconstitution. The first step in reconstitution of membrane proteins into liposomes is overnight dialysis into buffer containing liposomes without detergent. Digitonin and tritonX-100 do not dialyse away from protein fractions and as such must be removed before the dialysis step. Peak elution fractions were diluted 10-fold in buffer 1 (50 mM Hepes.KOH (pH 7.4), 5 mM DTT, 0.3% (v/v) tritonX-100 or digitonin) and incubated overnight on a roller at 4°C with 0.5 ml CM, Q or S-sepharose. The sepharose was packed into a 10 ml Poly prep (BIORAD) chromatography column and washed with 2 x 50 ml buffer 2 (50 mM Hepes.KOH (pH 7.4), 15% (v/v) glycerol, 5 mM DTT, 0.5% (w/v) deoxyBigCHAP) and eluted with 10 x 100 µl buffer 3 (50 mM Hepes.KOH (pH 7.4), 750 mM KOAc, 5 mM DTT, 15% (v/v) glycerol, 0.5% (w/v) deoxyBigCHAP).

2.25 Immunoprecipitation

100 ml of each yeast strain was grown, harvested and microsomes isolated as described in section 2.19a. Microsomes were solubilised and soluble protein

recovered from these as described in section 2.20b except 200 µl solubilisation buffer was used. 1 µl of polyclonal antibody was added to each 50 µl of soluble protein extract diluted to 500 µl in solubilisation buffer and incubated at 4°C on a roller for 1 hr. 10 µl of a 1:1 slurry of protein A sepharose: solubilisation buffer were added and the mixture incubated at 4°C on a roller for 1 hr. The protein bound sepharose was pelleted by centrifugation at 10,000 rpm for 1 min in a microcentrifuge and the pellet washed 2 x 0.5 ml with solubilisation buffer and 3 x 0.5 ml with TBS. Both pellet and supernate were reserved for analysis by SDS-PAGE and Western blotting.

2.26 Cross-linking

100 ml of each yeast strain was grown, harvested and microsomes isolated as described in section 2.19a. 50 µl of microsomes were used per cross-linking reaction. A final concentration of 5.43 mM DSS was added to 50 µl of microsomes and incubated at 24°C between 5 and 20 min. The reaction was quenched by the addition of an equal volume of 0.1 M Tris.HCl (pH 7.5). The protein was then precipitated by the addition of an equal volume of 30% (w/v) TCA or the microsomes solubilised and protein recovered for immunoprecipitation of cross-linked complexes (section 2.25).

2.27 RAMP sedimentation

1 l of each yeast strain was grown, harvested and microsomes isolated as described in section 2.19a. Microsomes were solubilised and soluble protein recovered from these as described in section 2.20b except 1 ml solubilisation buffer was used. Ribosome associated membrane proteins (RAMPs) were recovered by centrifugation at 60,000 rpm for 90 min at 4°C in a Beckman Ti70.1 rotor.

2.28 Preparation of yeast lysate

A protease-resistant yeast strain (RSY607) was grown in 10 l of YPD to an OD_{600} of 3. The cells were harvested by centrifugation at 5,000 rpm for 5 min in a Beckman JLA10.500 rotor at 4°C and washed with DEPC-treated water by resuspension and centrifugation as before. The cells were resuspended in 10 ml of ice cold buffer A (100 mM KOAc, 2 mM $Mg(OAc)_2$, 20 mM Hepes.KOH (pH 7.4)) and the cells frozen by pouring a thin stream of the yeast cell suspension from a 25 ml glass pipette into a canister of liquid nitrogen. The frozen cells were transferred to a Waring blender where they were lysed by blending in the presence of liquid nitrogen for 8-10 min continuously, adding liquid nitrogen when required. The cell powder was transferred to a beaker where the liquid nitrogen was allowed to evaporate and the powder thawed on ice. 30 ml of buffer A containing 0.5 mM PMSF was then added and the cell lysate transferred to 50 ml polycarbonate tubes. Unbroken cells and all debris were pelleted by centrifugation at 9,000 rpm for 10 min in a Beckman JA25.5 rotor at 4°C. The supernate of this step was collected and transferred to one or two Ti45.5 tubes and subjected to centrifugation at 36,000 rpm for 30 min in a Beckman Ti45.5 rotor at 4°C. The supernate of this step was applied to a 100 ml Sephadex G-25 column equilibrated in buffer A containing 14% (v/v) glycerol and the buffer A/glycerol buffer reapplied at a flow rate of 1 ml/min. Fractions of 3 ml were collected and the absorbance of a 1:200 dilution of each fraction at OD_{260} measured. Fractions with an OD_{260} of greater than 30 units were pooled, frozen in 1 ml aliquots in liquid nitrogen and stored at –80°C. The Sephadex G-25 column was then washed extensively with and stored in buffer A containing 14% (v/v) glycerol and 2 mM NaN_3.

2.29 Treatment of yeast lysate

Yeast lysate used to translate exogenously added mRNA must first be treated with micrococcal nuclease to remove endogenous mRNA. Each 1 ml of lysate had 5 µl of

micrococcal nuclease (1 mg/ml) and 12.5 µl 40 mM $CaCl_2$ added, this was incubated at 20°C for 20 min. 12.5 µl 100 mM EGTA was then added and the sample incubated at 20°C for a further 5 min after which time the lysate was frozen at –80°C in 100 µl aliquots.

2.30 Preparation of mRNA

Capped mRNA was transcribed from *Xba*I restriction endonuclease cut plasmids pDJ100 and pJD96 (table 2) using the Megascript SP6 transcription kit (Ambion) according to the manufacturer's instructions.

2.31 Preparation of phospholipids

Azolectin liposomes were prepared by dissolving 5 g azolectin lipid powder in 100 ml of 10 mM KPO_4 (pH 7), 2 mM β-mercaptoethanol at 24°C with vigorous stirring. Once dissolved the liposome suspension was frozen in liquid nitrogen in 1 ml aliquots and stored at –80°C.

2.32 Solubilisation of microsomes and reconstitution into proteoliposomes

Part 1: Solubilisation and removal of detergent

50 µl of the appropriate microsomes were added to 360 µl ice cold solubilisation buffer (100 mM KPO_4 (pH 7), 20% (v/v) glycerol, 10 mM DTT, 500 mM KOAc) and mixed well. To this, 30 µl of sonicated (1 min burst) azolectin liposomes were added and the mixture vortexed gently. 60 µl of 10% (w/v) octylglucoside dissolved

in distilled water was layered on top and the solution vortexed vigorously for 10 s at the highest setting to solubilise the microsomes. Insoluble material was pelleted by centrifugation at 60,000 rpm for 30 min at 4°C in a Beckman TLA 120.2 rotor alongside the previously sonicated azolectin liposomes to remove multilamellar vesicles not disrupted by sonication. The supernate of the sonicated azolectin liposomes was removed and stored overnight at 4°C for use in the reconstitution. The microsomal supernate containing the proteins to be reconstituted was removed and placed in a Spectra/Por dialysis membrane (molecular weight cut-off of 6-8000 Da) with additional purified proteins such as SR, Sec61p or Kar2p. If the additional proteins were not supplemented with lipid sonicated azolectin liposomes were added to the mixture to a final concentration of 3 mg/ml to maintain the protein: lipid ratio. This was necessary to maintain the activity of the added proteins. The volume of purified material added to the solubilised microsomal proteins was less than one third of the total volume for efficient reconstitution. Once all additions were made the mixture was dialysed against 0.5 l of dialysis buffer (150 mM KOAc, 20% (v/v) glycerol, 20 mM KPO_4 (pH 7), 2 mM β-mercaptoethanol) per dialysed sample at 4°C for 16-17 hr and no longer.

Part 2: Reconstitution

Following dialysis 350 µl of dialysate was mixed gently with 350 µl of sonicated and centrifuged azolectin liposomes in a glass test tube, providing the lipid necessary for reconstitution of proteins. This mixture was immersed in a dry ice/acetone bath for 20 s and then allowed to thaw at 24°C for approximately 10 min. The reconstituted proteoliposomes in the mixture were then pelleted by centrifugation at 55,000 rpm for 45 min at 4°C in a Beckman TLA120.2 rotor and the supernate removed. The proteoliposomes were resuspended in residual liquid for use in the translocation assay.

2.33 Translation assay

In vitro yeast translation reactions were carried out as follows. 1-2 μg of transcript mRNA, 9 μl of 3 x translation buffer (75 mM creatine phosphate, 2.25 mM ATP, 300 μM GTP, 120 μM amino acids minus methionine (Promega), 360 mM KOAc, 6 mM $Mg(OAc)_2$, 66 mM Hepes.KOH, 5.1 mM DTT), 1 μl of creatine phosphokinase, 20 U of RNasin (Promega), 9 μl of yeast cell lysate, 1 μl of ^{35}S-methionine (specific activity 37 TBq/mmol) and 38 μl of buffer 88 (see section 2.19a) in a total volume of 60 μl were mixed and incubated at 20°C for 20 min (or 40 min if translocation was to be monitored). The reactions were terminated by addition of an equal volume of 30% (w/v) TCA and centrifugation at 13000 rpm for 15 min at 4°C in a microcentrifuge. Pellets were washed in acetone before being subjected to SDS-PAGE and phosphoimage analysis.

2.34 Translocation assay

When translocation was monitored each reaction was supplemented with 4 μl of the appropriate microsomes or 10 μl of the appropriate proteoliposomes reducing the amount of buffer 88 appropriately. Upon completion, 20 μl of the reaction was precipitated by addition of 20 μl of 30% (w/v) TCA, 20 μl was supplemented with 5 μl of 1 mg/ml trypsin (dissolved in PBS pH 3), and 20 μl was supplemented with 5 μl of 1 mg/ml trypsin and 2 μl of 10% (v/v) tritonX-100. Samples supplemented with trypsin and/or tritonX-100 were incubated for 20 min on ice before precipitation with an equal volume of 30% (w/v) TCA. Samples were pelleted by centrifugation at 13,000 rpm at 4°C for 15 min and pelleted protein was washed with acetone before being subjected to SDS-PAGE and phosphoimage analysis.

2.35 Sec61-peptide test

Yeast strains were grown, harvested and microsomes recovered as described in sections 2.5 and 2.19a. Translation/translocation assays were carried out as described in sections 2.33 and 2.34 except 500 nM, 5 μM, 50 μM and 500 μM Sec61-Nterminal peptide (sequence SSNRVLDLFKPFESFLPEVIAP, corresponding to residues 2-22 of Sec61p) or 500 μM control peptide were added to the reactions.

2.36 Two-hybrid blue/white assay

The blue/white filter lift assay, to determine if proteins were interacting in a two-hybrid screen, was carried out according to the protocol described in Fromont-Racine et al. (1997).

Table 1

Bacterial Strain	**Genotype**	**References**
DH5α	*supE*44 Δ*lac*U169(ϕ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gryA*96 *thi*-1 *relA*1	Sambrook and Russell, 2001
M15pREP4	Not available	Qaigen
Yeast Strain	**Genotype**	**Reference**
JDY3	*trp1, lys2, his3, ura3, ade2*	Parker *et al.* (1988)
JDY516	As JDY3 but *SEC63*-TEV-ZZ::*his5*$^+$	This study
JDY72	As JDY3 but *srp101::ADE2*, *srp102::HIS3*	Dr J. Brown, University of Edinburgh, UK
ARY1	As JDY3 but *SRP102*-TEV-ZZ, *his5*$^+$	This study
ARY2	As JDY3 but *SRP102*-HIS_6, *URA3*	This study
ARY3	As JDY3 but *SRP102*-HIS_8, *URA3*	This study
ARY4	As JDY3 but *SRP102*-CBD-TEV-ZZ, *his5*$^+$	This study
ARY5	As JDY3 but *HMG1*-TEV-ZZ, *his5*$^+$	This study

RSY937	*leu2-3, 112, ura3-1, sec61::HIS3, his3-11, 15,* pRS316-N-terminal *SEC61(HIS$_6$) tag*	Gift of Dr M. Morrow and Dr J. Brodsky, Pittsburgh, USA
RSY607	*ura3-52, leu2-3, 112, pep4::URA3*	Gift of Dr M. Morrow and Dr J. Brodsky, Pittsburgh, USA
RSY586	*MATα, ura3-52, leu2-3, 113, ade2-101, kar2-159*	Gift of Dr M. Morrow and Dr J. Brodsky, Pittsburgh, USA
JDY638	Derived from BMA38a: *his3Δ200, leu2-3,112, ura3-1, trp1Δ1, ade2-1, can1-100, kan^R-pGAL-SEC61*	BMA38a was a gift of Prof. J. Beggs, University of Edinburgh, UK JDY638 created by Dr J. Brown, University of Edinburgh, UK
CG1945	*MATa, ura3–52, his 3–200, lys2–801, ase2–101, trp1–901, leu2–3, 112, Gal4–542, Gal80–538, cyh-2, Lys2::Gal1UAS -Gal1TATA -HIS3; URA3::GAL4 17mers(X3)-CyC1TATA -lacZ*	Clonetech, gift of Prof. J. Beggs, University of Edinburgh, UK
JDY225	*GAL4, GAL80, ura3-52, leu2-3, -112, reg1-510, gal1, pep4-3*	Gift from K. Weis (Berkeley)

Table 2

Bacterial plasmids	Background	Insert	Promoter	Reference
pMR2623	pQE9	*S. cerevisiae KAR2-HIS$_6$*	IPTG-inducible *lacIQ* promoter	Gift from Dr J. Brodsky, Pittsburgh, USA
pUV5-G1S	pOP95-15	β-1,3-glucanase	*lac*UV5	Gift from Dr J. Brodsky, Pittsburgh, USA; Shen *et al.* (1991)
pJE97	pGEX-3X	*S. cerevisiae SRP101* and *SRP102-GST* (bp 73-735) separated by a ribosome binding site	IPTG-inducible lac promoter	Dr J. Brown, University of Edinburgh, UK
pSO488	pGEX-3X	*S. cerevisiae SRP102-GST* (bp 73-735)	IPTG-inducible lac promoter	Gift from S. Ogg and P. Walter, San Francisco, USA
pGEXSRP101	pGEX-3X	*S. cerevisiae SRP101-GST*	IPTG-inducible lac promoter	Ogg et al., 1992; Ng et al., 1996

pJD96	pJD82	*S. cerevisiae* alpha factor with DPAP-B signal sequence	N/A	Ng *et al.*, 1996; Mason *et al.*, 2000
pDJ100	pSP65	*S. cerevisiae* alpha factor	N/A	Hansen *et al.*, 1986
pJDY96	pRS316	*S. cerevisiae* *SEC65*-HIS_6	N/A	Dr J. Brown, University of Edinburgh, UK
pJE39	pBluescript	TEV protease site, protein A "ZZ" motif and *S. pombe his5*$^+$	N/A	A kind gift of Dr Sean Munro, MRC-LMB, Cambridge, UK
pJE82	pJE39	Calmodulin binding domain, TEV protease site, protein A "ZZ" motif and *S. pombe his5*$^+$	N/A	Dr J. Brown, University of Edinburgh, UK
Yeast Plasmids	**Background**	**Insert**	**Promoter**	**Reference**
pAR143	pTR143	*S. cerevisiae* *SRP101* and *SRP102-CBD*-TEV-*ZZ*	GAL1/10	This study

pAR2H1	pAS-C	*SRP102* bp 73-735		This study
pAR2H2	pAS-C	*SEC61* bp 1-97		Chris Morrow, University of Edinburgh, UK
pJDY187	pACTII	*SRP101*		Dr J. Brown, University of Edinburgh, UK
pAR2H4	pACTII	*SRP102* bp 73-735		This study
pAR2H5	pACTII	*SEC61* bp 1-97		This study
pAR2H6	pACTII	*SEC61* bp 502-636		Chris Morrow, University of Edinburgh, UK
pAR2H7	pACTII	*SEC61* bp 781-886		Chris Morrow, University of Edinburgh, UK
pAR2H8	pACTII	*SEC61* bp 1143-1249		Chris Morrow, University of Edinburgh, UK
pAR2H9	pACTII	*SBH1* bp 1-102		Chris Morrow, University of Edinburgh, UK

Table 3

Antibody	Animal raised in	Useful dilution	Reference
Anti-SRα	Rabbit	Western – 1:1000	This study
Anti-SRβ	Rabbit	Western – 1:1000	This study
Anti-Sec61p	Rabbit	Western – 1:2000	Gift of P. Walter, San Francisco, USA
Anti-Sec62p	Rabbit	Western – 1:2000	Gift of P. Walter, San Francisco, USA
Peroxidase anti-peroxidase (PAP) (HRP-conjugated)	Rabbit	Western – 1:2000	Sigma-Aldrich
Anti-HA (3F10)	Rat	Western – 1:5000	Gift of K. Hardwick, Edinburgh, UK

Table 4

Oligo name	Sequence (5'-3')	Amplified DNA
SRBHISTAG5	GCTTTTGAGGGCAGTAT TAATAAAAGAAAAATTT CCCAATGGCGCGAATGG ATAGATGAAAAACTGAC TAGTATTGAAGGTAGAT CTCAC	3' flanking region of *SRP102*, HIS_6 of JDY96 and the *URA3* gene
SRBHISTAG3	GCTATGTTAAATGCTTGT TTTTAAACTATCTATCGT TCATCTAGTTATAAATTA TAATTATATTAGCGGCA TCAGAGCAGATTGTACT G	3' terminator of *SRP102* and the *URA* gene
SRBHISTAG25	GCTTTTGAGGGCAGTAT TAATAAAAGAAAAATTT CCCAATGGCGCGAATGG ATAGATGAAAAACTGAC TAGTATTGAAGGTCATC ATCACCACCAC	3' flanking region of *SRP102*, HIS_6 of JDY96 with 2 additional histidine codons and the *URA3* gene
5SRP102	GCTTTTGAGGGCAGTAT TAATAAAAGAAAAATTT CCCAATGGCGCGAATGG ATAGATGAAAAACTGGG AGCAGGGGCGGGTGCTA GC	3' flanking region of *SRP102*, the TEV cleavage site, the protein A "ZZ" motif and the *S. pombe HIS5*$^+$ gene
3SRP102	GCTATGTTAAATGCTTGT TTTTAAACTATCTATCGT	*SRP102* with the TEV

	TTTTAAACTATCTATCGT TCATCTAGTTATAAATTA TAATTATATTAGAGGTC GACGGTATCGATAAG	cleavage site and the protein A "ZZ" motif
5SRBSPPAI	GTTGCTTTTGAGGGCAG TATTAATAAAAGAAAAA TTTCCCAATGGCGCGAA TGGATAGATGAAAAACT GGCGAGCTCCAGCTCCA GCTCCAGCTCCACCGCG GCCATG	3' flanking region of *SRP102*, the calmodulin binding domain spacer, the TEV cleavage site, the protein A "ZZ" motif and the *S. pombe HIS5*$^{+}$ gene
5SFR6OX	GCCGAATTCATGCTTAG TAATACACTT	*SRP102*
3SRBOXNEW	GAATTCTACCGATCACG AGTTCGCGTC	*SRP102* with the calmodulin binding domain spacer, the TEV cleavage site and the protein A "ZZ" motif
2MAOX5	GCGGGATCCTCACCCCT CAGTATCCCT	*SRP101*
2MAOX3	GCGGGATCCTTAAGACA TTAATGTATTAAC	*SRP101*
5SEC63PRA	GCTAGCGATTATACTGA TATCGATACGGATACAG AAGCTGAAGATGATGAA TCACCAGAAGGAGCAGG GGCGGGTGCTTAGCG	3' flanking region of *SEC63*, the TEV cleavage site and the protein A "ZZ" motif

3SEC63PRA	AAGATGAAATATATACG TCTAAGAGCTAAAATGA AAAACTATACTAATCAC TTATATCTAGAGGTCGA CGGTATCGATAAG	3' terminator of *SEC63*, the protein A "ZZ" motif and the TEV cleavage site
5HMGCT	CAATTTGGACGCCACTG ATATAAATCGTTTGAAA GATGGGTCCGTCACCTG CATTAAATCCAGCTCCA CCGCGGCCATGGGAGCA GGGGCGGGTGCTAGC	3' flanking region of HMG-CoA reductase, the TEV cleavage site and the protein A "ZZ" motif
3HMGCT	GTACAAACTATAAAGTA AATATTTTACGTAACAC ATGGTGCTGTTGTGCTTC TTTTTCAAGAGAATACC AATGACGTATGACTAGA GGTCGACGGTATCGATA AG	3' terminator of HMG-CoA reductase, the protein A "ZZ" motif and the TEV cleavage site
5SRB2H	GTGCAAGAATTCAAGGC ATCCTCCAAGACAGGG	bp 73-735 of *SRP102*
3SRB2H	GTGCGGCAATTCTTACA GTTTTTCATCTATCCA	bp 73-735 of *SRP102*
5NTERM61	GAGGCCCCGGGGATGTC CTCCAACCGTGTTCTAG	bp 1-97 of *SEC61*
3NTERM61-2	CGGGGATCCTTATTTCTG GTTGTATGGAACCTT	bp 1-97 of *SEC61*

5LOOP2	GAGGCCCCGGGGGACGA ATTGCTATCTAAG	bp 502-636 of *SEC61*
3LOOP2-2	CGGGGATCCTTATTCGA ACTCCTTACCACGACC	bp 502-636 of *SEC61*
5LOOP3	GAGGCCCCGGGGCAAGG CTTCCGTTACGAATTG	bp 781-886 of *SEC61*
3LOOP3-2	CGGGGATCCTTACAACA TGATTGGGGTGTTGGA	bp 781-886 of *SEC61*
5LOOP4	GAGGCCCCGGGGGAAAT CTCCGGCACTTCC	bp 1143-1249 of *SEC61*
3LOOP4-2	CGGGGATCCTTACTTCTT CAATTCTCTGTAAAT	bp 1143-1249 of *SEC61*
5SBH1	GAGGCCCCGGGGATGTC AAGCCCAACTCCT	bp 1-102 of *SBH1*
3SBH1-2	CGGGGATCCTTAGTTCG TGTTTTTCTTTGGAGC	bp 1-102 of *SBH1*

Chapter 3

Purification of the SRP-receptor from yeast

Introduction

A main aim of my project was to purify the components necessary for, and to reconstitute, SRP-dependent targeting and co-translational translocation *in vitro*. This reconstituted system would then be used to look in more detail at interactions and processes that occur at the ER membrane during targeting. Previously, the translocon has been purified by several means (Panzner *et al.*, 1995; Beckmann *et al.*, 1997; Morrow and Brodsky, 2001) and shown to be active in either post-translational translocation and/or ribosome binding. However, the yeast SRP receptor had not been purified and this was considered the major obstacle to generation of a co-translational translocation system with yeast components. Here I describe a purification procedure for yeast SR, and other experiments that were carried out during its development.

Strategies

First conditions were established under which the intact SR complex could be efficiently solubilised from yeast ER membranes. Second, SR was tagged to facilitate purification. Two types of tag were used, poly-histidine and the IgG binding domain of protein A (the "ZZ" tag). Both have successfully been used to purify membrane proteins from yeast e.g. the translocon component Sec61p (Morrow and Brodsky, 2001) and Golgi resident mannosyltransferases (Rayner and Munro, 1998). Protein A has been used in the purification and visualisation of many proteins (Puig *et al.*, 2001) and binds with high affinity to IgG allowing in many cases a "one step" purification. Protein A cross-reacts with many secondary antibodies, anti-rabbit in particular, which results in visualisation of tagged proteins in Western blotting without incubation with primary antibody. The ZZ-tag used in this study contained, in addition, a tobacco etch virus protease (TEV protease) site facilitating cleavage of protein A from tagged proteins and thus isolation of native SR. Initially these tags were incorporated into the genomic SRβ locus allowing purification of SR

expressed at wild type levels. However, this was found to be insufficient and finally a protein A-tagged SR was over-expressed from a galactose-inducible plasmid construct. Last, as detergents used for solubilisation and purification steps are incompatible with reconstitution of protein into proteoliposomes the ability of SR to bind ion exchange resins was investigated. Purified SR bound to such resins allowing detergent exchange to be carried out. In addition, ion exchange chromatography while on its own unlikely to lead to a rapid purification of SR, may be useful for refining the purifications carried out herein.

3.1 Genome based methods

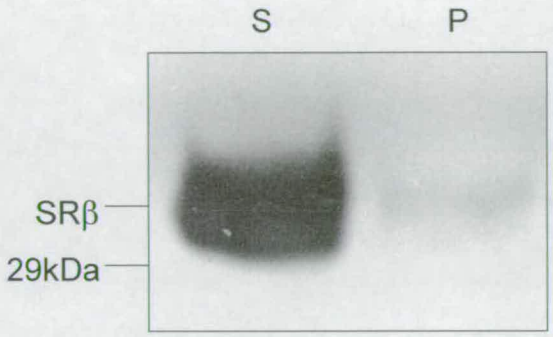
3.1.1 Solubilisation of SR

The first stage in extraction of SR from the ER and purification from other membrane proteins was determining which detergent would be the most effective at solubilising SR from the ER membrane whilst maintaining the complex intact. Detergents tested were octyl-G-glucoside, deoxyBigCHAP, BigCHAP, digitonin and tritonX-100. 1-3% (v/v) of detergent in solubilisation buffer were tested. Octyl-G-glucoside, deoxyBigCHAP and BigCHAP were less effective than digitonin at solubilising SR from ER membranes while triton X-100 was the most effective. Figure 3.1 shows a comparison between tritonX-100 and digitonin at 3% (v/v). While some SRβ can be seen in the pellet fraction of the digitonin extraction (3.1B) virtually all the protein is solubilised by tritonX-100 extraction (3.1A).

