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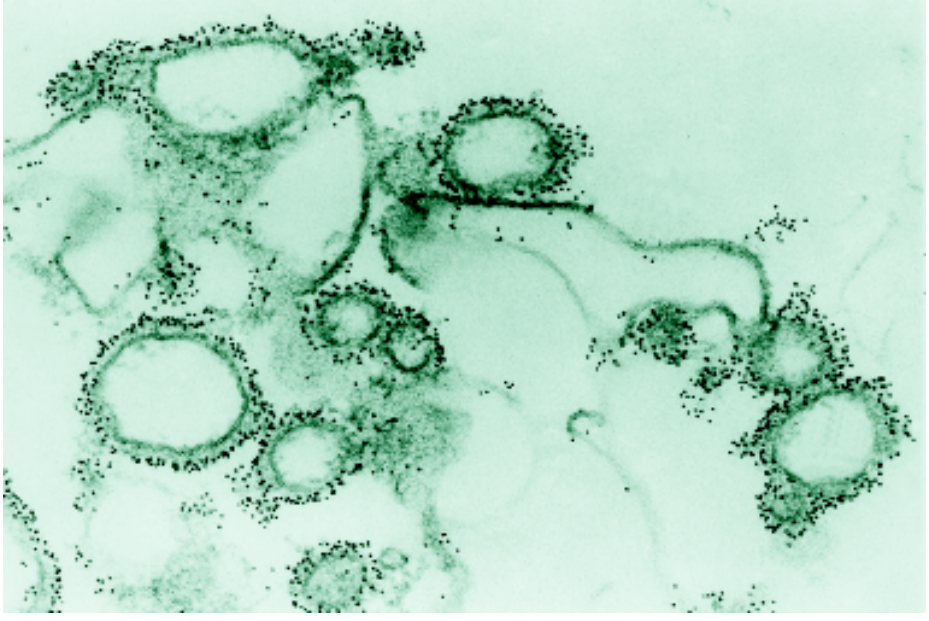

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Functional studies on components of the secretory pathway of *Saccharomyces cerevisiae*

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Abstract

SSO genes have been isolated as multicopy suppressors of *sec1-1*, a mutation in a late-acting *SEC* gene causing accumulation of post-Golgi vesicles at the restrictive temperature, and shown to encode the so-called target membrane SNAP receptors (t-SNAREs) in the plasma membrane of the yeast *Saccharomyces cerevisiae*. The Sso proteins are type II membrane proteins that are capable of posttranslational membrane insertion independent of signal recognition particles (SRPs). Thus, they might be inserted to the plasma membrane directly instead of entering the endoplasmic reticulum (ER) and being transported to their site of action along the biosynthetic pathway. The initial membrane insertion site of Sso2p in the secretory pathway was studied by transient expression of Sso2p in animal cells, which are superior to yeast for such morphological studies. Sso2p was shown to be first inserted into the ER and then transported to the plasma membrane via the secretory pathway.

The Sso proteins are likely to be rate-limiting factors in the secretory machinery of *S. cerevisiae* because their overproduction enhanced by several fold the secretion of both a yeast protein, invertase, and a heterologous reporter protein, *Bacillus* α-amylase. Secretion to the periplasm was enhanced in the Sso2p-overproducing strain, consistent with a previously suggested role for plasma membrane t-SNAREs as anchors in targeting/fusion of the secretory vesicles to the plasma membrane. The secretion enhancement by overproduction of components of the secretory machinery offers a novel, more generally applicable approach to increase the rather modest secretory capacity of *S. cerevisiae*. The secretory pathway of yeast has been considered to contain other bottle necks *e.g.* in the ER and the Golgi complex. Interestingly, overexpression of a newly discovered gene, *SEB1*, shown to encode a novel subunit of an ER translocon, also resulted in enhanced secretion.

SEB1 was cloned as a multicopy suppressor of *sec15-1*, a late-acting mutant gene. *SEC15* shows an extensive pattern of genetic interactions with other late-

acting *SEC* genes and is, thus, considered to have a central function in post-Golgi transport. It encodes a component of a multisubunit complex called the exocyst, which is implicated in targeting/fusion of the secretory vesicles to the plasma membrane as an effector of Sec4p. Surprisingly, the nonessential *SEB1* gene encodes the evolutionarily conserved β subunit of the Sec61p complex, which functions in ER translocation. This unexpected finding prompted further studies on genetic interactions between *SEB1* and the genes encoding exocyst subunits. Interestingly, *SEB1*, unlike its close homologue, *SEB2*, could suppress mutations in all of the exocyst genes. Furthermore, overproduction of the other two components of the ER translocon, Sec61p or Sss1p, could also suppress defects in many of the exocyst mutants. The double disruption *seb1Δseb2Δ* in combination with either of two mutant genes encoding exocyst components, *sec10* or *sec15*, caused synthetic lethality, further strengthening the evidence for genetic interactions.

The genetic interactions observed were rather specific for the two complexes, the ER translocon and the exocyst. In addition, overexpression of *SEC1* or *SSO2* could also suppress all the exocyst mutants except one and on the other hand all the genes encoding components of the Sec61p complex could suppress *sec1-1* when overexpressed. These results suggest a closer interplay between the ER translocon, exocyst complex and plasma membrane t-SNARE than has previously been anticipated.

Preface

This work was carried out at VTT Biotechnology and Food Research (formerly VTT Biotechnical Laboratory) during the years 1994 - 1999. I am grateful to Research Director, Professor Juha Ahvenainen and Research Professor Hans Söderlund for excellent working facilities and their supportive and encouraging attitude towards my thesis work. Hans Söderlund is also thanked for collaboration. I thank Professor Tapio Palva at the Department of Biosciences, Division of Genetics, University of Helsinki for his co-operation during the final stage of my studies. Doctor Marjo Simonen and Professor Hans Ronne are thanked for critical reading of my thesis and useful comments on it.

My warmest thanks are due to Docent Sirkka Keränen, my supervisor. She has taught me a great deal about yeast genetics and various other things during these years. Her never-ending enthusiasm has really impressed me. During her sabbatical 1994 - 1995 at Yale University I had a chance to join her there for four months. I am grateful for that experience and wish to thank Professor Pietro de Camilli for welcoming me to visit his group and for fruitful collaboration. I also thank Doctor Patrick Brennwald for helping me, a first-year graduate student by that time, with many practical problems dealing with my work.

I warmly thank my co-authors Christian Ehnholm, Evelina Gatti, Jussi Jäntti, Esa Kuismanen, Vesa Olkkonen, Mika Outola, Laura Ruohonen, Markku Saloheimo, Kohji Takei and Ville Tieaho for collaboration. Docents Vesa Olkkonen and Markku Saloheimo are also thanked for many useful discussions and for providing me with the support of a senior scientist during Sirkka's sabbatical. Riitta Lampinen and Outi Könönen are thanked for the skilled technical assistance and for being so pleasant and flexible to work with. The technical assistance of Hanna Karmakka is also acknowledged. The supporting services in the lab provided by Mirjami Pelkonen and Kati Sulin have been greatly appreciated. I am grateful to John Londesborough for revising the language of my thesis and to Oili Lappalainen for proficient secretarial assistance.

During these years the number of people working in the yeast/fungi lab has expanded a great deal. Thus, without mentioning the names, I want to thank all the members of the lab, especially those working in the group of Sirkka Keränen, and others working at the division of Genetic Engineering for creating such a nice, friendly and supportive working atmosphere. My dearest colleagues are Nina Aro and Jussi Jäntti. The humour we share has brightened many days and helped me to continue the struggle with my research. I also thank Mari Valkonen and Tuija Vasara, among others, for their friendship.

My thanks are due to my closest ones, both friends and family, for the support and understanding over these years. Also, all the fun and relaxing moments off-science have been greatly appreciated. Lastly, my dearest thanks are due to my parents, Eeva and Yrjö Toikkanen, for their caring, support and encouragement during these years.

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List of publications

This thesis is based on the following articles and a submitted manuscript which are referred to as I-IV in the text, and on unpublished results presented in the text.

I Jäntti, J., Keränen, S., Toikkanen, J., Kuismanen, E., Ehnholm, C., Söderlund, H. and Olkkonen, V. M. 1994. Membrane insertion and intracellular transport of yeast syntaxin Sso2p in mammalian cells. J. Cell Sci. **107**, 3623-3633.

II Toikkanen, J., Gatti, E., Takei, K., Saloheimo, M., Olkkonen, V. M., Söderlund, H., De Camilli, P. and Keränen, S. 1996. Yeast protein translocation complex: Isolation of two genes *SEB1* and *SEB2* encoding proteins homologous to the Sec61β subunit. Yeast **12**, 425-438.

III Ruohonen, L., Toikkanen, J., Tieaho, V., Outola, M., Söderlund, H. and Keränen, S. 1997. Enhancement of protein secretion in *Saccharomyces cerevisiae* by overproduction of Sso protein, a late-acting component of the secretory machinery. Yeast **13**, 337-351.

IV Toikkanen, J. and Keränen, S. Genetic screens reveal functional interactions between ER translocon, exocyst complex and plasma membrane t-SNARE in *Saccharomyces cerevisiae*. Submitted.

Contents

Appendices of this publication are not included in the PDF version. Please order the printed version to get the complete publication (http://www.inf.vtt.fi/pdf/publications/1999/)

Abbreviations

ADH1	gene encoding Alcohol dehydrogenase I
BFA	brefeldin A
BHK	baby hamster kidney
COP	coatomer protein
CPY	carboxypeptidase Y
ER	endoplasmic reticulum
ERAD	ER associated degradation
GEF	guanine nucleotide exchange factor
MDCK	Madin-Darby canine kidney
NAC	nascent-polypeptide-associated complex
NSF	N-ethylmaleimide sensitive fusion protein
OST	oligosaccharyl transferase complex
PAGE	polyacrylamide gel electrophoresis
PDI	Protein disulphide isomerase
RAMP 4	ribosome-associated membrane protein 4
RNC	ribosome-nascent chain complex
scR1	small cytoplasmic RNA
SDS	sodium dodecylsulphate
SFV	Semliki Forest virus
SEC	gene involved in protein secretion
sec	mutant *SEC* gene
SNAP	soluble NSF attachment protein
SNAP25	synaptosome associated protein of 25 kDa
SNARE	SNAP receptor
SPC	signal peptidase complex
SRP	signal recognition particle
SR	SRP receptor
TRAM	translocating-chain-associated membrane protein
TRAP	translocon-associated membrane protein
ts	temperature sensitive
t-SNARE	target membrane SNAP receptor
VAMP	vesicle associated membrane protein
v-SNARE	vesicle SNAP receptor
wt	wild type

1. Introduction

Yeast *Saccharomyces cerevisiae* is a good model organism for biological studies for several reasons. It is genetically the best known eukaryote and at the end of April 1996 became the first such organism whose complete genome sequence was public knowledge. Currently the genome is also under systematic functional analysis. *S. cerevisiae* is easy and inexpensive to grow in the laboratory and genetic and fermentation techniques are well developed for this organism. Many proteins and protein families involved in protein secretion are conserved from yeast to man. Therefore, the unicellular yeast *S. cerevisiae* serves as a good model and tool for studies of secretory transport in higher eukaryotes.

S. cerevisiae has been utilised by man for thousands of years in brewing beer, baking bread and making wine. More recently, *S. cerevisiae* has been used also as a host for production of heterologous proteins. Furthermore, the tractable genetic methods available are being utilised as tools for various screening procedures *e.g.* in drug development (see *e.g.* Broach and Thorner, 1996). Despite its many advantages as a production host, its secretory capacity is rather low. Therefore, understanding the molecular basis of the events taking place on the secretory pathway is of major importance also in order to be able to improve the secretory efficiency.

1.1 The yeast secretory pathway

Eukaryotic cells contain membrane-enclosed compartments, organelles, which have specialised functions and contain unique combination of proteins, lipids and cofactors. Proteins destined to these organelles can enter only a few of them directly from the cytosol. Thus, many proteins have to be sorted and further distributed to their final destination (Nunnari and Walter, 1996). The distribution takes place in transport vesicles and the transported proteins need to contain signal sequences in order to be first translocated into the endoplasmic reticulum (ER), the entry point of the secretory pathway, and then transported further to the target site. In addition to maintaining the cell dynamics, the secretory pathway is used for the delivery of proteins and membrane and cell wall components to the growing bud area as the cell divides and for secreting proteins out of the cell.

Today well over 100 genes involved in protein secretion in yeast have been identified (Figure 1). Also, an estimation that 10 - 20% of the approximately

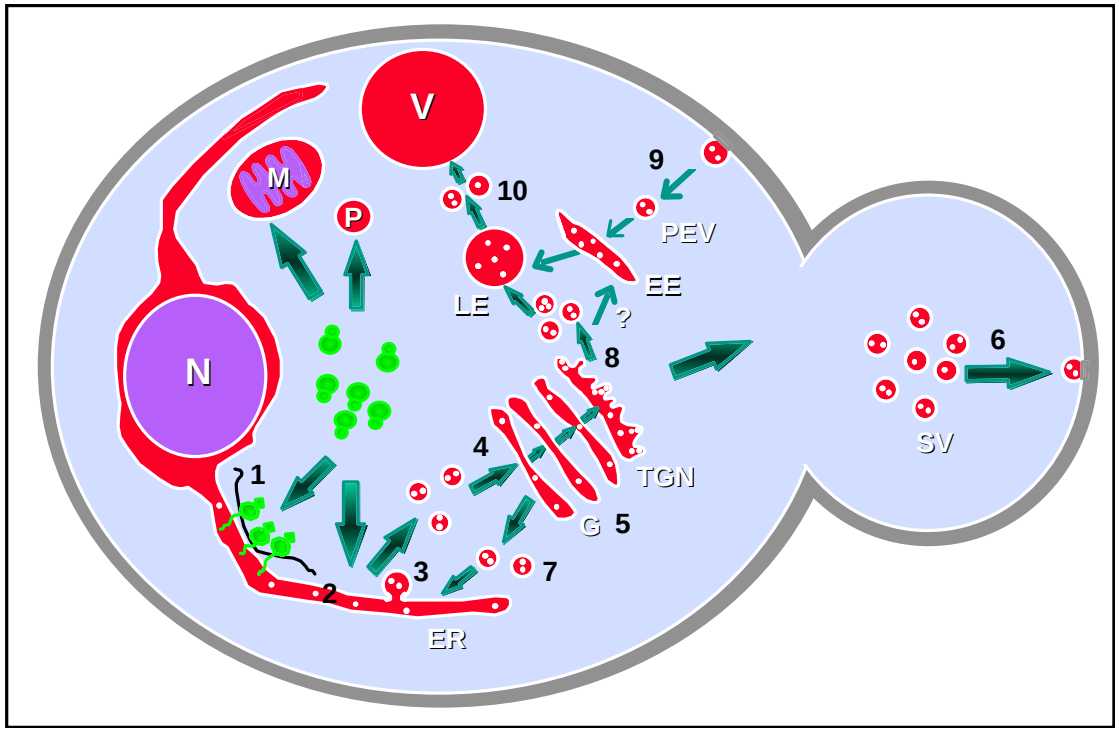

Figure 1. Schematic presentation of the secretory and endocytic pathway of S. cerevisiae *and the genes involved in it. Modified and updated from Kaiser* et al.*, 1997.*

1. Translocation	2. Maturation in ER	3. Vesicle Budding	4. Vesicle Fusion	5. Transport Through Golgi	6. Targeting and Fusion with Plasma Membrane
SSA1-4	*SEC11*	*SEC12*	*SEC17*	*SEC14*	*SEC1*
YDJ1	*PDI1*	*SED4*	*SEC18*	*SFT1*	*SEC2*
SRP54	*EUG1*	*SAR1*	*SEC22/SLP2*	*PMR1*	*SEC3*
SEC65	*MPD1*	*SEC16*	*BET1*	*MNN9*	*SEC4*
SRP14	*CNE1*	*SEC13*	*BOS1*	*VAN1*	*SEC5*
SRP21	*IRE1*	*SEC31*	*BET3*	*VRG4*	*SEC6*
SRP68	*KAR2*	*SEC23*	*YPT1*	*ANP1*	*SEC8*
SRP72	*LHS1*	*SEC24*	*BET2*	*ERD1*	*SEC9*
SCR1	*VMA12*	*BST1*	*BET4*		*SEC10*
SRβ	*VMA21*	*EMP24*	*MRS6*		*SEC15*
SRP101	*VMA22*	*ERV25*	*SED5*		*SNC1,2*
SEC61	*SHR3*		*SLY1*		*SSO1,2*
SSS1			*YKT6*		*ACT1*
SEB/SBH1,2			*USO1*		*MYO2*
SSH1			*SEC7*		*SMY1*
SEC63					*TPM1*
SEC62					*GDI1*
SEC71					*DSS4*
SEC72					*SEC4-GAP*
KAR2					*EXO70*
LHS1					*MSO1*
SCJ1					*SEM1*
SAC1					

7. Retrival	8. Golgi to Endosome	9. Endocytosis	10. Endosome to Vacuole
ERD2	*VPS10*	*END3*	*YPT7*
RET1	*VPS45*	*SLA2*	*YPT52, 53*
SEC26	*PEP12*	*VRP1*	*VAM3/PTH1*
SEC27	*VPS21/YPT51*	*END6*	*NYV1*
SEC21	*VPS1*	*RVS161*	*VPS34*
RET2	*MVP1*	*RVS167*	*SLP1/VPS33*
RET3	*CHC1*	*END8-11*	*DNM1*
ARF1,2	*CLC1*	*ACT1*	
GCS1	*APL2*	*SAC6*	
GEA1,2	*APL4*	*MYO3,5*	
UFE1	*APM1*	*CMD1*	
RER1	*APS1*	*CHC1*	
RER2	*PEP7*	*CLC1*	
RER3			

6300 genes of *S. cerevisiae* would encode proteins either traversing or functioning in the secretory pathway has been presented (Kaiser *et al*., 1997). The yeast secretory pathway was originally defined by secretory mutants. Peter Novick and Randy Schekman isolated a number of temperature sensitive (ts) secretory (*sec*) mutants, that failed to export active invertase or acid phosphatase at the restrictive temperature of 37°C although the protein synthesis could continue for several hours, and there was no secretory defect at a permissive temperature (25°C). Among these mutants were *sec1-1* and *sec2-1* (Novick and Schekman, 1979). The *sec1-1* cells became dense due to vesicle accumulation at the restrictive temperature. The increased cell mass enabled the use of Ludox density gradients for mutant enrichment and led to the isolation of a further set of *sec* mutants (Novick *et al*., 1980).

