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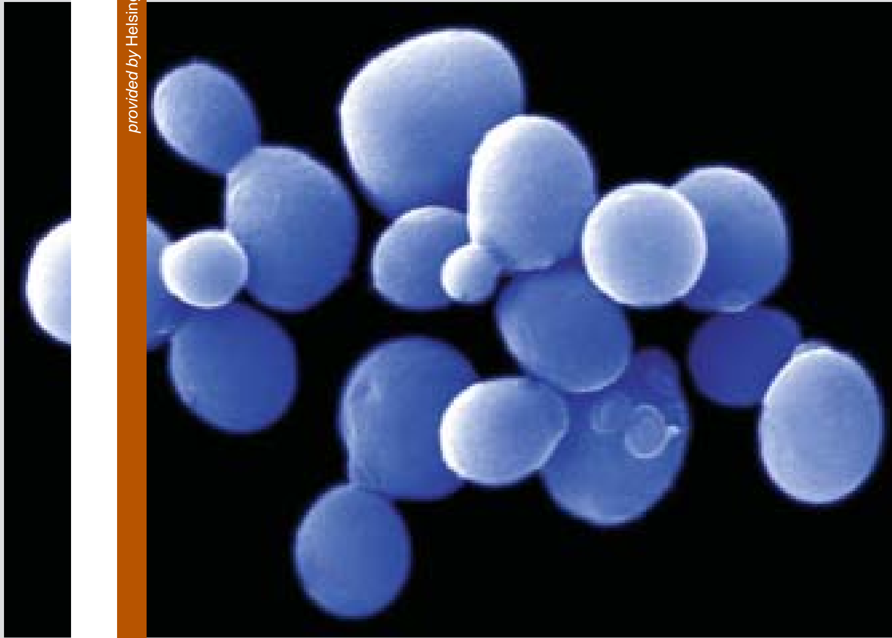

Glyc ation and Sorting of Secretory Proteins in Endoplasmic Reticulum of the Yeast *Saccharomyces cerevisiae*

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Gummerus Kirjapaino
Saarijärvi 2005

Results! Why, man, I have gotten a lot of results.
I know several thousand things that won't work.

Thomas A. Edison

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following two articles and a manuscript. In the text, they are referred to by their Roman numerals.

I
Karhinen, L. and Makarow, M. (2004). Activity of recycling Golgi mannosyltransferases in the yeast endoplasmic reticulum. *J.Cell.Sci.* **117**:351 - 358.

II
*Fatal, N., *Karhinen, L., Jokitalo, E. and Makarow, M. (2004). Active and specific recruitment of a soluble cargo protein for endoplasmic reticulum exit in the absence of functional COPII component Sec24p. *J.Cell.Sci.* **117**:1665 - 1673.

III
Karhinen, L., Nunes Bastos, R., Jokitalo, E. and Makarow, M. ER exit of a secretory glycoprotein in the absence of Sec24p family proteins in yeast, submitted manuscript.

*equal contribution

ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ATPase	ATP phosphatase
ALG	Asparagine-linked glycosylation gene
ARF	ADP ribosylation factor
BiP	Immunoglobulin heavy chain binding protein
COP	Coat protein
CPY	Carboxypeptidase Y
Dol-P	Dolichol pyrophosphate
ER	Endoplasmic reticulum
ERO	ER oxidoreductin
FAD	Flavin adenine dinucleotide
GalNAc	N-acetyl galactosamine
GAP	GTPase activating protein
GDP	Guanidine diphosphate
GFP	Green fluorescent protein
GMP	Guanidine monophosphate
Glc	Glucose
GlcNAc	N-acetyl glucosamine
GPI	Glycosylphosphatidylinositol
GTP	Guanidine triphosphate
GTPase	GTP phosphatase
Hsp	Heat shock protein
kDa	Kilodalton
Man	Mannose
M-Pol	Mannosyl polymerase
NSF	N-ethylmaleidimide-sensitive factor
OST	Oligosaccharyl transferase
PACE	Paired base amino acid-cleaving enzyme
PC	Prohormone convertase
PDI	Protein disulfide isomerase
PLD	Phospholipase D
PMT	Protein-mannosyl transferase
POMT	Protein-O-mannosyl transferase
RNA	Ribonucleic acid
RNC	RNA-nascent chain complex
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electroforesis
Sfb	Sed5 binding protein
SNAP	Soluble NSF attachment protein
SNARE	SNAP receptor
SPC	Signal peptidase complex
SR	SRP receptor
SRP	Signal recognition particle
TEM	Transmission electron microscopy
TGN	*trans*-Golgi network
UDP	Uracil diphosphate
UGGT	UDP-Glc::glycoprotein transferase
UPR	Unfolded protein response
VSV-G	Vesicular stomatitis virus glycoprotein
VTC	Vesicular-tubular cluster