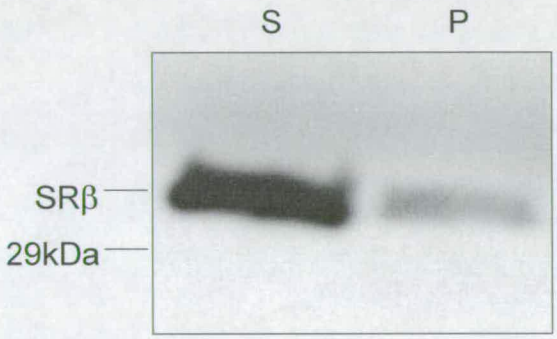
Figure 3.1 Comparison of extraction of microsomes with digitonin and tritonX-100

Strain JDY3 was used to prepare microsomes as described in section 2.19a. 12 OD_{280} units of microsomes were solubilised with 3% TritonX-100 (A.) or 3% digitonin (B.) in buffer 88 (section 2.19a) and afterwards insoluble material was pelleted by centrifugation at 13,000 rpm for 15 mins at 4°C in a microcentrifuge. Equal amounts of the supernate and pellet of each were loaded. An antibody specific for SRβ was used to detect soluble and insoluble SR. In both cases S is the supernate and P is the pellet of insoluble material.

A.



B.



3.1.2 Histidine tagging

A. Hexahistidine tagging

The first tagging strategy adopted was histidine tagging. The tag (HIS_6) was amplified from plasmid pJDY96 using oligos SRBHISTAG5 and SRBHISTAG3 (table 4) that contained 66 bases of homology to the 3' end of the SRβ ORF and 3' flanking DNA respectively. The amplified region also contained a *URA3* metabolic marker (Figure 3.2). Purified PCR product was transformed into strain JDY3 (table 1). Cells expressing Ura3p were identified by their ability to grow on media that did not contain uracil.

Colonies could be isolated which grew on –ura media due to non-homologous integration of the *URA3* gene or integration into the *ura3* locus. Thus, *URA3+* colonies were also screened by Western blotting for a molecular weight increase in SRβ indicating the presence of the tag. Unfortunately, antibodies against the histidine tag (Qaigen) failed to recognise the protein.

Only one colony was recovered from many ura+ colonies that expressed hexahistidine-SRβ indicating that the integration was not efficient. I also attempted to histidine tag SRα by the same method, but this was unsuccessful. Elements in the genome downstream of SRα or the function of the subunit itself may have prevented the genomic insertion of a tag.

Purification of SR via the HIS_6-tag at the C-terminus of SRβ is shown in Figure 3.3 (described in section 2.19c). As can be seen (compare lanes 1 and 3), SR bound to the Ni-NTA agarose but a proportion of SR is visible in the unbound fraction. In this case most of the SRα protein washed away from the agarose immobilised SRβ indicating that the salt concentration or pH of the wash buffer were inappropriate. The SRβ protein still present on the agarose eluted in fractions 1-3 (lanes 7-9). Although the composition of the wash buffers were varied for pH, salt and imidazole

Figure 3.2 Schematic of tagged SRβ constructs

Constructs were made by genomic insertion of a tag and a marker gene at the C-terminus of the SRβ subunit. The tag DNAs were PCR amplified from plasmids (table 2). PCR amplification and integration was carried out as described in sections 2.6 and 2.8 using oligonucleotides described in table 4. Selection of integrants was by their ability to grow on media lacking uracil, in the case of the hexa/octa-histidine tags or histidine, in the case of the ZZ-tags. Integrants isolated by the activity of the requisite metabolic marker had the presence of the correct protein tag confirmed by SDS-PAGE and Western blotting. (A) hexa-histidine construct containing the *S. cerevisiae URA3* gene, (B) octa-histidine construct containing the *S. cerevisiae URA3* gene, (C) ZZ-tag with an N-terminal TEV protease site containing the *S. pombe his*5^{+} gene and (D) ZZ-tag with an N-terminal TEV protease site with a calmodulin binding domain (CBD) as a spacer between the SRβ gene and the TEV protease site at its N-terminus with the *S. pombe his*5^{+} gene as metabolic marker.

Also shown, in orange, is the transmembrane domain of SR beta at the N-terminus of the protein.

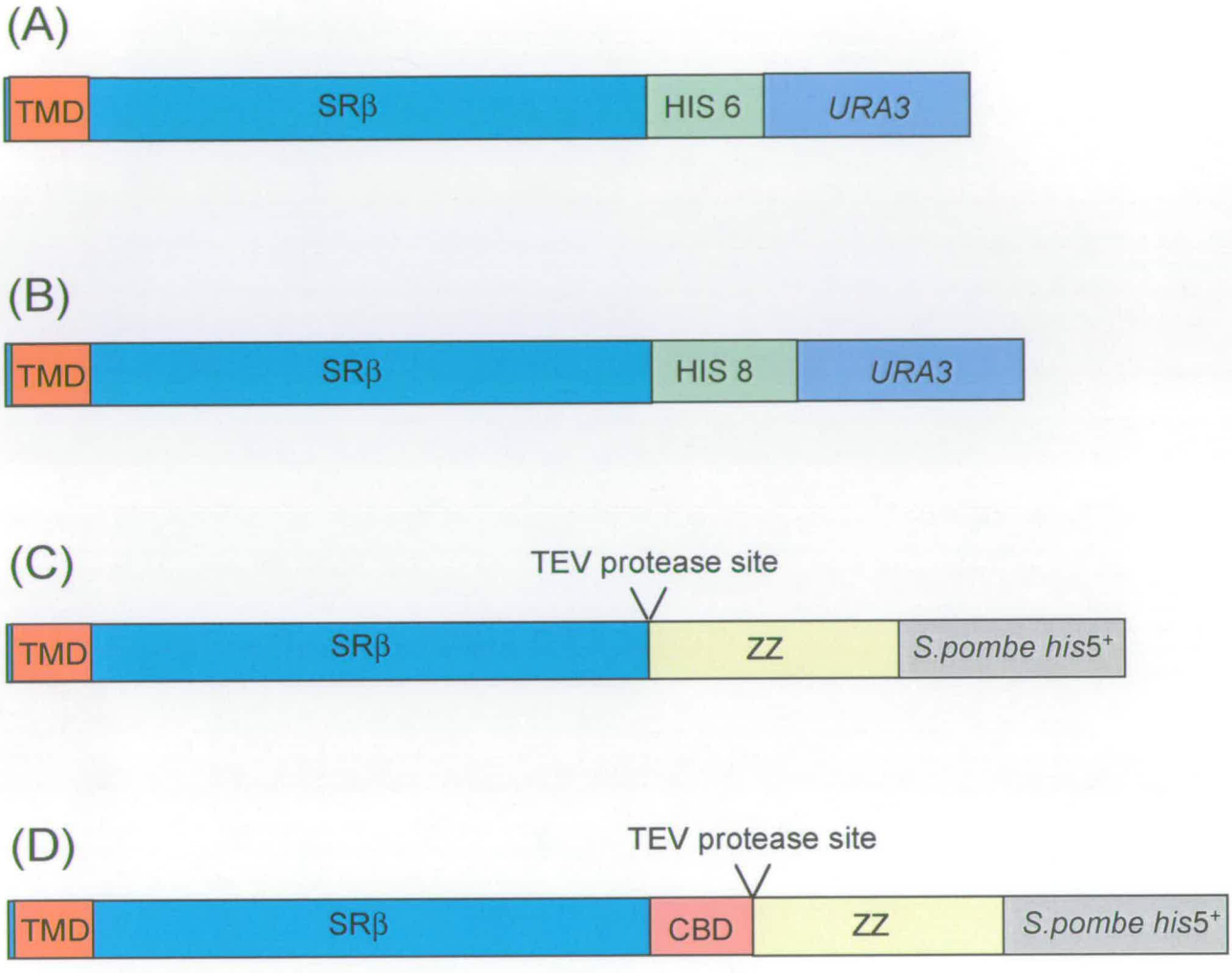

(A)
TMD
SRβ
HIS 6
URA3
(B)
TMD
SRβ
HIS 8
URA3
(C)
TEV protease site
TMD
SRβ
ZZ
S.pombe his5+
(D)
TEV protease site
TMD
SRβ
CBD
ZZ
S.pombe his5+

Figure 3.3 Purification of SR via a hexa-histidine tag

240 OD_{280} units of microsomes recovered from ARY2 were solubilised in digitonin buffer and insoluble material pelleted (section 2.19c). Ribosome associated membrane proteins were pelleted from the membrane extract (section 2.27) and the post-ribosomal supernate bound to Ni-NTA agarose overnight at 4°C on a roller. The sepharose was washed and eluted as described in section 2.19c. 1/200th (lanes 1-6) and 1/5th (lanes 7-11) of samples loaded were analysed by SDS-PAGE, Western blotting and silver staining. Antibodies specific for SRα and SRβ were used to detect SR. The Western blot is shown in panel A while the silver stain is shown in panel B. The lanes in panels A and B are loaded with identical samples.

Lane

1 Load fraction (post-ribosomal supernate)

2 Ribosomal pellet

3 Unbound fraction

4 Wash 1

5 Wash 2

6 Wash 3

7 Elution 1

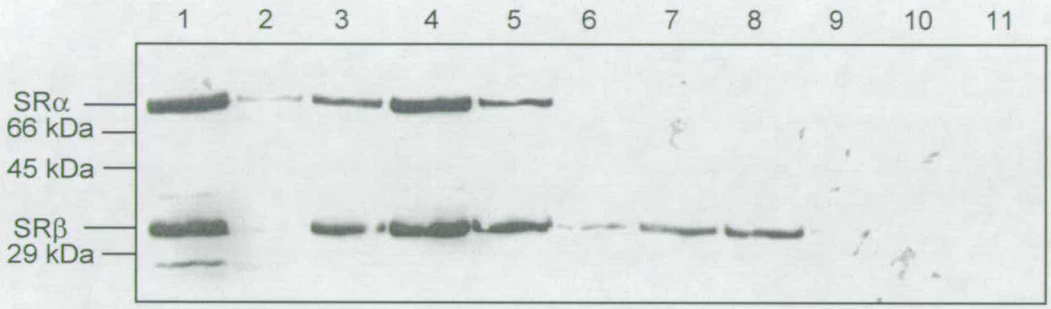
8 Elution 2

9 Elution 3

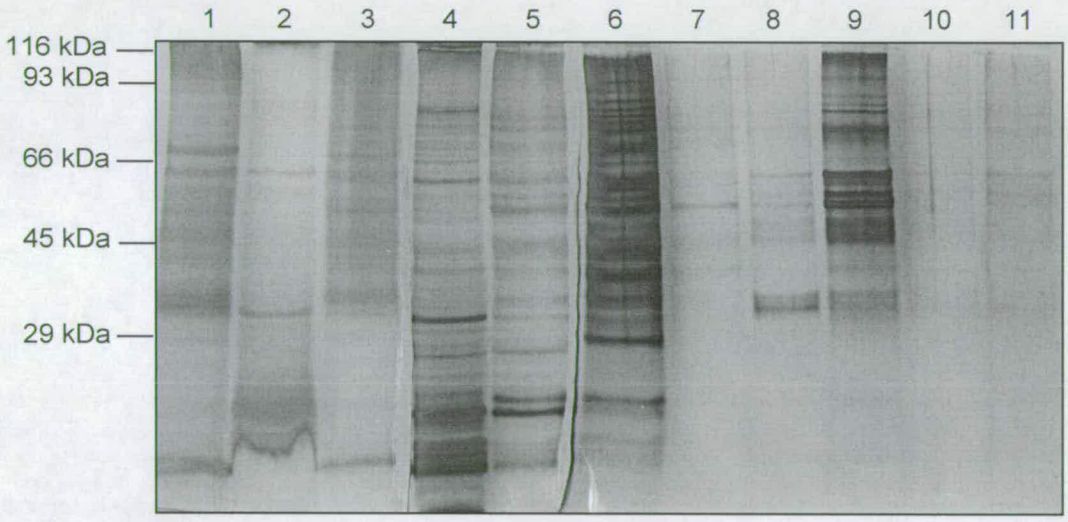
10 Elution 4

11 Elution 5

A.


1 2 3 4 5 6 7 8 9 10 11
SRα
66 kDa
45 kDa
SRβ
29 kDa

B.


1 2 3 4 5 6 7 8 9 10 11
116 kDa
93 kDa
66 kDa
45 kDa
29 kDa

concentration, in every case either SR would elute in the wash step or the subunits would dissociate into individual components rendering this technique useless for purification of intact receptor. It was concluded that as SR washing off the agarose before the elution step was a consistent problem, a tag of six histidine residues was not sufficient and that increasing the number of residues may prove beneficial.

Indeed, it had been suggested that when purifying proteins from yeast an octa-histidine tag could be more effective than a hexa-histidine tag (Alan Boyd, unpublished observations).

B. Octahistidine tagging

The method used previously to HIS_6-tag SRβ was used to generate the HIS_8-tag; however, two additional histidines were encoded by the oligonucleotide SRBHISTAG25 (table 4) used to generate the PCR product. Figure 3.2 outlines the structure of the octa-histidine tag. The PCR product was purified and transformed into a haploid yeast strain, as before, and putative positive transformants were selected for their ability to grow on -ura media. Positive transformants were detected by a molecular weight shift of SRβ-HIS_8 compared with SRβ-HIS_6 and SRβ wild type.

Once a positive SRβ-HIS_8 tagged strain was isolated, purification of SR proceeded as before. As hoped, the octa-histidine tag conferred greater affinity for the Ni-NTA resin, and SR remained bound during the washing steps (Figure 3.4A). However, as can be seen in Figure 3.4B the elution fractions contained many contaminating proteins. Ion-exchange chromatography was then used as a second purification step. In this experiment the eluate from the Ni-NTA agarose was incubated with DEAE-sepharose in an attempt to remove contaminants. Unfortunately, SR bound inefficiently to the DEAE resin under these conditions (50 mM KOAc) and no

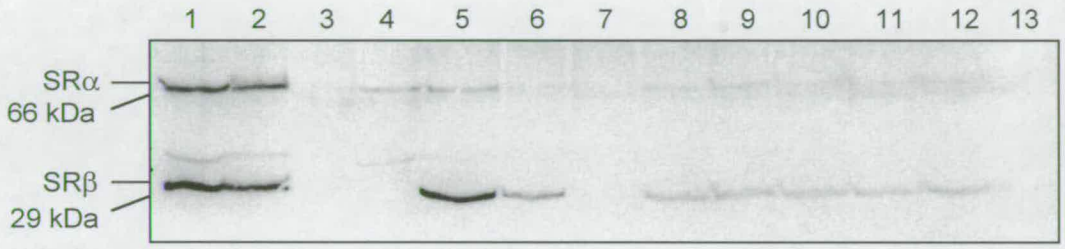
Figure 3.4 Purification of octa-hisitidine tagged SR by affinity and ion-exchange chromatography

240 OD_{280} units of microsomes recovered from ARY3 were solubilised in digitonin buffer and insoluble material pelleted (section 2.19c). Ribosome associated membrane proteins were pelleted from the membrane extract (section 2.27) and the post-ribosomal supernate bound to Ni-NTA agarose overnight at 4°C on a roller. The sepharose was washed and eluted as described in section 2.19c. The eluate from the Ni-NTA agarose was bound to DEAE sepharose overnight at 4°C on a roller. The sepharose was washed once in wash buffer (50 mM $NaPO_4$ (pH 8), 100 mM NaCl, 1%(v/v) tritonX-100). Protein was eluted in 2 x 1 ml elution buffer 1 (50 mM $NaPO_4$ (pH 8), 150 mM NaCl, 1%(v/v) tritonX-100), 2 x 1 ml elution buffer 2 (50 mM $NaPO_4$ (pH 8), 175 mM NaCl, 1%(v/v) tritonX-100) and 2 x 1 ml elution buffer 3 (50 mM $NaPO_4$ (pH 8), 200 mM NaCl, 1%(v/v) tritonX-100). $1/200^{th}$ (lanes 1-6) and $1/5^{th}$ (lanes 7-11) of samples loaded were analysed by SDS-PAGE, Western blotting and silver staining. Antibodies specific for SRα and SRβ were used to detect SR. The Western blot is shown in panel A while the silver stain is shown in panel B. The lanes in each are loaded with identical samples.

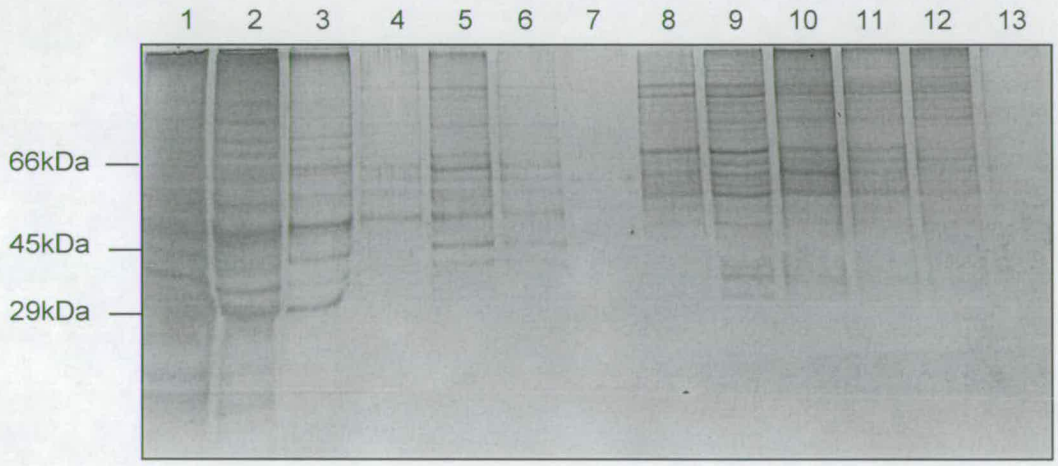
Lane

1 Load fraction (post-ribosomal supernate)
2 Unbound fraction
3 Wash 1
4 Wash 2
5 Elution from Ni-NTA agarose, load fraction of DEAE sepharose
6 Unbound fraction
7 Wash 1
8 Elution 1
9 Elution 1b
10 Elution 2
11 Elution 2b
12 Elution 3
13 Elution 3b

A.


1 2 3 4 5 6 7 8 9 10 11 12 13
SRα
66 kDa
SRβ
29 kDa

B.


1 2 3 4 5 6 7 8 9 10 11 12 13
66kDa
45kDa
29kDa

significant purification from contaminating proteins was achieved. Also apparent is a degree of dissociation of SRα from SRβ during the purification.

As no significant improvement in purification was achieved despite attempts using several ion exchange resins I concluded that histidine tagging of SRβ was not an efficient means of purifying SR and decided to purify SR using a protein A-tag.

3.1.3 Protein A-tagging

A. Protein A-tagging SRβ

Oligonucleotides were designed to amplify the protein A ZZ-tag with flanking regions of homology to SRβ for integration and a *S.pombe his5*$^{+}$ metabolic marker, which complements a *his3* mutation in *S. cerevisiae*, for selection from plasmid vector (pJE39) as described in section 2.6 and shown in Figure 3.2.

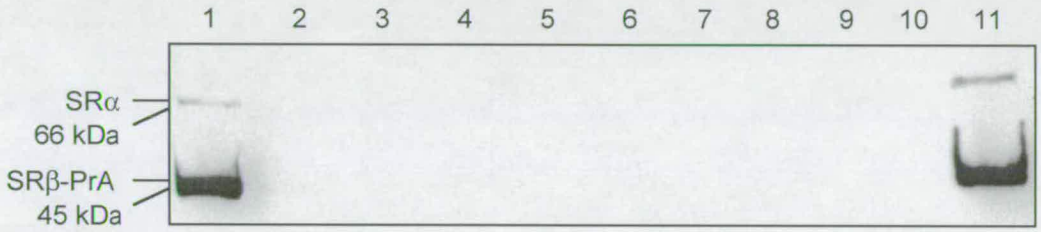
Expression of protein A-tagged SRβ was confirmed by Western blotting with anti-protein A antibodies (Sigma) which revealed a protein of the expected 45 kDa size. Purification of protein A-tagged SRβ was carried out as described (section 2.20b). After elution the fractions were analysed by SDS-PAGE and Western blotting. Purification of the protein A-tagged SR was complicated by the fact that the TEV protease was unable to cleave the protein A tag off SRβ successfully. Figure 3.5 shows a typical purification of SR via the protein A-tag. The protein A-tag conferred efficient binding of SR to IgG sepharose with very little SR remaining in the unbound fraction. Very little protein washed off the resin and all of the protein is still bound to the resin after incubation with TEV protease. Repeated attempts using buffers at different salt composition and concentration with different protein A-tagged strains were all unsuccessful. As the result was the same with several different strains this problem is unlikely to be due to a PCR induced mutation in the

Figure 3.5 TEV protease is insufficient to cleave PrA-tag from SR

240 OD_{280} units of microsomes recovered from strain ARY1 were dissolved in solubilisation buffer and isoluble material pelleted as described in section 2.20b except 0.5 ml solubilisation buffer was used. Ribosome associated membrane proteins were pelleted from the membrane extract (section 2.27) and the post-ribosomal supernate bound to 200 µl IgG sepharose overnight at 4°C on a roller. The sepharose was washed and eluted as described in section 2.20b except 10 units of TEV protease were added to 1 ml elution buffer and afterwards the sepharose was washed with 5x1 ml aliquots of wash buffer. 1/200th of all samples, including a sample of the IgG-sepharose were analysed by SDS-PAGE and Western blotting. Antibodies specific for SRα and protein A were used to detect SR.

Lane

1 Load fraction (post-ribosomal supernate)
2 Unbound fraction
3 Wash 1
4 Wash 2
5 Elution 1
6 Elution 2
7 Elution 3
8 Elution 4
9 Elution 5
10 Elution 6
11 IgG sepharose


1
2
3
4
5
6
7
8
9
10
11
SRα
66 kDa
SRβ-PrA
45 kDa

cleavage site sequence. Instead it suggests that the tag may fold into a conformation at the C-terminus of SRβ making the TEV site inaccessible to the protease.

It seemed reasonable that introducing some distance between the C-terminus of SRβ and the N-terminus of the protein A-tag would increase the space between SRβ and the protein A domain allowing the TEV protease access to the cleavage site.

B. Protein A-tagging SRβ with spacer

Having decided that a solution to cleavage by TEV protease was to add a spacer to the SRβ-PrA construct, the decision now was which form of spacer should be used. Other purifications had been published using the "TAP-tagging" system (Tandem Affinity Purification) in which a calmodulin binding domain is inserted between the C-terminus of the tagged protein and the TEV protease site at the N-terminus of the protein A-tag (Puig, *et al.*, 2001). A construct was thus made in which the sequence of the human calmodulin binding domain was inserted before the N-terminus of the protein A sequence in pJE39 (pJE82, J. Brown). Again, wild type yeast cells were transformed with a PCR product derived from the plasmid (using oligos 5SRBSPPAI and 3SRP102 (table 4)) and selected for their ability to grow on -his media. Transformants were screened by Western blotting for the presence of the fusion protein. Once positive transformants were selected purification of SR was as before. However, in this case the addition of TEV protease now eluted SR intact from the IgG sepharose. Figure 3.6 shows clearly that upon addition of TEV protease both subunits of SR are co-eluted from the resin with protein A remaining attached to the sepharose at the end of the experiment. In this experiment some SRβ-ZZ remained uncleaved and bound to the resin.

This purification was scaled-up in an attempt to purify sufficient protein to attempt reconstitution experiments. Figure 3.7 shows a typical purification of SR *via* the

Figure 3.6 TEV protease cleavage of CBD-PrA-tag from SR

120 OD_{280} units of microsomes recovered from ARY4 were dissolved in 0.5 ml solubilisation buffer and insoluble material pelleted (section 2.20b). Ribosome associated membrane proteins were pelleted (section 2.27) and the post-ribosomal supernate bound to 200 µl IgG sepharose overnight at 4°C on a roller. The sepharose was washed twice in wash buffer (1% (v/v) triton X-100, 0.4 M KOAc, 14% (v/v) glycerol, 20 mM Hepes.KOH (pH 7.4), 2 mM DTT, 1 mM PMSF). Sepharose was incubated in 1ml wash buffer containing 10 units of TEV protease and incubated at room tempterature for 1 hour on a roller. After which the elute was collected, the sepharose washed 3 x 1 ml with wash buffer and 3 x 1 ml TBS and the IgG sepharose pellet collected for analysis. 1/200th of all samples were analysed by SDS-PAGE and Western blotting. Antibodies specific to SRα and SRβ were used to detect SR (table 3).