The *sec* mutants were originally divided into three classes. Class A contains 200 mutants in 23 complementation groups and represents genes that are genuinely involved in secretion (Novick and Schekman, 1979; Novick *et al*. 1980; Novick, 1985). Instead, mutants of class B did not accumulate active invertase even though proteins were synthesised at the restrictive temperature, and protein synthesis as such was temperature sensitive in class C mutants (Novick *et al*., 1980). Further genes have since been identified and they are divided into distinct functional groups according to the transport step in which they are involved: ER entry, budding from ER, targeting to the Golgi complex, budding from Golgi or targeting to the plasma membrane in addition to the endosomal and vacuolar targeting steps (Figure 1).

The order of the transport events in the secretory pathway was first shown in mammalian cells (Palade, 1975). Novick *et al*. (1981) applied this to the yeast secretory pathway by using double mutant analysis. The order of three energy consuming transport steps, ER-to-Golgi, Golgi-to-vesicles and vesicles-to-cell surface, was independently confirmed by studies on glycosylation of invertase in the mutants (Esmon *et al*., 1981). The *sec* mutants are powerful tools in analysis of the secretory pathway and the small number of secretory precursors present in the yeast cells makes the experimental system sensitive in evaluation of the phenotypes caused by the mutations. In addition to these, *in vitro* techniques are important in studying the mechanisms of secretory transport in yeast (Schekman, 1982).

1.2 ER translocation

Protein synthesis takes place in the cytoplasm and the nascent polypeptides, which are destined for the cell organelles, plasma membrane or cell exterior, enter the secretory pathway via translocation into the ER. Eukaryotic cells can

transport proteins across the ER membrane either cotranslationally (*e.g.* invertase in yeast) or posttranslationally (*e.g.* prepro-α-factor in yeast) (Hansen *et al.*, 1986; Rothblatt and Meyer, 1986; Hansen and Walter, 1988). Cotranslational translocation is predominative in most mammalian cells, but in *S. cerevisiae* both pathways are widely used (reviewed by Rapoport *et al.*, 1996). Numerous proteins on the ER membrane participate in the translocation of nascent polypeptides, and *in vitro* techniques with proteoliposomes containing purified components have been used in combination with yeast genetics in detailed studies on the translocation events. Although the key components (Figure 2) have been identified and an image of the tertiary structure of the translocon has been obtained with electron microscopy, the exact functions of most of the subunits are still unclear (reviewed by Kalies and Hartmann, 1998).

1.2.1 Cotranslational translocation

In mammals the Sec61p complex, the signal recognition particle (SRP) receptor (SR) and, for most proteins, the translocating-chain-associated membrane protein (TRAM) are sufficient membrane protein components for the cotranslational translocation *in vitro* (Görlich *et al.*, 1992; Görlich and Rapoport, 1993), although additional components with possibly stimulatory role exist. According to the mammalian model the first step of ER targeting is the recognition of the signal sequence in a ribosome-bound nascent polypeptide by an SRP in the cytosol. The SRP is then bound to the ribosome. This causes GTP to bind to a subunit of SRP called SRP54, which binds also the signal sequence. Elongation of nascent chain is arrested and the complex of ribosome, nascent chain and the SRP becomes bound to the ER membrane via interactions of the SRP with its receptor SR, and of the ribosome with the Sec61p complex (Rapoport, 1992; Walter and Johnson, 1994; Rapoport *et al.*, 1996). Additional components like nascent-polypeptide-associated complex (NAC) may be involved in assisting proper targeting (Wiedmann *et al.*, 1994). The SRP is then released from the ribosome and the signal sequence, and the SRP-SR complex is dissociated in a reaction that hydrolyses GTP. The SRP is delivered to the cytosol for successive cycles of use. Until now, the ribosome-nascent chain complex has been bound to the Sec61p complex only weakly. Once the signal sequence is recognised in the membrane, the nascent chain will be inserted into the translocation site firmly. The signal sequence contacts the Sec61p complex, the TRAM and the phospholipids of the membrane and the translocation channel opens toward the lumen of the ER. While the nascent chain is elongated, it is transferred across the membrane through the protein conducting channel formed by the Sec61p complex (Rapoport, 1992; Walter and Johnson, 1994; Rapoport *et al.*, 1996).

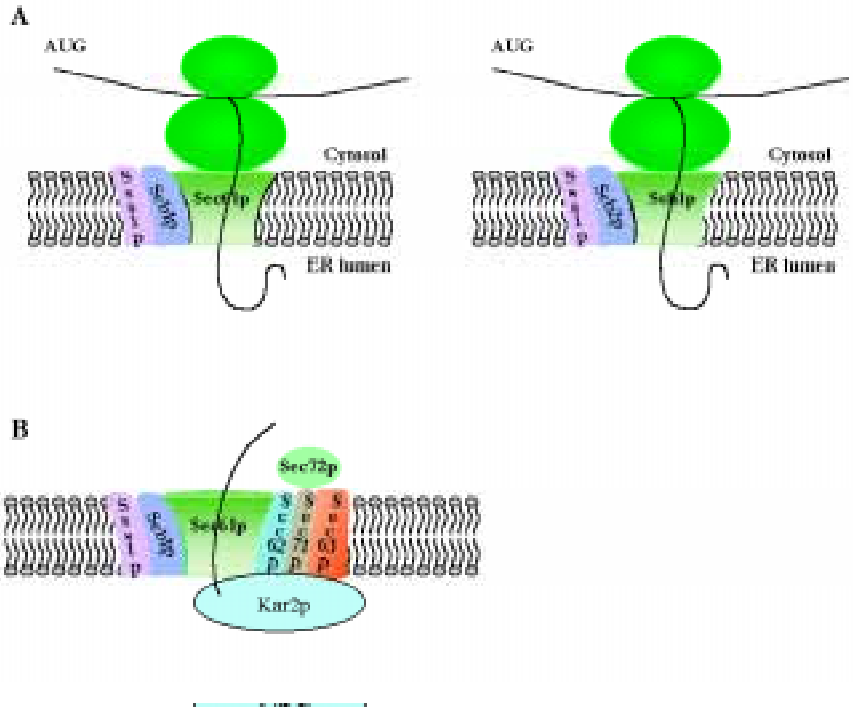

Figure 2. The subunit composition of the co- and posttranslational ER translocation complexes in yeast. The Sec61p complex functions both in cotranslational (A) and post-translational (B) translocation. In the former it is tightly connected with the ribosome and in the latter constitutes together with the Sec62p-Sec63p complex a heptameric Sec complex. The SRP and its receptor SR, Kar2p and Sec63p are other participants in the cotrans-lational translocation. Additional components may facilitate the translocation of a nascent chain. A homologous translocon, the Ssh1p complex, is reported to also function in cotranslational translocation. In addition to the Sec complex, Kar2p, ATP and possibly cytosolic chaperone proteins to keep the nascent chain in a translocation competent conformation are in-volved in the posttranslational translocation.

The TRAM protein needed for the early steps of the cotranslational transport (Görlich *et al*., 1992) was recently shown to have novel regulatory roles in the translocational pausing of a secretory protein and possibly in preventing exposure to the cytosol of the already translocated domains of the nascent polypeptide (Hedge *et al*., 1998). The signal peptidase complex (SPC) and the oligosaccharyl transferase complex (OST) modify the nascent chain during its translocation. Other factors of presently unknown function involved in cotranslational translocation are ribosome-associated membrane protein 4 (RAMP 4) (Görlich and Rapoport, 1993), the translocon-associated membrane protein (TRAP) complex (Hartmann *et al*., 1993), and the proteins p180 (Savitz and Meyer, 1990; 1993) and p34 (Tazawa *et al*., 1991). In yeast the presence of TRAM and RAMP 4 homologues is currently unknown, and there are no homologues of TRAP, p180 or p34 (reviewed by Kalies and Hartmann, 1998). Recently, an initial characterisation of the yeast NAC was reported (Reimann *et al*., 1999). Moreover, yeast has another trimeric translocon functioning only in the cotranslational translocation, the Ssh1p complex, which is a duplicated copy of the Sec61p complex (Finke *et al*., 1996).

1.2.2 Posttranslational translocation

The targeting in posttranslational translocation in yeast is independent of SRP and SR. Instead, the Sec62p-Sec63p complex is required (Rothblatt *et al*., 1989; Hann and Walter, 1991; Ng *et al*., 1996) and forms together with the Sec61p complex a heptameric Sec complex (Deshaies *et al*., 1991; Panzner *et al*. 1995). *In vitro*, the Sec complex, together with ATP and Kar2p, the yeast Bip homologue (Normington *et al*., 1989; Rose *et al*., 1989), was shown to be sufficient for posttranslational translocation both in presence and absence of microsomal membranes (Panzner *et al*., 1995; Matlack *et al*., 1997). When translation of the precursor in the cytosol is ended and the precursor is released from the ribosome, the signal sequence targets the precursor polypeptide to the binding site on the cytosolic face of the Sec complex. The binding of the nascent chain to the Sec complex is independent of ATP. However, in the subsequent step Kar2p interacts with the lumenal side of the Sec complex, ATP is hydrolysed, and the precursor passes through the translocon to be released into the lumen (Lyman and Schekman, 1997; Matlack *et al*., 1997).

In addition to the Sec complex, both cytosolic and lumenal chaperones are implicated in the posttranslational ER translocation. The cytosolic heat shock proteins of the Hsp70 family may be required for keeping the polypeptide in a translocation competent conformation (Chirico *et al*., 1988; Deshaies *et al*., 1988). Kar2p is indicated to be essential for both co- and posttranslational translocation in an ATP-consuming manner (Vogel *et al*., 1990; Sanders *et al*.,

1992, Brodsky *et al*., 1995; Panzner *et al*., 1995), although a fraction of prepro-α factor has been reported to translocate *in vitro* even in the absence of Kar2p and ATP (Panzner *et al*., 1995).

Kar2p is needed for several functions during the translocation event: association of the precursor with the translocation channel, release of the precursor from the docking site formed by the Sec complex, translocation of the precursor through the translocon and completion of the passage through it (Sanders *et al*., 1992; Lyman and Schekman, 1995; Lyman and Schekman, 1997; Matlack *et al*., 1997). However, according to a recent report of Plath *et al*. (1998), the insertion of the precursor chain into the translocation channel does not require Kar2p *in vitro*. Kar2p interacts with the lumenal J domain of Sec63p (Brodsky and Schekman, 1993; Scidmore *et al*., 1993; Schlenstedt *et al*., 1995; Corsi and Schekman, 1997; McClellan *et al*., 1998) and is suggested to determine the directionality of the translocation. Two different models for accomplishing this task have been suggested. According to the molecular ratchet model, Kar2p binds to the polypeptide chain when it emerges in the lumen and prevents the chain from sliding back (Simon *et al*., 1992). Another model suggests a more active, pulling role for the Kar2p (Glick, 1995). Furthermore, the yeast Kar2p assists folding of proteins (Simons *et al*., 1995), and the mammalian Bip has been shown to act as a permeability barrier on the lumenal end of the translocon (Hamman *et al*., 1998).

1.2.3 The signal sequence, SRP and SR

The basic mechanism of cotranslational translocation appears to be conserved from prokaryotes to eukaryotes. This is evidenced by the interchangeability of the signal sequences, signal recognition particles and their receptors among different species *in vitro* (Gierasch, 1989; Powers and Walter, 1997). Signal sequences function in targeting of the proteins to the secretory route. They are very heterogeneous, but contain certain common features that are essential for protein export. These features are a hydrophobic core of 7-15 amino acids, that is preceded by an amino-terminal positively charged region and followed by a polar region where signal peptidase cleavage occurs (von Heijne, 1985; 1986). The exact amino acid sequence is not crucial for the signal peptide to be functional. Kaiser *et al*. (1987) have shown that numerous varied sequences can replace the signal sequence of invertase and lead to correct transport.

The signal sequence is suggested to participate in the opening of the translocation channel (Simon and Blobel, 1992; Crowley *et al*., 1994; Jungnickel and Rapoport, 1995). The amino acid composition of the hydrophobic core of the signal sequence and the length of the preceding amino-

terminal domain can affect the TRAM dependency of a nascent chain (Voigt *et al*., 1996). According to Ng *et al*. (1996) the hydrophobicity of a signal sequence determines the mode of translocation of the precursor peptide. A more hydrophobic signal sequence is likely to lead to the cotranslational SRP-dependent translocation whereas a less hydrophobic sequence is used in an SRP-independent posttranslational manner. In addition, they found that some precursors can be translocated via both pathways.

Recognition of the signal sequence is suggested to include two steps that affect the translocation efficiency: an SRP-mediated targeting step and a step involving components of the translocon at the ER membrane (Jungnickel and Rapoport, 1995; Belin *et al*., 1996). According to recent reports the SRP enhances the binding of the ribosome-nascent chain complex (RNC) to the ER membrane irrespective of the presence or absence of the NAC (Neuhof *et al*., 1998; Raden and Gilmore, 1998) thus bringing into question the importance of the NAC in this process (Wiedmann *et al*., 1994). The function and subunit composition of the SRP is conserved in evolution. However, unlike the eukaryotic SRPs, the bacterial SRP is reported to be required for the membrane insertion of certain polytopic integral membrane proteins (Ulbrandt *et al*., 1997). The composition of the SRP, – 7S RNA and six polypeptides denoted by their molecular masses in kilo Daltons (kDa) SRP9, SRP14, SRP19, SRP54, SRP68 and SRP72, – was first uncovered in a mammalian system (Walter and Blobel, 1980; 1982). The signal sequence binding ability of the 54 kDa subunit was shown with cross-linking experiments (Krieg *et al*., 1986; Kurzchalia *et al*., 1986). Consistent with the important function, the sequence homology of this subunit is the highest among different species, and has allowed isolation of the yeast homologues (Bernstein *et al*., 1989; Hann *et al*., 1989; Römisch *et al*., 1989; Amaya *et al*., 1990).

Srp54p, Sec65p and small cytoplasmic RNA (scR1) were the first SRP components identified in *S. cerevisiae* (Hann *et al*., 1989; Hann and Walter, 1991; Hann *et al*., 1992; Stirling and Hewitt, 1992). The use of immunoaffinity chromatography with antibodies against Sec65p enabled the purification of yeast SRP and isolation of five additional proteins (Brown *et al*., 1996). Four of them are homologous to the mammalian SRP subunits and were, unlike the previously identified subunits, shown to be required for the stability of the complex in yeast. The lack of functional yeast SRP, however, is not lethal. Instead, it leads to reduced growth rate of the cells and a defect in translocation of certain proteins (Hann and Walter, 1991; Brown *et al*, 1996). Interestingly, adaptation to this situation has been observed. Ogg *et al.* (1992) showed that after extended growth cells with disrupted genes encoding SRP subunits had only mild defects in translocation. The growth rate, though, was not restored to

the normal level. The yeast SRP receptor, which is a heterodimer formed by α and β subunits, is not essential for viability either. However, depletion of its subunits leads to the same phenotype as observed when SRP components are depleted (Ogg *et al*., 1992; Miller *et al*., 1995).