Single letter code	*Three letter code*	*Amino acid*
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

SUMMARY

In eukaryotic cells, newly synthesized polypeptides enter the secretory pathway *via* translocation into the endoplasmic reticulum (ER). In the ER, the polypeptides acquire glycan moieties, which are extended when the proteins reach the Golgi complex. The transport of secretory proteins from the ER to the Golgi complex is carried out in vesicles generated by the coat protein complex II (COPII). Another coat protein complex, COPI, mediates retrograde transport from the Golgi complex to the ER. In this study, the COPII-transport, and its effect on glycosylation of proteins in the ER were investigated using the yeast *Saccharomyces cerevisiae* as a model organism. Our study revealed that proteins that were retained in the ER lumen acquired Golgi-specific glycan modifications. This was found to be due to the normally Golgi-localized N- and O-glycosylating mannosyltransferases that recycled through the ER *via* the COPI-mediated retrograde transport mechanism. The Golgi-specific modifications occurring in the ER could only be detected when the forward traffic from the ER was blocked, allowing the transferases and substrate glycoproteins to interact for a longer time. The recycling of Golgi enzymes may contribute to a mechanism maintaining integrity of the dynamic Golgi complex. In this study we also found that the yeast secretory glycoprotein Hsp150 could exit the ER when the essential COPII protein Sec24p was nonfunctional, and its two non-essential homologues, Sfb2p and Sfb3p were lacking. In the same conditions other cargo proteins remained in the ER indicating that sorting of Hsp150 was active and specific. The sorting signal that guided Hsp150 for Sec24p-independent ER exit was mapped to the C-terminal domain of Hsp150. When the C-terminal domain was replaced with a horse radish peroxidase portion, the fusion protein could be localized to the ER by electron microscopy. Furthermore, the sorting signal actively recruited another normally Sec24p-dependent reporter protein for Sec24p-independent ER exit, when the C-terminal domain was fused to the reporter protein. Since Hsp150 is a soluble protein, the sorting signal is likely to interact with a putative trans-membrane adapter protein that may directly bind to COPII components. Hsp150 was also found to be slowly secreted even in the absence of all Sec24 family members. This work demonstrated that ER exit of glycoproteins can occur using different pathways, characterized by different compositions of the COPII coat components, and that cargo proteins carry specific signatures, which guide them to the different ER exit routes.

INTRODUCTION

1. The secretory pathway

The contents of eukaryotic cells are organized into membrane-bounded compartments. Each of these compartments consists of a distinct set of proteins and lipids and thus provides a specialized environment for different biochemical reactions to occur. The compartments are interconnected by vesicular transport mechanisms that deliver cargo from one organelle to another.

The secretion process consists of a sequence of events that take place in the different organelles composing the secretory pathway. Proteins are synthesized on ribosomes in the cytoplasm. Those destined to be secreted, or to function in the organelles of the secretory route, are translocated into the endoplasmic reticulum (ER), the entry point to the secretory pathway. Inside of the ER lumen several modifications take place: the signal peptide is cleaved off, the polypeptide is folded to its correct conformation, disulfide bonds are formed, and glycans are added to the newly synthesized protein. Once the protein has reached its appropriate, native form that is approved by the quality control machinery, it is