Lane

L Load fraction

U Unbound fraction

E Eluate

P IgG sepharose pellet

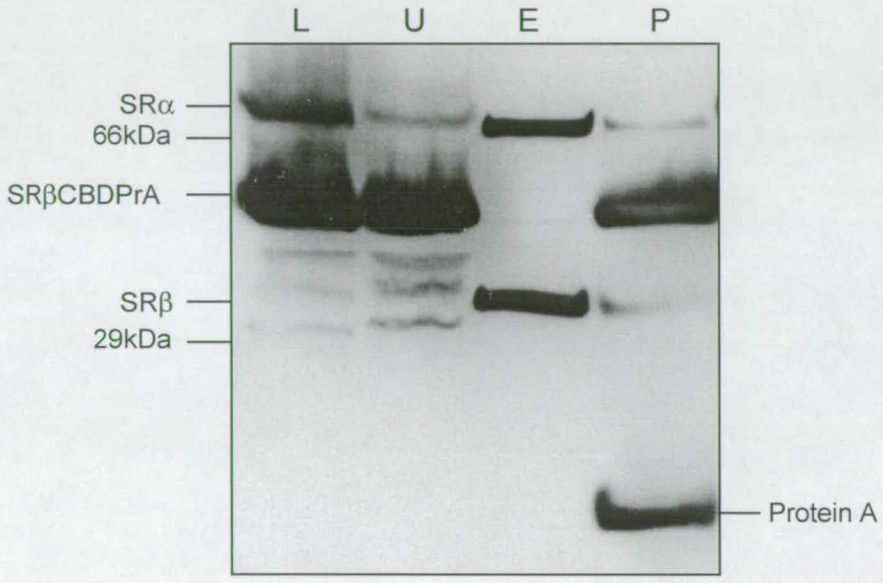

L
U
E
P
SRα
66kDa
SRβCBDPrA
SRβ
29kDa
Protein A

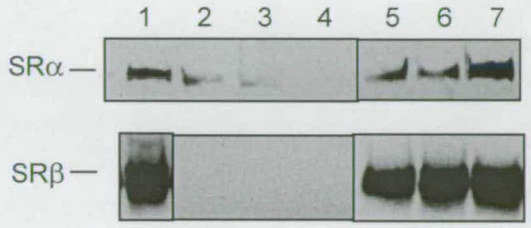
Figure 3.7 Purification of SR from yeast via Protein A-tag

7,680 OD_{280} units of microsomes prepared from strain ARY4 were solubilised in solubilisation buffer and insoluble material pelleted (section 2.20b). Ribosome associated membrane proteins were pelleted (section 2.27) and the post-ribosomal supernate bound to IgG sepharose overnight at 4°C on a roller (section 2.20b). The sepharose was washed twice in wash buffer (1% (v/v) triton X-100, 0.4 M KOAc, 14% (v/v) glycerol, 20 mM Hepes.KOH (pH 7.4), 2 mM DTT, 1 mM PMSF) and incubated at room temperature for 1 hour with 1ml of wash buffer containing 50 units of TEV protease. The eluate was collected and 1/200th (lanes 1-4), 1/10th (lane 5), 1/5th (lane 6) and 1/2 (lane 7) of the samples were analysed by SDS-PAGE, Western blotting (A) and coomassie staining (B). Antibodies specific for SRα and SRβ were used to detect SR.

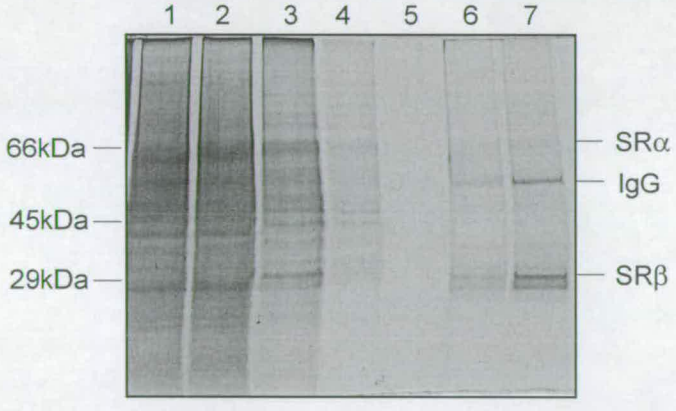
Lane

1 Load fraction (post-ribosomal supernate)
2 Unbound fraction
3 Wash 1
4 Wash 2
5 Elution 1
6 Elution 2
7 Elution 3

A.



B.



protein A-tag. Bands of 69 kDa and 30 kDa were expected size for SRα and SRβ respectively. Contaminating bands of 50 kDa and 25 kDa were of the size expected for IgG and TEV protease. IgG can be removed from the elution fractions by incubation with protein A-sepharose and TEV protease can be removed by incubation with Ni-NTA agarose since it is provided in hexa-histidine tagged recombinant form (GIBCO-BRL). Although bands on the coomassie stained gel can be putatively identified as corresponding to SR subunits, the bands are extremely faint. The SR proteins are clearly not major constituents of the elution fraction as these samples comprised one fifth of the total elution from 200 g of yeast cells, the product of a 32 l fermenter of yeast cell culture. Thus, either these proteins are not in abundance in the cell, or cell lysis, membrane solubilisation or SR recovery were inefficient. Despite sphaeroplasting cells before dounce homogenisation to lyse them (section 2.19a), a significant proportion of cells remain unbroken. This is also true for cells lysed using a Waring blender in the presence of liquid nitrogen (section 2.28). Membrane solubilisation is less likely to be a source of lost SR as membrane dispersion was monitored by sedimenting insoluble membranes and analysing for the presence of SR by SDS-PAGE and Western blotting. It is also unlikely to be due to insufficient SR recovery after membrane solubilisation as little SRβ-PrA was ever seen in the unbound fraction. However, although no data is published on the concentration of SR in ER membranes it does not appear to be as well expressed as SRP (data not shown), which is consistent with SR having a regulatory function in protein targeting where stoichiometric amounts of SR to SRP would not be required.

3.1.4 Ion-exchange chromatography

Previous work on mammalian SR described its ability to bind and elute specifically from an ion-exchange resin, for the purpose of exchanging detergent present in the column buffer. This detergent exchange was a necessary step before reconstitution of the subunits could go ahead. To examine the pattern of proteins eluting with or without yeast SR from several different ion-exchange resins I attempted to identify a

particular resin that would allow binding and elution of SR with a minimum of other proteins. The four resins chosen were CM, DEAE, Q and SP sepharoses for their strong or weak anion or cation exchange properties.

Solubilised ER membrane fractions were bound to each ion-exchange resin and the pattern of proteins eluting from this examined by SDS-PAGE and silver staining (section 2.15). In each case almost all of the proteins present after binding eluted in the same fraction as the SR subunits. This was despite many variations of salt concentration and pH in binding, wash and elution buffers. It was clear that ion-exchange chromatography would not be efficient as a second stage in the purification of SR if the first stage eluate was heavily contaminated with other proteins.

However, binding of SR to these resins identified the conditions under which SR could bind and elute for the future step of detergent exchange.

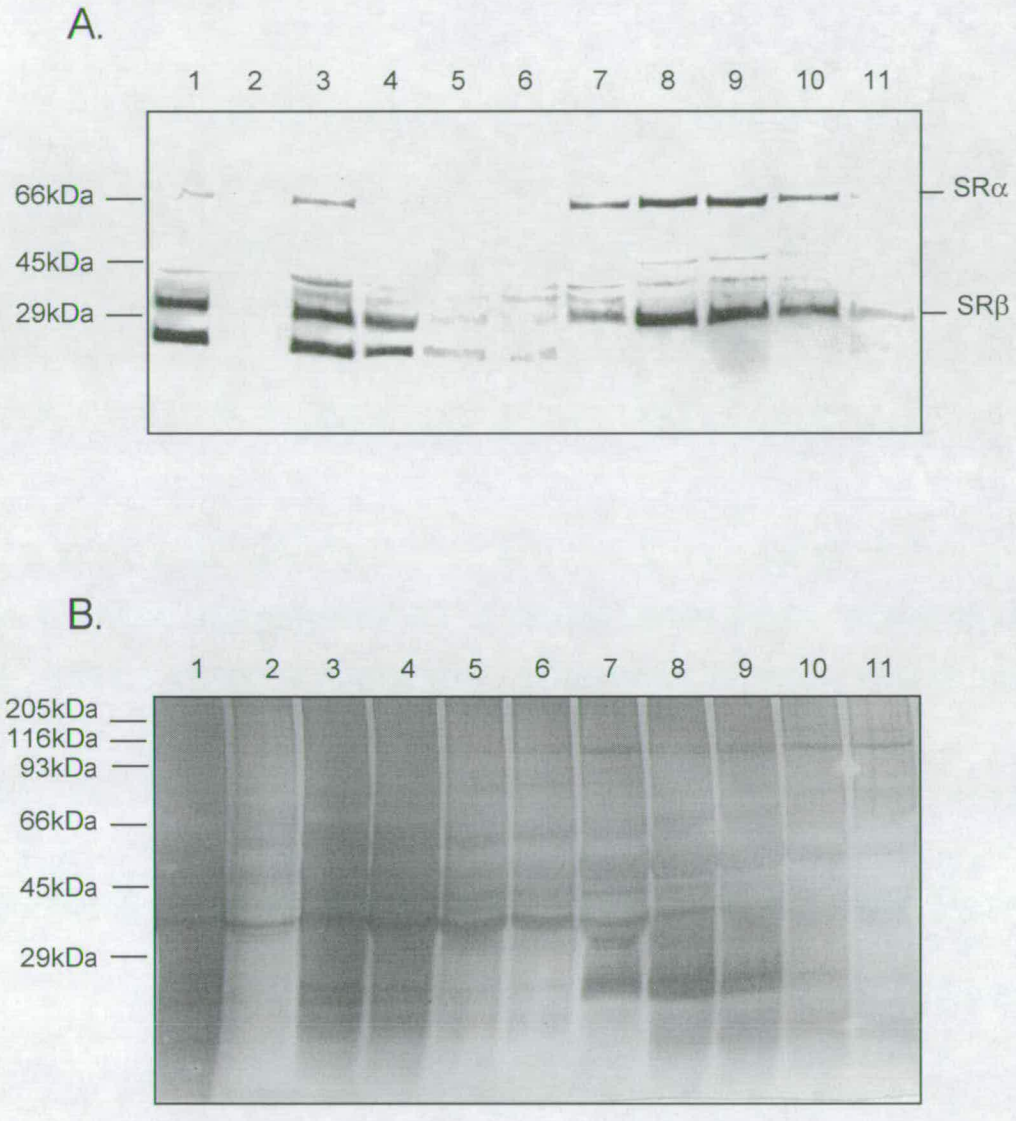
As described, if the eluate containing SR was heavily contaminated ion-exchange could not be used to further purify the complex. Therefore, it was thought that removal of as many contaminating proteins as possible before binding would be beneficial. After solubilisation of ER-derived microsomal membranes many of the proteins present are ribosomal or ribosome-associated. Ultracentrifugation can remove ribosomal proteins and ribosome-associated membrane proteins (RAMPs). As a result, this ultracentrifugation step was included in the strategy for purification of SR. Figure 3.8 shows a typical ion-exchange mediated purification of SR. The Western blot (A) shows that SR bound to the resin, a little protein was in the unbound fraction, and bound SR eluted in fractions 1 to 4. The silver stain (B) highlights proteins co-eluting with SR. As in this case a previous purification step had not been attempted there are many contaminating proteins with SR in the eluate. However, the ribosomal pellet fraction (lane 2) illustrates that many proteins are removed by the ultracentrifugation step that would otherwise be free to bind to the ion-exchange resin. Also, in this experiment it is clear that SR does bind to and elute from CM-sepharose as a complex and as such this could be used for the detergent exchange step.

Figure 3.8 Isolation of SR by ion-exchange chromatography using CM sepharose

240 OD_{280} units of microsomes recovered from ARY2 were solubilised in digitonin buffer and insoluble material pelleted (see section 2.19b). Ribosome associated membrane proteins were pelleted from the membrane extract (section 2.27) and the post-ribosomal supernate bound to CM sepharose overnight at 4°C on a roller in buffer containing 50 mM KOAc. The sepharose was washed with buffer containing 50 mM KOAc and eluted in buffer containing between 0.1 and 1 M KOAc as described in section 2.19b. 1/200th (lanes 1-6) and 1/5th (lanes 7-11) of samples loaded were analysed by SDS-PAGE, Western blotting and silver staining. Antibodies specific for SRα and SRβ were used to detect SR. The Western blot is shown in panel A while the silver stain is shown in panel B. The lanes in each are loaded with identical samples.

Lane

1 Load fraction (post-ribosomal supernate)
2 Ribosomal pellet
3 Unbound fraction
4 Wash 1
5 Wash 2
6 Wash 3
7 Elution 1
8 Elution 2
9 Elution 3
10 Elution 4
11 Elution 5


A.
1 2 3 4 5 6 7 8 9 10 11
66kDa
45kDa
29kDa
SRα
SRβ
B.
1 2 3 4 5 6 7 8 9 10 11
205kDa
116kDa
93kDa
66kDa
45kDa
29kDa

3.1.5 Discussion (Part 1)

I have shown that polyhistidine tags are insufficient to purify intact SRP receptor free from contaminants. I have also shown that protein A-tagging allows the purification of SR free of almost all contaminants, and that this tag, with the addition of a spacer, can be cleaved from SR allowing purification of native intact complex. Last, I have determined which of the four ion-exchange resins tested bind SR with sufficient affinity that they may be used to exchange detergent before reconstitution. However, it was clear from these experiments that SR expressed from its endogenous promoters and isolated from yeast was not in significant quantity to allow reconstitution or testing its activity. As a result over-expression and purification of SR from yeast was carried out.

Part 2: Over-expression of SR-protein A

3.2.1 Over-expression of SR-Protein A

The decision was made to over-express both SRα and SRβ in yeast under control of the *GAL1/10* promoter with the aim of obtaining large amounts of the complex containing SRβ-PrA. *GAL1/10* divergent promoters have been shown to induce up to 1000-fold over-expression of proteins compared to expression from their endogenous promoters (West *et al.*, 1987). SRβ is required for SRα to fold correctly *in vivo* (Young and Andrews, 1996) and co-expression of both subunits is necessary for solubility in *E.coli* (Fulga *et al.*, 2001). As a result, I decided to express both *SRP101* (SRα) and *SRP102* (SRβ) from the same plasmid and thus the *GAL1/10* divergent promoters were chosen. SRβ-CBD-PrA and SRα were amplified from genomic DNA of ARY1 used previously to purify SR (section 3.1.3-B). The products were cloned into pTR143 (Roberts *et al.*, 1994) as described (section 2.6 and Figure 3.9) under control of the divergent *GAL1/10* promoters yielding plasmid

Figure 3.9 Plasmid map of pAR143 and diagnostic digests

(A) Plasmid map of pAR143 (not to scale).

(B) Restriction endonuclease cleavage of three possible clones of SRα and SRβ-CBD-PrA.

Approximately 2 μg of each plasmid was digested with the restriction endonucleases *BamH*I and *EcoR*I according to the manufacturers instructions. The samples were electrophoresed through a 1% (w/v) agarose gel as described in section 2.6.

Lanes for (B)

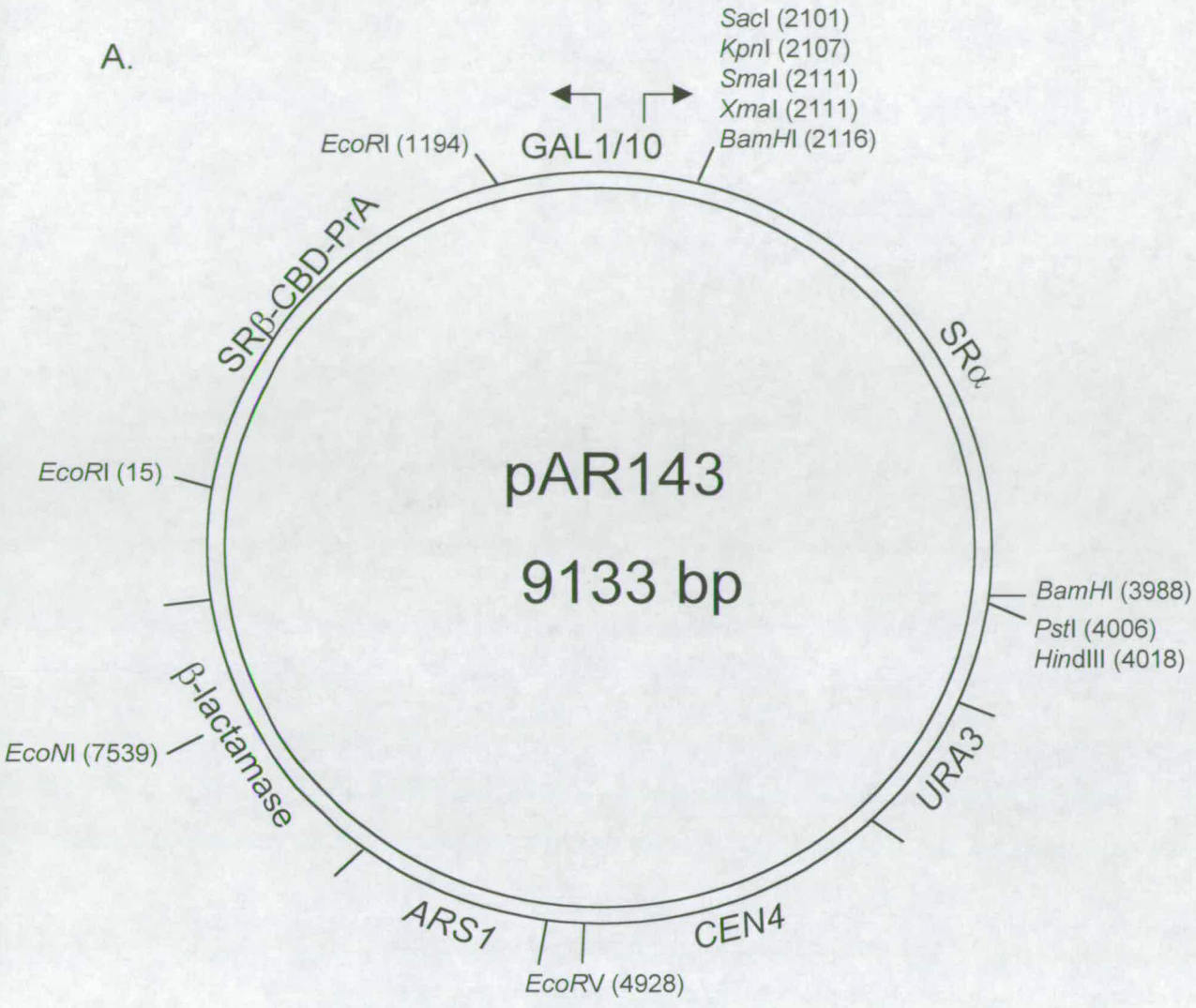
1 1 kb DNA ladder (NEB)

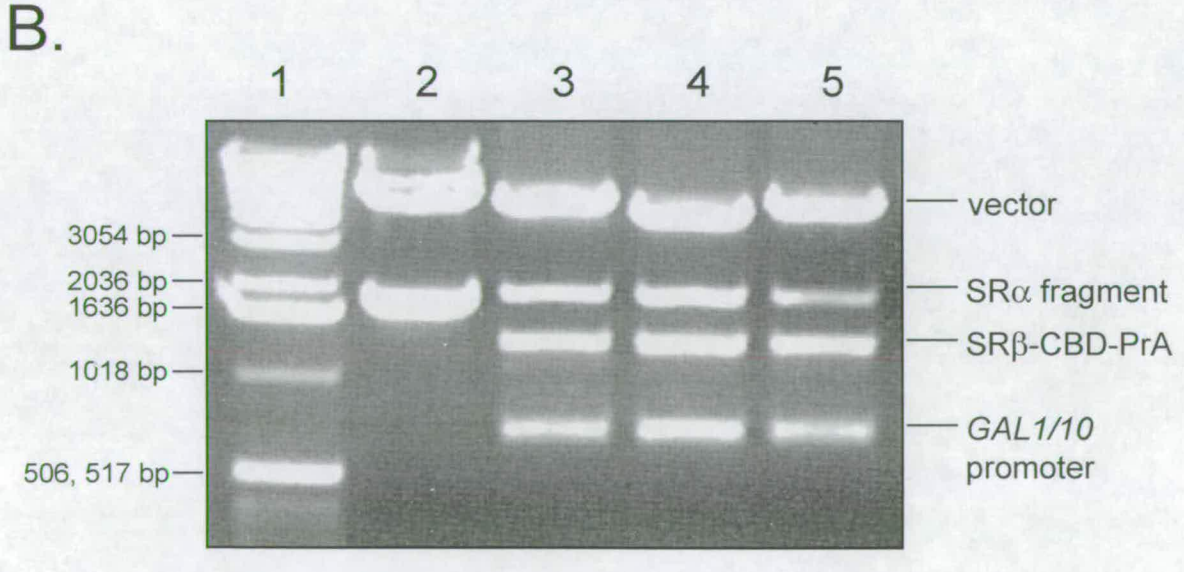
2 pTR143(SRα)

3 pAR143a

4 pAR143b

5 pAR143c


A.
SacI (2101)
KpnI (2107)
SmaI (2111)
XmaI (2111)
BamHI (2116)
EcoRI (1194)
GAL1/10
SRβ-CBD-PrA
SRα
EcoRI (15)
pAR143
9133 bp
BamHI (3988)
PstI (4006)
HindIII (4018)
β-lactamase
EcoNI (7539)
URA3
ARS1
CEN4
EcoRV (4928)


B.
1
2
3
4
5
3054 bp
2036 bp
1636 bp
1018 bp
506, 517 bp
vector
SRα fragment
SRβ-CBD-PrA
GAL1/10
promoter

pAR143. Sequencing confirmed that the coding sequences for SRβ-CBD-PrA and SRα in pAR143 contained no mutations from the wildtype *SRP101, SRP102* and CBD-PrA (Appendix 1). pAR143 was then transformed into several yeast strains to characterise expression. These were a wildtype strain (JDY3), a strain that lacked SR (JDY72) and a *reg1-501* strain (JDY225) which is deficient in glucose repression, thereby allowing expression from the *GAL1/10* promoter in glucose containing media. This was considered desirable as it would by-pass the need for a change of media during large-scale culture. In addition, it would allow maximum growth rates in glucose rather than slower growth on sucrose or raffinose, the most commonly used non-repressing carbon sources. Initial tests of induction (data not shown) indicated that the JDY225 gave, as expected, the most rapid response to the inducing carbon source galactose, and did not require shift to a non-repressing sugar before addition of galactose. Thus, this strain was chosen for further experiments. For reasons that are not clear all strains tested expressed the SRβ-CBD-PrA fusion at all times, whereas SRα was only expressed under inducing conditions (Figure 3.10a).

Optimisation of expression was carried out by inducing expression at several cell densities, for different times and with increasing concentrations of galactose. Figure 3.10a shows that induction of SRα and β was optimum when started at OD_{600} 0.2. Figure 3.10d shows that induction of SRα was increased by the addition of increasing galactose concentration up to 1% (w/v), at which point it was saturated. Thus, 1% (w/v) galactose was used in all subsequent experiments. Induction time courses revealed that induction of SRα is rapid and saturated by 1 hour (Figure 3.10e). One advantage of allowing induction to proceed beyond saturation at one hour is that the cells will multiply, increasing the final mass of cells harvested and, thus, the concentration of protein purified. This is especially relevant as maximum induction was achieved when the cells were induced when at a very low cell density (OD_{600} 0.2). Typically, cells were induced with galactose for 8-12 hours.

Figure 3.10 Characterisation of plasmid over-expression of SR

(A), (B) and (C) Determination of optimum optical density of yeast cells for maximum over-expression of SR. 10 ml cultures of strain JDY225(pAR143) were grown in glucose containing selective media to OD_{600} 0.2, 0.5 or 1.0 at which time protein over-expression was induced by the addition of galactose to a final concentration of 1% (w/v). Protein over-expression was induced for 6 hours after which time the cells were harvested, washed, lysed (section 2.19a) and proteins analysed by SDS-PAGE and Western blotting (section 2.15). Antibodies specific to SRα and SRβ were used to detect SR (table 3). In all cases a pre-induction sample was taken at the zero time point. (A) induction of cells at OD_{600} 0.2. (B) induction of cells at OD_{600} 0.5. (C) induction of cells at OD_{600} 1.0.

(D) Determination of optimum galactose concentration for maximum over-expression of protein. 10 ml cultures of strain JDY225(pAR143) were grown in glucose containing selective media to OD_{600} 0.2 at which time protein over-expression was induced by the addition of galactose to final concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 and 2.0% (w/v). Protein expression was allowed to proceed and proteins analysed as in (A).

(E) Determination of optimum length of induction. A 100 ml culture of strain JDY225(pAR143) was grown and protein over-expression induced as in (A) and the cells sampled every hour after induction. A pre-induction sample was taken at the zero time point. Proteins were analysed as in (A).