1.2.4 The Sec61p and Ssh1p complexes

Protein translocation into the ER has been suggested to occur via a hydrophilic protein conducting channel (Simon and Blobel, 1991). The first evidence involving Sec61p in this channel in both mammals (Mothes *et al*., 1994) and yeast (Müsch *et al*., 1992; Sanders *et al*., 1992) came from crosslinking experiments. *In vitro* experiments using proteoliposomes containing purified protein components then showed that the trimeric Sec61p complex is necessary for the translocation, thus further confirming its channel-forming role (Görlich and Rapoport, 1993; Panzner *et al*., 1995). Finally, Hanein *et al.* (1996) directly visualised oligomers representing mammalian and yeast translocation pores. These oligomeric structures were formed by 3 to 4 trimeric Sec61p complexes and contained a pore with a diameter of approximately 20 Å.

The pore formation was induced by binding of the ribosome or the Sec62p-Sec63p complex to the Sec61p complex. Thus, Sec61p is considered to be the core component of the translocation machinery: it assembles with the Sec62p-Sec63p complex for posttranslational translocation, and it forms a tight conjunction with a ribosome in order to build the translocation channel for cotranslational transport (Deshaies *et al*., 1991; Crowley *et al*., 1994; Kalies *et al*., 1994; Panzner *et al*, 1995; Hanein *et al*., 1996). Also free trimeric Sec61p complexes have been found (Hanein *et al*., 1996), and the channel is capable of releasing proteins laterally into the ER membrane itself (Simon and Blobel, 1991; Martoglio *et al*., 1995). The diameter of mammalian translocon pores is reported to alter depending on the presence of ribosomes and the functional state of the pore (Hamman *et al*., 1998). Since the Sec61p complex can exist as part of the Sec complex and in ribosome-bound or free form, the pore size of the yeast translocons is likely to vary in the same way.

The components of the Sec61p are evolutionarily conserved (Görlich *et al*., 1992; Stirling *et al*., 1992; Hartmann *et al*., 1994) with respect to their function and sequence similarity. Sec61α/Sec61β/Sec61γ are subunits of the mammalian Sec61p complex, Sec61p, Sbh1p/Seb1p and Sss1p those of yeast and SecY/SecG/SecE of bacteria. Sec61p is a 52 kDa integral membrane protein that spans the membrane ten times (Stirling *et al*., 1992; Wilkinson *et al*., 1996). Consistent with the important function of Sec61p, the gene encoding it is essential (Stirling *et al*., 1992).

The conditional lethal mutants in yeast have been valuable in studies on the function and interactions of the Sec61p. The previously isolated ts *sec61-2* and *sec61-3* mutations, however, have been shown to destabilise the structure of Sec61p rather than to impair its function in translocation (Sommer and Jentsch, 1993; Esnault *et al*., 1994; Biederer *et al*., 1996). Recently, several *sec61* mutants were isolated with defects both in co- and posttranslational ER translocation and in export from the ER (Pilon *et al*., 1997; Pilon *et al*., 1998). These cold sensitive mutants were defective because of malfunction of the protein rather than reduced stability, and they were divided into two classes according to the stage at which they manifest their defect. In the mutants of the first class, the interactions of the nascent polypeptide precursor with Sec72p of the precursor docking site formed by the Sec complex were almost abolished. The mutants of the second class were able to crosslink the precursor to the Sec72p, but with reduced efficiency, and the subsequent release of the precursor in the presence of ATP was blocked. Thus, with these mutants, Pilon *et al*. (1998) were able to characterise the involvement of Sec61p in two early stages of translocation before the precursor protein transport across the membrane, and they suggested a role for Sec61p in transition of the translocon pore from a closed to an open state.

The other two subunits are small tail-anchored membrane proteins of less than 10 kDa. The yeast β subunit (Sbh1p/Seb1p) has been identified independently by both biochemical and genetic means (Panzner *et al*., 1995; II). Unlike the other two components, Sbh1p/Seb1p is encoded by a nonessential gene that in yeast has a homologue (Finke *et al*., 1996; II). The homologous gene *SBH2*/*SEB2* is also nonessential, but simultaneous disruption of both *SBH*/*SEB* genes causes a temperature sensitive growth defect with only mildly impaired protein translocation to the ER. Therefore, the role of Sbh/Seb proteins in ER translocation in yeast is still unclear.

The role of β subunits in other organisms is also rather ambiguous. The mammalian Sec61β is not essential for the cotranslational translocation, but it has been suggested to have a stimulatory role in it. Kalies *et al*. (1998) have shown that Sec61β is not required for the docking of ribosome-nascent chain complex to the Sec61p complex, but surmise that it is needed later for insertion of the nascent chain to the translocation channel. In addition, the protein interacts with a subunit of the signal peptidase complex (SPC). Based on this, Kalies *et al*. (1998) have suggested that Sec61β transiently recruits SPC to the translocation site which leads to the enhanced signal peptidase activity and translocation of nascent chains. The prokaryotic counterpart, SecG, also known as Band 1 and p12, is a polytopic membrane protein structurally quite different from eukaryotic β subunits and is not required for protein translocation under

standard growth conditions although its presence accelerates translocation across the cytoplasmic membrane (Brundage *et al*., 1990; Douville *et al*., 1994; Akimaru *et al*., 1991; Nishiyama *et al*., 1993; Nishiyama *et al*., 1994).

The essential gene encoding the yeast γ subunit was cloned as a multicopy suppressor of a *sec61-2* ts mutation, thus the name *SSS1* for **s**uppressor of ***s****ec* **s**ixty one. The essentiality of Sss1p in translocation was demonstrated with depletion experiments in which the translocation of α factor, carboxypeptidase Y (CPY) and invertase was defective (Esnault *et al.* 1993). The suppression of mutant Sec61p has been shown to be due to a stabilising effect of Sss1p on Sec61p. Furthermore, the interactions of the α and γ subunits are mutually stabilising: the *sec61* mutation destabilises Sss1p (Esnault *et al*., 1994; Biederer *et al*., 1996). Recently, Sss1p was shown to bind to the same regions on Sec61p as do the signal sequences (Plath *et al*., 1998). In addition, it has been hypothesised that, together with the channel-forming Sec61p, Sss1p forms the most basic translocation machinery (Finke *et al*., 1996; Plath *et al*., 1998).

Yeast has another trimeric complex functioning in ER translocation. The α subunit of it is Ssh1p, a homologue of Sec61p, and the β subunit, Sbh2p/Seb2p, is a homologue of Sbh1p/Seb1p. Interestingly, Sss1p was isolated also as a subunit of the Ssh1p complex (Finke *et al*., 1996). Despite the sequence similarity, the α and β subunits of the Sec61p and Ssh1p complexes are not normally interchangeable and the *SSH1* gene is not essential for viability although its deletion reduces the growth rate of the cells. However, the β subunit of the Ssh1p complex is able to interact the α subunit of the Sec61p complex if *SSH1* and *SBH1*/*SEB1* are deleted. The Ssh1p complex is believed to function only in cotranslational translocation as judged by translocation studies and its ability to interact with ribosomes but not with the Sec62p-Sec63p complex. Thus, the Ssh1p complex has been suggested to be required when cotranslational function of the Sec61p complex becomes limiting (Finke *et al*., 1996).

1.2.5 The Sec62p-Sec63p complex

Sec62p, Sec63p, Sec71p and Sec72p form together with the Sec61p complex a heptameric Sec complex that in addition to Kar2p is required for post-translational ER translocation (Deshaies *et al*., 1991; Brodsky and Schekman, 1993; Panzner *et al*., 1995). Sec62p and Sec63p are integral membrane proteins that are encoded by essential genes (Deshaies and Schekman, 1989; Rothblatt *et al*., 1989; Sadler *et al*., 1989; Deshaies and Schekman, 1990; Feldheim *et al*., 1992). The nonessential *SEC66*/*SEC71* and *SEC72* genes encode integral and

cytosol-facing peripheral membrane proteins, respectively (Feldheim *et al*., 1993; Feldheim and Schekman, 1994). Their deletion or ts mutations cause defects of variable extent in translocation of certain secretory proteins such as α factor precursor, CPY, invertase and Kar2p (Green *et al*., 1992; Feldheim *et al*., 1993; Kurihara and Silver, 1993; Fang and Green, 1994; Feldheim and Schekman, 1994).

Sec63p contains an ER lumenal DnaJ-like domain that interacts with the DnaK-like domain of the lumenal Hsp70 homologue Kar2p (Brodsky and Schekman, 1993; Scidmore *et al*., 1993). Furthermore, the lumenal DnaJ-like domain is sufficient to prompt the hydrolysis of ATP by Kar2p, which is essential for ER translocation (Corsi and Schekman, 1997). Sec63p and Kar2p have been suggested to be involved in early events of translocation (Sanders *et al*., 1992; Lyman and Schekman, 1995). Lyman and Schekman (1997) have later specified their roles in them by showing that Sec63p-Kar2p interaction is required for ATP-dependent release of a nascent chain from the Sec62p-Sec63p complex. According to Brodsky *et al.* (1995), Sec63p and Kar2p are required also for cotranslational translocation *in vitro*. In accordance, overproduction of Sec63p was able to suppress the cotranslational translocation defect in cold sensitive *sec61* mutant cells more efficiently than the posttranslational defect (Pilon *et al*., 1998). Panzner *et al*. (1995), however, were unable to detect the association of Sec62p-Sec63p complex with the membrane-bound ribosomes in the re-constituted system. Therefore, the role of Sec63p in cotranslational translocation needs further examination.

The heptameric Sec complex is necessary for precursor docking to the ER membrane in posttranslational translocation *in vitro*. Only the intact Sec complex, and neither Sec61p complex nor Sec62p-Sec63p complex, is sufficient for forming the oligomeric structures and binding the signal sequences. This suggests that the channel formation may be a prerequisite for the binding of a signal sequence (Hanein *et al*., 1996; Matlack *et al*., 1997). Furthermore, the presence of Sec61p in the complex is not enough. It must also be functional (Pilon *et al*., 1998). In addition to Sec61p, all the subunits of the Sec62p-Sec63p complex have been shown to be present in the binding site (Lyman and Schekman 1997; Pilon *et al*., 1998). Based on studies *in vitro* with dominant *KAR2* mutants, an ATP-dependent conformational change of Kar2p has been suggested to be necessary both for the Sec63p binding and the forwarding of the translocation reaction (McClellan *et al*., 1998). Similarly, conformational changes of Sec63p and Sec61p or the other subunits of the complex may be necessary for the release of precursor from the binding site (Corsi and Schekman, 1997; Lyman and Schekman, 1997; Pilon *et al*., 1998).

1.3 Protein maturation

Several events in the ER, which is an oxidising environment with a high concentration of chaperones, are responsible for maturation and secretion competence of the translocated precursor protein. The signal sequence is cleaved off, disulphide bonds are formed and the nascent chain is folded, oligomerized, and possibly acylated or glycosylated. Furthermore, there is so-called quality control machinery to ensure that an immature or malfolded protein will not escape the ER for further transport along the secretory pathway (Hurtley and Helenius, 1989; Hammond and Helenius, 1995).

The signal sequence of the translocated nascent chain is cleaved by a signal peptidase complex consisting of four subunits of which only two are essential (Böhni *et al*., 1988; YaDeau *et al*., 1991; Fang *et al*., 1996; Mullins *et al*., 1996; Fang *et al*., 1997). At least two classes of chaperones assisting the folding are known. The first class consist of enzymes, *e.g.* protein disulphide isomerase (PDI), that catalyse specific isomerization steps and the second class are chaperones that bind unfolded or partially folded precursors and prevent their inappropriate interactions. Representatives of the second class include several members of the Hsp70 family which despite their common features are not interchangeable (reviewed by Gething and Sambrook, 1992).

Kar2p, in addition to its requirement for translocation, is needed for folding of secretory proteins as was demonstrated for CPY by Simons *et al*. (1995). The functions of Kar2p in translocation and as a folding chaperone seem to be clearly separable as some proteins have been shown to require only one of these functions (Holkeri *et al*., 1998). Another lumenal Hsp70-related protein, Lhs1p, is involved both in translocation and folding of proteins (reviewed by Craven *et al*., 1997). Unlike Kar2p, it is not essential for viability, but its depletion impairs translocation of several precursors. Furthermore, null mutation of *LHS1* is lethal in combination with certain *kar2* mutants, and can partially rescue another *kar2* mutation, which becomes more deleterious when *LHS1* is overexpressed. These genetic interactions led to the suggestion that Kar2p and Lhs1p have partly overlapping functions. Homologues of Lhs1p from other species have been found and together they are presumed to form a novel subclass of Hsp70-like proteins (reviewed by Craven *et al*., 1997).

Mammalian calnexin and calreticulin are chaperones of different functional principle. They are lectins that bind N-glycosylated polypeptides through the sugar moieties. It is currently unclear whether binding also involves protein-protein interactions. In addition to transient binding to glycoproteins, calnexin can bind for a prolonged time to innative proteins and so retain them in the ER.

This suggests a role in quality control (reviewed by Helenius *et al*., 1997). A nonessential homologue of calnexin, Cne1p, has been identified in yeast by sequence homology, but it is not clear whether its function is similar. The chaperone function of Cne1p seems dispensable or can possibly be substituted by another chaperone. However, disruption of *CNE1* leads to transport of two proteins to the cell surface that would normally be expected to remain in the ER, indicating a role in quality control for Cne1p (Parlati *et al*., 1995). A yeast homologue for calreticulin has not been identified.

Both O- and N-linked glycosylation of proteins are initiated in the yeast ER and the processing continues in the Golgi. The early stages of N-glycosylation are conserved among eukaryotes reflecting the important contribution of these glycans to protein stability, conformation and targeting. The N-linked core oligosaccharide unit is attached to the amide group of an asparagine residue that is part of the tripeptide sequence asn-x-ser/thr, in which x denotes any amino acid residue but proline. The later stages differ between higher eukaryotes and fungal species. In mammalian cells several mannnoses are cleaved during processing of a glycan and either new residues of different oligosaccharides are added to form complex or high-mannose type glycans, or hybrids between them (Kornfeld and Kornfeld, 1985). In yeast only a single mannose is removed and a great number of mannose units can be added to the glycan to form so-called outer chain units. The yeast O-linked glycans are added to serine or threonine residues and consist of mannose residues only (reviewed by Herscovics and Orlean, 1993; Orlean, 1997).

1.4 Protein maintenance in the ER and ERAD

1.4.1 Retrotranslocation and ERAD

ER associated protein degradation (ERAD) was previously assumed to take place in the lumen or membrane of the ER (Knittler *et al*., 1995). In recent years, however, a great deal of evidence has been gathered to support the view that the aberrant or malfolded proteins are transported in an ATP-consuming manner to the cytosol to be degraded by proteasomes. ERAD is very selective for specific, aberrant or misfolded, proteins. The substrate selectivity has been proven by studies on monomers that fail to assemble into proper complexes such as the subunits of the T-cell receptor. A proteasome inhibitor, lactacystin, has been utilised in studies to show that proteasome is indeed responsible for ERAD. In addition, the use of a ubiquitin mutant has proved that ubiquitination is a prerequisite in most cases for degradation mediated by proteasomes (Sommer and Jentsch, 1993; reviewed by Brodsky and McCracken, 1997).

Both lumenal and membrane proteins are degraded by the ubiquitin-proteasome pathway. The human cytomegalovirus products US2 and US11 were shown to target major histocompatibility complex class I (MHCI) molecules selectively to degradation in the cytosol. The degradation was evidently mediated by proteasomes because addition of lactacystin revealed a complex formed between MHCI and a proteasome (Wiertz *et al*., 1996a; 1996b). In yeast, similar results have been obtained with the help of lactacystin and mutant forms of ubiquitin-conjugating enzymes or proteasomes (Biederer *et al*., 1996; Hiller *et al*., 1996; Werner *et al*., 1996). In *sec61-2* cells the mutant Sec61p possibly leads to enhanced dissociation of the Sec61p complex. In those cells Sec61p in addition to another wild type (wt) subunit of the Sec61p complex, Sss1p, was targeted for degradation at a restrictive temperature (Biederer *et al*., 1996). Two ubiquitin-conjugating enzymes, Ubc6p and Ubc7p, were essential for proteasome-mediated degradation of mutant Sec61p and unfolded CPY (Biederer *et al*., 1996, Hiller *et al*., 1996) but not that of mutant pro-α factor (Werner *et al*., 1996). Both these ubiquitin-conjugating enzymes and the proteasome are on the cytosolic side of the ER. Thus, the degradation machinery is considered to be tightly coupled to retrotranslocation (Brodsky and McCracken, 1997).