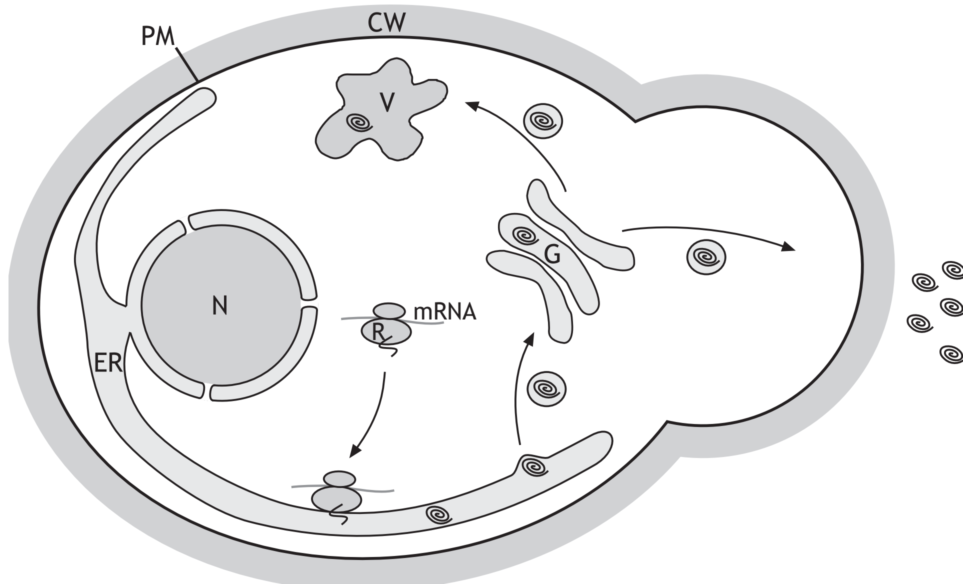

Figure 1. A schematic illustration of the polarized secretory pathway of a yeast cell. Protein transalation is initiated on free ribosomes in the cytoplasm. Polypeptides are translocated into the ER lumen, modified and folded, and packaged into vesicular transport carriers. The vesicles migrate and release their cargo to the Golgi complex, where the proteins are further modified and finally sorted for their final destinations, to the growing bud (PM, CW, culture medium), or to the vacuole. (R) ribosome, (N) nucleus, (ER) endoplasmic reticulum, (G) Golgi complex, (V) vacuole, (PM) plasma membrane, (CW) cell wall.

packaged into vesicular carriers and delivered to the next compartment, the Golgi complex. In the Golgi, further modifications occur: the glycan side chains are extended, and proteins synthesized as precursors are processed. Finally, proteins are sorted in the *trans*-Golgi network (TGN), and then transported to their final destinations, to the vacuole/lysosome or to the exterior of the cell.

The cellular organisation of the unicellular yeast *Saccharomyces cerevisiae* is very similar to that of a mammalian cell. Furthermore, mechanisms of vesicular transport are highly conserved from yeast to mammalian cells. Since yeast is a well characterized model organism, safe and easy to manipulate, and whose genome has been completely resolved, much of the current understanding of the secretory pathway has been obtained using yeast as a model. In **Figure 1**, the secretory pathway of a yeast cell is illustrated.

In the following chapters, individual events along the secretory pathway that take place in the different organells, and mechanisms of vesicular transport between the ER and Golgi, are discussed.

1.1 Entering the ER

Secretory proteins are targeted to the ER by a signal peptide, a usually cleavable amino-terminal (N-terminal) extension of the nascent polypeptide chain (Blobel & Dobberstein, 1975, Walter & Johnson, 1994). In mammalian cells, translocation to the ER lumen occurs simultaneously with protein synthesis, i.e. cotrans-lationally, whereas in yeast, proteins may also enter the ER after completion of synthesis, i.e. posttranslationally. Co-translational and posttranslational translocation mechanisms are illustrated in **Figure 2**.

1.1.1 Signal peptides

The overall lengths of signal peptides vary from 15 to more than 50 amino acid residues, yet they have several common features. The most crucial element of a signal peptide lies in its hydrophobic central region of 6 - 15 amino acids (h-region). Mutations in this region result in a partial or complete mistargeting. The h-region is flanked by a polar, in most cases positively charged N-terminal region (n-region) that varies in length, and by a polar carboxy-terminal (C-terminal) region (c-region). The c-region contains a signal peptidase cleavage site determined by two small uncharged residues in positions -3 and -1 (Reviewed by Martoglio & Dobberstein, 1998).