Lanes for (A), (B) and (C)

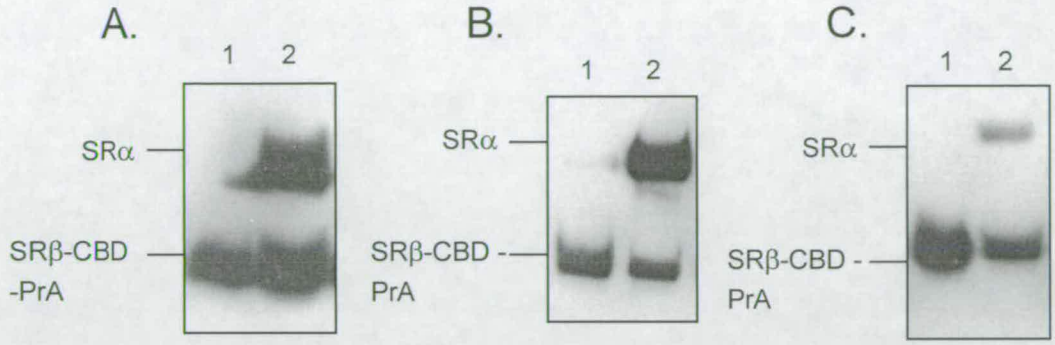
1 Uninduced JDY225(pAR143)
2 Induced JDY225(pAR143)

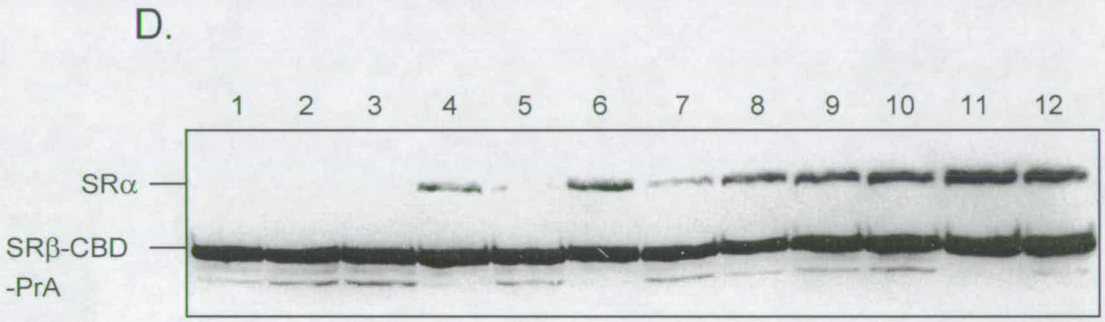
Lanes for (D)

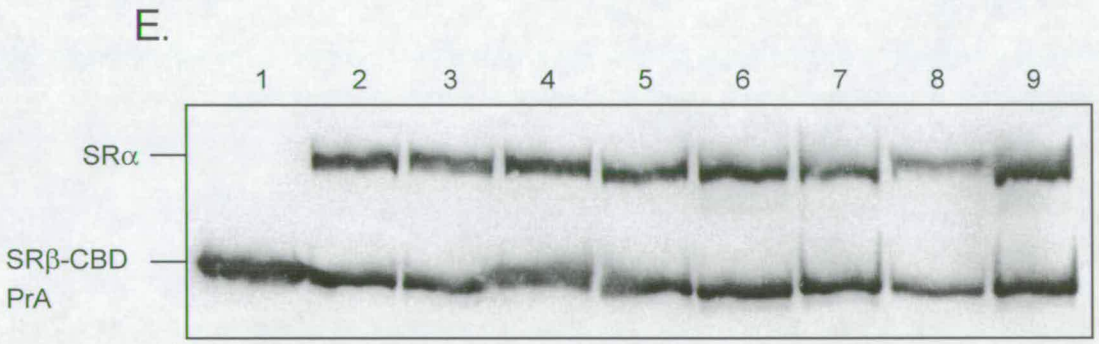
1 Uninduced JDY225(pAR143)
2 0.1% (w/v) galactose induction
3 0.2% (w/v) galactose induction
4 0.3% (w/v) galactose induction
5 0.4% (w/v) galactose induction
6 0.5% (w/v) galactose induction
7 0.6% (w/v) galactose induction
8 0.7% (w/v) galactose induction
9 0.8% (w/v) galactose induction
10 0.9% (w/v) galactose induction
11 1.0% (w/v) galactose induction
12 2.0% (w/v) galactose induction

Lanes for (E)

1 Uninduced JDY225(pAR143)
2 1 hour after induction
3 2 hours after induction
4 3 hours after induction
5 4 hours after induction
6 5 hours after induction
7 6 hours after induction
8 7 hours after induction
9 8 hours after induction


A.
1 2
SRα
SRβ-CBD
-PrA
B.
1 2
SRα
SRβ-CBD -
PrA
C.
1 2
SRα
SRβ-CBD -
PrA


D.
1 2 3 4 5 6 7 8 9 10 11 12
SRα
SRβ-CBD
-PrA


E.
1 2 3 4 5 6 7 8 9
SRα
SRβ-CBD
PrA

In order to characterise expression of SR from pAR143 further two additional experiments were carried out. The first judged the fold over-expression of SRα and β that was obtained compared to wild type expression. The second determined whether over-expression of SR was detrimental. To assess the amount of SR over-expressed from pAR143 microsomes were prepared from strain ARY4 (table 1) which expresses SRβ-CBD-PrA and non-tagged SRα from their normal chromosomal promoters, and from strain JDY225(pAR143). Dilutions of each were analysed by SDS-PAGE and Western blotting. The concentration of microsomes analysed in OD_{280} units per ml are indicated on figure legend 3.11. The asterix in lanes 2 and 7 of panel B indicates bands of protein corresponding to SRβ-CBD-PrA of approximately equal intensity, indicating that SRβ-CBD-PrA expressed from pAR143, was approximately 8-fold over-expressed compared with wild type. Comparing over-expression of SRβ-CBD-PrA with wild type expression of SRβ-CBD-PrA eliminated any artificial increase in signal intensity generated by protein A itself. The arrows in Figure 3.11 indicate two bands of protein corresponding to SRα. The band in lane 2 was that of over-expressed SRα and appears approximately 4-fold darker than the band in lane 9 (SRα expressed at wild type levels). As 64-fold more protein was analysed in lane 9 than in lane 2 it can be conservatively estimated that SRα was 250-fold over-expressed from pAR143 compared with wild type. Despite SRα and β not being present in equal amounts SRα appears to be stably associated with the microsomal membrane. Previous work indicated a membrane-binding site within SRα, also found to be necessary for binding to SRβ (Young and Andrews, 1996). In the absence of sufficient SRβ this binding site would be free to associate with microsomes. This was seen previously when in the absence of SRβ a little SRα was found associated with the ER membrane (Ogg *et al.*, 1998). As deletion mutants of SRβ no longer have functional SRP-dependent targeting

Figure 3.11 Estimation of fold over-expression

480 and 240 OD_{280} units of microsomes were recovered from strain ARY4 (wild type expression) and JDY225(pAR143) (over-expressed) respectively as described in section 2.19a. Increasing volumes of microsomes from both strains were analysed by SDS-PAGE and Western blotting. Antibodies specific for SRα (A) and SRβ (B) were used to detect SR. Microsomes isolated from strain JDY225(pAR143), panels A and B, lanes 1-5. Microsomes isolated from strain ARY4, panels A and B, lanes 6-10.

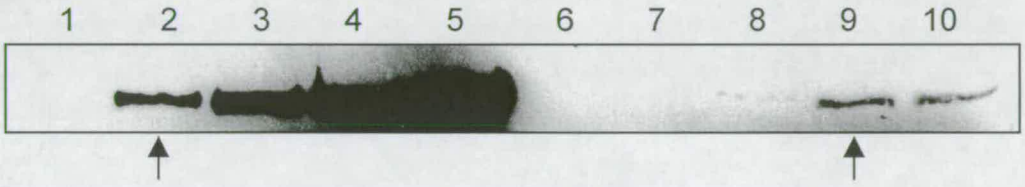
Lanes for (A)

1 0.04 OD_{280} units
2 0.2 OD_{280} units
3 0.4 OD_{280} units
4 0.8 OD_{280} units
5 2.0 OD_{280} units
6 0.64 OD_{280} units
7 3.2 OD_{280} units
8 6.4 OD_{280} units
9 12.8 OD_{280} units
10 32 OD_{280} units

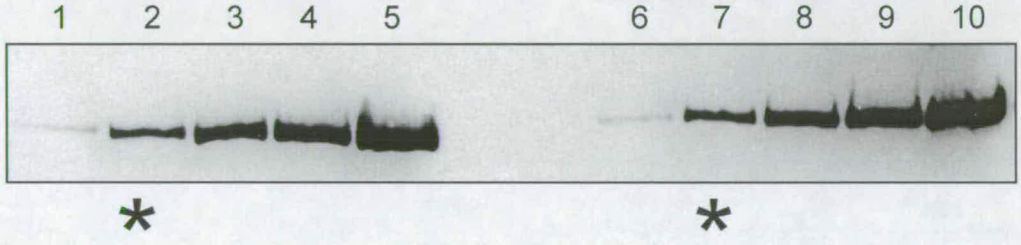
Lanes for (B)

1 0.04 OD_{280} units
2 0.2 OD_{280} units
3 0.4 OD_{280} units
4 0.8 OD_{280} units
5 2.0 OD_{280} units
6 0.32 OD_{280} units
7 1.6 OD_{280} units
8 3.2 OD_{280} units
9 6.4 OD_{280} units
10 16 OD_{280} units

A.



B.



pathways it can be assumed that any excess SRα attached to the membrane is inactive.

To assess whether overproduction of SR was detrimental, growth rates of strain JDY3(pAR143) were monitored in media containing one of three combinations of carbon sources: glucose; sucrose and raffinose or sucrose, raffinose and galactose. Only in the presence of galactose would over-expression of SRα be permitted, with SRβ over-expressed at all times (Figure 3.10). Figure 3.12 shows no significant difference in growth rate between cells with slightly over-expressed SRβ (square and rhombus data sets) and those with over-expressed SR (round data set). It was concluded that over-expression of SR was not detrimental to the cell.

3.2.2 Large-scale purification of SR

Having characterised over-expression from pAR143 large-scale (32 l) cultures of JDY225(pAR143) were grown, induced and SR purified from the cells (section 2.20). Figure 3.13 shows a typical purification of SR using this method. From the Western blot (A) it was estimated that approximately half SR in the load bound to the IgG sepharose with endogenous wild type SRβ also present that does not bind IgG as it is expressed without the "ZZ-tag" and all the SR eluted in eluates 1 and 2. Also in eluates 1 and 2 are 2 bands of lower molecular weight than SRβ-CBD. These can be attributed to degradation, one of these species appears to co-migrate with endogenous SRβ (compare panel A, lanes 1 and 5) and is presumably generated by degradative cleavage of the calmodulin binding domain creating "wild-type" SRβ. The slightly lower molecular weight band in lane 6 must be due to further degradation of the "wild-type" species. Silver staining (B) revealed several additional proteins in the elution fractions. The major contaminants in the purification are putatively identified as IgG and TEV protease from their predicted

Figure 3.12 Growth rates of yeast containing pAR143

3 x 100 ml cultures of yeast strain JDY3 (table 1) containing pAR143 were grown in rich growth medium containing glucose (square data set), sucrose and raffinose (rhombus data set) and sucrose, raffinose and galactose (round data set). Each culture was sampled at 2 hourly intervals and the OD_{600} of the cells measured. The $\log_{10}OD_{600}$ for each culture was plotted against time.

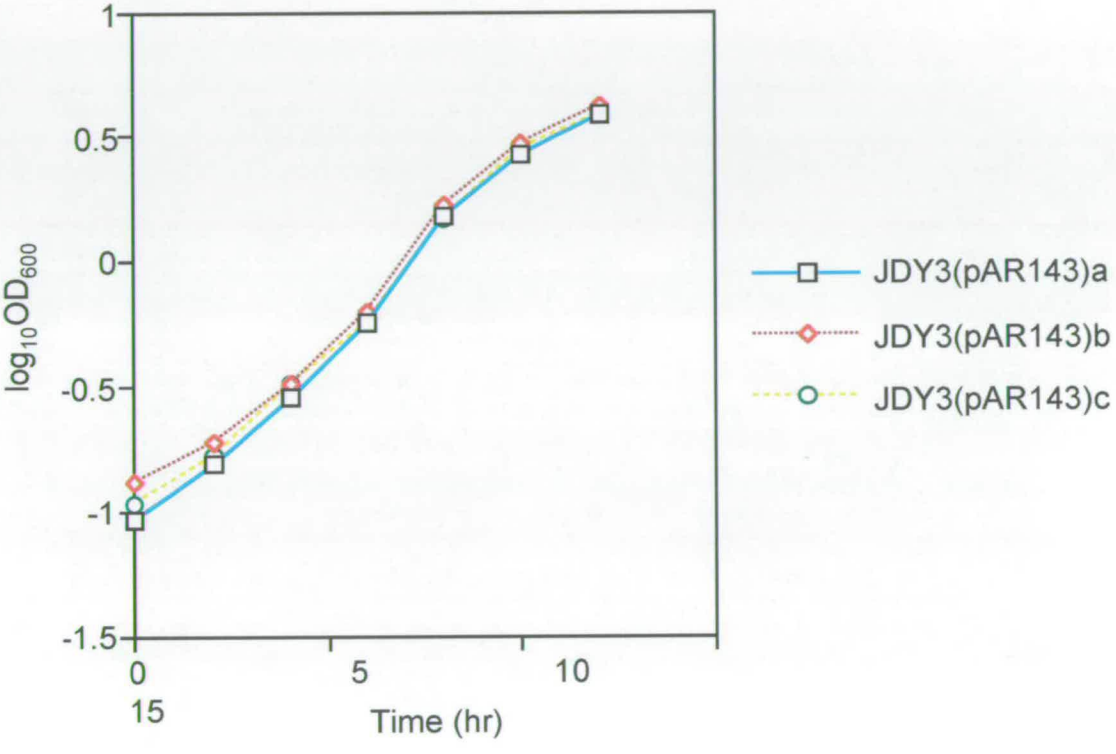

1
0.5
0
-0.5
-1
-1.5
log10OD600
0
5
10
15
Time (hr)
JDY3(pAR143)a
JDY3(pAR143)b
JDY3(pAR143)c

Figure 3.13 Purification of over-expressed SR

SR was over-expressed and purified from strain JDY225(pAR143) as described in section 2.20. 1/1000th (lanes 1-4) and 1/50th (lanes 5 and 6) of each sample were TCA precipitated and analysed by SDS-PAGE, Western blotting (A) and silver staining (B). Antibodies specific for SRα and SRβ were used to identify SR. Only the first 2 of the 5 elutions are shown as eluates 3-5 did not contain any SR.

Lanes for (A) and (B)

1 Load (solubilised membrane fraction minus RAMP proteins)

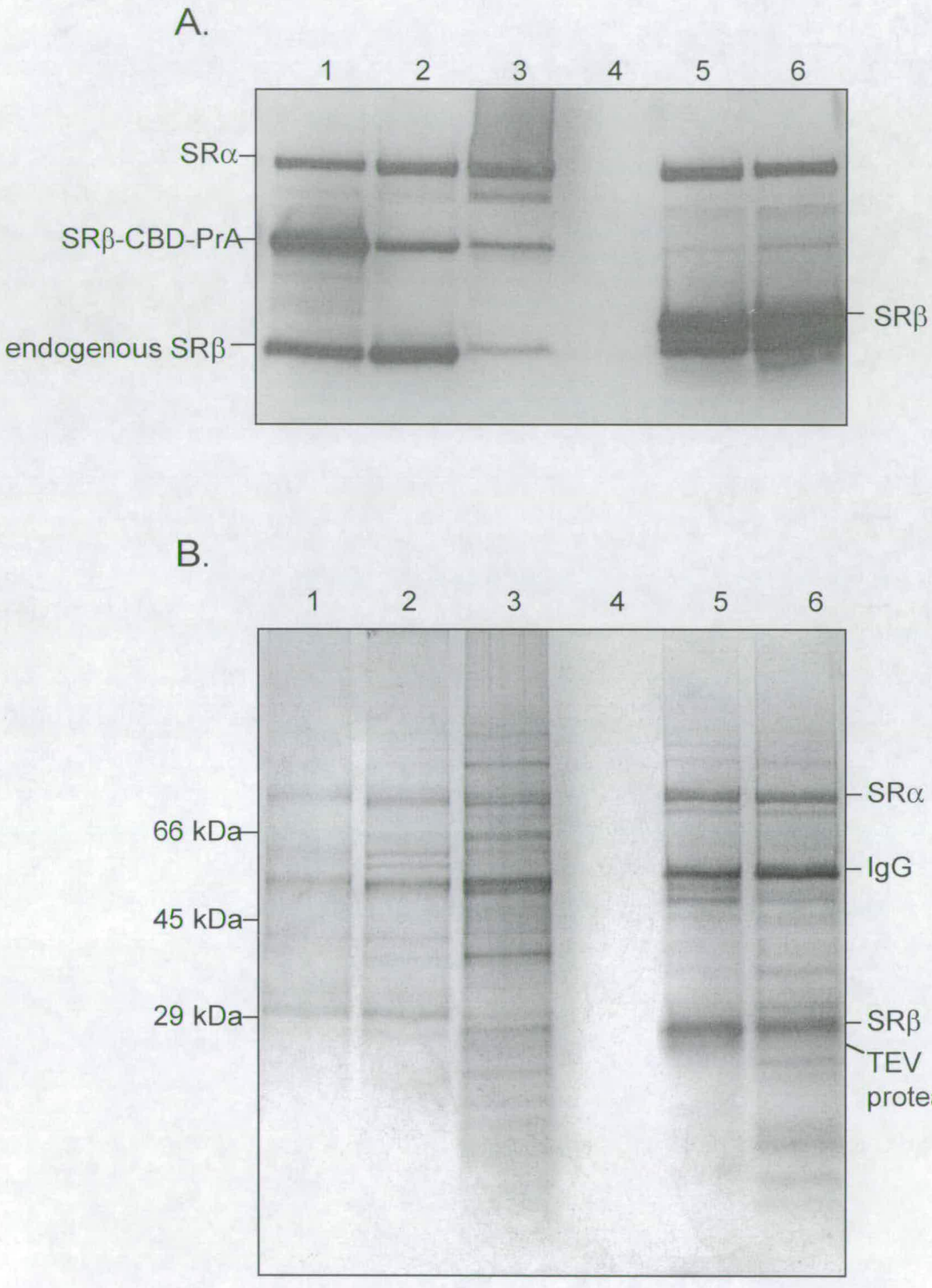
2 Unbound

3 Wash A

4 Wash B

5 Elution 1

6 Elution 2


A.
1
2
3
4
5
6
SRα
SRβ-CBD-PrA
endogenous SRβ
SRβ
B.
1
2
3
4
5
6
66 kDa
45 kDa
29 kDa
SRα
IgG
SRβ
TEV
protease

molecular weight. Other, less abundant contaminating proteins are present but in much smaller amounts than either subunit of SR. Almost 100 μg of protein was isolated, as estimated by Bradford protein assay, and available for reconstitution.

3.2.3 Discussion (Part 2)

In this Chapter protocols for purification of SR from large-scale culture of yeast strain ARY4 were developed and the conclusion made that over-expression was necessary. I detailed how SR was over-expressed. I described the purification of sufficient quantity of SR to allow reconstitution experiments to be undertaken. These experiments (Chapter 4) revealed that the purified material retained activity. To continue this work in the future my first concern would be to over-express each subunit of SR at the same level, thereby potentially allowing purification of greater amounts of SR and, perhaps, a reduction in the scale of cultures necessary to provide sufficient material for reconstitution experiments. Second, it should be possible to remove the two major contaminating proteins from the purified material (IgG and TEV protease) by incubation of the eluates with protein A sepharose and Ni-NTA agarose. Preliminary attempts to remove these contaminants failed due to protein degradation (data not shown); however, this should be easily rectified. Despite the necessity for further steps in this purification, it can be concluded that a protocol for the purification of active SR from yeast has been developed to the point at which this material can be tested *in vitro*.

Chapter 4

In vitro reconstitution of SRP-dependent translocation

4.1 Introduction

The development of strategies for the purification of yeast SR provided a crucial tool for the reconstitution of SRP-dependent targeting and translocation. A long-term goal of this work was reconstitution of this entire pathway. Thus, in addition to SR, other components necessary for SRP-dependent targeting also had to be purified. To this end, published protocols for the purification of the translocon and Kar2p (Beckmann *et al.*, 1997; McClellan *et al.*, 1998) were used and/or adapted. The Sec61p complex is necessary for all modes of translocation across the ER. Kar2p is known to be essential for post-translational translocation, but as discussed (introduction) has also been suggested to be necessary for co-translational translocation. In this chapter the preparation of components necessary for *in vitro* translation/translocation are described along with activity tests of the purified factors. Unfortunately, due to time constraints, the final experiments, testing translocation with only purified proteins, were not carried out.

4.2 Preparation and titration of essential components

In vitro translation/translocation reactions require a number of components. These are translation extract (YTE), capped mRNA (whether SRP-dependent or -independent) and microsomes (yRM) from appropriate yeast strains. Established protocols were used for the preparation of YTE and yRM, and mRNAs were prepared using commercial transcription kits (section 2.30).

Endogenous mRNA in YTE competes with exogenously added mRNA for translationally active ribosomes, and thus must be removed. YTE is therefore treated with micrococcal nuclease before use (section 2.29). As this nuclease requires calcium ions for activity, addition of the chelating agent EGTA effectively inhibits the enzyme and prevents degradation of subsequently added synthetic mRNA. The activity of the treated YTE was tested with mRNAs encoding either SRP-dependent or –independent proteins and compared with a previously prepared extract (a gift of

Jeff Brodsky, University of Pittsburgh) for comparison (data not shown). To ensure that the reactions were efficient each batch of mRNA was titrated against the YTE (data not shown).

Figure 4.1 shows titration of purified microsomes into translation reactions. mRNAs used in every translation reaction encoded a dipeptidylaminopeptidase-B-pro-alpha factor fusion ($D_{HC}\alpha F$) which is SRP-dependent (Ng *et al.*, 1996), and pre-pro-alpha factor (ppαF), which is SRP-independent (Hansen and Walter, 1988). Panel A shows the titration of microsomes isolated from a wild type yeast strain. These support translocation of both $D_{HC}\alpha F$ and ppαF substrates, observed by the signal sequence cleavage of ppαF and the appearance of glycosylated forms of both proteins. In panel B titrated into a translation reaction are microsomes derived from the SR-deficient strain JDY72 (table 1). Hence, translocation of $D_{HC}\alpha F$ is not seen as this protein requires SR (lanes 2-5, panel B; Ng *et al.*, 1996). However, the post-translational pathway is still active in these microsomes and ppαF is translocated (lanes 7-10, panel B). Often a low level of translocation of $D_{HC}\alpha F$ into SR-deficient microsomes was observed. This was presumably due to "leakage" through the post-translational translocation pathway as substrate fidelity within targeting pathways is often not absolute. A phenomenon observed here and seen with many different microsome preparations (compare panel A, lanes 1 and 4 and panel B, lanes 1 and 5) was inhibition of protein synthesis upon addition of a high concentration of microsomes. For that reason it was important to titrate each microsome preparation to determine the amount that gave maximal translocation while not impairing translation.

4.3 Purification of active bacterially over-expressed SR

While I was optimising the conditions for purification of over-expressed SR from yeast, a plasmid was generated in the laboratory from which SR could be

Figure 4.1 Titration of translocationally active microsomes

480 OD_{280} units of microsomes were prepared from yeast strains JDY3 (wild type) and JDY72 (*srp101::ADE2, srp102::HIS3)* (section 2.19a). The translocation activity of the prepared microsomes was assessed in an *in vitro* translation/translocation assay (sections 2.33 and 2.34). Microsomes prepared from strains JDY3 (A) and JDY72 (B) were tested.

(A) Lanes

1 O microsomes, $D_{HC}\alpha F$ mRNA

2 0.16 OD_{280} units, $D_{HC}\alpha F$ mRNA

3 0.32 OD_{280} units , $D_{HC}\alpha F$ mRNA

4 0.48 OD_{280} units , $D_{HC}\alpha F$ mRNA

5 0 microsomes, ppαF mRNA

6 0.16 OD_{280} units , ppαF mRNA

7 0.32 OD_{280} units , ppαF mRNA

8 0.48 OD_{280} units , ppαF mRNA

(B) Lanes

1 0 microsomes, $D_{HC}\alpha F$ mRNA

2 0.16 OD_{280} units , $D_{HC}\alpha F$ mRNA

3 0.32 OD_{280} units , $D_{HC}\alpha F$ mRNA

4 0.48 OD_{280} units , $D_{HC}\alpha F$ mRNA

5 0.64 OD_{280} units , $D_{HC}\alpha F$ mRNA

6 0 microsomes, ppαF mRNA

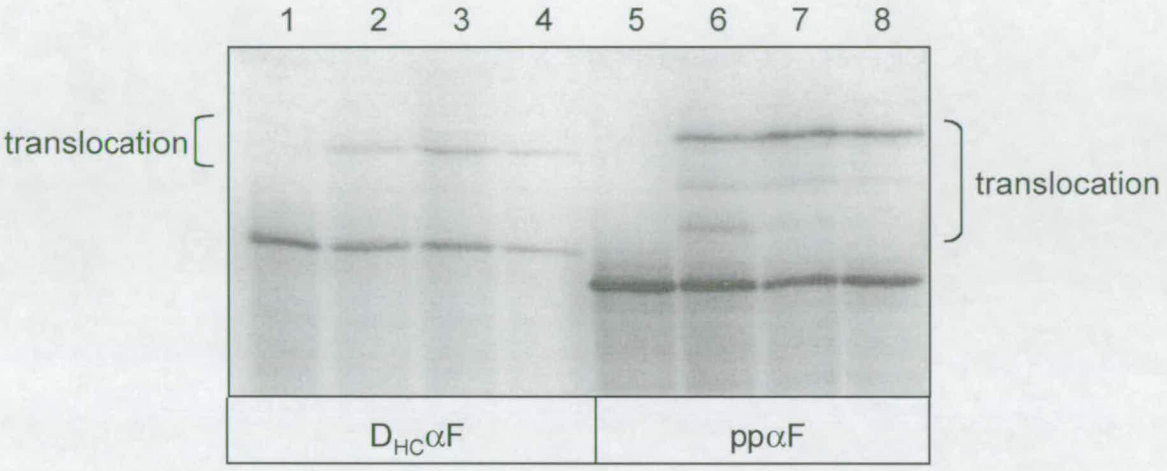
7 0.16 OD_{280} units , ppαF mRNA

8 0.32 OD_{280} units , ppαF mRNA

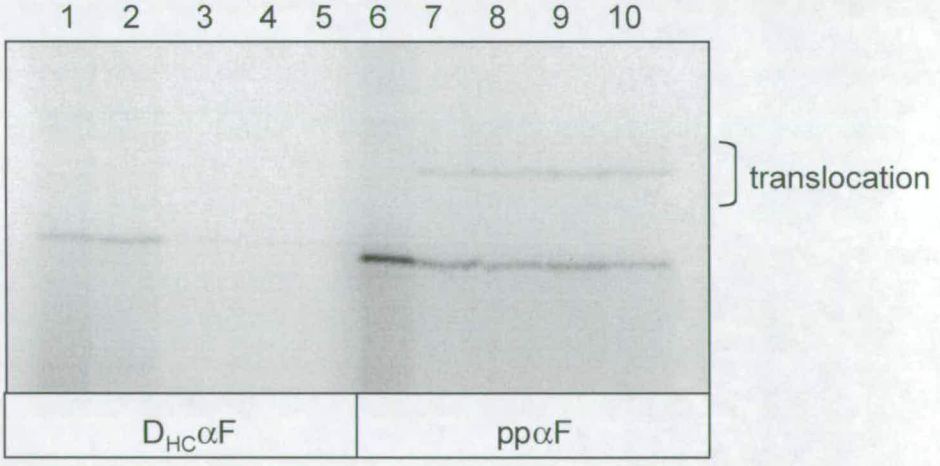
9 0.48 OD_{280} units , ppαF mRNA

10 0.64 OD_{280} units , ppαF mRNA

A.