The export of proteins destined for degradation from the ER has been shown to be mediated by the Sec61p complex both in mammals (Wiertz *et al*., 1996b) and in yeast (Pilon *et al*., 1997; Plemper *et al*., 1997). In reducing conditions unfolded MHCI was detected bound to Sec61p complex (Wiertz *et al*., 1996b). In yeast, the cold-sensitive *sec61* mutants and ts *sec61-2* cells have been shown to accumulate unfolded precursors in the ER even at a temperature that is permissive for growth and protein import into the ER. This indicates that in addition to having defects in translocation, the mutants are unable to export the aberrant proteins to the cytosol (Pilon *et al*., 1997; Plemper *et al*., 1997; Pilon *et al*., 1998).

Sec63p and Kar2p have also been proposed to function in protein export from the ER, although their role may be indirect (Plemper *et al*., 1997). As the translocon has been thought to have no directional bias (Ooi and Weiss, 1992), accessory factors in the ER, such as calnexin (McCracken and Brodsky, 1996), Kar2p (Plemper *et al*., 1997), Der1p (Knop *et al*., 1996) and Der3p/Hrd1p (Bordallo *et al*., 1998), are likely needed for retrotranslocation of proteins destined for degradation. Similarly to the role of Kar2p in posttranslational translocation, chaperones in the cytosol are suggested to assist the retrotranslocation either by pulling or acting as a ratchet in an ATP-hydrolysing manner (Brodsky and McCracken, 1997).

1.4.2 ER retention and retrieval

Many proteins resident in the lumen of the ER contain the carboxy-terminal tetrapeptide sequence of KDEL in mammals and HDEL in yeast. The essential and sufficient role for this sequence in ER retention was revealed by experiments showing that addition of it to the carboxy-terminus of secretory or lysosomal proteins led to their ER localisation and that its deletion induced secretion of Bip (reviewed by Pelham, 1989). The proteins containing this retention signal had acquired modifications for which enzymes of the Golgi are required. Thus, the proteins must had first exited the ER and then been retrieved back. The retrieval was postulated to be mediated by a receptor and this was further confirmed by identification of Erd2p, which was shown to be necessary for retrieval of lumenal ER resident proteins in yeast (Lewis *et al*., 1990; Semenza *et al*., 1990).

Recent studies on animal cells, though, have questioned the role of KDEL as an essential retrieval signal. B-fragments of Shiga toxin containing either wild type or mutated KDEL signal were delivered to the ER with the same kinetics (Johannes *et al*., 1997). Similarly, studies with vesicular stomatitis virus G protein called ts045 indicate that the Golgi to ER retrograde traffic is not entirely signal-mediated (Cole *et al*., 1998). The results from these studies rather suggest that KDEL would be important for retention in the ER (Johannes *et al*., 1997; Cole *et al*., 1998). In yeast, however, the previously shown requirement of the HDEL signal and its receptor Erd2p for ER retrieval *in vivo* was recently confirmed by *in vitro* studies (Spang and Schekman, 1998).

Like the lumenal-resident proteins, proteins resident in the ER membrane contain a signal that can lead to retrieval to the ER from as far as from the trans Golgi (reviewed by Nilsson and Warren, 1994). The intermediate compartment between the ER and Golgi, which was first reported in animal cells (Saraste and Kuismanen, 1984), is considered to be the major recycling site for ER-resident proteins (Lippincott-Schwartz *et al*., 1990). The retrieval signal of type I membrane proteins (amino-terminus in the lumen of the ER) is a double lysine motif in KKXX or KXKXX sequences (X denotes any amino acid residue) in their carboxy-termini, which face the cytosol (Gaynor *et al*., 1994; Townsley and Pelham, 1994). Instead, two arginines (RR) in the amino-terminus are essential for ER retrieval of type II membrane proteins (carboxy-termini in the lumen of the ER). In addition, other less well known retrieval signals exist (reviewed by Nilsson and Warren, 1994). Interestingly, the COPI coat proteins recognise the KKXX signal and thus the retrieval is likely mediated by COPI-coated vesicles. Consistently, a functional double lysine ER retention signal, but not a mutated form, could bind to COPI coatomer *in vitro* (Cosson and Letourneur, 1994), and the yeast mutants with defective COPI coatomer

components were shown to be unable to retain a reporter protein in the ER *in vivo* (Letourneur *et al*., 1994).

There are probably ER retention signals in addition to retrieval signals. Thus, although proteins containing ER retention sequences can reach the intermediate compartment, an ER lumenal protein which lacks its retrieval signal is still unlikely to exit the ER. The exact retention signal is unknown, but it could be a membrane-spanning domain as has been shown for the nuclear and Golgi resident membrane proteins. Furthermore, both retention and retrieval signals may exist in one protein and function independently. Thus, the retrieval signal could act as safe-guard to ensure the correct localisation of the ER resident proteins (reviewed by Nilsson and Warren, 1994).

1.5 Exocytosis

The term exocytosis is used in a narrow meaning to describe only the regulated release of proteins from the secretory granules (Palade, 1975). In a wider use, it means also the constitutive transport of secretory proteins and the last step of the secretory pathway, from Golgi to plasma membrane and release to cell exterior. Today more than twenty genes essential for this protein transport step have been identified in yeast (Figure 1). However, the exact function of most of the proteins encoded by them is still unknown, in part because there is no *in vitro* assay for this transport step.

The transport of secretory proteins to the plasma membrane has been presumed to occur without requirement for any specific signal (Pfeffer and Rothman, 1987; Rothman, 1987). This so-called bulk flow model was experimentally evidenced by the transport of a tripeptide that is a target for N-glycosylation. Wieland *et al*. (1987) suggested that the tripeptide was transported to the cell surface through the secretory pathway although faster than the secretory protein, albumin. The bulk flow theory was further supported by the existence of retention signals for the ER and Golgi resident proteins (Munro and Pelham, 1987; Pelham, 1989; Nilsson and Warren, 1994 and references therein) and a specific signal, mannose 6-phosphate, in soluble proteins targeted to the lysosome of animal cells (reviewed by Farquhar, 1985). Also the regulated secretion by secretory granules (Burgess and Kelly, 1987) that release their contents only after appropriate stimulus is considered as an evidence that constitutive secretion occurs by default. Furthermore, constitutive secretion without a specific signal seems a more economical choice for the cells than tagging secretory proteins with several signal sequences that would allow them to pass different cellular compartments before they reach their final destination (Pfeffer and Rothman, 1987).

The trans-Golgi network (TGN) is considered to be the major sorting site of proteins targeted to the lysosome and those secreted either constitutively or in regulated manner (Griffiths and Simons, 1986). One experimental evidence for this was offered by showing that both insulin, whose transport is regulated, and constitutively secreted viral membrane protein, influenza hemagglutinin, follow the same route to the TGN whereafter their fate is different (Orci *et al*., 1987). Also in yeast the TGN is the major sorting station between the prevacuolar or vacuolar- and plasma membrane-targeted protein traffic (Rothman and Stevens, 1986). Constitutive protein secretion to the plasma membrane is mediated by coated vesicles (Payne and Schekman, 1985; Orci *et al*., 1986). The coat is different from clathrin as the yeast cells with deletion of the gene encoding the clathrin heavy chain manifested only a minor defect in the transport and secretion of invertase (Payne and Schekman, 1985).

The bulk flow theory has more recently been challenged. Römisch and Schekman (1992) showed that the tripeptide, whose transport was used as the initial evidence for the signal-independent transport through the secretory pathway, is not transported in vesicles to the Golgi and is consistently unable to acquire Golgi-dependent modifications. Exit from the ER is considered to imply selective sorting and concentration of the cargo and to require a di-acidic sorting signal (reviewed by Bannykh *et al*., 1998). Also, at least two different post-Golgi vesicle populations, whose cargo contents are different, have been suggested to exist (Bretscher *et al*., 1994; Govindan *et al*., 1995) and have now been isolated (Harsay and Bretscher, 1995; Mulholland *et al*., 1997; David *et al*., 1998). Furthermore, studies of the nitrogen source-influenced and Sec13p-dependent post-Golgi transport of amino acid permeases, Gap1p and Put4p (Roberg *et al*., 1997a; 1997b), and of the dispensability of the late-acting vesicle SNAP receptors (v-SNAREs), the Snc proteins (David *et al*., 1998), have suggested that also post-Golgi traffic can occur in a regulated manner in yeast. The regulated secretion of the amino acid permeases to the plasma membrane is suggested to occur in a third vesicle population, whose coat component may be Sec13p (Roberg *et al*., 1997a; 1997b).

1.5.1 The SNARE hypothesis and membrane fusion

In order to maintain compartmentalisation in cells it is important that a vesicle budding from a donor membrane fuses only with the appropriate target membrane. According to the SNARE hypothesis this fidelity of targeting and fusion is acquired by specific recognition between a membrane protein on a vesicle (v) and its receptor on the appropriate target (t) membrane (Bennett and Scheller, 1993; Söllner *et al*., 1993). The proteins that fulfil these functions were first isolated from neuronal cells as a 20S complex containing the vesicle-

associated membrane protein VAMP/Synaptobrevin, the target membrane receptor syntaxin and the synaptosome-associated protein of 25kDa (SNAP25). In addition, the N-ethylmaleimide sensitive fusion protein (NSF) and the soluble NSF attachment proteins (SNAPs) are needed as general factors for the targeting and fusion events. Synaptobrevin/VAMP, syntaxin and SNAP25 bind αSNAP, thus the term SNARE for SNAP receptor. The *in vivo* evidence for the functioning of this complex in the neuronal exocytosis originates from experiments showing specific proteolysis of the SNAREs by botulinum and tetanus toxins, and their ability to bind the general factors NSF, α and γ SNAP needed for the fusion (Bennett and Scheller, 1993; Söllner *et al*., 1993; Ferro-Novick and Jahn, 1994).

The vesicle fusion machinery is conserved in all eukaryotes and used both in constitutive and regulated secretion (Figure 3) (Ferro-Novick and Jahn, 1994). The yeast equivalents of NSF, Sec18p (Wilson *et al*., 1989), and αSNAP, Sec17p (Clary *et al*., 1990; Griff *et al*., 1992), are needed for multiple steps along the exocytic pathway (Kaiser and Schekman, 1990; Graham and Emr, 1991). The v-SNAREs in the exocytic step of yeast are the Snc proteins (Gerst *et al*., 1992; Protopopov *et al*., 1993) and the t-SNAREs are the Sso proteins (Aalto *et al*., 1993) and Sec9p localised on the plasma membrane (Brennwald *et al*., 1994). Today, many other SNAREs functioning at various secretory steps are known both in yeast and mammals. According to the simplest model, the fidelity of the targeting and fusion is obtained by pairing of the v- and t-SNAREs specific to these different secretory steps (Bennett and Scheller, 1993; Söllner *et al*., 1993).

Because the SNAREs function both in constitutive and regulated secretion *e.g.* in neuronal cells in which the membrane fusion is triggered by a calcium stimulus (reviewed by Bennett and Scheller, 1993), additional specificity determinants are likely needed (Galli *et al*., 1995). According to a more recent view the SNAREs are the minimal requirement for the membrane fusion to occur (Weber *et al*., 1998) but do not probably regulate the specificity of the reaction. This view is supported by several findings (reviewed by Götte and Fisher von Mollard, 1998). The yeast plasma membrane t-SNAREs localise on the entire plasma membrane even in small-budded cells in which the bud area is the active site of exocytosis (Brennwald *et al*., 1994). Similarly, neuronal syntaxin 1 is not restricted to the membrane of the presynaptic active zone but is found along the axonal plasma membrane (Garcia *et al*., 1995). Furthermore, a v-SNARE can pair with more than one t-SNARE *in vitro* (Fisher von Mollard *et al*., 1997; Holthuis *et al*., 1998). Thus, despite intensive studies on SNAREs, their exact function is still unsolved (Schekman, 1998).

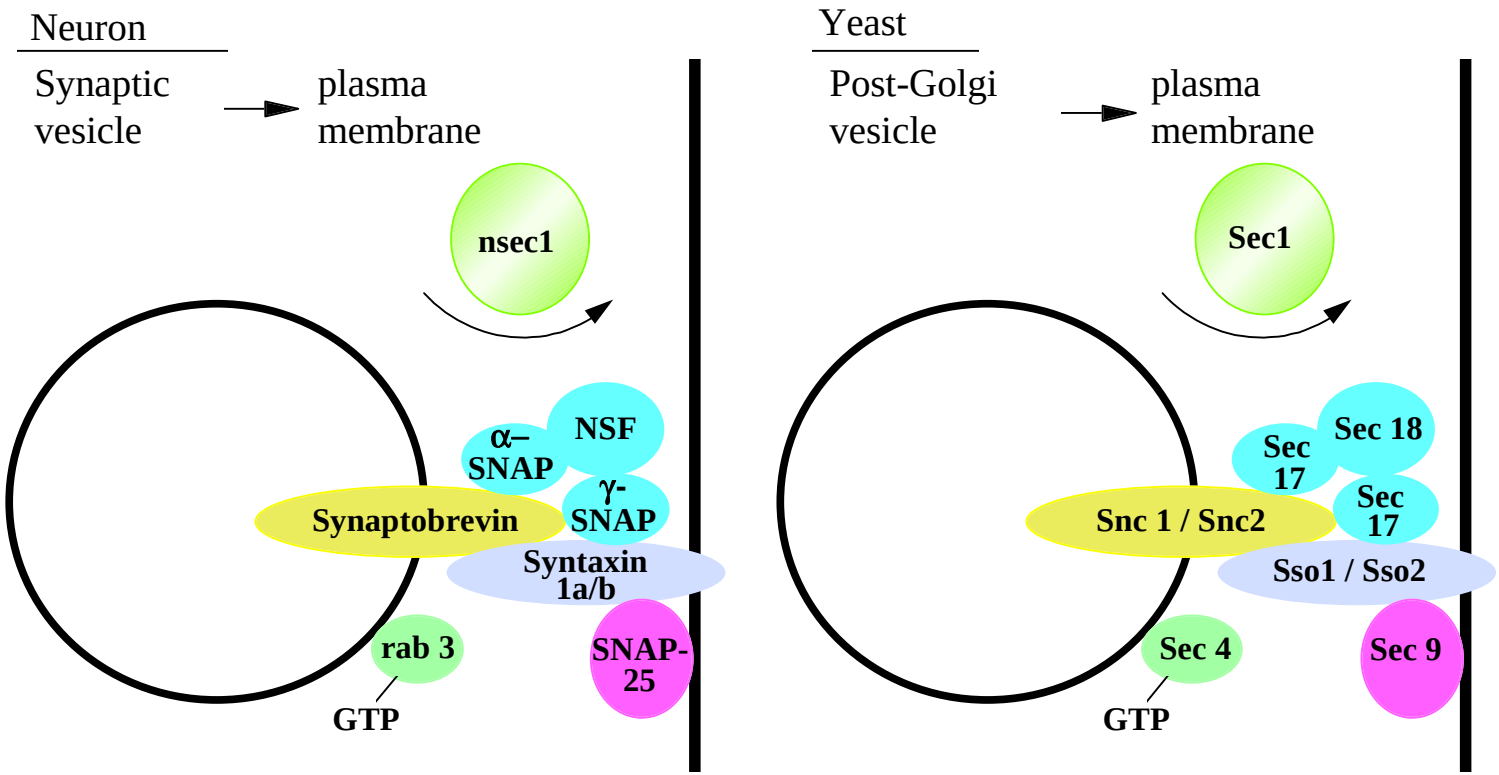

Figure 3. The membrane fusion machineries are conserved in evolution from yeast to mammals. Modified from Ferro-Novick and Jahn (1994).

The pairing specificity may be mediated by regulatory proteins such as members of the Sec1p family and the small GTP-binding proteins (Lupashin and Waters, 1997; Rothman and Söllner, 1997). Sec4p, that functions in vesicle transport from the Golgi to the plasma membrane in yeast, was the first GTP-binding protein shown to be involved in secretory transport (reviewed by Ferro-Novick and Novick, 1993). The essential Sec4p is homologous to the mammalian Rab proteins and the yeast Ras and Ypt proteins, and is a member of the Ypt/Rab subfamily of the RAS superfamily (reviewed by Olkkonen and Stenmark, 1997). The role of Sec4p in secretion was directly evidenced with a ts *sec4-8* mutation which was shown to arrest the post-Golgi vesicle traffic (Salminen and Novick, 1987). Sec4p is presumed to cycle between forms bound to the surface of a secretory vesicle or the plasma membrane and a cytosolic, free form (Goud *et al*., 1988; Walworth *et al*., 1989). This cycle is linked to another cycle, the sequential binding and hydrolysis of GTP and exchange of nucleotides, that involves additional factors (reviewed by Ferro-Novick and Novick, 1993; Novick and Brennwald, 1993).