In yeast, the signal peptides have a key role in selection for the co- and posttranslational translocation pathways. The selection correlates with the hydrophobicity of the respective signal peptide. A signal peptide of relatively low hydrophobicity prefers to enter the posttranslational pathway, whereas a stronger hydrophobic signal peptide leads to the cotranslational pathway (Ng *et al*, 1996).

1.1.2 Cotranslational translocation

When a protein targeted to the cotranslational translocation pathway is being synthesized by a ribosome, it is directed to the ER by the signal recognition particle (SRP) (reviewed by Keenan *et al*, 2001, Rapoport *et al*, 1996). Initially, SRP was discovered in mammalian cells. It was shown to consist of six proteins, SRP9, SRP14, SRP19, SRP54, SRP68 and SRP72K, and 7SL RNA (Walter & Blobel, 1980, Walter & Blobel, 1982). The yeast SRP components Srp68p, Srp72p, Srp65p, Srp54p, Srp21p, Srp14p, and scR1 RNA (Hann & Walter, 1991, Hann *et al*, 1992, Brown *et al*, 1994, Mason *et al*, 2000) are homologous to their

mammalian counterparts, except that there is no homologue for Srp21p in mammalian cells, no yeast homologue for mammalian SRP9, and there are two Srp14p subunits in yeast (Mason *et al*, 2000). The SRP recognizes and tightly binds the hydrophobic signal peptide of a nascent protein as it emerges from the ribosome (Walter *et al*, 1981). The binding is accomplished by the SRP54 component that is a GTP-binding protein (Bernstein *et al*, 1989). SRP also interacts directly with the ribosome itself (Walter *et al*, 1981), which results in a transient delay in translation, i.e. elongation arrest (Mason *et al*, 2000, Siegel & Walter, 1988). For this activity, the heterodimer of SRP9 and SRP14, that together with the RNA component form the "Alu" domain, is required (Strub *et al*, 1991). Particularly important for the elongation arrest is the C-terminal region of the SRP14, or Srp14p in yeast, since a SRP lacking a stretch of C-terminal amino acids of SRP14 lacks elongation arrest activity (Mason *et al*, 2000, Thomas *et al*, 1997). In yeast, instead of SRP9/SRP14, two copies of Srp14p form a homodimer that comprises part of the Alu domain (Mason *et al*, 2000, Strub *et al*, 1999).

The ribosome-nascent chain (RNC)-SRP complex is targeted to the ER membrane *via* interaction of the SRP and the ER membrane-bound SRP receptor (SR) (Walter & Blobel, 1982, Meyer & Dobberstein, 1980, Gilmore *et al*, 1982a). Elongation arrest has been proposed to function in this step by providing a longer time frame for the interaction to take place. *In vitro*, elongation arrest has been shown to enhance translocation efficiency, but not to be essential (Thomas *et al*, 1997, Siegel & Walter, 1985). In mammalian cells, SR is a heterodimeric complex that consists of the 69 kDa protein SRα (Gilmore *et al*, 1982b) and the 30 kDa SRβ (Tajima *et al*, 1986). SRα is a GTPase closely related to the SRP54 GTPase domain. It is a peripheral membrane protein and it is anchored to the ER through its association with the integral membrane protein SRβ. SRβ is also a GTPase but it is related to the ARF and Sar1p families rather than the SRP54 and SRα (Miller *et al*, 1995). The GTPase activity of SRβ is required for the stable formation of the SR complex (Schwartz & Blobel, 2003). The yeast SRα/Src101p (Ogg *et al*, 1992) and SRβ/Src102p (Ogg *et al*, 1998) are homologous to the mammalian SR components. SRα is important, but not essential for yeast, since lack of *SRC101* results in a six-fold slowing down in growth rate (Ogg *et al*, 1992). Also in yeast, SRβ binds SRα thus connecting the complex to the ER membrane. The binding as well as GTPase activity are important for the SR function, but deletion of the SRβ ER anchor results in no significant defect of function, suggesting that SR only needs to contact the ER transiently (Ogg *et al*, 1998).