1 2 3 4 5 6 7 8
translocation
translocation
$D_{HC}\alpha F$
ppαF

B.


1 2 3 4 5 6 7 8 9 10
translocation
$D_{HC}\alpha F$
ppαF

over-expressed in bacteria as a soluble complex lacking the transmembrane domain of, and with GST fused to, the N-terminus of SRβ (Jeremy Brown). The plasmid was created initially for purification of SR for crystallisation trials. If addition of this purified SR to microsomes recovered from a SR deficient yeast strain restored translocation activity it would provide an important additional tool for analysis of SRP-dependent targeting *in vitro*. Figure 4.2 shows the purification of the SR-GST from *E. coli.* (section 2.14).

The purified SR-GST protein was tested *in vitro* to determine if it was active and would stimulate translocation of an SRP-dependent substrate. Figure 4.3 shows that addition of purified SR-GST to translation reactions containing microsomes lacking endogenous SR resulted in increased translocation of the SRP-dependent substrate D_{HC}αF (compare lane 3 with 5 and 7) while the translocation of ppαF was unaffected (compare lane 4 with 6 and 8). Addition of 2 μg of SR-GST increased the translocation of D_{HC}αF over two-fold (compare lanes 3 and 5) while addition of 4 μg resulted in a three-fold increase (compare lanes 3 and 7). Thus, despite lack of a transmembrane domain in SRβ-GST the protein, together with SRα, is able to stimulate the translocation of an SRP-dependent substrate. There is a precedent for the activity of SR-ΔTMβ in yeast (Ogg *et al.*, 1995) as a yeast strain containing this allele grew well and had no observable defects in translocation. In addition, while this work was in progress a similar construct of mammalian SR was also shown to be active *in vitro* (Fulga *et al.*, 2001).

It was of interest to determine if SR(ΔTM)-GST associated with components of the microsomal membrane or only interacted with the membranes during the SRP-dependent targeting cycle. To test this, purified SR-GST alone, SR-deficient microsomes alone and purified SR-GST mixed with SR-deficient microsomes were incubated on ice for 30 min, after which they were subjected to centrifugation at 13,000 rpm for 15 min to pellet microsomes and any insoluble material. Figure 4.4 shows that in the absence of microsomes SR(ΔTM)-GST remains entirely in solution

Figure 4.2 Purification of SR-GST

SRα and SRβ(ΔTM)-GST were over-expressed in strain M15pREP4(pJE97) and purified according to the protocol described (section 2.14). 1/2000th (lanes 1-5) and 1/50th (lanes 6-8) of samples were analysed by SDS-PAGE and Coomassie staining.

Lanes

1 Pre-induction

2 Induced cells

3 Load

4 Flow through

5 Wash

6 Elution 1

7 Elution 2

8 Dialysate

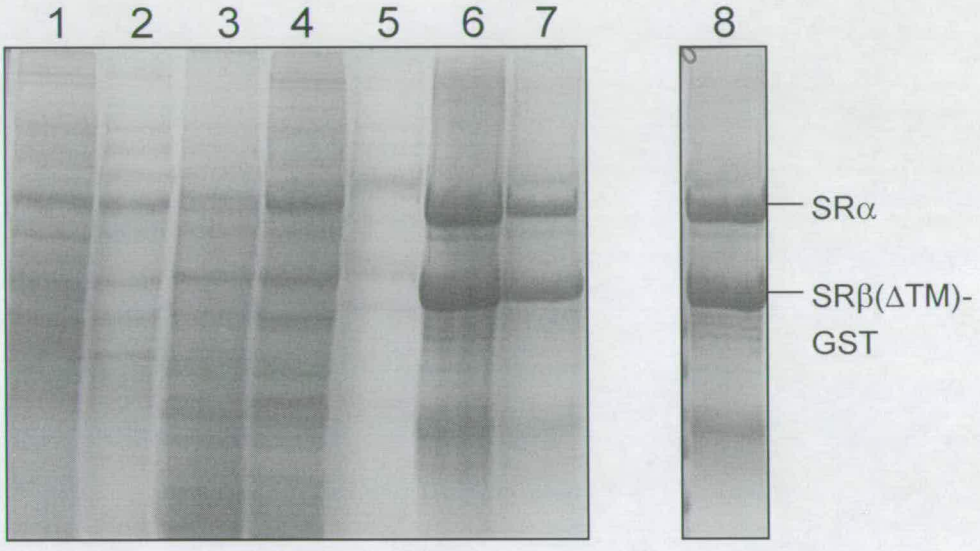

1
2
3
4
5
6
7
8
SRα
SRβ(ΔTM)-
GST

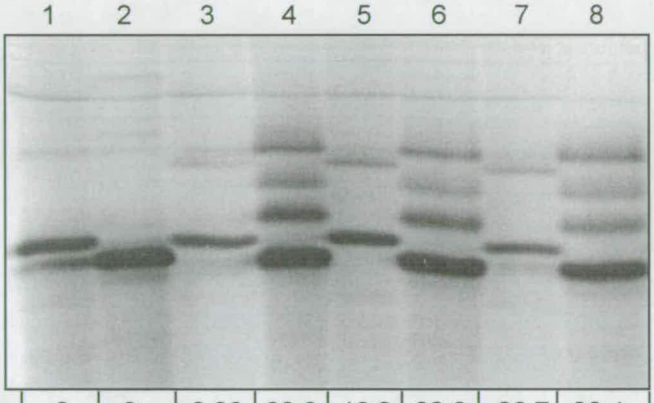
Figure 4.3 Activity test of purified SR-GST

SR was bacterially over-expressed and purified (section 2.14, Figure 4.2). Zero (lanes 1-4), 2 μg (lanes 5 and 6) and 4 μg (lanes 7 and 8) of the proteins were added to *in vitro* translation/translocation reactions (section 2.33 and 2.34) containing 0 OD_{280} units (lanes 1 and 2) or 0.32 OD_{280} units (lanes 3-8) of microsomes recovered from yeast strain JDY72 (table 1) (section 2.19a). $1/3^{rd}$ of each reaction were analysed by SDS-PAGE and phosphoimaging.

Lanes

1, 3, 5 and 7 $D_{HC}\alpha F$ mRNA

2, 4, 6 and 8 ppαF mRNA



% translocation	0	0	6.96	29.9	16.2	22.2	20.7	26.4
microsomes	-	-	+	+	+	+	+	+
SR-GST (μg)	0	0	0	0	2	2	4	4

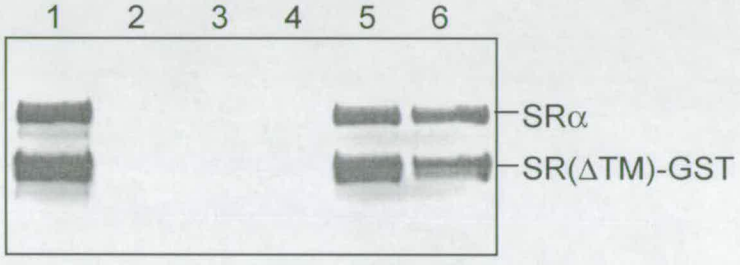
Figure 4.4 Association of SR-GST with microsomal membranes

2 μg SR(ΔTM)-GST alone (lanes 1 and 2), 0.32 OD_{280} units of microsomes recovered from JDY72 (SR deficient) alone (lanes 3 and 4) and 2 μg SR(ΔTM)-GST mixed with 0.32 OD_{280} units SR deficient microsomes (lanes 5 and 6) were incubated on ice for 30 min. Microsomes were pelleted by centrifugation at 13,000 rpm for 15 min at 4°C in a microcentrifuge. All of the supernate and pellet were analysed by SDS-PAGE and Western blotting. Antibodies specific for SRα and SRβ were used to detect SR.

Lanes

1, 3 and 5 Supernate

2, 4 and 6 Pellet


1
2
3
4
5
6
SRα
SR(ΔTM)-GST

and does not pellet upon centrifugation. However, in the presence of microsomes SR(ΔTM)-GST is found in equal quantity in the supernatant as in the pellet confirming that the proteins associated with microsomal membranes.

4.4 Reconstitution of translocation using purified yeast ER membrane proteins

4.4.1 Titration of lipid for protein reconstitution

Previously post-translational translocation has been reconstituted with purified yeast proteins (Panzner *et al.*, 1995). Co-translational translocation has been reconstituted with purified mammalian proteins (Görlich and Rapoport, 1994). However, co-translational translocation with yeast components had not been accomplished. Therefore, demonstrating co-translational translocation with proteoliposomes prepared with total yeast microsomal membrane proteins was imperative before embarking on assays using purified SR or other membrane proteins. Thus, yeast ER-derived microsomes were solubilised and proteins reconstituted into proteoliposomes using a protocol previously used to demonstrate activity of proteins involved in post-translational translocation (Brodsky *et al.*, 1995).

As noted earlier (section 4.2) high concentrations of microsomes inhibit translation reactions. Initial assays of translocation in the presence of proteoliposomes showed severely reduced translocation. This effect was substantially more pronounced with proteoliposomes than microsomes and was also seen when liposomes were added to *in vitro* translation reactions, indicating that the presence of lipids was the detrimental factor (data not shown).

Proteoliposomes are generated by mixing purified and/or solubilised proteins with azolectin (the source of lipid for the proteoliposomes) and carrying out a freeze-thaw cycle (section 2.32). Given that translation is inhibited by proteoliposomes it was considered that the volume of lipid added before the freeze-thaw step and thus the

lipid: protein ratio, as well as the volume of proteoliposomes added to the translation/translocation reactions might be crucial. Therefore, titration experiments were carried out to determine a) the volume of proteoliposomes that could be added to the reaction without substantially decreasing the translation rate and b) whether the lipid: protein ratio in the reconstitutions could be reduced. Figure 4.5 shows the comparative levels of translation obtained in a reaction with decreasing lipid: protein ratios used to reconstitute protein and with increasing volumes of proteoliposomes added to the translation/translocation reaction. It is apparent that decreasing the lipid: protein ratio of the proteoliposomes added to the reaction results in a corresponding increase in translation (Figure 4.5, panel A, compare lanes 3, 6 and 9 with 12). In addition, decreasing the volume of proteoliposomes added to the reaction leads to a corresponding increase in translation (panels A and B, compare lanes 4, 7, 10 and 13 with lanes 2, 5, 8 and 11). The effect is more pronounced when translating the co-translationally dependent substrate $D_{HC}\alpha F$ than the post-translationally dependent substrate ppαF. However, when one third of the reaction volume consists of proteoliposomes (as described in McClellan *et al.*, 1998) translation of both substrates diminishes almost entirely (Figure 4.5, panels A and B, lanes 4, 7, 10 and 13). As this is the case for both SRP-dependent and –independent substrates, this suggests a universal inhibition of ribosome function in the presence of high concentrations of lipid and not a specific substrate-related defect.

Decreasing the lipid: protein ratio when reconstituting proteins would be expected to lead to a decrease in incorporation of protein into proteoliposomes and thus, a corresponding decrease in translocation. However, the higher the concentration of proteoliposomes in a translation reaction the lower the level of translation that is observed. Therefore, a balance is necessary between observable translocation and necessary translation. From the titration experiments detailed here it was concluded that using a lipid: protein ratio of 1:1 and adding one-sixth the total reaction volume of proteoliposomes was a suitable compromise (section 2.33 and 2.34).

Figure 4.5 Titration of lipid

4 OD_{280} units of microsomes each, recovered from strain JDY3 (wild type) were solubilised and the protein fraction reconstituted into proteoliposomes (section 2.32). Four different ratios of lipid:protein were analysed: 2:1 (panels A and B, lanes 2-4), 1.6:1 (panels A and B, lanes 5-7), 1.3:1 (panels A and B, lanes 8-10) and 1:1 (panels A and B, lanes 11-13). 5, 10 and 20 µl of the proteoliposomes generated were added to translation reactions containing $D_{HC}\alpha F$ (A) and ppαF (B) mRNA and their effect on its translation monitored by SDS-PAGE and phosphoimaging.

Lanes for (A) and (B)

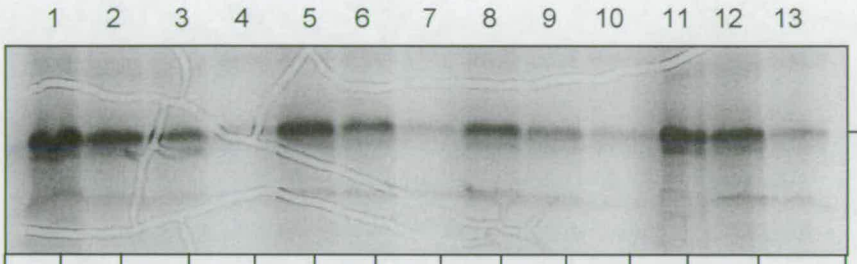
1 no proteoliposomes

2, 5, 8 and 11 5 µl proteoliposomes

3, 6, 9 and 12 10 µl proteoliposomes

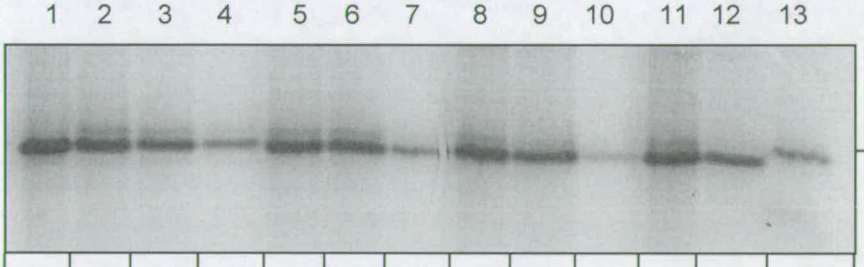
4, 7, 10 and 13 20 µl proteoliposomes

A.



Proteoliposomes (μl)	0	5	10	20	5	10	20	5	10	20	5	10	20
Lipid:protein ratio	0	2:1			1.6:1			1.3:1			1:1		

B.



Proteoliposomes (μl)	0	5	10	20	5	10	20	5	10	20	5	10	20
Lipid:protein ratio	0	2:1			1.6:1			1.3:1			1:1		

4.4.2 Reconstitution of Kar2p

The ER lumenal Hsp70 homologue Kar2p is essential for post-translational translocation (Lyman *et al.*, 1995; Panzner *et al.*, 1995; Matlack *et al.*, 1997). As discussed (introduction) this protein binds the translocon component Sec63p and is thought to provide a driving force for translocation (Matlack *et al.*, 1999). Recent evidence suggests that it is also essential for co-translational translocation both *in vitro* and *in vivo* (Brodsky *et al.*, 1993; Young *et al.*, 2001). To test the requirement for Kar2p in co-translational translocation Kar2p was purified and tested for its post-translational translocation promoting activity before incorporation into proteoliposomes to test its ability to stimulate co-translational translocation. Kar2p was purified from bacterial cells expressing pMR2623 (McClellan *et al.*, 1998) (see section 2.13). Such a purification is shown in Figure 4.6 (panels A, B and C). To test the activity of this purified protein without endogenous Kar2p activity microsomes were isolated from a yeast stain defective in Kar2p (RSY586; *kar2-159*). Although generation of a deletion mutant of Kar2p is not possible (Kar2p is essential in yeast) certain temperature sensitive mutations allow the isolation of microsomes with insufficient Kar2p activity to stimulate translocation, of which *kar2-159* is one (Vogel *et al.*, 1990; Brodsky *et al.*, 1995).

Purified Kar2p was included in a reconstitution of proteins from solubilised RSY586 microsomes at 30 μg per reaction (as in the published protocol (McClellan *et al.*, 1998)). Figure 4.6d shows translocation of ppαF only in the presence of added Kar2p. In addition to showing that the purified Kar2p was active *in vitro*, this experiment also demonstrates that identification of translocated species is different when using proteoliposomes than when using native microsomes. Protein translocated into microsomes are typically recognised as species lacking signal sequences and/or through N-glycosylation. When using proteoliposomes these species do not appear, indicating that the solubilisation and reconstitution process removes and/or inactivates the enzymes from the ER lumen responsible for signal sequence cleavage (SPase) and glycosylation (OSTase). Therefore, proteins translocated into proteoliposomes were detected by incubating the

Figure 4.6 Purification of Kar2p

Kar2p was over-expressed and purified from strain DH5α(pMR2623) (table 2) as described in section 2.13. (A) Induction, (B) Ni-NTA affinity chromatography, (C) Ion-exchange chromatography on Q- sepharose. 1/200th of all fractions in (A), (B) and (C) were analysed by SDS-PAGE and Coomassie staining.

Lanes for (A)

1 Uninduced cells

2 Induced cells

Lanes for (B)

1 Load (clarified cell extract)

2 Wash 1

3 Wash 2

4 Wash 3

5-14 Elutions 1-10

Lanes for (C)

1 Load (Ni-NTA agarose eluate)

2 Unbound

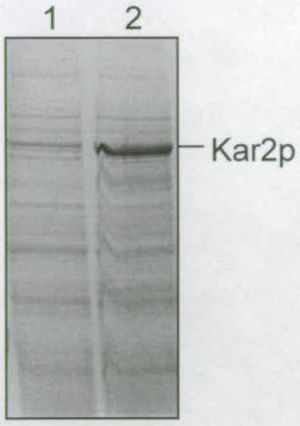
3 Wash 1

4 Wash 2

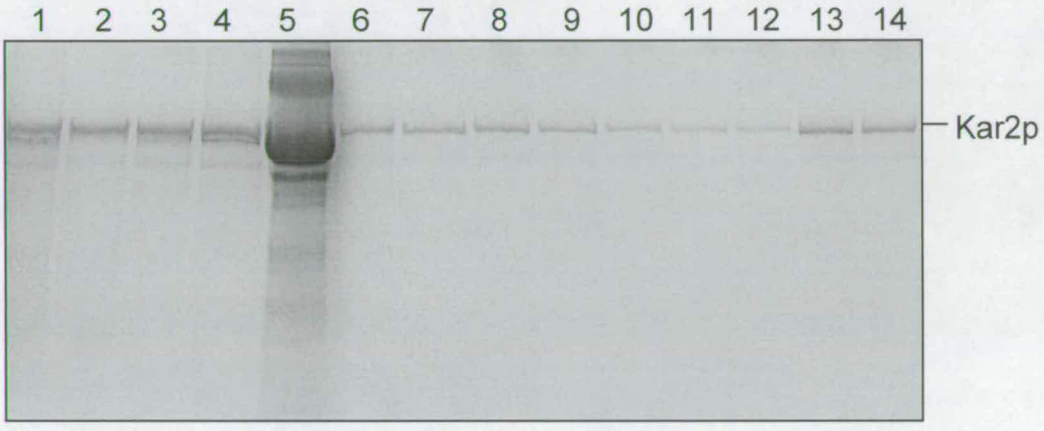
5-15 Elutions 1-11

16 Dialysate of pooled elutions 4-8 (lanes 8-12)

A.


1
2
Kar2p

B.


1
2
3
4
5
6
7
8
9
10
11
12
13
14
Kar2p

C.

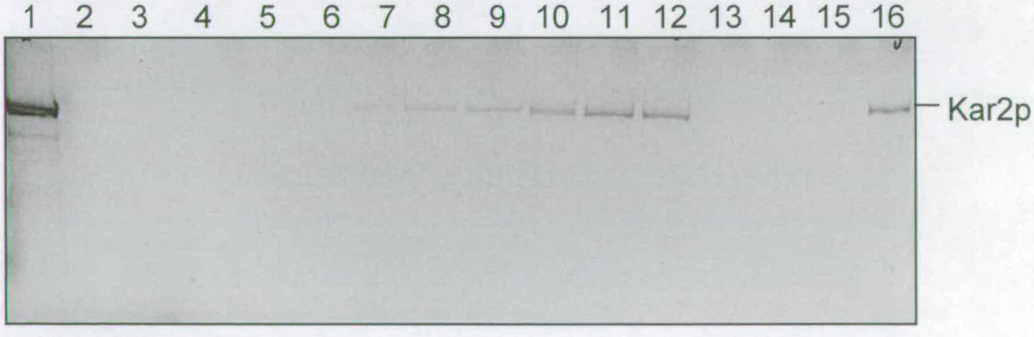

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Kar2p

Figure 4.6(D) Reconstitution of Kar2p

Proteoliposomes were reconstituted with or without 30 μg of Kar2p and proteins solubilised from 4 OD_{280} units of microsomes recovered from strain RSY586 (table 1) as described in section 2.32. No proteolipsomes (panel D, lanes 1-3), 10 μl of proteolipsomes without additional Kar2p (panel D, lanes 4-6) and 10 μl of proteolipsomes with additional Kar2p (panel D, lanes 7-9) were included in translation/translocation reactions (sections 2.33 and 2.34) containing ppαF mRNA. Translocation of the *in vitro* radiolabelled protein was monitored by the addition of trypsin and/or tritonX-100 to the reactions, after which 1/3rd of each was analysed by SDS-PAGE and phosphoimaging.

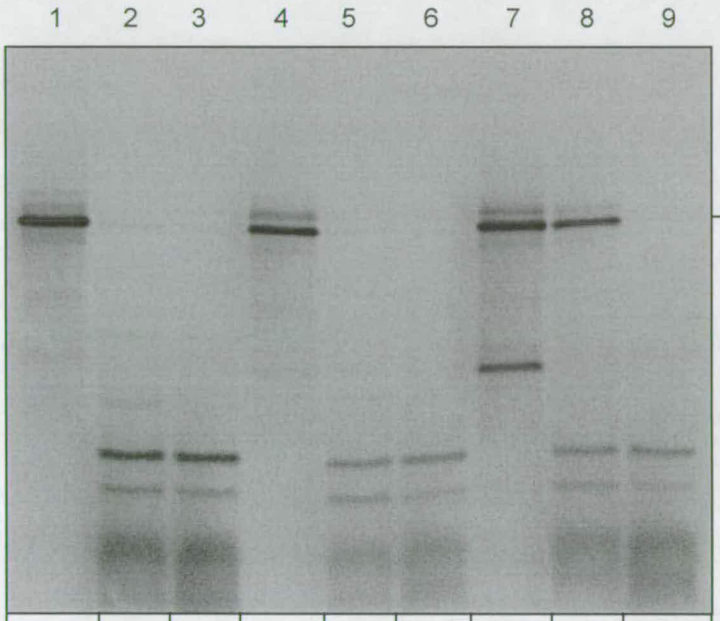
Lanes

1, 4 and 7 No additions

2, 5 and 8 5 μg trypsin

3, 6 and 9 5 μg trypsin, 1% (v/v) tritonX-100

D.



Trypsin (μg)	0	5	5	0	5	5	0	5	5
TritonX-100 (% (v/v))	0	0	1	0	0	1	0	0	1

translation/translocation reaction with trypsin, to degrade non-translocated and thus non-protected protein. Addition of trypsin and tritonX-100 (to disrupt the proteoliposomes) results in degradation of all protein. Thus, in Figure 4.6d comparison of lanes 2, 5 and 8 reveals that only lane 8 contains ppαF that is protected from the addition of trypsin to the reaction. The ppαF was translocated into the lumen of the proteoliposomes only in the presence of microsomal proteins and purified Kar2p. This confirms that the Kar2p purified was active in promotion of the translocation of an SRP-independent substrate.