The GTP-bound Sec4p is assumed to activate a downstream effector. The clue to the possible candidate for such an effector came from genetic studies. Salminen and Novick (1987) had shown that an extra copy of *SEC4* could suppress the ts phenotype of *sec2-4*, *sec8-9* and *sec15-1*, and that *sec4-8* in combination with *sec2-41*, *sec3-2*, *sec5-24*, *sec8-9*, *sec10-2*, *sec15-1* or *sec19-1* caused synthetic lethality. Furthermore, overexpression of *SEC9*, which was cloned as a multicopy suppressor of the *sec4* effector domain mutation, could partially suppress the temperature sensitivity of *sec1-1*, *sec3-2*, *sec8-9* and *sec15-1* (Brennwald *et al*., 1994). These genetic interactions (see Table 5) suggested that the exocyst complex (1.5.5) could be a mediator of the regulatory effect of Sec4p on the Sec9-Sso-Snc protein complex (Bowser *et al*., 1992; Ferro-Novick and Novick, 1993; Brennwald *et al*., 1994). A recent study by Guo *et al*. (1999) has experimentally established this role for the exocyst complex.

A question has lately risen concerning whether the t- and v-SNAREs are both universally required for the vesicle docking and fusion. David *et al*. (1998) have shown that under certain conditions the exocytic v-SNAREs, the Snc proteins, are dispensable. However, under these conditions, the t-SNAREs were still required. The *snc* mutant cells are conditionally lethal and their secretory capacity is defective (Protopopov *et al*., 1993). The viability and secretion in these cells could be restored by providing the cells with mutations in genes *VBM1* or *VBM2* which encode proteins involved in synthesis of long chain fatty acids and sphingolipids in the ER (Oh *et al*., 1997; David *et al*., 1998). The absolute requirement for v-SNAREs is further questioned by results showing that both vacuolar (Nichols *et al*., 1997) and ER membrane fusion (Patel *et al*., 1998) can occur in the absence of the appropriate v-SNAREs.

1.5.2 *SEC1*

sec1-1, that accumulates post-Golgi vesicles of 80 - 100 nm in diameter at the restrictive temperature, was the first secretory mutant identified in yeast (Novick and Schekman, 1979; Novick *et al*., 1980). The essential wild type gene encoding Sec1p was cloned from a chromosomal library by complementation of *sec1-1* at 37°. The size of the Sec1p protein predicted from the amino acid sequence is 83 kDa and the protein does not contain any clearly hydrophobic sequences. Thus it is considered to be a soluble cytosolic protein needed for the post-Golgi traffic (Aalto *et al.*, 1991). However, in subcellular fractionation studies it was reported to behave rather as though it was strongly coupled to a sedimentable subcellular fraction (Egerton *et al.*, 1993). *SEC1* displays genetic interactions with other late-acting *SEC* genes. Its overexpression has been shown to suppress *sec3-2*, *sec15-1* and *sec10-2*, which all encode components of the exocyst (1.5.5) (Aalto *et al*., 1993). *SEC1* homologues are known in nematode, *Drosophila* and several mammalian species (1.5.4).

1.5.3 *SSO* genes and *MSO1*

Suppressors of ***s****ec* **o**ne, *SSO1* and *SSO2*, were isolated from a cDNA library on a multicopy plasmid (Aalto *et al*., 1993). Overexpression of either *SSO* gene suppresses the ts growth and secretory defect of *sec1-1*. However, Sso proteins cannot rescue the depletion of Sec1p. Thus they seem to have an independent function rather than to replace that of Sec1p when overproduced. The Sso1 and Sso2 proteins consist of 290 and 295 amino acids, respectively, and are 72% identical at the amino acid level. They contain a single transmembrane region close to their carboxy-termini followed by a short tail of three or four amino acids (Aalto *et al*., 1993). Thus, they represent type II membrane proteins and localise to the plasma membrane with their amino-termini facing the cytosol (Kutay *et al*., 1993; Brennwald *et al*., 1994).

Disruption of either *SSO* gene does not cause a detectable phenotype, but simultaneous deletion of both *SSO* genes is lethal. Thus, the proteins perform together an essential function. Overexpression of *SSO* genes can suppress also other late-acting *sec* mutations *sec3-2*, *sec5-24*, *sec9-4* and *sec15-1*. The suppression pattern of the two *SSO* genes is similar. This further confirms that the Sso proteins together are responsible for a function in the late secretory transport, which according to the genetic studies appears to be a central one.

MSO1, another **m**ulticopy suppressor of ***s****ec* **o**ne, was cloned more recently (Aalto *et al*., 1997). Disruption of the *MSO1* gene does not affect viability under any growth conditions tested. Instead, it leads to the accumulation of secretory

vesicles of 60 nm in diameter in the bud area. Unlike the *SSO* genes, *MSO1* is a specific suppressor of *sec1* and cannot suppress the ts phenotype of any other late-acting *sec* mutants. However, disruption of *MSO1* shows synthetic lethality with *sec2-41* and *sec4-8* similarly as with *sec1-1* and *sec1-11*. Furthermore, it reduces the viability of *sec3-2*, *sec5-24*, *sec6-4*, *sec8-9*, *sec9-4*, *sec10-2* and *sec15-1* mutants but has no effect on that of the *sec7-1*, *sec18-1* or *sec19-1* mutant cells. The size of the Mso1p estimated from the sequence is 23 kDa and the sequence of this hydrophilic protein is not obviously similar to that of any known proteins. Mso1p has been shown to bind Sec1p, which in addition to the other results suggests a role for it in the last step of the secretory pathway. The exact function it carries out in late secretory transport, however, is still unsolved (Aalto *et al*., 1997).

1.5.4 Conserved protein families in vesicular transport

Yeast has several Sec1p related proteins that together form a protein family. It consists of Sly1p, Slp1p/Vps33p, Sec1p (Aalto *et al*., 1992) and a more recently identified member Vps45p (Cowles *et al*., 1994; Piper *et al*., 1994). Sly1p functions in the transport step between ER and Golgi (Dascher *et al*., 1991; Ossig *et al*., 1991), and Slp1p/Vps33p and Vps45p are needed for vacuolar delivery (Banta *et al*., 1990; Wada *et al*., 1990; Cowles *et al*., 1994). Mutation in genes encoding these proteins leads to vesicle accumulation at the transport step in question analogously to that observed for *sec1* mutants. Thus, the members of the yeast Sec1p family appear to have similar tasks in protein secretion at different transport steps. Furthermore, they seem to be factors providing the specificity for these sorting events. Since Sec1p and Sly1p are more homologous with each other than with Slp1p, they are assumed to have diverged from each other later in evolution than their common ancestor diverged from Slp1p (Aalto *et al*., 1992).

Other members of the Sec1p family have been identified from higher eukaryotes: *unc-18* gene was identified in the nematode *Caenorhabditis elegans* (Hosono *et al*., 1992), *Rop* in *Drosophila melanogaster* (Salzberg *et al*., 1993) and mammalian homologues specific for neuronal (Hata *et al*., 1993; Garcia *et al*., 1994; Pevsner *et al*., 1994) or other type of tissue (Hodel *et al*., 1994; Katagiri *et al*., 1995; Tellam *et al*., 1995; Hata and Südhof, 1995; Riento *et al*., 1996). Both positive and negative roles have been suggested for Sec1p (reviewed by Halachmi and Lev, 1996), but its exact function is unknown.

The t-SNARE family of syntaxins includes also members both from yeast and other eukaryotes. Aalto *et al.* (1993) found significant sequence similarities between Sso proteins and six other eukaryotic proteins. Two of them were found

from yeast: Sed5p that is needed for ER to Golgi transport (Hardwick and Pelham, 1992) and Pep12p functioning in vacuolar targeting (Becherer *et al.*, 1994). The other members are mammalian syntaxin A/HPC-1 and syntaxin B (Bennett *et al.*, 1992; Inoue *et al.*, 1992), which are assumed to function in synaptic vesicle docking and/or fusion to the plasma membrane, and epimorphin that has been suggested to function in epithelial morphogenesis (Hirai et al., 1992). The role of epimorphin in secretory transport, however, has been under debate (Pelham, 1993; Hirai et al., 1993). Lastly, a nematode cDNA (Ainscough *et al.*, 1991) was found to be homologous to syntaxins. The family was enlarged by Bennett *et al*. (1993) who reported isolation of four new members, syntaxins 2-5. Today, eight syntaxin-like t-SNARE proteins have been identified in yeast. In addition to the previously described, an ER-specific Ufe1p (Lewis and Pelham, 1996), vacuolar Vam3p (Wada *et al.*, 1997) and two t-SNAREs which have effect on late Golgi and seem to function in endocytosis, Tlg1p and Tlg2p (Holthuis *et al.*, 1998; Séron *et al.*, 1998), have been reported.

Small GTP-binding proteins that function at various transport steps also form a family. The yeast family contains 11 members of which Sec4p and Ypt1p were the first ones identified (Aalto *et al*., 1992; Bennett and Scheller, 1993; Novick and Zerial, 1997). Ypt1p is needed for ER to Golgi transport (Segev *et al*., 1988), Sec4p for post-Golgi transport (Salminen and Novick, 1987), Ypt31p and Ypt32p for intra-Golgi transport and most of the other members for vacuolar targeting (Wichmann *et al*., 1992; Horazdovsky *et al*., 1994; Novick and Zerial, 1997; Sato and Wickner, 1998). The fact that proteins with homologous sequences and similar functions are found in different organisms and at distinct steps of the transport route in the same organism (Table 1) suggests that the function they carry out is of fundamental nature. The evolutionary conservation also enables the use of simpler organisms for studies of corresponding events in the higher organisms (Bennett and Scheller, 1993).

Table 1. Conserved protein families functioning at different steps of vesicular transport in yeast.

Transport step	Sec1p family	Small GTP-proteins	v-SNAREs	t-SNAREs
ER-Golgi	Sly1p	Ypt1p	Bos1p, Bet1p Sly2p/Sec22p	Sed5p, Ufe1p
Golgi-plasma membrane	Sec1p	Sec4p	Snc1p, Snc2p	Sso1p, Sso2p
Golgi-endosome	Vps45p	Vps21p/Ypt51p	Vti1p	Pep12p, Tlg1p?, Tlg2p?
endosome-vacuole	Slp1p/Vps33p	Ypt7p	Nyv1p	Vam3p/Pth1p

1.5.5 The exocyst complex

Two late-acting Sec proteins, Sec8p (Bowser *et al*., 1992) and Sec15p (Salminen and Novick, 1989), have been shown to reside on a large complex, that is found both free in the cytosol and peripherally associated with the plasma membrane (Bowser and Novick, 1991; Bowser *et al*., 1992). Part of the sequence of Sec8p is homologous to that of the adenylate cyclase domain required for response to Ras. Thus, Sec8p was presumed to respond to Sec4p, the post-Golgi GTP-binding protein of the Ras superfamily. Based on this and the genetic evidence (Salminen and Novick, 1987; Bowser *et al*., 1992), it was suggested that the Sec8p/Sec15p particle could form the downstream effector of Sec4p. Furthermore, a fraction of Sec4p co-eluted with the Sec8p/Sec15p complex in gel filtration. This was not observed in cells containing either *sec8-9* or *sec15-1* mutations that cause instability of the complex. These results implied that Sec4p could directly associate with the complex (Bowser *et al*., 1992).

TerBush and Novick (1995) further studied the complex which they localised to the tip of the bud and suggested to be involved in targeting of secretory vesicles to the plasma membrane of the bud. Using 6-histidine-tagged Sec8p combined with immunoprecipitations and affinity chromatography they could show that it contains at least eight polypeptides of molecular masses between 70 and 144 kDa. Sec6p was found to be the third component of the complex, which cofractionated with the tagged form of Sec8p and Sec15p. On the contrary, Sec1p, Sec2p and Sec4p, whose genes interact with *SEC8* and *SEC15*, did not cofractionate with the tagged form of Sec8p. Thus, they are not stable subunits of the complex.

Synthetic phenotypes had previously been detected in combinations of *sec8-9* with most of the late-acting *sec* mutants and *sec19-1* (Bowser *et al.*, 1992; Potenza *et al*., 1992). The stability of the Sec6/Sec8/Sec15 complex was altered in these mutant cells, although at a permissive temperature of 25°C *sec1-1*, *sec2-41*, *sec4-8*, *sec6-4*, *sec8-9* and *sec15-1* had only minor effect on the subunit composition of the complex. *sec6-4* and *sec15-1* mutations, instead, caused almost total instability of the complex at a restrictive temperature, and in strains containing *sec3-2*, *sec5-24* or *sec10-2* mutations the complex was unstable even at the permissive temperature (TerBush and Novick, 1995).

Another purification of the complex with the aid of triple c-myc-tagged Sec8p yielded enough protein for microsequencing of the subunits of the complex. This in addition to comparing the relative molecular masses of the proteins led to the identification of four new components (TerBush *et al.*, 1996). Three of them were encoded by *SEC3*, *SEC5* and *SEC10*. An essential gene *EXO70*, for

exocyst component of **70** kDa, encodes the fourth component identified. Moreover, the seven known hydrophilic protein components of the complex are present in single copies. As the complex is only required for the exocytosis it was named the exocyst (Figure 4). A mammalian complex with homologous subunits has been isolated from rat brain (Ting *et al*., 1995; Hsu *et al*., 1996; Guo *et al*., 1997; Hazuka *et al*., 1997; Kee *et al*., 1997). The complex contains eight subunits and seems to be involved in synaptic vesicle docking and fusion (Hsu *et al*., 1996).

Sem1p, a novel small hydrophilic protein was recently identified in yeast (Jäntti *et al*., 1999). The gene encoding it is highly conserved in evolution and has been shown to interact with several genes encoding the components of the exocyst. Overexpression of the nonessential *SEM1* can suppress the temperature sensitivity of *sec3-2*, *sec8-9*, *sec10-2* and *sec15-1*. Interestingly, deletion of *SEM1* from these same mutant strains eliminated their temperature sensitivity. Thus, a regulatory role for Sem1p on exocyst function has been proposed. Furthermore, Sem1p is indicated to regulate also the pseudohyphal growth in yeast. Deletion of both copies of *SEM1* from a diploid strain induced pseudohyphal growth which the strain normally does not display.

1.5.6 Polarised secretion

Surface growth and protein secretion by yeast cells is restricted to the bud area during most of the cell cycle. When the bud enlarges exocytic secretion is directed towards the tip of the bud, and after the bud has reached about two-thirds of the size of the mother cell secretion is directed to the entire bud area. This is followed by directing the vesicles carrying new cell wall material to the neck area between the mother and daughter cells, which leads to cytokinesis and septation (Byers, 1981). The correct distribution of vesicles to the sites of exocytosis is presumed to rely on both actin-dependent vesicle transport and creation of vesicle-docking sites independently of actin (reviewed by Finger and Novick, 1998).

The actin cytoskeleton participates both in the creation of polarised secretion in yeast and its maintenance (Ayscough *et al*., 1997). Unlike mammalian cells, yeast does not use microtubules to transport vesicles. In this respect, it reminds more of a plant cell (Novick, 1985). Actin mutants have been shown to accumulate post-Golgi vesicles and partially arrest secretion of invertase (Novick and Botstein, 1985). Furthermore, the cells become enlarged and lack polarised secretion. The vesicle accumulation phenotype suggests that actin is not only involved in polarised secretion but can also facilitate exocytosis. Mutant form of profilin, a protein that regulates actin assembly, also causes

depolarisation of secretion but not accumulation of secretory vesicles (Finger and Novick, 1998). However, the gene encoding it shows genetic interactions with several late acting *sec* mutations, *e.g.* with *sec3* (Haarer *et al.*, 1996; Finger and Novick, 1997).

Myosin, tropomyosin and Aip3p/Bud6p among several other factors are involved in cytoskeleton-dependent polarised secretion (reviewed by Drubin and Nelson 1996; Amberg *et al.*, 1997; Finger and Novick, 1998). Mutations in genes encoding myosin and tropomyosin, an actin filament-binding protein, have been shown to display genetic interactions with late-acting *SEC* genes and with each other (Liu and Bretscher, 1992; Govindan *et al.*, 1995). At a restrictive temperature (37°C) cells containing either *myo2-66* mutation or *tpm1Δ* disruption accumulated vesicles similar to those in the late-acting *sec* mutants. Interestingly, export of most of their cargo proteins did not seem affected. In comparison to the wild type cells, only secretion of a-agglutinin, an α-factor-inducible cell surface glycoprotein in *MATa* cells, was slower and partly delocalised in *myo2-66* or *tpm1Δ* mutant cells at 37°C (Liu and Bretscher, 1992). Thus, the secretion continues although in a not accurately polarised way. Myo2p, a class V myosin, has been localised to the active sites of exocytosis. These results support the hypothesis according to which Myo2p could be the motor bringing the secretory vesicles to the bud tip along the actin cables (reviewed by Finger and Novick, 1998).