Interaction of the RNC-SRP complex with the SR at the ER membrane is GTP-dependent. The SRP GTPase SRP54 and the SR subunit SRα both bind GTP upon formation of the SRP-SR complex thus stabilizing it. This initiates the transfer of the signal sequence from SRP54 to the Sec61α component of the Sec61 complex at the translocation site. Hydrolysis of GTP by both SRP54 and SRα is required for dissociation of the SRP-SR complex and for the polypeptide synthesis to resume (Rapiejko & Gilmore, 1994, Rapiejko & Gilmore, 1997). The hydrolysis is controlled by the Sec61 complex (Song *et al*, 2000).

The Sec61 complex forms the core of the protein translocation channel, i.e. translocon. The mammalian Sec61 complex consists of Sec61α (Görlich *et al*, 1992), Sec61β and Sec61γ (Görlich &

Rapoport, 1993) subunits that are homologous to the yeast Sec61p (Stirling *et al*, 1992), Sbh1p (Panzner *et al*, 1995) and Sss1p (Esnault *et al*, 1993), respectively. In yeast, homologues of Sec61p and Sbh1p, Ssh1p and Ssh2p, together with Sss1p can form the Ssh1p complex. Since the Ssh1p complex does not associate with components of the posttranslational translocation machinery, it most likely has a function in cotranslational translocation (Finke *et al*, 1996).

Sec61p is the ribosome-biding unit of the translocon (Görlich *et al*, 1992, Kalies *et al*, 1994). It has been suggested that the binding of Sec61p and the ribosome is tight, resulting in a seal so that the peptide emerging from the aqueous pore of the ribosome is in no contact with the cytosol. As protein synthesis resumes after elongation arrest, the nascent peptide could only pass through the translocation channel (Crowley *et al*, 1993, Crowley *et al,* 1994). However, recent data describing the crystal structure of the Sec61 complex reveals that the ribosome binds to the Sec61α subunit in an open manner, and that the seal separating the cytosol and the ER lumen is provided by a pore ring formed by the same α-subunit (see **Figure 2A**). The translocon channel is plugged by a short helix of the α-subunit and it is opened upon binding of a ribosome and the signal peptide to the α-subunit (Van den Berg *et al*, 2004).

Passage of the newly synthesized polypeptide to the ER lumen appears to be driven by peptide synthesis itself. However, completion of cotranslational translocation may require some extra energy, since lack of BiP (or Kar2p, in yeast), an ER-resident chaperone, impedes all protein translocation (Vogel *et al*, 1990, Brodsky *et al*, 1995). BiP has been suggested to provide the driving force for posttranslational translocation (Matlack *et al*, 1999).

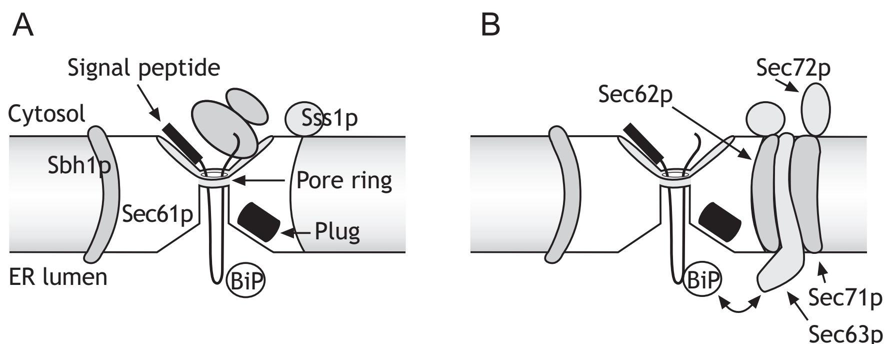

Figure 2. Co- and posttranslational translocation. A) In cotranslational translocation, ribosome is bound to the translocon (Sec61 complex; Sec61p, Ss1p and Sbh1p), and translocation and translation occur simultanously. The key component of the translocon, Sec61p, contains a signal peptide binding region, a pore ring that separates cytoplasm and ER lumen, and a plug. The binding of the ribosome and the signal peptide results in opening of the pore and in displacement of the plug. Protein synthesis itself drives translocation. B) Posttranslational translocation occurs after completion of the polypeptide synthesis. Sec61 complex associates with the Sec62/63 complex, and BiP, activated by Sec63p, provides the energy for translocation. See text for details.