4.5 Reconstitution of co-translational translocation

4.5.1 Reconstitution of purified yeast Kar2p and SR

Having determined that the purified Kar2p was active in promoting post-translational translocation the next step was to attempt to reconstitute co-translational translocation. In order to restore Kar2p activity to microsomes isolated from strain RSY586 30 μg of purified protein must be incorporated. However, when adding purified Kar2p to wild type microsomes to ensure adequate Kar2p activity is retained only 15 μg of protein need be reconstituted (McClellan *et al.*, 1998). Microsomes isolated from wild type and SR deficient yeast strains (section 2.19a) were solubilised and reconstituted in the presence of purified Kar2p into proteoliposomes that were assayed for their ability to translocate D_{HC}αF and ppαF. Figure 4.7, panel A shows translocation of D_{HC}αF and ppαF into proteoliposomes reconstituted from proteins resident in wild type microsomes. Both SRP-dependent and –independent substrates are translocated. Figure 4.7, panel B shows the translocation of D_{HC}αF and ppαF into proteoliposomes. Even in the presence of Kar2p proteoliposomes reconstituted from the proteins resident in SRΔ microsomes cannot support SRP-dependent translocation (panel B, lane 2). However, as seen previously

Figure 4.7 Reconstitution of microsomal membrane proteins

Membrane proteins solubilised from microsomes recovered from strains JDY3 (wild type) and JDY72 (*srp101::ADE2, srp102::HIS3*) (table 1) were reconstituted into proteolipsomes in the presence of 15 μg purified Kar2p. 10 μl proteoliposomes reconstituted with wild type proteins (A) and 10 μl proteoliposomes reconstituted with SR-deficient proteins (B) were included in translation/translocation reactions containing D_{HC}αF (panels A and B, lanes 1-3) or ppαF (panels A and B, lanes 4-6) mRNA. Translocation was monitored by addition of trypsin and/or tritonX-100 and analysis by SDS-PAGE and phosphoimaging.

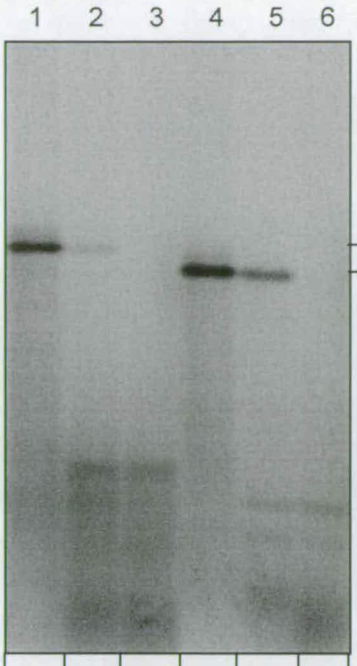
Lanes for (A) and (B)

1 and 4 No additions

2 and 5 5 μg trypsin

3 and 6 5 μg trypsin, 1% (v/v) tritonX-100

A.



Trypsin (μg)	0	5	5	0	5	5
TritonX-100 (% (v/v))	0	0	1	0	0	1
% translocation	11.1			28.2		

B.



Trypsin (μg)	0	5	5	0	5	5
TritonX-100 (% (v/v))	0	0	1	0	0	1
% translocation	6.9			26.9		

with intact SRΔ microsomes (Figure 4.1, panel B) they do support the translocation of SRP-independent substrates (lane 5). In this experiment it is clear that the efficiency of translocation of $D_{HC}\alpha F$ is poor relative to the translocation of ppαF (compare panel A, lanes 2 and 5). This is typical of *in vitro* reconstituted co-translational translocation but despite the low efficiency of the reaction it is reproducible. One method of increasing the efficiency of the reaction may be to increase the concentration of SRP in the translation extract by supplementing with purified SRP. This experiment showed that it is possible to reconstitute microsomal membrane proteins and retain the activity of those proteins required for co-translational translocation.

Having established conditions under which co-translational translocation could be reconstituted using solubilised ER membrane proteins, the next step was to assay the activity of purified yeast SR. The purification of SR and concentration of protein added *in vitro* are detailed in Chapter 3 and Section 2.20. Proteins solubilised from microsomes without endogenous SR (SRΔ) were used in the reconstitution along with SR, Kar2p or both. Translocation of both $D_{HC}\alpha F$ and ppαF was assayed and, as before, detected by the presence of protease-protected material (Figure 4.8). PpαF was translocated in all of the reactions except when Kar2p was omitted from the reconstitution or when SR was added. As Kar2p is absolutely required for translocation of SRP-independent substrates this result was not surprising. However, SR inhibited the translocation of ppαF. This was surprising, and perhaps the presence of a high concentration of SR resulted in generation of translocation sites specific for SRP-dependent translocation thereby limiting the capacity of the membranes for SRP-independent translocation. In contrast, translocation of $D_{HC}\alpha F$ was stimulated by addition of purified SR and Kar2p. The purified receptor is thus active in promoting SRP-dependent translocation and translocation is dependent also on the presence of active Kar2p. The basal level of translocation in lane 2 (6.9%) is enhanced substantially by the addition of SR and Kar2p (lane 20, 14.5%). Therefore, SR over-expressed and purified from yeast by the protocol described yields active

Figure 4.8 Reconstitution of purified SR

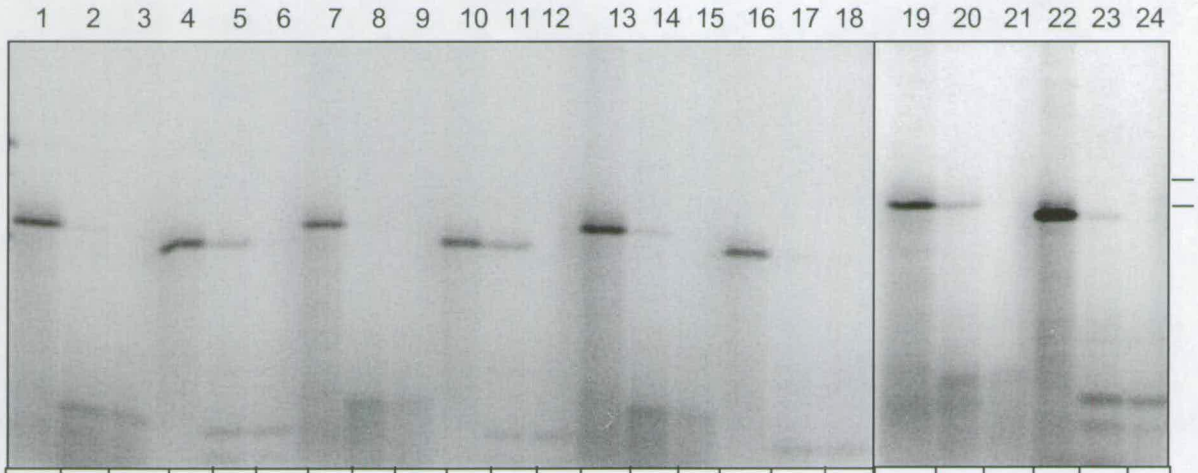
SR was over-expressed and purified from strain JDY225(pAR143) (table 1) as described in section 2.20 (Figure 3.13). Membrane proteins solubilised from microsomes recovered from strain JDY72 (table 1) were reconstituted into proteoliposomes with approximately 9.5 μg purified protein containing SR in the absence or presence of 15 μg purified Kar2p (section 2.32). 10 μl of proteoliposomes without any additional purified proteins (lanes 1-6), 10 μl of proteoliposomes containing only 15 μg Kar2p (lanes 7-12) and 10 μl of proteolipsomes containing only SR (lanes 13-18) or SR and Kar2p (lanes 19-24) were included in translation/translocation reactions containing $D_{HC}\alpha F$ (lanes 1-3, 7-9, 13-15 and 19-21) or ppαF (lanes 4-6, 10-12, 16-18 and 22-24) mRNA. Translocation was monitored be addition of trypsin and/or tritonX-100 to each reaction. 1/3rd of each reaction was analysed by SDS-PAGE and phosphoimaging.

Lanes

1, 4, 7, 10, 13, 16, 19 and 22 No additions

2, 5, 8, 11, 14, 17, 20 and 23 5 μg trypsin

3, 6, 9, 12, 15, 18, 21 and 24 5 μg trypsin, 1% (v/v) tritonX-100



Trypsin (μg)	0	5	5	0	5	5	0	5	5	0	5	5	0	5	5	0	5	5	0	5	5	0	5	5
TritonX-100 (% (v/v))	0	0	1	0	0	1	0	0	1	0	0	1	0	0	1	0	0	1	0	0	1	0	0	1
Kar2p (μg)	0						15						0						15					
SR (μg)	0						0						9.5						9.5					
% translocation	6.9			18.5			5.8			27.4			7.7			6.9			14.5			4.0		

protein that can restore the SRP-dependent targeting activity to microsomes derived from an SRΔ yeast strain only in the presence of active Kar2p. It should be noted that the preparation of SR used contained a number of contaminants. Of these, the major contaminants are IgG and TEV protease that would not be expected to stimulate membrane targeting or translocation. The other contaminants have yet to be identified. While it is possible that these are responsible for the protein targeting activity that is seen upon addition of the purified protein to the assay this is considered unlikely, particularly as the only factor missing from the membranes used in the reconstitution was SR itself.

In an attempt to titrate the amount of added SR necessary for translocation of $D_{HC}\alpha F$ the experiment was repeated from purification through to reconstitution. Unfortunately, this preparation failed to yield sufficient material for this analysis and time constraints prevented further experiments from being carried out.

4.5.2 Reconstitution of the Sec complex

The second protein complex purified for inclusion into the *in vitro* assay was the Sec complex. Beckmann *et al.* (1997) described a purification procedure for the Sec61 complex that was used to generate a cryo-EM structure of the Sec61-ribosome complex. This purification was modified to yield the entire Sec complex. The purification utilises Sec63p modified to include a cleavable Protein A-tag at its C-terminus similar to that generated for SRβ (section 2.6). A yeast strain expressing Sec63-PrA was generated and used to purify the Sec complex (described in section 2.21) which is shown in Figure 4.9. The Western blot reveals that binding of this complex to IgG sepharose was efficient (lane 2) and that the majority of the complex eluted from the resin following treatment with TEV protease (lanes 6-9). Western blotting identified Sec63p, Sec61p and Sec71p. As solubilisation of the Sec complex requires digitonin and elution of the complex is into buffer containing digitonin an additional detergent exchange step had to be undertaken before reconstitution could

Figure 4.9 Purification of intact Sec complex

Sec complex was purified from strain JDY516 as described in section 2.21. 1/200th of each fraction from the purification were analysed by SDS-PAGE and Western blotting (A) and 1/100th of each fraction from the detergent exchange step were analysed by SDS-PAGE and silver staining (B). Antibodies specific for Sec61, Sec63 and Sec71p were used to identify intact Sec complex.

Lanes for (A)

1 Load (post-ribosomal supernate)

2 Unbound

3 Wash 1

4 Wash 2

5-10 Elutions 1-6

Lanes for (B)

1 Load (elutions 2-5 of the purification)

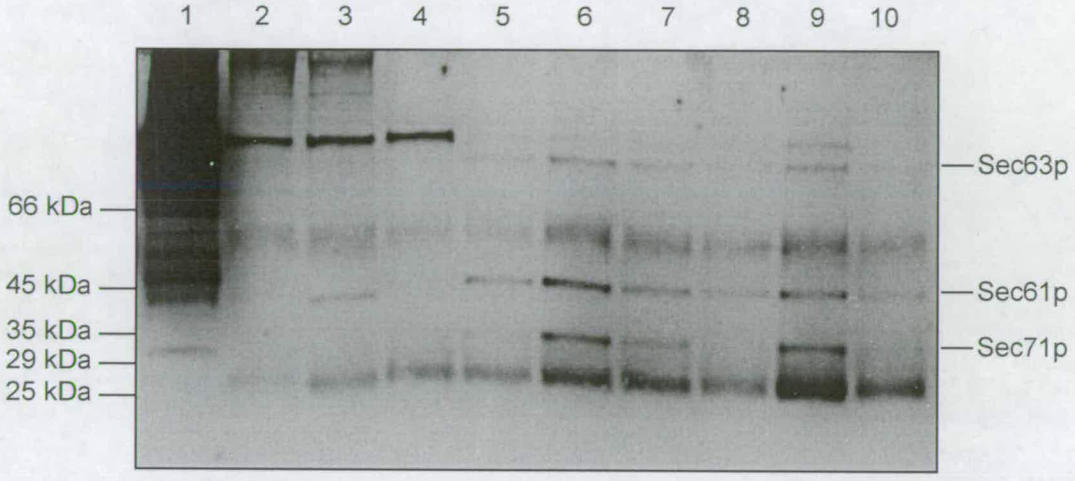
2 Unbound

3 Wash 1

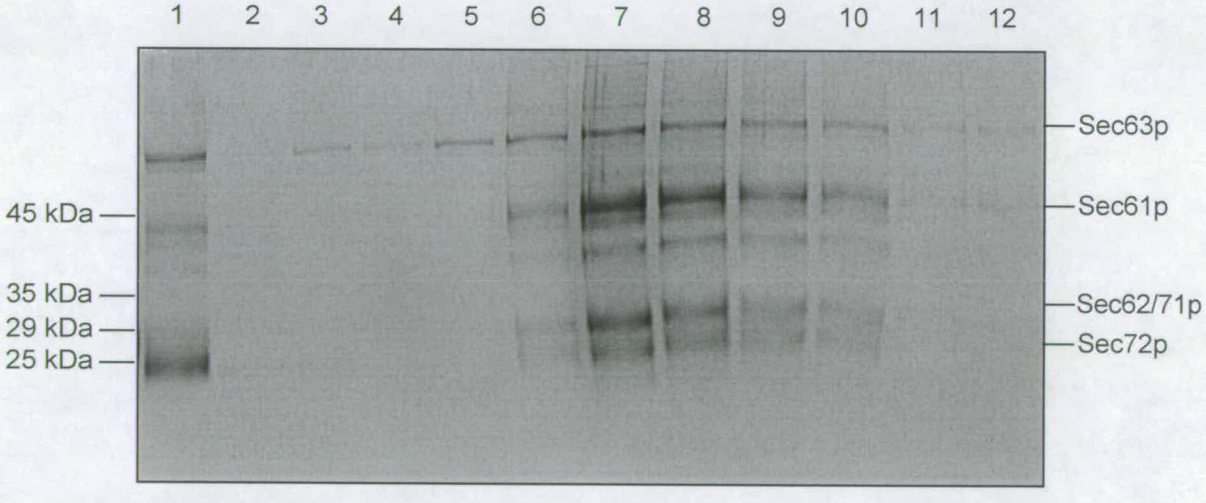
4 Wash 2

5-12 Elutions 1-8

A. Western blot



B. Silver stain



be carried out (section 2.24 and Figure 4.9). Silver stain analysis of the fractions collected during the detergent exchange of digitonin for deoxyBigCHAP revealed very little protein in the unbound or wash fractions, the complex eluting in fractions 3-7 (lanes 6-10). Approximately 6μg of Sec complex was recovered for reconstitution into proteoliposomes.

To assay the purified Sec complex it was necessary to isolate microsomes from a yeast strain deficient in Sec61p. Sec61p is essential in yeast therefore a construct was created in which *SEC61* was constitutively expressed only in the presence of galactose. When the cells were grown in glucose Sec61p was depleted to undetectable levels within 6-8 hours (Jeremy Brown). Microsomes were isolated from this strain (JDY638, table 1) after 6-8 hours of glucose depletion of endogenous Sec61p. Solubilised proteins from these microsomes were used in the reconstitution of purified Sec complex into proteoliposomes in the presence or absence of purified Kar2p. Figure 4.10, lane 2 shows very little translocation of ppαF in the absence of purified Sec complex or Kar2p. Upon addition of purified Sec complex no increase in translocation of ppαF can be detected (lane 5). However, in the presence of purified Sec complex and Kar2p an increase in translocation of ppαF can be seen (lane 8). This increase may have been due to the addition of Kar2p to the reaction and not to the presence of active purified Sec complex. Western blot analysis of all the proteoliposomes used in the experiment showed Sec61p was only present in fractions where purified Sec complex was added (Figure 4.10b). The data suggest that Sec complex, purified from yeast and reconstituted into proteoliposomes, was active.

Figure 4.10 Activity test of purified Sec complex

Sec complex, purified from strain JDY516 (Figure 4.9), was subjected to a detergent exchange step (section 2.24) and included in a reconstitution of membrane proteins from microsomes recovered from strain JDY638(pGAL-SEC61) (Sec61p-deficient; Table 1) (section 2.32). Proteoliposomes without added purified proteins (panel A, lanes 1-3), proteolipsomes with added purified Sec complex (panel A, lanes 4-6) and proteoliposomes with added Kar2p and added Sec complex (panel A, lanes 7-9) were included in translation/translocation reactions containing ppαF mRNA. Translocation was monitored by addition of trypsin and/or tritonX-100 to the reactions and 1/3rd of each reaction was analysed by SDS-PAGE and phosphoimaging.

Lanes for (A)

1, 4 and 7 No additions

2, 5 and 8 5 μg trypsin

3, 6 and 9 5 μg trypsin, 1% (v/v) tritonX-100

Proteolipsomes added to translation/translocation reactions were subjected to centrifugation at 55,000 rpm for 45 min at 4°C in a Beckman TLA 120.2 rotor. The supernate and pellet of this centrifugation step were TCA precipitated and analysed by SDS-PAGE and Western blotting. Antibodies specific for Sec61p were used to identify the Sec complex.

Lanes for (B)

1 Supernate of proteoliposomes included in panel A, lanes 1-3

2 Pellet of proteoliposomes included in panel A, lanes 1-3

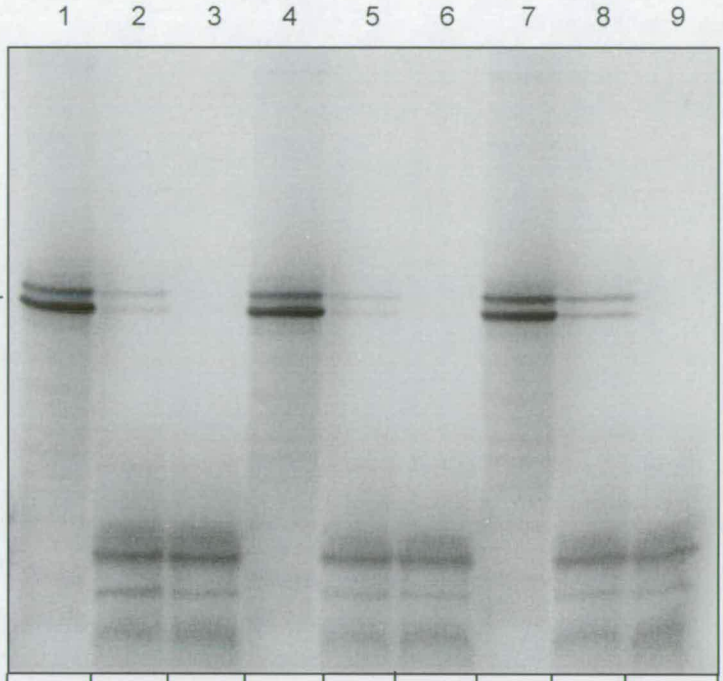
3 Supernate of proteoliposomes included in panel A, lanes 4-6

4 Pellet of proteoliposomes included in panel A, lanes 4-6

5 Supernate of proteoliposomes included in panel A, lanes 7-9

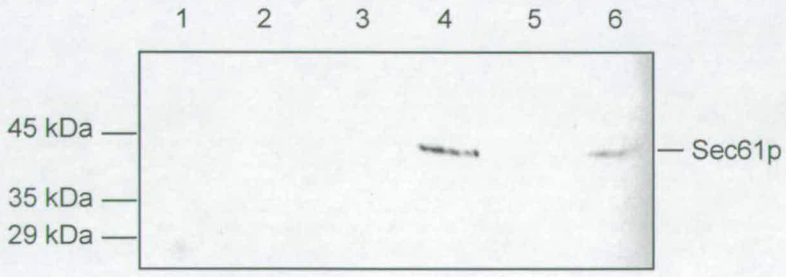
6 Pellet of proteoliposomes included in panel A, lanes 7-9

A.



Trypsin (μg)	0	5	5	0	5	5	0	5	5
TritonX-100 (% (v/v))	0	0	1	0	0	1	0	0	1
Kar2p (μg)	0			0			15		
Sec complex (μg)	0			3			3		

B.



4.6 Discussion

In this Chapter I have described the preparation of all of the materials necessary to reconstitute SRP-dependent targeting and the optimisation of each step in the translation/translocation reaction. I describe the purification of active yeast SR (Chapter 3), Kar2p, the Sec complex and bacterially over-expressed SR-ΔTMβ and their reconstitution. Finally, I have shown the reconstitution of SRP-dependent targeting using yeast components and demonstrated that Kar2p is necessary to stimulate SRP-dependent translocation in this *in vitro* system.

I have shown that SR-ΔTMβ associates with microsomal membranes and retains activity despite the absence of a transmembrane domain on SRβ (Figure 4.4). Interestingly, approximately 25% of SR-ΔTMβ associates with the ER membrane *in vivo* (Ogg *et al.*, 1995). This *in vitro* experiment extends this finding and suggests that SR-ΔTMβ associates with an ER membrane component thus providing a binding site for SR. The finding that SRβ interacts with Sec61p in a two-hybrid assay supports this possibility (section 5.3). Alternatively, and like the *E. coli* SRα homologue FtsY, SR may have an affinity for the lipid components of the membrane (de Leeuw *et al.*, 2000). Thus, SR-GST provides a tool to investigate interactions of SR-ΔTMβ and how SR-ΔTMβ is tethered to the membrane during protein targeting and translocation *in vitro*.

It was observed that the presence of SR inhibited post-translational translocation (Figure 4.8). The observed interaction between SRβ and Sec61p (Chapter 5) suggests that SR may compete with Sec62p for available translocons. In this scenario a translation/translocation reaction with excess SR would titrate out available translocons thereby favouring SRP-dependent over SRP-independent translocation.

The purified Sec complex was shown to be active. However, as this experiment was carried out only once it must be repeated. Although SRP-dependent translocation was reconstituted, fulfilling one of the aims of this study, the assay was not established using only purified components. A necessary step before completion of this aim will be to establish firmly the activity of purified Sec complex. While it was established that purified yeast SR was present in the proteoliposome fraction included in the translation/translocation assays it was not demonstrated conclusively whether SR was associated with or was incorporated into proteoliposomes. In order to establish this proteoliposomes composed of lipid and purified SR must be either sedimented upon high salt and urea treatment or subjected to flotation gradient analysis under the same conditions. In the presence of high salt and urea protein associated with the proteoliposomes will not be found in the same fraction with them, however, protein incorporated into the liposome will.

Once it has been established that active Sec complex can be reproducibly purified reconstitution of SRP-dependent targeting using only purified components can be attempted. Sec61p can be purified and reconstituted separately from the Sec62/63p complex. This will allow three questions to be addressed: what is the minimum translocation apparatus necessary to support co-translocational translocation *in vitro*, what role has SRβ in the pathway and is Sec63p absolutely required?

Chapter 5

SRβ interacts with Sec61p

5.1 Introduction

The sequence of events that must occur to transfer RNC from SRP to the translocon is incompletely understood. As SR and the translocon provide the contact points for the SRP-ribosome complex on the membrane it is perhaps likely that they do interact, either providing a joint "landing site" or in a regulated "gating" function to promote transfer of nascent chains from SRP to the translocon. With this hypothesis as a starting point the experiments described in this chapter were carried out in attempts to determine if such an interaction does indeed occur. The results obtained strongly support an interaction between SRβ and Sec61p.