Activated Sec4p is suggested to regulate polarised exocytosis. It can be transported to the active sites of exocytosis via attachment to the surface of the secretory vesicles. However, *act1-3* and *myo2-66* mutants fail to distribute Sec4p correctly. Similarly, mutants of *SEC2*, that encodes the guanine nucleotide exchange factor (GEF) of Sec4p, but not the other late-acting *sec* mutants mislocalise Sec4p (Walch-Solimena *et al.*, 1997). In *sec2-78* mutant Sec4p was found coupled to the post-Golgi vesicles which accumulated in both mother cell and the bud. This further strengthens the regulatory role for Sec2p-activated Sec4p in exocytosis.

Several lines of evidence suggest that the exocyst functions in determination of the vesicle docking site. The role was supported by identification of rsec6/8 complex from rat brain (Hsu *et al.*, 1996). The rsec6/8 complex, which consists of eight polypeptides, is localised to plasma membrane of the neuronal cells, and rSec8p specific antibodies were shown to coimmunoprecipitate syntaxin suggesting either direct or indirect interaction between the complex and syntaxin. The mammalian Sec6/8 complex is also suggested to determine the specificity of vesicle transport to the basolateral membrane in Madin-Darby

canine kidney (MDCK) cells and thus to be essential for generation of the epithelial cell surface polarity (Grindstaff *et al*., 1998).

The exocyst complex in yeast is suggested to act as a targeting patch (Drubin and Nelson, 1996). First indications for this were the Sec8p localisation and the patch forming phenotype of the *SEC15* overexpression strain (reviewed in Finger and Novick, 1998). Mutations in Sec3p, unlike in the other components of the complex, lead to depolarisation of the cell (Finger and Novick, 1997). Moreover, homozygous *sec3* diploid cells display random budding patterns, and their chitin location is depolarised in addition to defective secretion and cytokinesis (Haarer *et al*., 1996; Finger and Novick, 1997). Thus, Sec3p was considered as a candidate for a spatial determinant of exocytosis.

Finger *et al.* (1998) fused green fluorescent protein (GFP) to the carboxy terminus of Sec3p and showed the functional fusion protein to localise to the sites of exocytosis at each stage of the cell cycle. The fusion protein was also presumed to associate stably with the plasma membrane. In addition to Sec5p (Mondésert *et al*., 1997), Sec4p and Sec8p were shown to colocalise with Sec3p but their transport to the site, unlike that of Sec3p, depended on the secretory pathway. Interestingly, in all ER to Golgi, intra-Golgi or post-Golgi *sec* mutants examined Sec3-GFP was correctly localised. Similarly, its transport did not require a polarised actin cytoskeleton. These results further supported the idea that Sec3p could be a regulator of the exocytic fusion site.

A model for the establishment of polarised secretion in yeast has been proposed (Finger and Novick, 1998). According to it Sec3p that defines the site of exocytosis is transported there independently of the actin cytoskeleton and secretory machinery but likely under guidance of the cell cycle machinery. The post-Golgi secretory vesicles bind Sec4p, that becomes activated in a reaction catalysed by Sec2p, and vesicles are transported to the site of exocytosis on actin cables, possibly with the aid of Myo2p. Sec15p would bind Sec4p and dock the vesicles in response to the activated Sec4p at the appropriate exocytic site that is marked by Sec3p. This could lead to recruitment of the other components of the exocyst to the site and assembly of the complex. Finally v- and t-SNAREs would interact leading to the fusion of the vesicles to plasma membrane.

Sec15p was recently shown to cofractionate with Sec4p and Snc2p, a post-Golgi v-SNARE, in cellular fractionation (Guo *et al*., 1999). Its localisation to the vesicles was supported by immunoelectron microscopy and physical interaction with GTP-bound Sec4p. These results, thus, experimentally establish the role for Sec15p as an effector of Sec4p. The coimmuprecipitations and results from

the yeast two-hybrid assay with pairwise combinations of the exocyst components has led to a model of molecular interactions that could link the secretory vesicles to the site of exocytosis (Figure 4). The protein-protein interactions suggest that Sec5p is the core component of the complex. It interacts with Sec3p, Sec6p, Sec10p and Exo70p. Furthermore, the association of Sec15p with the complex depends on the presence of Sec10p. Interestingly these two proteins, Sec10p and Sec15p, were also found to form an independent subcomplex *in vivo* (Guo *et al*., 1999).

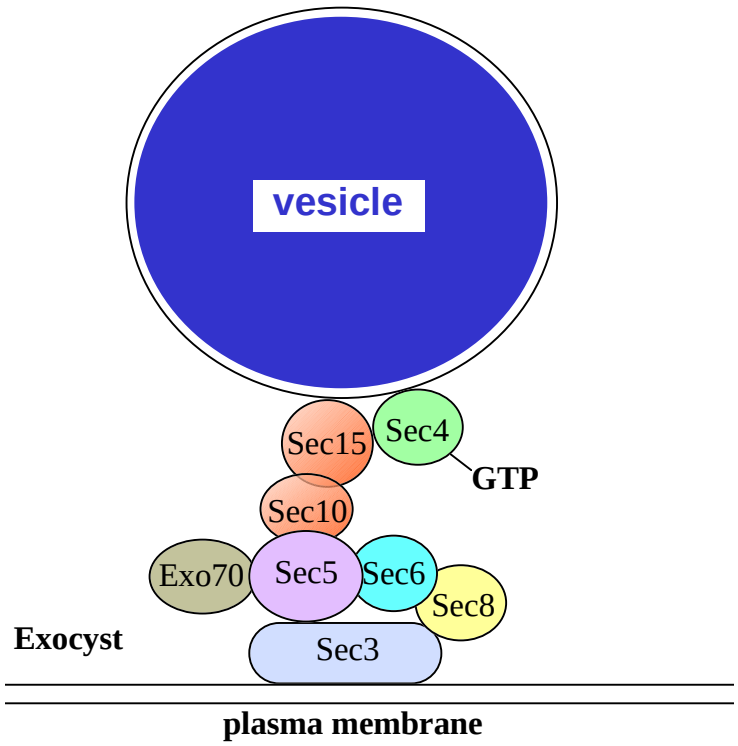

Figure 4. The exocyst complex regulates the polarised vesicle transport in yeast. According to Guo et al. *(1999) the molecular interactions between Sec15p and GTP-bound Sec4p on the surface of the vesicle and those between the subunits of the complex link the secretory vesicle to the plasma membrane at the site that is marked by Sec3p. Modified from Guo* et al., *1999.*

1.6 The production of heterologous proteins in the yeast *Saccharomyces cerevisiae*

1.6.1 *Saccharomyces cerevisiae* as a production host

The yeast *S. cerevisiae* has several advantages as a production organism. Its whole genome is known, which in combination with the well developed genetic and other methods enables engineering of desired modifications to the yeast genome in order to gain a more powerful host for production of heterologous proteins. Moreover, cultivation and fermentation of yeast is easy and inexpensive. Unlike animal or many bacterial cells, yeast does not possess a possible risk of containing pyrogens, viral or oncogenic DNA. This non-pathogenic organism has acquired a GRAS (**g**enerally **r**egarded **a**s **s**afe) status and can be utilised *e.g.* for production of enzymes that will be used in the food industry. As a eukaryote yeast is capable of most if not all posttranslational modifications that normally take place in animal cells: disulphide bond formation, proteolytic processing of precursor proteins, glycosylation, fatty acid acylation, phosphorylation, myristylation, isoprenylation and removal of amino-terminal methionine. Yeast's capacity to secrete proteins to the medium makes the further purification easy because only up to 0.5% of yeast's own proteins are secreted. In practise, only trace amounts of proteases, if any, are secreted to the medium. The secreted proteins, thus, can be maintained rather stably (reviewed by Romanos *et al*., 1992).

1.6.2 Factors affecting the production and secretion of heterologous proteins

Many factors need to be considered when designing protein production in yeast. Production yields of heterologous proteins are influenced by the choice of promoter for expression. There are several constitutive and inducible promoters available in yeast. In case the heterologous protein is toxic for the host, an inducible promoter that will be turned on once the cell density in the culture is high can prove useful. A foreign signal sequence can lead to successful secretion from yeast (Ruohonen *et al*., 1987), but usually yeast's own signal sequences are used to provide the targeting to the secretory pathway. Most often the sequence encoding the heterologous protein is fused to the prepro region of the yeast mating factor α (Romanos *et al*., 1992), but *e.g.* the signal sequence of invertase and the prepro region of Hsp150 (Simonen *et al*., 1994) have also been used. The codon usage may also affect production output which has been evidenced by comparing the product yield and quality to the degree of optimal codon usage (reviewed by Romanos *et al*., 1992).

Glycosylation in yeast differs from that in animal cells and can lead to extensive glycosylation of foreign glycoproteins and create immunogenic problems (reviewed by Romanos *et al*., 1992). The hyperglycosylation problem can, however, be circumvented by mutagenising the potential glycosylation sites in the sequence or using a glycosylation mutant yeast if the outer chain glycosylation is not required for the functionality of the protein product. Another drawback of *S. cerevisiae* is its rather moderate secretory capacity. However, small peptides *e.g.* epidermal growth factor and insulin are secreted well. The yield may be as high as hundreds of milligrams per litre in high-density cultures.

1.6.3 Attempts to enhance the secretion of heterologous proteins

First optimisation of production and secretion can be done at the level of choosing and adjusting appropriate promoter and signal sequence for expression and targeting of the secretory product (Baldari *et al*., 1987; Martegani *et al*., 1992; Ruohonen *et al*., 1995). Modulation of the expression plasmid copy number and tuning of the growth conditions (Ruohonen *et al*., 1991) can also improve the product yield. One commonly used strategy to enhance secretion of heterologous proteins has been mutagenesis and screening for supersecretory phenotype (Smith *et al*., 1985; Sakai *et al*., 1988; Kaisho *et al*., 1989; Shuster *et al*., 1989; Suzuki *et al*., 1989; Kotylak and El-Gewely, 1991; Sleep *et al*., 1991; Wingfield and Dickinson, 1993). In some cases overexpression of a gene has improved protein production and secretion (Chow *et al*., 1992; Chen *et al*., 1994; Robinson *et al*., 1994). For example, integration of an extra copy of the gene encoding PDI (protein disulphide isomerase) into the genome was reported to enhance secretion of human platelet derived growth factor B, which contains eight disulphides, up to ten fold (Robinson *et al*., 1994). Deletion of a gene can also elevate secretion. Disruption of *CNE1*, which encodes a putative yeast homologue of mammalian calnexin, did not affect secretion of yeast's own secretory proteins, α-pheromone or acid phosphatase, but enhanced that of α_1-antitrypsin, a heterologously expressed soluble glycoprotein, up to 2 - 2.6 fold (Parlati *et al*., 1995). Null mutation of *PMR1* gene encoding a Ca^{2+}pump (Rudolph *et al*., 1996) was also reported to enhance secretion of bovine proteins from yeast (Smith *et al*., 1985; Rudolph *et al*., 1996).

1.7 Aims of the study

The main goal of the study was to gain more information on the molecular mechanism of the yeast secretory pathway. This was to include cloning of new genes involved in protein secretion and studying the functions and interactions of the secretory components by genetic and biochemical methods.

The other purpose was to attain knowledge on the mechanism of the enhancement of protein production in secreted form. Previously an observation had been made in this laboratory that overproduction of the Sso proteins leads to enhanced production of a marker protein in secreted form. This phenomenon was to be studied in more detail. In addition, other secretory genes were to be screened for their possible enhancing effect on protein secretion.

2. Materials and methods

Table 2. Methods used in this study. The detailed descriptions of the methods are presented in the original publications and the manuscript.

Method	Described and used in
Animal cell culture	I
DNA methods and southern blotting	III, IV
Expression of Sso2p in animal cells	I
Genetic methods	III, IV
Immunoelectron microscopy of animal cells	I
Immunoelectron microscopy of yeast cells	III
Immunofluorescence microscopy of animal cells	I
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The *sec* mutant strains used in these studies were obtained from Peter Novick and Randy Schekman. The wild type yeasts to which the growth and secretion properties of the *sec* mutants were compared were of the same background as the corresponding mutants. Secretion enhancement was studied in the wild type strain DBY746 of David Botstein. The SKY strains are diploids that were constructed by crossing the haploid *sec* mutants with wild type yeast or disruptant derivatives of it. For integration of an α-amylase expression cassette at the *HIS3* locus of DBY746, the 3.35 kilo base fragment containing the *Bacillus* α-amylase gene between the shortened, 700 base pairs (bp) long *ADH1* promoter and the *ADH1* terminator, was released from YEpαa6 (Ruohonen *et al.*, 1995) as a *BamH*I-*Sal*I fragment and cloned into pRS403 (Sikorski and Hieter, 1989) between *BamH*I and *Sal*I yielding YIpαaH. For integration YIpαaH was linearized with *PstI* in the *HIS3* sequence. The integrant strain thus obtained, H1374, was used in secretion enhancement studies (unpublished).

3. Results and discussion

3.1 Transport of Sso2p to the plasma membrane along the secretory pathway

3.1.1 The site of membrane insertion of Sso2p in the secretory pathway

The Sso proteins are type II membrane proteins (amino-termini on the cytosolic side) residing in the plasma membrane (Aalto *et al*., 1993; Brennwald *et al*., 1994). They lack the amino-terminal signal sequence that would interact with SRP in cotranslational ER translocation. Instead, they have a carboxy-terminal hydrophobic region which has been shown to mediate the membrane insertion of such tail-anchored type II membrane proteins (Kutay *et al*., 1993 and refs. therein). Several cellular compartments contain tail-anchored proteins and they probably insert into the membrane by a mechanism independent of the SRP-mediated targeting. Thus they might be inserted directly to their target membrane. It was, therefore, interesting to study the primary membrane insertion site of these proteins. SNARE proteins, in particular, were of interest in this respect because their regulatory role on membrane traffic might create problem on possible transport to their target site. The membrane insertion site and mode of Sso2p was investigated in baby hamster kidney (BHK) cells because animal cells are superior for morphological studies in comparison to yeast. The recombinant Semliki Forest virus (SFV) vector was used for transient expression of the protein because it provides a means to synchronise the expression of proteins in a time scale of few hours.

Localisation of Sso2p in BHK cells was studied by immunofluorescence microscopy using affinity-purified polyclonal antibodies against Sso2p. The antibodies raised in rabbits were specific for Sso2p, which was evidenced by immunoblotting of cell lysates both from yeast and from BHK cells expressing Sso2p (I). The antibodies did not detect any band in the untransfected BHK cells that served as a negative control. However, they recognised Sso1p well (unpublished results). The antiserum recognised a single band of estimated size of 38 kDa (I). This is in reasonable agreement with the size of Sso proteins estimated from the sequence, 32.5 kDa. The antibodies are very potent and can be used in high dilution.

After three hours post-infection (p.i.) a perinuclear Golgi staining was detected with the antibodies. The staining colocalised with that of mannosidase II, a marker protein for the Golgi stack. Subsequently, a strong staining of plasma

membrane was observed in addition to that in the Golgi. Immunoelectron microscopy was used for more detailed study of the intracellular localisation of Sso2p. With this technique the Sso2p was localised to the entire Golgi stack and plasma membrane at five hours p.i. The Golgi staining observed was due to transit of Sso2p through it on the way to the plasma membrane. A thermal block of 20°C, which inhibits transport from the trans-Golgi network (Matlin and Simons, 1983; Saraste and Kuismanen, 1984; Kuismanen and Saraste, 1989), was utilised to evidence the post-Golgi transport of Sso2p. After Sso2p was arrested to the Golgi by this treatment, its onward passage to the plasma membrane was studied by subsequent incubation of the cells at 37°C in the presence of cycloheximide. Immunofluorescence microscopy of cells fixed after 15 - 30 min incubation showed staining of vesicle-like structures, scattered throughout the cytosol, and the plasma membrane itself. After longer incubation times the Golgi staining disappeared and Sso2p was seen accumulated at the plasma membrane (I). The localisation observed for Sso2p in BHK cells was in agreement with the results of Brennwald *et al*. (1994) who have localised the Sso2p on the yeast plasma membrane by immunofluorescence microscopy.

It was demonstrated so far that Sso2p was transported from the Golgi to plasma membrane via the normal transport route. However, it still remained open whether Sso2p was inserted to the membrane at the ER or at the Golgi. This question was approached by treating the cells with brefeldin A (BFA), which arrests the exit of secretory proteins from the ER and causes extensive retrograde movement of Golgi membranes to the ER (Lippincott-Schwartz *et al*., 1989, 1990; Doms *et al*., 1989). The BFA treatment at 37°C caused Sso2p accumulation in a reticular structure resembling the ER. This was confirmed by immunoelectron microscopy that showed clear staining of the outer nuclear membrane and associated ER cisternae. Wash-out of BFA led to movement of Sso2p along the secretory pathway and the protein was chased to the Golgi and then the plasma membrane (I).