1.1.3 Posttranslational translocation

Posttranslational translocation of fully synthesized proteins occurs *via* a heptameric Sec complex consisting of the Sec61 and Sec62/63 subcomplexes (**Figure 2B**). The yeast Sec61 complex components are Sec61p, Sbh1p and Sss1p, and those of the Sec62/63 subcomplex are Sec62p, Sec63p, Sec71p and Sec72p (Panzner *et al*, 1995, Deshaies *et al*, 1991). In addition, the ER luminal ATPase BiP is also necessary for posttranslational translocation (Panzner *et al*, 1995). As in cotranslational translocation, the Sec61 complex serves as the protein conducting channel (Reviewed by Matlack *et al*, 1998).

Posttranslational translocation is initiated by targeting the completely synthesized polypeptide to the translocon *via* its signal sequence (Matlack *et al*, 1997, Lyman & Schekman, 1997). The signal sequence binds to the Sec61p component of the translocon in a BiP- and ATP-independent manner. The binding requires an intact signal sequence as well as an intact Sec61 complex. This initial binding directs the polypeptide to the translocation channel (Plath *et al*, 1998). Simultaneously with the binding to Sec61p, the signal sequence makes contact with the Sec62p component in one single signal recognition step (Plath *et al*, 2004). The passage of the polypeptide through the channel initially occurs *via* passive diffusion. When the peptide emerges at the lumenal side of the translocon, it is bound by a BiP molecule. The binding of BiP requires an interaction between BiP and the luminal J-domain of Sec63p (Matlack *et al*, 1999, Lyman & Schekman, 1995). The Sec63p J-domain binds to ATP-bound BiP and stimulates ATP hydrolysis resulting in ADP-bound BiP. This enables BiP to bind to the translocating polypeptide with higher affinity. BiP binds essentially to any part of the peptide emerging from the translocon (Misselwitz *et al*, 1998). The Sec63p - BiP interaction is transient, and the J-domain-induced ATP hydrolysis does not require a peptide (Misselwitz *et al*, 1999). Several BiP molecules bind to the translocating poypeptide as it enters the ER lumen. As a result, the peptide can no longer diffuse backwards in the channel, eventually resulting in completed translocation. Thus, BiP provides a molecular ratchet that facilitates translocation by maintaining the translocated peptide in the ER lumen. Even though the ratcheting is sufficient for translocation to occur, it has not been ruled out that BiP may also actively pull the peptide (Matlack *et al*, 1999). Such extra energy may be required for instance if the substrate is folded to a stable conformation in the cytosol prior to translocation. For instance, the *Eschericia coli* β-lactamase, expressed as a part of a recombinant secretory protein in yeast, has been found to fold into an enzymatically active globular form in the cytoplasm. Prior to translocation, β-lactamase is unfolded, and translocation requires BiP (Paunola *et al*, 1998, Paunola *et al*, 2001). BiP molecules dissociate from the translocated peptide upon nucleotide exchange thus liberating the polypeptide to the ER lumen, and rendering BiP ready for a new cycle of J-domain activation (Misselwitz *et al*, 1998).

1.2 Modifications in the ER

Several modifications take place in the ER lumen during and after translocation. The signal peptide is cleaved off by the signal peptidase complex (SPC). The nascent polypeptide is folded to its correct three-dimensional conformation with help of assistant proteins, i.e. chaperones. Disulfide bonds are formed

stabilizing the conformation, and glycosylation is initiated.

1.2.1 Signal peptide cleavage

During, or shortly after translocation to the ER lumen, the signal peptide of a newly synthesized polypeptide is proteolytically released. In yeast, the signal peptidase complex (SPC) responsible for the cleavage consists of four components: Sec11p, Spc1p, Spc2p and Spc3p (Bohni *et al*, 1988, YaDeau *et al*, 1991), whereas mammalian SPC consists of five components: SPC18, SPC21, SPC22/23, SPC25 and SPC12 (Evans *et al*, 1986). Spc3p and Sec11p are essential proteins and they form the catalytical core of SPC that is functional even in the absence of the other two components (Fang *et al*, 1997, VanValkenburgh *et al*, 1999, Mullins *et al*, 1996). Spc1p and Spc2p are noncatalytic, but they are tightly associated to Sec11p and Spc3p. Spc2p enhances the enzymatic activity of the SPC and it facilitates interactions between different components of the translocation site (Antonin *et al*, 2000). The SPC component SPC21 has been chemically cross-linked to the Sec61β subunit of the Sec61 complex, demonstrating that the SPC is localized at close proximity to the translocon. The cross-linking requires a membrane-bound ribosome, suggesting that SPC is recruited to the translocon upon initiation of translocation (Kalies *et al*, 1998). Similarly, the yeast Spc2 has also been shown to be in complex with the Sec61β homologues Sbh1p and Sbh2p (Antonin *et al*, 2000).