5.2 Co-isolation of Sec61p and SR-PrA

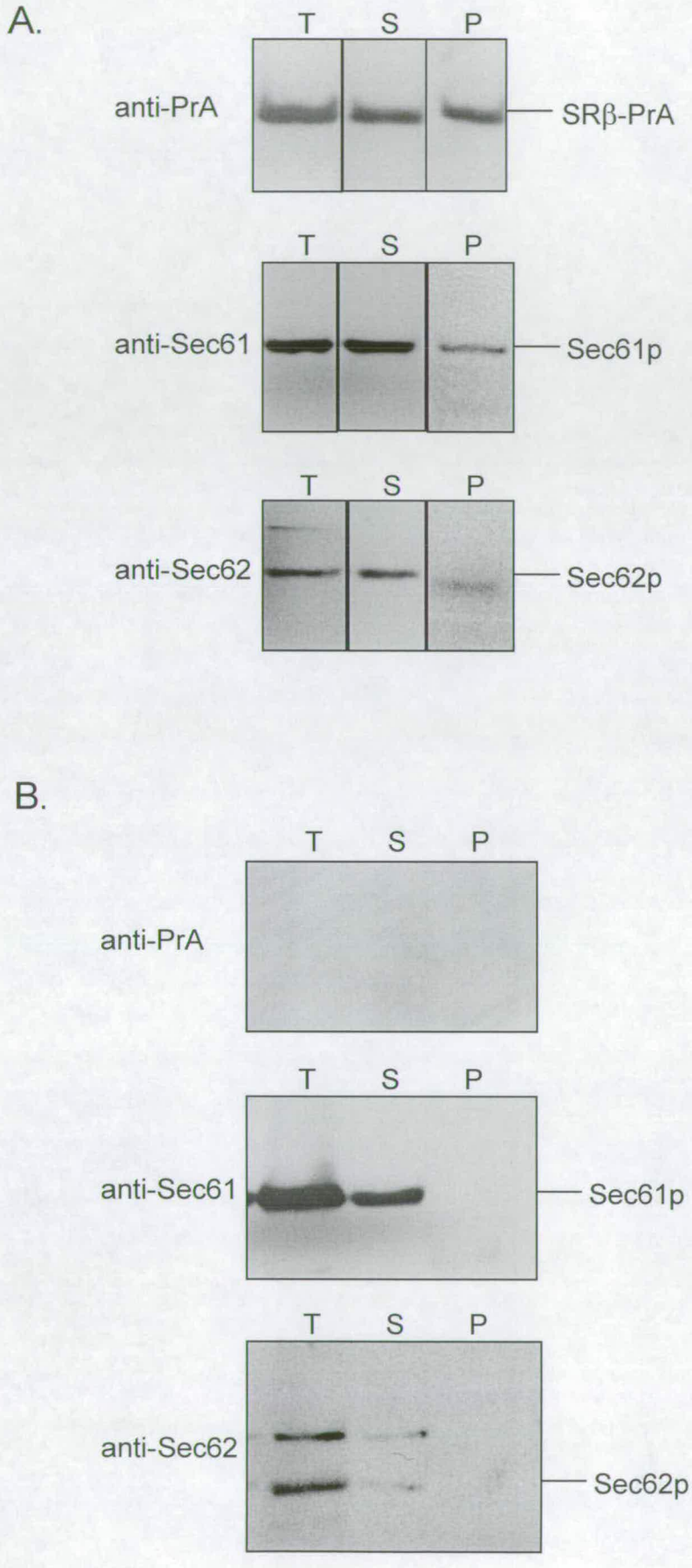
While optimising conditions to purify native SR complex (see Chapter 3), I observed that a small proportion of Sec61p co-isolated with SR. The co-isolation of Sec61p only occurred when microsomal membranes used to recover SR were solubilised with digitonin. Figure 5.1 shows Western blots of fractions from an isolation of SR-PrA (Chapter 3 section 3.1.3) (A) and a control experiment using non-tagged strain JDY3 (B). The nitrocellulose was cut using molecular weight markers as a guide and incubated with antibodies raised against protein A, Sec61p and Sec62p (table 3). The association of Sec61p with SRβ-PrA in the pellet fraction can be clearly seen (panel A, compare pellet lanes). Sec62p was also detected, at a very low level, in the pellet fraction. Subsequent attempts to detect Sec62p co-isolating with SR failed (data not shown). Neither Sec61p nor Sec62p was detected in the pellet of the control experiment (figure 5.1b).

The interaction between SR-PrA and Sec61p could have been due to an interaction between Sec61p and the protein A-tag at the C-terminus of SRβ. To test this hypothesis 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), an integral ER membrane protein involved in lipid, fatty-acid and sterol

Figure 5.1 Co-isolation of Sec61 and Sec62p with SR-PrA

240 OD_{280} units of microsomes recovered from strains ARY1 (A) and JDY3 (B) (section 2.19a) were solubilised in solubilisation buffer which contained 1% (v/v) digitonin and 150 mM KOAc (section 2.20b). Insoluble material was pelleted by centrifugation at 17,000 rpm for 20 min at 4°C in a Beckman JA25.5 rotor. The soluble fractions were bound to 200 µl IgG sepharose for 1 hr at 4°C and the supernates and pellets recovered. Both pellet fractions were washed 3 x 1 ml solubilisation buffer and 2 x 1 ml TBS. 1/4 (lanes T and S, both panels) and 1/2 (lanes P, both panels) of the fractions were analysed by SDS-PAGE and Western blotting. Antibodies specific for protein A, Sec61p and Sec62p were used to identify SRβ, Sec61p and Sec62p.

Lanes for (A)	Lanes for (B)
T Total	T Total
S Supernate	S Supernate
P Pellet	P Pellet


A.
T
S
P
anti-PrA
SRβ-PrA
T
S
P
anti-Sec61
Sec61p
T
S
P
anti-Sec62
Sec62p
B.
T
S
P
anti-PrA
T
S
P
anti-Sec61
Sec61p
T
S
P
anti-Sec62
Sec62p

metabolism, was genomically protein A-tagged, the fusion protein being expressed from the endogenous HMG-CoA reductase promoter (section 2.6 and 2.8). Microsomal membranes were isolated and solubilised from this strain and HMG-CoA reductase-PrA isolated as described for SR-PrA (section 2.20). Under these conditions Sec61p could not be co-isolated with HMG-CoA reductase-PrA (data not shown). Therefore, the co-isolation of Sec61p with SR was not the result of non-specific interactions between Sec61p and IgG Sepharose or the protein A-tag itself, but due to an interaction direct or indirect between Sec61p and SRα and/or SRβ.

The interaction between SR and Sec61p was sensitive to salt concentration. When the co-isolation experiment was carried out in buffer containing 500 mM KOAc (Figure 5.2) as opposed to 150 mM KOAc (Figure 5.1) the interaction was lost and Sec61p was no longer detected in the pellet fraction (Figure 5.2b, lane P). This suggested that the interaction between SR and Sec61p was relatively weak. However, the interaction between SR and Sec61p may have a regulatory role or only be a transient contact. In this case, a relatively weak interaction may be all that is required and might be expected. Co-isolation of Sec62p with SR suggested the possibility that both the SRP-dependent and –independent translocation apparatus form complexes in the ER membrane.

SRβ is an ER integral membrane protein with no previously characterised function. Therefore, it seemed plausible that SRβ was the Sec61p interacting component. Deletion of either SRα or SRβ results in a complete loss of SRP-dependent targeting. Therefore, removing one subunit of SR to study the effects on translocation complexes was not possible. To study the interaction in more detail two-hybrid analysis was employed (Section 5.3).

Figure 5.2 The SR-Sec61p interaction is not seen at high salt concentration

240 OD_{280} units of microsomes recovered from strain ARY1 were solubilised in solubilisation buffer containing 500 mM KOAc (sections 2.19a and 2.20b). Insoluble material was pelleted by centrifugation at 17,000 rpm for 20 min at 4°C in a Beckman JA25.5 rotor. The soluble fraction was bound to 200 µl IgG sepharose for 1 hr at 4°C and the supernate and pellet recovered. The pellet fraction was washed 3 x 1 ml solubilisation buffer and 2 x 1 ml TBS. 1/4 (lanes T and S, both panels) and 1/2 (lanes P, both panels) of the fractions were analysed by SDS-PAGE and Western blotting. Antibodies specific for SRα, SRβ and Sec61p were used to identify SR and Sec61p.

Lanes for (A)

T Total

S Supernate

P Pellet

Lanes for (B)

T Total

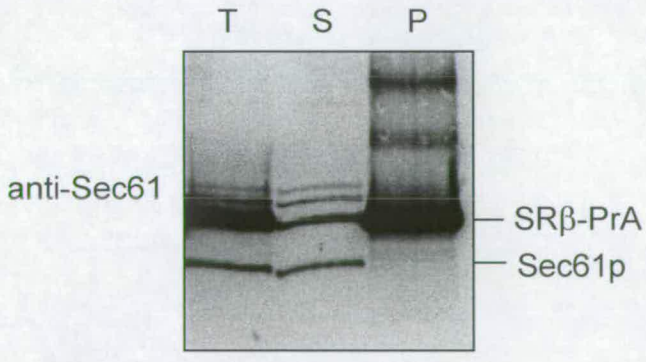
S Supernate

P Pellet

A.


T
S
P
anti-SRα
anti-SRβ
SRα
SRβ-PrA

B.


T
S
P
anti-Sec61
SRβ-PrA
Sec61p

5.3 Dissection of Sec61p-SRβ interaction

Two-hybrid analysis allows the identification of protein-protein interactions through the reconstitution of the activity of a transcriptional activator. The method (Fields and Song, 1989; Chien *et al.*, 1991) is based on the properties of the yeast GAL4 protein, which consists of separable domains responsible for DNA-binding and transcriptional activation. Once separated these domains cannot interact. Plasmids encoding two hybrid proteins, one (the bait fusion) consisting of the GAL4 DNA-binding domain fused to protein X and the other (the prey fusion) consisting of the GAL4 activation domain fused to protein Y, are constructed and introduced into yeast. Interaction between proteins X and Y leads to the transcriptional activation of a reporter gene containing a binding site for GAL4.

Initially, all but the transmembrane domain of SRβ was cloned into a two-hybrid bait vector (pAS-C) while the cytosolic domains of a variety of ER integral and peripheral membrane proteins were cloned into two-hybrid prey vectors (pACT II) illustrated in Figure 5.3. Strain CG1945 (table 1) was used to assay the plasmid constructs and contained *lacZ* and *HIS3* reporter genes. A feature of cloned prey fusion proteins is a HA-tag (Figure 5.3). Western blotting and incubation with anti-haemagglutanin (HA) antibodies revealed that all but 1 of the prey fusions were expressed (Figure 5.4, lanes 1-7).

All combinations of bait and prey vectors were transformed into strain CG1945 for analysis (section 2.8). The cells were grown on solid media lacking leucine, tryptophan and histidine (selecting for activation of the *HIS3* reporter) and media lacking all three but containing varying concentrations of 3-aminotriazole (3-AT). 3-AT is an inhibitor of the product of the *HIS3* gene and thus monitors the level of transcriptional activity of the *HIS3* reporter. Weak transcriptional activation of the *HIS3* reporter results in growth of strains with non-interacting bait and prey fusions. Low levels of 3-AT suppress growth of these "false positives" (Bartel and Fields (ed.), 1997). Figure 5.5 shows a typical result of this experiment. In panel A the

Figure 5.3 Schematic of two-hybrid bait and prey constructs

Schematic of constructs used in two-hybrid analysis. All bait fusions (A.) have the *GAL4* DNA binding domain (GAL4DBD) (pink) at the N-terminus of the test protein (yellow). All prey constructs (B.) have haemagglutanin (HA) (green) at the C-terminus of the test protein (yellow) and the N-terminus of the *GAL4* activating domain (GAL4AD) (blue).

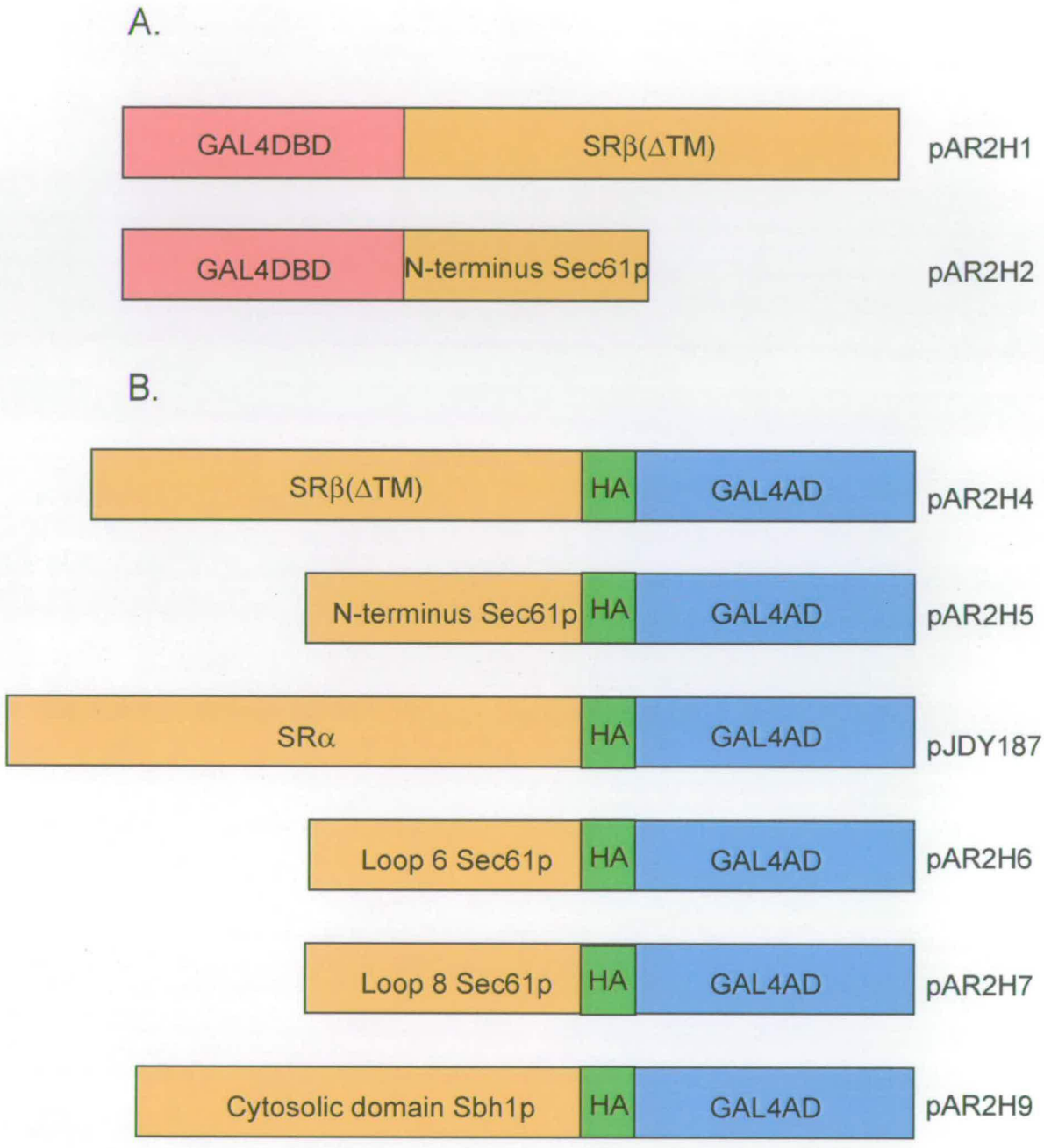

A.
GAL4DBD
SRβ(ΔTM)
pAR2H1
GAL4DBD
N-terminus Sec61p
pAR2H2
B.
SRβ(ΔTM)
HA
GAL4AD
pAR2H4
N-terminus Sec61p
HA
GAL4AD
pAR2H5
SRα
HA
GAL4AD
pJDY187
Loop 6 Sec61p
HA
GAL4AD
pAR2H6
Loop 8 Sec61p
HA
GAL4AD
pAR2H7
Cytosolic domain Sbh1p
HA
GAL4AD
pAR2H9

Figure 5.4 Expression and visualisation of two-hybrid prey fusion proteins

Combinations of bait and prey two-hybrid fusions were expressed in strain CG1945 (table 1) and visualised by SDS-PAGE and Western blotting. Antibodies specific for haemagglutanin were used to identify expressed prey fusions. Bait fusions were not identified.

Lanes

1 Sec61-N terminus prey (pAR2H5)

2 Loop 6 Sec61p prey (pAR2H6)

3 Loop 8 Sec61p prey (pAR2H7)

4 Cytosolic domain of Sbh1p prey (pAR2H9)

5 Empty vector

6 SRα prey (pJDY187)

7 SRβ(ΔTM) prey (pAR2H4)


1 2 3 4 5 6 7
HA-prey fusions
HA-prey fusions

Figure 5.5 Growth of cells on selective media

Combinations of two-hybrid bait and prey fusions were expressed in strain CG1945. Growth of each strain described on -HLW media containing 50 mM 3-AT was monitored and tabulated (A) and plate growth of 3 strains on -HLW media containing 100 or 150 mM 3-AT recorded (B).

Key to table

N/A = growth of strains with these combinations of bait and prey fusions not determined

+ = minimal growth

++ = good growth

+++ = thick growth

- = no growth

Panel 4 (key for (B))

1 pAR2H1 + pJDY187 (SRβ bait + SRα prey fusions)

2 pAS-C + pAR2H5 (empty bait vector + Sec61-Nterm peptide prey fusion)

3 pAR2H1 + pAR2H5 (SRβ bait + Sec61-Nterm peptide prey fusions)

Media

1 -leu, -trp, -his (-HLW)

2 -HLW, 100 mM 3-AT

3 -HLW, 150 mM 3-AT

A.

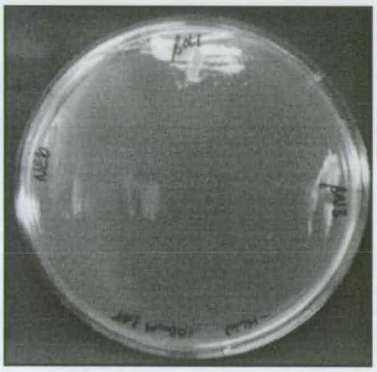
Prey plasmids	Bait plasmids		
	pAR2H1	pAR2H2	pAS-C
pAR2H4	N/A	++	-
pAR2H5	++	N/A	-
pJDY187	+++	N/A	-
pAR2H6	-	N/A	-
pAR2H7	-	N/A	-
pAR2H9	-	N/A	-
pACTII	-	-	-

B.

1



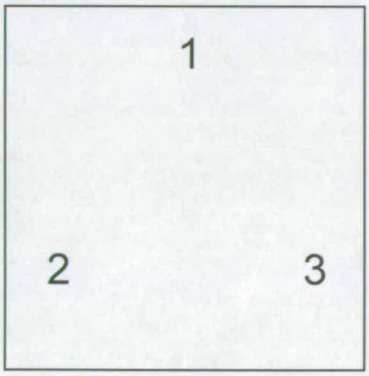
2



3



4



growth of strains containing combinations of bait and prey plasmids is recorded in a table. Growth of each strain was on media lacking leucine, tryptophan and histidine (-HLW media) and containing 50 mM 3-AT. In panel B growth of 3 strains (containing SRβ bait and SRα prey fusions, empty bait vector and N-terminus of Sec61p prey fusion and SRβ bait and N-terminus of Sec61p prey fusions) on –HLW media alone or containing 100 mM 3-AT and 150 mM 3-AT is shown. Cells expressing the SRβ-GAL4BD bait fusion and the SRα prey fusion grew on media containing up to 200 mM 3-AT concentrations (data not shown) indicating a strong interaction between these two proteins. Cells expressing the SRβ bait fusion and the Sec61-Nterm prey fusion grew on media containing up to 150 mM 3-AT (Figure 5.5, panel B). Cells expressing the reciprocal combination of Sec61-Nterm bait fusion and SRβ prey fusion grew on media containing up to 50 mM 3-AT (Figure 5.5, panel A). Hence, the interaction between SRβ and the Sec61-N terminal domain is weaker than that between SRβ and SRα. Only strains expressing the SRβ-bait fusion with either the Sec61-Nterm peptide or SRα grew on media containing 3-AT. Growth of the strain containing the empty bait vector and the Sec61-Nterm peptide prey fusion on –HLW media must be due to auto-activation.

Figure 5.6 shows the results of a blue/white test for activation of the *lacZ* reporter carried out on the SRβ-GAL4BD bait fusion and the Sec61p/Sbh1p cytosolic loop prey fusions. In the presence of both the SRβ-GAL4BD fusion and either the SRα or Sec61-Nterm prey fusion proteins β-galactosidase was expressed resulting in these patches being bright blue on incubation in Z-buffer (section 2.36). All other patches remained white indicating that the *lacZ* gene was not activated. Combining the results of growth on 3-AT and the β-galactosidase assay only three combinations of bait and prey fusions showed significant interaction. These are those containing the Sec61p N-terminal cytosolic domain (bait or prey fusion) and SRβ (prey or bait fusion), and the SRα prey fusion with the SRβ bait fusion (positive

Figure 5.6 Blue/white test of two-hybrid assay positives

Two-hybrid blue white assay was carried out as described in section 2.36. Combinations of bait and prey fusions expressed in CG1945 were tested. Colour photo of test filter (A.), black and white photo of test filter (B.) and key to fusions expressed in each patch of cells (C.) are shown.

Fusions tested

1 pAR2H1 + pACTII (SRβ bait fusion and empty prey vector)

2 pAR2H1 + pAR2H5 (SRβ bait fusion and Sec61-Nterm peptide prey fusion)

3 pAR2H1 + pJDY187 (SRβ bait fusion and SRα prey fusion)

4 pAR2H1 + pAR2H6 (SRβ bait fusion and Sec61-loop 6 prey fusion)

5 pAR2H1 + pAR2H7 (SRβ bait fusion and Sec61-loop 8 prey fusion)

6 pAR2H1 + pAR2H9 (SRβ bait fusion and Sbh1-cytosolic domain prey fusion)

7 pAS-C + pAR2H5 (empty bait vector and Sec61-Nterm peptide prey fusion)

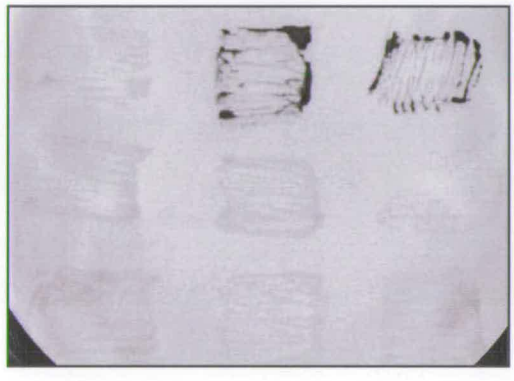
8 pAS-C + pJDY187 (empty bait vector and SRα prey fusion)

9 pAS-C + pACTII (empty bait vector and empty prey vector)

A.



B.



C.

1	2	3
4	5	6
7	8	9

control). Therefore, out of all the prey fusion proteins tested only SRα and the Sec61-N terminal domain interact with SRβ.

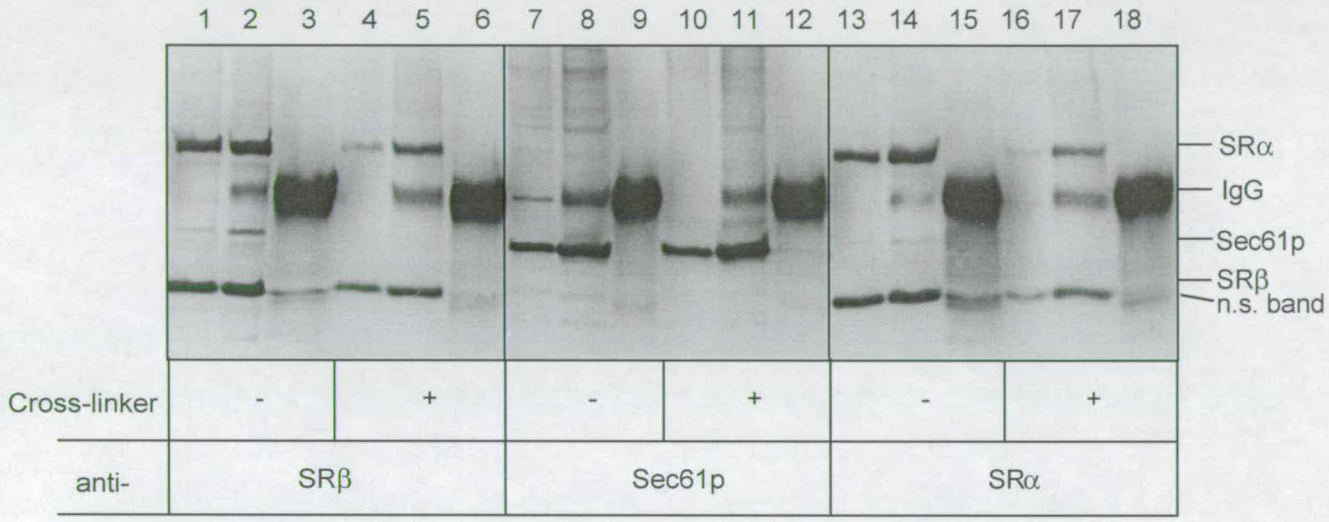
With the caveat that two-hybrid analysis can yield false positive results these data indicate that the N-terminal cytosolic domain of Sec61p interacts with the cytosolic domain of SRβ. This interaction may be transient *in vivo* but is maintained long enough in a two-hybrid assay to support growth on high concentrations of 3-AT and express β-galactosidase. One model of the functional significance of this interaction may be that the cytosolic domains of the two proteins interact in a regulatory capacity, allowing the transfer of RNC to the translocon only when a vacant site is available. It has been suggested that Sec61p may act as a GEF for SRβ (Fulga *et al.*, 2001) while ribosomal proteins acts as a GAP (Bacher *et al.*, 1999). Perhaps the N-terminal domain of Sec61p recruits SRP-SR-RNC complexes through the interaction with SRβ, the ribosome acting as a GAP for SRβ while Sec61p acts as a GEF once transfer of the RNC is complete or to stimulate the transfer itself.