These results showed that Sso2p can be transported from the ER to the Golgi but did not prove that it was first inserted into the ER membrane. The lack of ER-specific staining might be because the protein exits from the ER very rapidly. In order to increase the expression level of Sso2p the cells were first incubated in the presence of cycloheximide to allow accumulation of Sso2p-specific messenger RNA. After removal of the drug transient staining of vesicular ER-like structures was seen. This proved that membrane insertion of Sso2p occurred at the ER from where it was rapidly transported to the Golgi. Taken together, these results thus confirmed that Sso2p is transported along the biosynthetic pathway to its site of action, the plasma membrane.

3.1.2 Membrane association and posttranslational insertion of Sso2p

The carboxy-terminal hydrophobic region of type II membrane proteins is thought to anchor the proteins into membranes because removal of it has been shown to cause cytosolic accumulation of the truncated proteins (Kutay *et al*., 1993 and refs. therein). Previously, the membrane insertion of cytochrome b_5 had been shown to be SRP-independent (Anderson *et al*., 1983) and the insertion of type II membrane proteins in general is considered to take place in a posttranslational fashion (Kutay *et al*., 1993).

The mode of membrane insertion of Sso2p was investigated by expressing in BHK cells a shortened form of the protein, lacking the carboxy-terminal membrane anchor region, in addition to the wild type (wt) protein. Both forms of Sso2 protein were immunoprecipitated from the total microsomes and the cytosolic fraction of pulse-labelled cells, and the proportion of membrane association was analysed. More than 90% of the wild type Sso2p was found in the total microsomal fraction and most of it resisted washing with carbonate buffer pH 11.3. The truncated form of Sso2p, instead, was mainly found in the cytosolic fraction and the high pH wash removed the trace amount of the protein present in the microsomal membranes (I). The Sso2p is, thus, an integral membrane protein, which is retained in the membrane by its carboxy-terminal anchor region.

The mechanism of insertion of Sso2p into membranes was studied *in vitro* using dog pancreatic microsomes. Insertion of Sso2p was assayed by collecting the microsomes from the translation mixture by centrifugation through a sucrose cushion. This approach was chosen because the standard assays, signal sequence cleavage, glycosylation or protection against proteolytic digestion, could not be used to examine the membrane insertion of Sso2p. The wt Sso2p was able to associate with the microsomes, whereas the truncated form lacking the membrane anchor could not. When the microsomes were prior solubilised with 1% Triton-X, only trace amounts of Sso2p were present in the pellet, indicating that the protein was truly microsome-associated. Unlike the control proteins, interferon-α1 or β-lactamase, Sso2p was able to associate similarly with the membranes when translation had been inhibited by treatment of the translation mixture with either nucleases or cycloheximide prior to addition of the membranes (I), thus implying that it was predominantly inserted to the membrane posttranslationally. Similarly, both *Aplysia californica* and neuronal synaptobrevin, a post-Golgi v-SNARE, have been shown to insert to the membrane posttranslationally and the insertion site of the protein in neuroendocrine cells is the ER (Yamasaki *et al*., 1994; Kutay *et al*., 1995).

The mechanism by which retention and activation of SNAREs in the appropriate compartment is achieved is an interesting question. It has been suggested to be provided by interactions of SNAREs with other membrane proteins in the target organelle. According to an alternative view SNARE proteins may be kept nonfunctional by interactions with certain other proteins until they reach their correct site of action (Kutay *et al*., 1995). A candidate binding protein in the case of Sso2p could be Sec1p (III) since its neuronal homologue n-Sec1p has been shown to bind syntaxin and thus prevent binding of syntaxin to VAMP or SNAP25 (Pevsner *et al*., 1994). The targeting of t-SNAREs to the plasma membrane seems to depend on both the length and the amino acid composition of the transmembrane region (TMR) but not on the exact amino acid sequence. The targeting role of the TMR has been suggested by an experiment in which replacement of the TMR of the ER t-SNARE, Ufe1p, by that of Sso1p led to transport of Ufe1p to the plasma membrane (Rayner and Pelham, 1997). The transmembrane region cannot clearly be the sole determinant of the targeting to the correct location because Sso1p with a heterologous TMR could still be found in the plasma membrane. The cytosolic domain is also considered to influence the targeting (Rayner and Pelham, 1997).

3.2 *SEB1* encodes the β subunit of the yeast Sec61p complex

3.2.1 Cloning of *SEB* genes and characterisation of their encoded proteins

SEC15 encodes a component of the exocyst complex involved in regulation of the targeting/fusion of the secretory vesicles at the plasma membrane and shows an extensive pattern of genetic interactions with other late-acting *SEC* genes. Multicopy suppressors of *sec15-1*, a ts mutation, were isolated to find possible new genes functioning at the exocytic step. The genes thus isolated include *SEB1* (II) and *SEM1* (Jäntti *et al*., 1999), which regulates both exocytosis and pseudohyphal growth in yeast. *SEB1*, however, was presumed to encode the β subunit of the Sec61p complex functioning in the ER translocation, because its sequencing revealed similarity (II) to the genes encoding the equivalent component in mammals and *Arabidopsis thaliana* (Hartmann *et al*., 1994). Thus the name *SEB1* for **SE**c61 **B**eta. The fact that overexpression of *SEB1* could suppress temperature sensitivity of *sec61-2* and *sec61-3* mutants further confirmed the presumption. Localisation of Seb1p to the ER was established with cell fractionation and immunofluorescence microscopy, which showed a clear ER-specific staining for the Seb1p with antibodies raised against its

amino-terminal peptide (II). Furthermore, in an independent study Panzner *et al*. (1995) isolated Seb1p/Sbh1p as a component of the trimeric Sec61p complex containing the previously identified subunits Sec61p and Sss1p.

SEB1 was disrupted by replacing most of its coding region with *URA3* gene. Unlike the other components of the Sec61p complex Seb1p is encoded by a nonessential gene (II). Because disruption of *SEB1* did not show any detectable phenotype it was likely that there was a homologous gene in yeast encoding a redundant function. The results from heterologous hybridisation supported this view and led to isolation of a similar gene, *SEB2*, by using a *SEB1*-specific probe (II). Disruption of *SEB2* also did not alter cell viability, but simultaneous disruption of both *SEB* genes made the yeast temperature sensitive at 38°C. A search of the yeast genome data base did not reveal any gene likely to be a third member of the family. Seb1p and Seb2p are type II membrane proteins of 82 and 88 amino acids, respectively. In accord, immunoelectron microscopy showed that Seb1p resides on the ER membrane with its amino-terminus facing the cytosol (II and the figure in the cover) and the protein was able to attach posttranslationally to membranes *in vitro*. Seb2p is a component of a novel Ssh1p complex presumed to function in cotranslational translocation into the ER (Finke *et al*., 1996; Table 3). Despite the 53% sequence identity at the amino acid level (II), the Seb proteins are specific for their complexes and thus not interchangeable (Finke *et al*., 1996).

Table 3. The evolutionarily conserved components of the protein translocation complexes.

Mammals	Yeast		Bacteria
Sec61α	Sec61	Ssh1	SecY
Sec61β	Seb1/Sbh1	Seb2/Sbh2	SecG
Sec61γ	Sss1	Sss1	SecE

3.2.2 *SEB1* interacts genetically with *SEC61* and genes encoding the components of the exocyst

Overexpression of *SEB1*, similarly to that of *SSS1*, which encodes the γ subunit of the Sec61p complex (Esnault *et al*., 1993), rescued the growth of the *sec61-2* and *sec61-3* mutants at the restrictive temperature (II). Moreover, the *sec61-2* mutation in combination with *seb1Δ* resulted in a synthetic phenotype (IV),

which is observed when a combination of two different mutant genes in a haploid strain enhances the severity of the phenotype more than either of the mutant genes do by themselves (Sherman, 1997). However, suppression of *sec62-1* or *sec63-1*, mutant genes encoding components which interact with the Sec61p complex in posttranslational translocation machinery, could not be obtained.

Because *SEB1* was cloned as a multicopy suppressor of *sec15-1*, a mutant gene encoding a component of the exocyst complex, it was of interest to study whether overexpression of *SEB1* could also suppress defects in other exocyst mutants. Interestingly, elevated expression of *SEB1* suppressed all the exocyst mutants (IV and Table 5). In addition to the growth defect of *sec8-9* and *sec15-1* at restrictive temperature, the defective secretion of invertase in these cells was rescued by overexpression of *SEB1*. Furthermore, overexpression of *SEB1* abolished the accumulation of α factor precursor in *sec3-101* cells shifted to restrictive temperature. The genetic interaction between *SEB1* and the genes encoding exocyst components was further demonstrated by synthetic phenotypes observed in combination of *seb1Δseb2Δ* with *sec10-2* or *sec15-1* (IV). Interestingly, in addition to being subunits of the exocyst, the Sec10 and Sec15 proteins were recently shown to form a separate subcomplex *in vivo* (Guo *et al*., 1999).

3.2.3 Simultaneous disruption of *SEB* genes with *SEM1* deletion causes a synthetic phenotype (unpublished)

SEM1 is a novel gene that was isolated as a suppressor of *sec15-1* and has been shown to interact genetically with a subset of genes encoding the exocyst components (Jäntti *et al.* 1999). Therefore, it was tested whether interaction could also be detected between the *SEB* genes and *SEM1*. The *seb* deletion strains were crossed with a *sem1* disruptant that does not have any detectable phenotype on its own in our standard yeast strain under normal growth conditions. A majority of the tetrads from the crosses *sem1Δ* x *seb1Δ* and *sem1Δ* x *seb2Δ* had four viable spores (Table 4). Three different diploid clones were analysed from the cross *sem1Δ* x *seb1Δseb2Δ* because unexpectedly high lethality of the spores in this cross was observed (Table 4). Only 57 of the 80 tetrads analysed yielded viable spores. The viability of the spores varied between the three clones studied being especially low in the strains SKY16 and SKY17. In addition, all the spores grew slowly at 24°C and a number of spores had a serious growth defect at 35°C.

Table 4. Synthetic phenotype analysis. seb1Δ, seb2Δ *or* seb1Δseb2Δ *strains were crossed with a* sem1Δ *strain of opposite mating type and at least 20 tetrads were analysed from each cross.* [a]*One tetrad had only one viable spore and in 18 tetrads all the spores were inviable.* [b]*Eight tetrads had only one viable spore and in five tetrads all the spores were inviable.*

Cross	Strain	Number of tetrads	Viable : inviable spores		
			4:0	3:1	2:2
sem1Δ x *seb1Δ*	SKY13	20	14	5	1
sem1Δ x *seb2Δ*	SKY14	20	17	3	-
sem1Δ x *seb1Δseb2Δ*	SKY15	20	17	1	2
sem1Δ x *seb1Δseb2Δ*	SKY16	40[a]	2	1	18
sem1Δ x *seb1Δseb2Δ*	SKY17	20[b]	-	2	5

Simultaneous disruption of the two *SEB* genes is known to cause inability to grow at 38°C. The *seb1Δsem1Δ* spores of SKY13 and the *seb2Δsem1Δ* spores of SKY14 grew normally at 38°C and 37°C. Therefore, the predominant phenotype of the slowly growing spores from SKY15, 16 and 17 was likely due to disruption of all the three genes *SEB1, SEB2, SEM1*. This was confirmed by Western analysis of strains derived from the spores. The strains unable to grow at 35°C lacked all the three proteins (Seb1p, Seb2p and Sem1p) whereas at least one of these proteins was present in the temperature insensitive strains. However, the surprisingly low spore viability observed in two of the clones of this cross cannot be explained by these gene disruptions only, because the distribution of the markers of the viable spores was as statistically expected. In a cross like this, four or eight descendant types are possible depending on the extent of recombination between *SEB1* and *SEB2*, which both reside in the same chromosome, and the number of ts *seb1Δseb2Δsem1Δ* spores among all the viable ones was about 15%.

Two haploid strains, SKY15-6C and SKY15-13A, which lack the Sem1 and Seb proteins were taken for further analysis. They were transformed with multicopy plasmids containing a *SEB1*, *SEB2* or *SEM1* gene, or their vector controls. The ts growth defect of the strains grown on plates at 37°C was rescued by overexpression of any of these three genes. A set of transformants was grown also in liquid culture at restrictive temperature and secretion of invertase was studied. In addition to abolishing the growth defect of these disruptants,

overexpression of the *SEB* genes also relieved the defect in secretion of invertase (Figure 5).

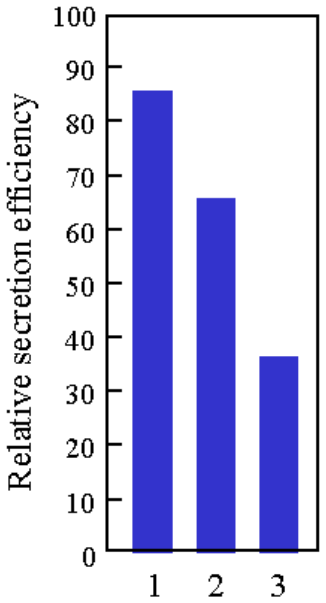

Figure 5. Partial rescue of secretion of invertase in seb1Δseb2Δsem1Δ *strain SKY15-6C by overexpression of* SEB *genes. The disruptant strain transformed with YEpT-SEB1L, YEpSEB2L or their vector control, and the wild type strain NY179 carrying vector pRS425 were cultivated at 37°C and secretion of invertase after one hour derepression of* SUC2 *was studied. The amount of secreted invertase in the transformants of the disruptant strain was compared to that of the wild type transformant and the value for the wild type transformant was set at 100. SKY15-6C transformed with YEpT-SEB1L (1), YEpSEB2L (2) or their vector control (3). The bars indicate the mean value of two independent experiments*.

3.2.4 Overproduction of components of the ER translocon can rescue the temperature sensitivity of the exocyst mutants

The components of the Sec61p complex interact with each other both genetically and physically. Since multiple genetic interactions had been observed for *SEB1*, it was studied whether overexpression of the other components of the ER translocon could suppress mutations in genes encoding the exocyst components. Remarkably both *SEC61* and *SSS1* suppressed several of them (IV and Table 5). *SEC61* rescued the growth defects of *sec3-101*, *sec6-4*, *sec8-9* and *sec10-2* at the restrictive temperature in addition to a weak suppression observed for *sec5-24*. *SSS1* suppressed *sec5-24*, *sec6-4* and *sec8-9*. Suppression of *sec6-4* was weaker than that of the other mutants. It was only clear on YPD plates and not on selective medium. Thus, elevated levels of ER translocon components could rescue the growth of several ts mutants with defects in components of the exocyst complex.

To study whether the suppression obtained by overproduction of ER translocon components was specific to exocyst mutants, all the late-acting *sec* mutants,

SEC1 homologues *sly1* and *slp1*, and a few other mutants were transformed with multicopy plasmids carrying *SEC61*, *SEB1* or *SSS1*, or with the empty vector controls. The only other late-acting mutant that was clearly suppressed by overexpression of any one of these three genes was *sec1-1* (IV). In addition, *SEB1* suppressed *sec2-41*, which encodes a truncated form of a protein mediating nucleotide exchange on Sec4p (Nair *et al*., 1990; Walch-Solimena *et al*., 1997), and *sec19-1*, which appears to cause secretory block at multiple steps (Novick *et al*., 1980). *sec19-1* was later shown to be allelic to *GDI1*, which encodes the GDP dissociation inhibitor of Sec4p which functions in releasing the GTP-binding Sec4 protein from its target membrane (Garrett *et al*., 1994).

Possible suppression of *kar2* and *sec13* by overexpression of *SEB1* was also tested, but was not detected. Overexpression of *SEB2*, the close homologue of *SEB1* encoding a subunit of the Ssh1p complex, did not suppress ts mutations in either genes encoding the exocyst components or *SEC61*, *SEC62* or *SEC63*. Overexpression of *SEC62* or *SEC63* could not suppress the exocyst mutations either (IV and Table 5). The genetic interactions, thus, appeared only between the Sec61p complex, Sec1p and the exocyst. Furthermore, the suppression occurred only in one direction. ER translocon components could suppress defects in exocytosis, but *sec61* mutants were not suppressed by overexpression of *SEC1*, *SEC3* or *SEC15* (IV). A model for the functional interplay between the components of the Sec61p complex and those needed for exocytosis will be discussed at 3.4.