5.4 Cross-linking and immunoprecipitation of microsomal membrane proteins

The data in sections 5.2 and 5.3 support a weak and/or transient interaction between SR and Sec61p. To attempt to demonstrate this interaction or the proximity of SR and Sec61p directly cross-linking and immunoprecipitation were carried out. The non-cleavable cross-linker disuccinimidyl suberate (DSS) was added to microsomal membranes for varying lengths of time (section 2.26). The membranes were then solubilised and proteins immunoprecipitated from the soluble fraction (section 2.25). Figure 5.7 shows a Western blot of a typical cross-linking reaction and immunoprecipitation of protein with anti-SRβ antibodies. The load, supernate and pellet fractions from the immunoprecipitation were analysed by SDS-PAGE

Figure 5.7 Cross-linking and immunoprecipitation of SR

Cross-linking of and immunoprecipitation from 240 OD_{280} units of microsomes recovered from strain JDY3 were carried out as described in section 2.25 and 2.26. Microsomes with cross-linker (lanes 4-6, 10-12 and 16-18) and microsomes without (lanes 1-3, 7-9 and 13-15) were solubilised and immunoprecipitated with anti-SRβ. 1/8 of the total fraction (lanes 1, 4, 7, 10, 13 and 16), 1/4 of the supernate fractions (lanes 2, 5, 8, 11, 14 and 17) and 1/4 of the pellet fractions (lanes 3, 6, 9, 12, 15 and 18) were analysed by SDS-PAGE and Western blotting. Antibodies specific for SRβ (lanes 1-6), Sec61p (lanes 7-12) and SRα (lanes 13-18) were used to identify cross-linked complexes containing SR and Sec61p.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
SRα
IgG
Sec61p
SRβ
n.s. band
Cross-linker
-
+
-
+
-
+
anti-
SRβ
Sec61p
SRα

separately three times so that the nitrocellulose could be incubated with three different primary antibodies at once. Ponceau S staining of the nitrocellulose prior to antibody incubation revealed that addition of cross-linker resulted in a shift of low molecular weight proteins to larger, presumably cross-linked complexes. This was also seen on the immunoblots as monomeric SRα and β were all reduced in abundance. However, it is not clear where these cross-linked protein complexes migrate to on the protein gel. Also, comparing lanes 3 and 6 it is evident that upon addition of cross-linker there is no longer any immunoprecipitation of free SRβ. It appears that after addition of cross-linker the anti-SRβ antibodies lose their ability to recognise SRβ. The antibodies also do not immunoprecipitate any complexes containing SRβ as none are seen with any of the antibodies tested. This may be due to the epitopes recognised by the antibodies being modified by cross-linker and/or cross-linked to other proteins. To circumvent this problem the cross-linking reaction was repeated with microsomal membranes isolated from a strain expressing SRβ-PrA. The presence of the protein A-tag allowed purification of cross-linked protein complexes using IgG Sepharose. The problem with this technique involved the reactivity of protein A to secondary antibodies and enhanced chemiluminescence (ECL). The presence of the protein A-tag allowed the identification of complexes of cross-linked protein that migrated to higher molecular weight positions on the protein gel. However, it was impossible to determine which proteins were in the complexes as the protein A-tag cross-reacted strongly with anti-rabbit secondary antibodies. The conclusions drawn from these experiments were that upon cross-linking of microsomal membrane proteins anti-SRβ antibodies no longer recognise free or SRβ-containing protein complexes, and that protein A-tags render cross-linking patterns uninterpretable.

Cross-linking of microsomal membranes and immunoprecipitation of complexes containing SRβ may still be a useful technique to isolate proteins that interact transiently with SRβ. Other approaches could involve the use of a hexa-histidine

(HIS_6) or glutathione-S-transferase (GST) tag on SRβ. These would allow immunoprecipitation of cross-linked complexes containing SRβ while eliminating the problem of cross-reactivity encountered with the protein A-tag. Another approach might be to raise another antibody against SRβ in the hope that these antibodies might not be so affected by the presence of cross-linker in the samples.

5.5 Discussion and future work

An association between SRβ and Sec61p was demonstrated using a pull down assay (section 5.2) and has been dissected by two-hybrid analysis (section 5.3). The interaction was shown to involve the N-terminal cytosolic domain of Sec61p and the cytosolic domain of SRβ. However, cross-linking and immunoprecipitation of complexes containing SRβ and other proteins was unsuccessful (section 5.4). The next step should be to try and demonstrate the interaction using other biochemical methods. One possibility would be to immobilise an N-terminal peptide of Sec61p on a chromatography column and to examine any interaction between the Sec61p N-terminal domain and recombinant SRβ. This could enable several important questions to be studied regarding the interaction. Does the interaction require GTP? Can the complex be trapped or dissociated by non-hydrolysable analogues of GTP or GDP respectively? Does the interaction require the presence of other factors such as ribosomal proteins, ribosomes or RNCs?

A related experiment was attempted using a 10- and 100-fold molar excess of N-terminal peptide of Sec61p, over endogenous Sec61p, added to a translation/translocation assay (section 2.33/2.34). This was carried out to determine if the peptide would exhibit any inhibitory effects through its interaction with SRβ. Translocation of SRP-dependent and –independent substrates was monitored (Figure 5.8). No inhibition was observed for either SRP-dependent or –independent

Figure 5.8 Inhibition of protein translocation by Sec61p-Nterminal peptide

The effects of the N-terminal peptide of Sec61p (sequence SSNRVLDLFKPFESFLPEVIAP, corresponding to residues 2-22 of Sec61p) on translocation of *in vitro* radiolabelled $D_{HC}\alpha F$ (lanes 1, 3, 5, 7, 9, 11 and 13) and ppαF (lanes 2, 4, 6, 8, 10 and 14) were tested as described in section 2.35. Translocation was monitored by SDS-PAGE and phosphoimage analysis.

Lanes

1 0 microsomes, 0 peptide

2 0 microsomes, 0 peptide

3 microsomes, 0 peptide

4 microsomes, 0 peptide

5 microsomes, 5 μM peptide

6 microsomes, 5 μM peptide

7 microsomes, 500 nM peptide

8 microsomes, 500 nM peptide

9 microsomes, 50 μM peptide

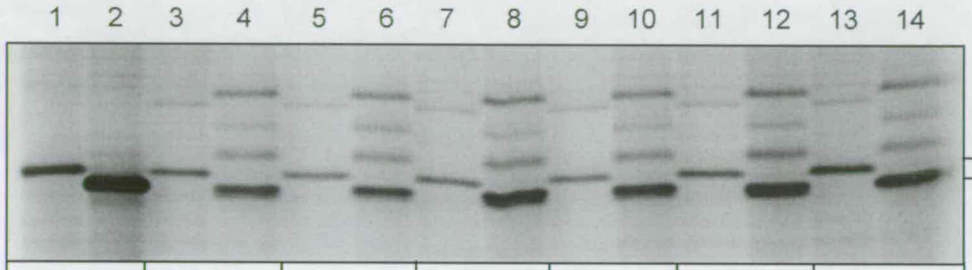
10 microsomes, 50 μM peptide

11 microsomes, 500 μM peptide

12 microsomes, 500 μM peptide

13 microsomes, 500 μM control peptide

14 microsomes, 500 μM control peptide



Microsomes added	-	+	+	+	+	+	+
Sec61p peptide added (mM)	0	0	5	0.5	50	500	0
Control peptide added (mM)	0	0	0	0	0	0	500

substrates indicating that the N-terminal domain of Sec61p alone in solution is insufficient to affect protein targeting. However, once immobilised it may be possible to determine the conditions required for its interaction with SRβ.

Another possibility would be to carry out a synthetic lethal screen with Sec61ΔNterm and/or SRβΔTM and other cold sensitive alleles of *SEC61*. Synthetic lethal screens can allow the identification of proteins that interact within the same pathway in the cell and may lead to the identification of other domains within Sec61p important for the interaction with SRβ. A synthetically negative interaction has been identified between Sec61ΔNterm and SRβΔTM (B. Wilkinson and C. Stirling, personal communication) supporting the existence of a physiologically important interaction between SRβ and Sec61p. In addition, recent work utilising the split ubiquitin assay (coupled to growth on –URA media) provided evidence of close physical proximity between SRβ and both Sec61p and Ssh1p (Wittke *et al.*, 2002). As Ssh1p is proposed to comprise the major subunit of an entirely SRP-dependent translocon (Wilkinson *et al.*, 2001) the observed proximity to SRβ strengthens the argument for Ssh1p in this role. The observed interaction between SRβ and Sec61p in the split ubiquitin assay provides further evidence that this interaction has a significant role in SRP-dependent targeting *in vivo*.

Discussion and future work

The broad aims of this study were to identify the functions of SRβ and to identify and study interactions of SRβ that take place during SRP-dependent targeting. A major goal was the purification of proteins necessary for and reconstitution of SRP-dependent co-translational translocation. Previous work with mammalian proteins established the requirement for Sec61p and SR in SRP-dependent co-translational translocation (Gorlich and Rapaport, 1993). In addition, there is genetic evidence in yeast for a requirement for Kar2p and Sec63p in co-translational translocation (Young *et al.*, 2001). However, the role of SRβ in co-translational translocation has not been established save as a membrane anchor for SRα with a GTPase domain essential for its function (Ogg *et al.*, 1998). Post-translational translocation was reconstituted using purified yeast components establishing the requirement for the Sec62/63p complex in SRP-independent targeting (Panzner *et al.*, 1995). Therefore, reconstitution of protein translation and translocation is an established method used to elucidate involvement of a protein in targeting.

Protocols for the over-expression and large-scale purification of SR were developed and described. Conditions under which SR can be bound and eluted from a variety of ion-exchange resins for further purification and buffer exchange are also detailed. Finally, the purification of sufficient quantity of SR to allow reconstitution experiments to be undertaken was described (Chapter 3).

The preparation of all of the materials necessary to reconstitute SRP-dependent targeting and the optimisation of each step in the translation/translocation reaction are summarised. I describe the purification of active yeast SR (Chapter 3), Kar2p, the Sec complex and bacterially over-expressed SR-ΔTMβ and their reconstitution. I have discussed previously the belief that over-expressing each subunit of SR at the same level would facilitate purification of larger quantities of protein for reconstitution and thus should be the next logical step. Also, reconstitution of a mock purified fraction from a strain with non-tagged SR should be carried out to be

convinced that purified SR, and not an unknown contaminant, is stimulating SRP-dependent translocation in the *in vitro* assay. Finally, I have shown the reconstitution of SRP-dependent targeting using yeast components and demonstrated that Kar2p is necessary to stimulate SRP-dependent translocation in this *in vitro* system.

An interaction between SRβ and Sec61p was demonstrated and dissected using two-hybrid analysis (sections 5.2 and 5.3). This interaction is localised to the N-terminal cytosolic domain of Sec61p and the cytosolic domain of SRβ. An interaction was also identified between SR and Sec62p. It could be speculated that the components involved in each translocation pathway form a specific complex (of Sec61p, Sec62/63p and SR) in the membrane until contacted by either a post-translational dependent signal sequence or a translating ribosome. The complexes would then dissociate allowing co- or post-translational translocation to proceed. While this may be the case, I was unable to co-isolate Sec62p with SR again suggesting that this result was somehow artifactual. Despite many attempts, cross-linking and/or immunoprecipitation of complexes containing SRβ and other proteins failed to provide further evidence of the interaction with Sec61p. Cross-linking may be a useful tool in the future, as new antibodies against SRβ have been generated. Previously, upon cross-linking the antibodies could no longer recognise SRβ or complexes containing SRβ. As this was likely due to the antibodies new antibodies may now be able to recognise SRβ epitopes even after addition of cross-linker.

The work that I have carried out in this study can be related to the model for SRP-dependent targeting that I proposed in the introduction. The model was based on all of the relevant data in the field and my own discovery of an interaction between SRβ and Sec61p. In this model, (Figure 1.5) binding of SRβ to a ribosomal protein stabilises the empty conformation of the GTPase domain of SRβ. The stable empty conformation leads to dissociation or significant structural rearrangement of SRα and SRβ but stable binding between GTP-bound SRα and GTP-bound Srp54p ensures

the RNC-SRP-SR complex remains intact on the membrane. At the point in the targeting cycle when SRβ is bound not only by the ribosomal protein but also by Sec61p this stimulates GTP binding and hydrolysis by SRβ allowing dissociation of the RNC-SRP-SR complex and docking of the RNC on the translocon. SRα and Srp54p reciprocally stimulate each other's GTPase activities, SRα and Srp54p dissociate and SRα and SRβ re-associate or regain their usual conformation on the ER membrane. This mechanism of protein targeting ensures that transfer of the RNC to the translocon can only take place when each are in close proximity to one another. Presumably, binding of SRβ to Sec61p would be inhibited should the translocon be occupied by another translating ribosome or engaged in translocation of a post-translational substrate. This model can also be applied to the sequence of events leading up to translocation through an Ssh1p translocon. The recently demonstrated proximity of SRβ to Ssh1p strongly suggests that binding of SRβ to Ssh1p or Sec61p is interchangeable (Wittke *et al.*, 2002). In the case of targeting to the Ssh1p translocon, binding of SRβ to Ssh1p and the ribosome would stimulate GTP binding and hydrolysis and allow transfer of the RNC to the translocon. Dissociation of SRα and SRβ during protein targeting has not been observed; indeed the subunits have previously only been purified as a stable complex. It can be assumed that if the proteins do dissociate during each cycle of targeting then it is only transient and you should not see it as they are still held within the targeting complex. Presumably, as dissociation has not been observed the empty state of the GTP binding domain of SRβ, stabilised by the ribosome, must be transient. Should cell lysis occur when this empty state is stable dissociation of SRβ and the ribosome would lead to GTP binding by SRβ and re-association of the subunits of SR.

Despite an enormous amount of analysis of the targeting and translocation pathways, there are still many questions to be answered. SRP is conserved throughout all three kingdoms of life but differences still exist in the composition of the proteins involved

in and the mechanisms of SRP-dependent co-translational translocation. It will be very interesting to learn fully which aspects of the pathway are specific to a particular organism and which are general to all.

Appendix 1 Sequence information

DNA sequence of *SRP101*

1 ATGTTCGACCAATTAGCAGTCTTTACCCCTCAAGGTCAAG

41 TACTTTACCAATATAACTGTTTAGGAAAAAAGTTTTCTGA

81 AATACAAATTAACAGCTTTATATCCCAGCTGATTACTTCC

121 CCAGTAACTAGAAAAGAAAGTGTTGCAAACGCAAATACAG

161 ACGGATTTGATTTCAATCTTTTAACAATCAACAGCGAACA

201 CAAAAATTCTCCTTCATTTAATGCACTATTTTATTTGAAT

241 AAGCAACCAGAATTGTATTTCGTAGTGACTTTTGCCGAGC

281 AGACTTTAGAGCTTAATCAAGAAACTCAACAAACACTTGC

321 ACTGGTGTTAAAACTCTGGAACTCATTGCATTTAAGTGAA

361 TCCATTCTAAAAAATCGTCAGGGCCAAAACGAAAAGAACA

401 AGCATAACTACGTCGATATTCTTCAGGGAATTGAAGACGA

441 CCTGAAGAAATTTGAGCAATATTTTAGGATAAAATATGAA

481 GAGTCAATAAAACAAGACCATATCAATCCAGATAATTTTA

521 CCAAAAATGGATCAGTACCCCAATCGCATAATAAAAATAC

561 CAAGAAAAAATTGAGGGATACAAAAGGTAAGAAGCAATCT

601 ACAGGAAATGTTGGTAGTGGGAGAAAGTGGGGCCGTGATG

641 GTGGCATGCTTGATGAAATGAATCATGAAGATGCAGCCAA

681 ATTAGATTTCTCATCATCCAACAGCCACAATAGTAGCCAA

721 GTAGCTCTAGACTCCACTATTAATAAAGATTCTTTTGGGG

761 ATAGAACAGAAGGAGGCGATTTCTTAATCAAAGAAATTGA

801 TGATCTTTTGTCTTCTCATAAAGACGAGATAACTAGTGGG

841 AACGAAGCTAAAAATTCTGGATACGTCAGTACAGCTTTTG

881 GATTCCTTCAAAAACACGTTCTAGGTAACAAAACGATCAA

921 TGAAAGTGATTTGAAATCTGTATTAGAGAAGTTAACACAA

961 CAATTGATAACCAAAAATGTAGCACCAGAGGCAGCAGACT

1001 ATTTAACACAGCAAGTCTCACATGATCTTGTAGGCTCAAA

1041 AACTGCAAATTGGACCAGTGTTGAGAATACTGCTCGTGAA

1081 TCTTTAACAAAAGCATTAACTCAAATATTAACGCCTGGTG

1121 TATCCGTTGATCTCCTCCGTGAAATTCAGAGCAAAAGAAG

1161 CAAAAAGGATGAAGAAGGTAAATGTGATCCCTATGTGTTC

1201 TCTATAGTTGGTGTTAATGGTGTTGGTAAGTCAACAAATC

1241 TTTCAAAGCTAGCGTTTTGGTTACTGCAAAATAATTTCAA

1281 GGTCTTAATTGTTGCTTGTGATACGTTTAGGTCTGGTGCA

1321 GTTGAGCAACTAAGGGTCCATGTTGAAAATTTGGCACAGC

1361 TAATGGATGATTCACACGTTCGTGGCTCCAAGAACAAAAG

1401 GGGTAAAACTGGTAATGACTACGTTGAACTATTTGAAGCT

1441 GGTTATGGTGGATCTGACTTGGTAACCAAAATTGCAAAGC

1481 AAGCCATCAAATATTCTCGTGATCAAAACTTCGATATAGT

1521 GTTAATGGATACTGCCGGGAGAAGGCATAATGATCCTACT

1561 TTAATGTCGCCATTGAAGTCTTTCGCTGACCAAGCCAAGC

1601 CAGATAAAATCATTATGGTTGGAGAGGCTTTAGTAGGTAC

1641 TGATTCTGTCCAGCAAGCCAAAAATTTTAATGATGCCTTC

1681 GGAAAGGGAAGAAATCTTGACTTCTTTATTATCTCCAAGT

1721 GTGACACAGTTGGTGAAATGCTGGGTACTATGGTAAATAT

1761 GGTTTATGCTACGGGGATTCCTATCTTATTCGTTGGCGTG

1801 GGACAAACCTATACCGATTTGAGGACATTAAGCGTAAAAT

1841 GGGCTGTTAATACATTAATGTCTTAA

DNA sequence of *SRP102*

1 ATGCTTAGTAATACACTTATTATTGCCTGTTTATTGGTGA

41 TAGGGACAACCATAGCGCTAATAGCAGTGCAAAAGGCATC

81 CTCCAAGACAGGGATCAAGCAAAAAAGTTATCAACCATCT

121 ATTATCATTGCAGGTCCTCAAAATTCTGGAAAAACGAGCT

161 TGCTTACGCTGCTAACCACAGATTCAGTAAGACCAACTGT

201 TGTTTCTCAAGAACCGTTATCAGCAGCGGATTACGATGGT

241 TCCGGCGTCACGTTAGTGGACTTCCCAGGCCATGTCAAGT

281 TGCGTTATAAACTCTCAGATTATTTGAAAACAAGAGCCAA

321 ATTTGTTAAAGGGTTGATATTTATGGTAGACTCAACAGTT

361 GATCCTAAGAAATTAACAACAACAGCTGAGTTCTTGGTAG

401 ACATATTATCAATTACTGAATCTAGTTGCGAAAATGGTAT

441 TGATATCTTAATTGCATGCAATAAAAGCGAATTGTTCACT

481 GCAAGACCACCATCAAAGATCAAGGATGCTTTAGAAAGCG

521 AAATTCAAAAGGTTATTGAAAGAAGGAAAAAATCGTTGAA

561 CGAGGTTGAAAGAAAAATTAACGAAGAAGATTATGCAGAG

601 AATACACTAGACGTTTTACAGTCTACCGACGGGTTCAAAT

641 TTGCAAATTTGGAAGCATCTGTAGTTGCTTTTGAGGGCAG

681 TATTAATAAAAGAAAAATTTCCCAATGGCGCGAATGGATA

721 GATGAAAAACTGTAA

Appendix 2 Production of affinity purified polyclonal antibodies

During this study polyclonal antibodies were raised against SRα and SRβ in order that these proteins might be immunoprecipitated and/or visualised by immunoblotting.

Plasmids containing fusions of the genes encoding SRα (pGEXSRP101) and SRβ (pSO488) with glutathione-S-transferase (GST) were kind gifts of Prof. P. Walter (U.C. San Francisco) (table 2). The fusion proteins were over-expressed in bacteria, purified on glutathione agarose (section 2.11c) and used to immunise rabbits.

The polyclonal antibodies were affinity purified as described (section 2.18b) using GST-fusions of SRα and SRβ, identical to those originally used for rabbit inoculation. Affinity purified antibodies were tested by using them to probe Western blots of proteins from wild type and SRΔ microsomes. The affinity purified antibody raised against SRα recognises a protein of the correct molecular weight in samples of microsomes prepared from yeast cells. This is also the case for the affinity purified antibody raised against SRβ. This recognition is absent when samples of microsomes that do not contain SRα or SRβ are used. Both antibodies were titrated for optimum dilution for Western blotting and were found to be 1:1000 for both antibodies. These results are shown in Figure A2.1.

Figure A2.1 Titration of affinity purified antibodies

0.8 OD_{280} units of microsomes recovered from strain JDY3 (wild-type) and JDY72 (SR deficient) (table1) were analysed by SDS-PAGE and Western blotting (JDY3, panel A, lanes 1, 3, 5, 7, 9, 11,13 and 15; JDY72, panel A, lanes 2, 4, 6, 8, 10, 12,14 and 16). Fractions collected from the affinity purification of these antibodies were also analysed (materials and methods, lanes 5-10) Dilutions of antibodies specific for SRα (panel B, lanes 1-5) and SRβ (panel A, lanes 1-16; panel B, lanes 6-10) were used to identify SR.

Lanes for (A)

1 and 2 Pre-immune serum

3 and 4 Production bleed

5 and 6 Flow through of GST column

7 and 8 Flow through 2 of GST column

9 and 10 Wash

11 and 12 Dialysate 1:5000

13 and 14 Dialysate 1:1000

15 and 16 Dialysate 1:500

Lanes for (B)

1 1:10,000 anti-SRα

2 1:5000 anti-SRα

3 1:2000 anti-SRα

4 1:1000 anti-SRα

5 1:500 anti-SRα

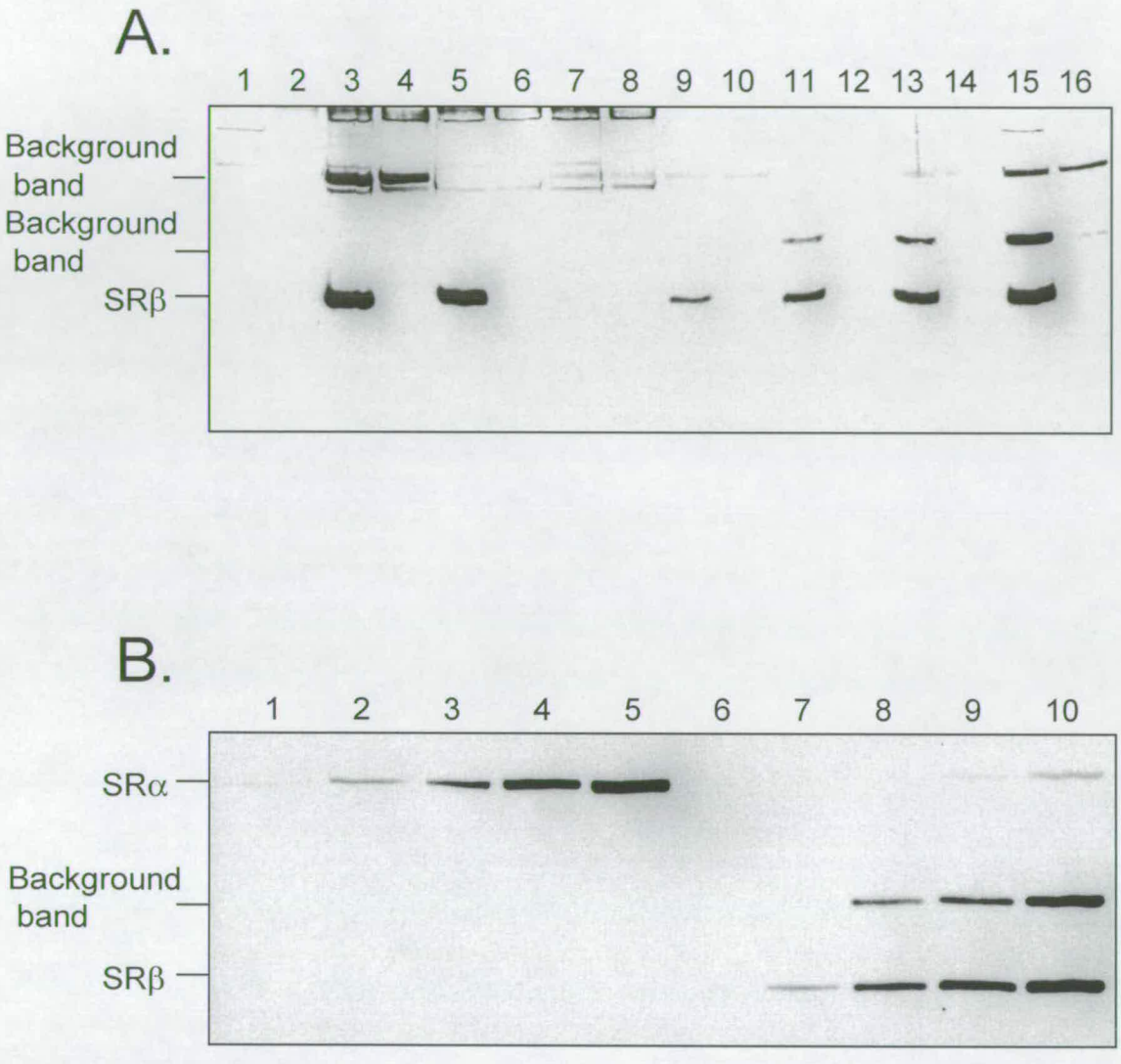
6 1:10,000 anti-SRβ

7 1:5000 anti-SRβ

8 1:2000 anti-SRβ

9 1:1000 anti-SRβ

10 1:500 anti-SRβ


A.
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Background band
Background band
SRβ
B.
1 2 3 4 5 6 7 8 9 10
SRα
Background band
SRβ

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