3.3 Enhancement of protein secretion in *S. cerevisiae*

3.3.1 Secretion enhancement by overproduction of components of the yeast secretory pathway

The *SSO* genes were isolated as multicopy suppressors of *sec1-1*. During characterisation of the Sso proteins it was noticed that overexpression of *SSO1* or *SSO2* suppressed the secretion defect of *sec1-1* at 37°C and moreover led to elevated levels of secretion of a reporter protein (Aalto *et al*., 1993) whereas the growth was rescued only at lower temperatures of 36°C and 35°C. Even more pronounced enhancement in the secretion of a reporter protein was obtained by overexpression of *SSO* genes at the permissive temperature when *sec1-1* cells do not manifest a growth or secretion defect (III). These studies were repeated in wild type cells to exclude the possibility that *sec1-1* cells had a defect even at the permissive temperature which overexpression of *SSO* genes would suppress. The secretion enhancement in the wild type yeast was similar to that in *sec1-1*

cells. The enhancement was 2 - 4 fold in comparison to the control strain carrying an empty vector instead of *SSO* expression plasmid (III).

The reporter protein used in these studies was *Bacillus amyloliquefaciens* α-amylase, which has been shown to be secreted efficiently by its own signal sequence. Over 70% of the enzyme activity is found in the culture medium (Ruohonen *et al*., 1987; III). The Sso1p and Sso2p overproduction strains secreted more α-amylase to the culture medium than either the control strain or segregant strains that had lost the respective overexpression plasmid. Western analysis confirmed that the amount of α-amylase and not its specific activity was increased in the culture medium. *SSO2* overexpression resulted in a slightly bigger enhancement effect than did overexpression of *SSO1*. In the further studies only *SSO2* was thus used.

The effect of *SSO2* on secretion of yeast's own secreted protein, invertase, and another heterologous protein, endoglucanase I of *Trichoderma reesei*, was also studied (III). When invertase was produced from the chromosomal *SUC2* gene, 1.5 fold enhancement of its secretion was obtained in the Sso2p overproduction strain. If the vesicle transport was loaded with invertase overexpressed from a multicopy plasmid, a 2 - 6 fold enhancement occurred in comparison to the control strain. The amount of endoglucanase activity in the culture medium, on the contrary, was not affected by elevated production of Sso2p. Endoglucanase I seems to accumulate in the yeast ER since only core-glycosylated protein has been detected indicating that it has not reached the Golgi (Penttilä *et al*., 1987). Overexpression of *SSO2*, thus, was not able to rescue secretion of heterologous protein that is trapped in the ER.

The enhancement of α-amylase secretion is not probably caused by enhanced transcription. Northern analysis of Sso2p overproducing and the control strains did not detect any clear difference between the levels of messenger RNA for α-amylase. This method, however, may not be sensitive enough to detect slight differences in the levels. These results, thus, should be confirmed with the more sensitive sandwich hybridisation assay (Syvänen *et al*., 1986). The mechanism of the secretion enhancement by overexpression of *SSO* genes is not yet known. Pulse labelling experiments suggested that secretion in the *SSO2* transformant continued longer than in the control strain and resulted in higher levels of secreted α-amylase (III). According to the cell fractionation studies, elevated levels of Sso2p accelerated secretion of proteins to the periplasmic space. This is in good agreement with the suggested function for Sso proteins, the yeast syntaxins, in the targeting/fusion of transport vesicles to the plasma membrane. The finding suggests that exocytosis is a rate-limiting step in protein secretion in *S. cerevisiae*.

In the original library plasmid YEpSSO2 (Aalto *et al*., 1993; McKnight and McConaughy, 1983) used in the above studies, *SSO2* was expressed from the alcohol dehydrogenase I (*ADH1*) promoter. Ruohonen *et al*. (1991; 1995) have previously shown that the promoter ceases to function when glucose is consumed from the growth medium because of a longer non-functional transcript initiating upstream of the normal transcription initiation site. To remove the upstream transcription initiation site the promoter region was shortened to 700 bp (Ruohonen *et al*., 1995). This promoter is denoted the middle *ADH1* promoter to differentiate it from the original, long form. The middle promoter is functional during both glucose and ethanol consumption phase which resulted in higher level of the Sso2 protein especially in the latter half of the growth period (III).

Using this new construct the enhancement effect obtained by overexpression of *SSO2* was even more pronounced. Approximately 10 - 20 fold overproduction of Sso2p resulted in 2 - 4 fold enhanced secretion of the reporter protein. These results suggested that other component(s) must become limiting. Thus, yeast was presumed to contain other bottle necks in the secretory pathway. In agreement with this, overexpression of *SEB1*, a gene encoding a component of the ER translocon, led to enhanced secretion of both α-amylase and invertase (IV). Overexpression of the two components of the bacterial translocation machinery at the plasma membrane, SecY and SecE, has been shown to enhance secretion of human interleukin-6, suggesting that the translocation machinery may become rate-limiting during heterologous protein production (Pérez-Pérez *et al*., 1994). However, no evidence of an altered rate of protein translocation into the ER or transport via the secretory pathway was seen in the Seb1p overproduction strain in comparison to the control strain (IV). Thus it remains open which reaction in the secretory process is accelerated by *SEB1* overexpression.

The secretion enhancement was studied in yeast carrying two different plasmids: YEpαa6 for expression of α-amylase and another for *SSO2* or *SEB1* expression, or the respective empty vector control. Since these were multicopy plasmids, the possibly varying plasmid copy numbers and stability became of concern. To confirm the results obtained with two plasmids, the α-amylase expression cassette was integrated into the yeast genome (IV). The cassette was integrated to the *HIS3* or *URA3* locus of wild type yeast. The secretion enhancement in these integrant strains was similar to that obtained in the two-plasmid strains (IV and unpublished results).

3.3.2 A novel approach for enhancement of secretion

The ER has often been considered to form the major bottle neck in the secretion of heterologous proteins in yeast (Moir and Dumais, 1987; Elliott *et al*., 1989; Biemans *et al*., 1991; McCracken and Kruse 1993; Robinson *et al*., 1994). The factors mediating protein maturation have been presumed to be rate limiting. For one such factor, PDI, it has indeed been shown (Robinson *et al*., 1994) that overexpression leads to enhanced secretion of proteins containing several disulphide bonds. The degree of acceleration of secretion obtained by PDI overproduction is assumed to reflect the extent to which the secretory protein requires the disulphide bond interchange. Overexpression of Bip/Kar2p has also been reported to enhance secretion of heterologous protein. Furthermore, by simultaneous overproduction of both PDI and Kar2p even more elevated levels of secreted single chain antibody fragments were obtained (Shusta *et al*., 1998) indicating a need for higher amounts of both these components. Deletion of *CNE1* was also reported to enhance secretion of heterologously expressed protein (Parlati *et al*., 1995). However, depletion of this component of the quality control machinery in the ER enabled secretion of malfolded proteins. A bottle neck at Golgi or post-Golgi transport has been reported for human insulin-like growth factor (Steube *et al*., 1991). The results of this study further confirm that there may be several rate-limiting steps in the yeast secretory pathway. One would be targeting/fusion of secretory vesicles to the plasma membrane mediated by Sso proteins and another the reaction facilitated by excess of Seb1p.

Since the Sso proteins and Seb1p are components of the secretory machinery in yeast they are likely to enhance the secretory process itself. Problems with many previous attempts to enhance secretion include that the enhancement obtained is specific for the reporter protein used in the screen (Kotylak and El-Gewely, 1991) or that the mechanism by which the accelerated secretion is obtained remains elusive (Sleep *et al*., 1991; Chow *et al*., 1992). For example, *PSE1* was uncovered in a screen for enhanced secretion of killer toxin. Its overexpression caused also elevated secretion of α factor and acid phosphatase (Chow *et al*., 1992). The gene has later been shown to encode a protein functioning in nuclear import of ribosomal proteins (Rout *et al*., 1997). Thus, it probably has no direct role in protein secretion. In many supersecretory strains obtained through random mutagenesis other processes than secretion as such have been altered (Sakai *et al*., 1988; Wingfield and Dickinson, 1993). Also, in many cases the mutations causing the supersecretory phenotype are recessive (Smith *et al*., 1985; Sakai *et al*., 1988; Shuster *et al*., 1989) thus making it difficult to apply them to industrial strains, which are polyploid or aneuploid. Secretion enhancement by overexpression of secretory genes, instead, is a novel, more

generally applicable approach to increase the moderate secretory capacity of *S. cerevisiae*.

3.4 A model for the functional interplay between ER translocon, exocyst and Sso2p

The Sso proteins and Seb1p probably carry out reactions that are rate-limiting in the secretory pathway, because often such components are identified by their overexpression (Rine, 1991). Lew and Simon (1991) suggested that the release sites on the yeast plasma membrane are rate-limiting, which is further supported by the results obtained with *SSO2* overexpression (III). *SEB1* encodes the β subunit of the Sec61p complex, which functions in the ER translocation (II). Electron microscopic analysis has suggested that the three subunits are not present in always the same stoichiometric ratio in the Sec61p complex (Hanein *et al*., 1996). Since Seb1p is more easily dissociable and can remain stable on its own (Biederer *et al*., 1996) it is considered to be probably absent under certain conditions (Hanein *et al*., 1996). The most basic translocation machinery may, thus, comprise only the channel-forming Sec61p itself and Sss1p, which has been suggested to serve as a gate for the translocation pore (Plath *et al*., 1998), while Seb1p would play an accessory or regulating role. The fact that overproduction of Seb1p does not alter the levels of the other two components of the Sec61p complex (IV) is in agreement with a regulatory role for Seb1p.

The exact function of the nonessential β subunit of the complex, Seb1p, is not known. Disruption of *SEB1* only mildly impairs translocation of α factor precursor (Finke *et al*., 1996) and does not affect that of *Bacillus* α-amylase (discussed in IV). Even disruption of both *SEB* genes leads only to a slight reduction in protein translocation into ER (Finke *et al*., 1996). The mammalian Sec61β is not essential but facilitates cotranslational translocation *in vitro* (Kalies *et al*., 1998). Overexpression of SecG, a component of the *E. coli* translocation machinery thought to be homologous to the eukaryotic β subunits of the Sec61p complex, has been reported to facilitate protein export (Bost and Belin, 1995). Our finding that *SEB1* overexpression in *sec3-101* cells relieved accumulation of a presumably untranslocated precursor of α factor (IV) is consistent with the assumption that *SEB1* may facilitate ER translocation in yeast.

Genetic interactions between genes imply that they contribute to the same pathway, but do not prove that the encoded proteins physically interact. Both Seb1p (II) and Sss1p (Esnault *et al*., 1993) reside in the ER even when

overproduced whereas the exocyst is indicated to function at the plasma membrane. Physical interaction between components of the Sec61p and exocyst complexes seems unlikely, though the ER and plasma membrane are not necessarily spatially far apart from each other (Preuss *et al*., 1991). Several secretory proteins have been shown to function at more than one step along the secretory pathway (Graham and Emr, 1991; Griff *et al*., 1992, Roberg *et al*., 1997a; b; Finger and Novick, 1997). Seb1p could also have a dual function: one as a component of the Sec61p complex and another possibly on its own, but still in the ER. This would be in accord with the results showing that, unlike overexpression of *SEC61* or *SSS1*, that of *SEB1* was able to relieve the defective secretion of invertase by *sec8-9* and *sec15-1* cells at restrictive temperature (IV).

One possibility is that suppression of the exocyst mutations by *SEB1* overexpression would be caused by more efficient translocation of secreted proteins into the ER. However, no experimental evidence was obtained to support this. Therefore it seems likely that the suppressions are mediated by other factors. It was reasoned that increased levels of Seb1 protein could facilitate translocation/transport of components that would enhance the exocyst function and that would normally be present in limiting amounts. Such components could function *e.g.* as receptors docking the exocyst at the membrane. It was further reasoned that it should be possible to identify such receptor proteins as suppressors of exocyst mutations, independent of ER function.

Good candidates for such factors would be the Sso proteins, the plasma membrane t-SNAREs. Overexpression of the *SSO2* gene suppresses all the exocyst mutants that cause structural instability of the complex (TerBush and Novick, 1995) except *sec6-4* (Aalto *et al*., 1993; IV and Table 5). Similarly, another plasma membrane t-SNARE, Sec9p (Brennwald *et al*., 1994), as well as Sec1p, a protein regulating the SNARE function, is known to suppress exocyst mutants (Aalto *et al*., 1993; IV and Table 5). In wild type cells *SSO2* overexpression leads to enhanced protein secretion, which indicates that Sso2p may be present in limiting amounts on the plasma membrane (III). These results strongly suggest that Sso2p may facilitate the exocyst function at the plasma membrane. This conclusion is further supported by results from animal cells. Antibodies raised against the rsec8 component of the rat exocysts were shown to co-immunoprecipitate the plasma membrane t-SNARE implying physical interaction between exocyst components and the t-SNARE (Hsu *et al*., 1996). Since Sso2p is transported to its site of action via the biosynthetic pathway (I) the elevated levels of Seb1p might facilitate insertion of Sso2p into the ER membrane. The suppression of exocyst mutants and the enhancement of

Table 5. Multicopy suppression of late acting sec *mutants. The genes encoding the exocyst components are marked in bold. The* SEC3 *mutant allele in studies of* SEC4 *or* SEC9 *overexpression was* sec3-2. [a]*Aalto* et al., *1993;* [b]*Salminen and Novick, 1987.* SEC4 *was expressed from a CEN plasmid. The results obtained with* SEC4 *multicopy plasmid were essentially the same (ibid).* [c]*Brennwald* et al., *1994;* [d]*Aalto* et al., *1993 and this study. ND = not determined.*

Mutation	Suppressor gene									
	SEB1	*SEC61*	*SSS1*	*SEB2*	*SEC62*	*SEC63*	*SEC1*[a]	*SEC4*[b]	*SEC9*[c]	*SSO2*[d]
sec1-1	+	+	+	ND	ND	ND	+	+/-	+	+
sec2-41	+	-	-	ND	ND	ND	-	+	-	-
sec3-101	+	+	-	-	-	-	+	-	+	+
sec4-8	-	-	-	-	ND	ND	-	+	+/-	-
sec5-24	+	+	+	-	-	-	+	+/-	-	+
sec6-4	+	+	+	-	-	-	-	-	-	-
sec8-9	+	+	+	-	-	-	+	+	+	+
sec9-4	-	-	-	ND	ND	ND	-	-	+	-
sec10-2	+	+	-	-	-	-	+	+/-	-	+
sec15-1	+	-	-	-	-	-	+	+	+	+
sec19-1	+	-	-	ND	ND	ND	ND	+	ND	ND
sso2-1	-	-	-	ND	ND	ND	ND	ND	+	+

secretion in wild type yeast by overexpression of *SEB1* could, thus, be mediated by increased amounts of the Sso proteins at the plasma membrane. However, the preliminary cell fractionation studies did not reveal an elevated level of membrane-bound Sso2p after overexpression of *SEB1* (discussed in IV). This method cannot disclose possible local differences in concentration, so further studies are still needed to elucidate this question. The role of Sec1p in these interactions is more difficult to define. The strong genetic interactions (Table 5), suppression of exocyst mutants by *SEC1* overexpression and suppression of *sec1-1* by elevated levels of the translocon components or plasma membrane t-SNAREs, suggest that the *SEC1* has a role in this play. The nature of this role must await further elucidation of the function of Sec1p itself.

Another possibility is that the influence of *SEB1* overexpression is mediated by other components than proteins, *e.g.* by membrane lipids. Interestingly, David *et al*. (1998) recently reported that mutations in genes involved in the long chain fatty acid elongation and sphingolipid synthesis that occur in the ER abolished the need for post-Golgi v-SNARE function, giving strong support for interactions between ER functions and those at the site of exocytosis. This raises the intriguing possibility that *SEB1* overexpression might enhance the activity rather than the absolute amount of Sso2p in the plasma membrane, the enhancement being mediated by a change in membrane lipid composition.

4. Conclusions

The novel genetic interactions revealed in this study indicate a functional connection between components of the early and late steps of the yeast secretory pathway. A model was suggested to explain the mechanism by which over-expression of *SEB1* leads to enhanced protein secretion and suppression of all available exocyst mutations. The model predicts that exocytosis is a rate-limiting step in protein secretion in yeast. This step can be accelerated by overproduction of proteins functioning in targeting/fusion at the plasma membrane and, interestingly, by a component, Seb1p, of the ER translocation machinery. Overexpression of *SEB1* may increase the amount of Sso2p at the plasma membrane or enhance the activity, perhaps in a membrane lipid-dependent manner. Several aspects of this model, however, need to be experimentally tested. Most important would be to demonstrate physical interaction between the exocyst and t-SNARE components.

The exact mechanism by which overproduction of Sso proteins and Seb1p execute their secretion enhancing effect will be an interesting target of future studies. Moreover, other rate-limiting factors are likely to be found and the balance between levels of different secretory components will also require consideration. The effects on protein secretion of overproduction of other components of the post-Golgi SNARE complex should also be studied in the future.

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