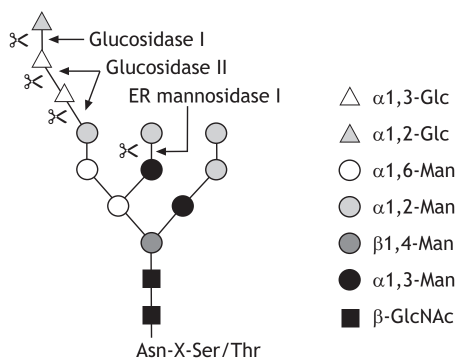

Figure 3. The structure of the N-glycan core. Cleavage sites of the ER-resident trimming glycosidases are indicated.

1.2.2 Glycosylation

Secretory proteins are usually modified by addition of glycan side chains to the polypeptide. Glycan modifications serve for a variety of functions. For instance, they affect folding and stability of a polypeptide, and function as signals for quality control and for targeting. Most commonly, both in yeast and in mammalian cells, glycans are added to amino (N-glycosylation) or hydroxyl groups (O-glycosylation) of specific amino acid residues. Proteins targeted to the cell surface may be modified by glypiation, i.e. addition of an anchoring glycolipid moiety. In addition, mammalian proteins may also be decorated with C-linked mannosyl residues, i.e. a carbohydrate group is linked to the peptide *via* a C-C bond and does not involve any amino acid functional group (reviewed by Spiro, 2002). Next, N- and O-glycosylation, and glypiation are discussed in more detail.

N-Glycosylation

N-glycosylation is intiated in the ER lumen by the transfer of a pre-assembled core oligosaccharide to the newly synthesized polypeptide. In both yeast and mammalian cells, the branched core oligosaccharide consists of two N-acetyl glucosamine (GlcNAc), three glucose (Glc), and nine mannose (Man) residues (**Figure 3**), and it is assembled by sequential addition of glycosyl-residues to a lipid carrier, dolichol pyrophosphate

(Dol-P). In yeast, the assembly is carried out by proteins coded by the *ALG* family members (Burda & Aebi, 1999). The assembly initially takes place on the cytosolic side of the ER membrane and is continued inside the ER lumen. Nucleotide-activated monosaccharide donors, UDP-Glc, GDP-Man and UDP-GlcNac, are first synthesized in the cytoplasm. They may then directly act as donors for the core oligosaccharide assembly, or they may first donate the monosaccharide portion to Dol-P carriers that provide these precursors for the later steps of the core oligosaccharide synthesis (Burda & Aebi, 1999). After the first seven glycosyl-residues are added to the core, the heptameric oligosaccharide is flipped to the ER lumen by the flippase Rft1p (Helenius *et al*, 2002). The biosynthetic steps of the core oligosaccharide formation, and the loci required for each step in yeast are illustrated in **Figure 4**.

The core oligosaccharide is transferred, *en bloc*, from the Dol-P donor to the polypeptide chain by the ER-resident oligosaccharyl transferase complex (OST). The yeast OST has been identified by blue native electrophoresis to be a 240 kDa complex that contains nine components (Knauer & Lehle, 1999a). The OST consists of 3 sub-complexes: the first including Wbp1p, Swp1p and Ost2p, the second Ost1p and Ost5p, and the third, Stt3p, Ost3p and Ost4p and perhaps Ost6p. Ost3p, Ost4p, Ost5p, and the Ost3p homologue Ost6p are coded by non-essential genes. However, yeast cells lacking these genes exhibit defects in N-glycosylation and growth. The genes coding for the other

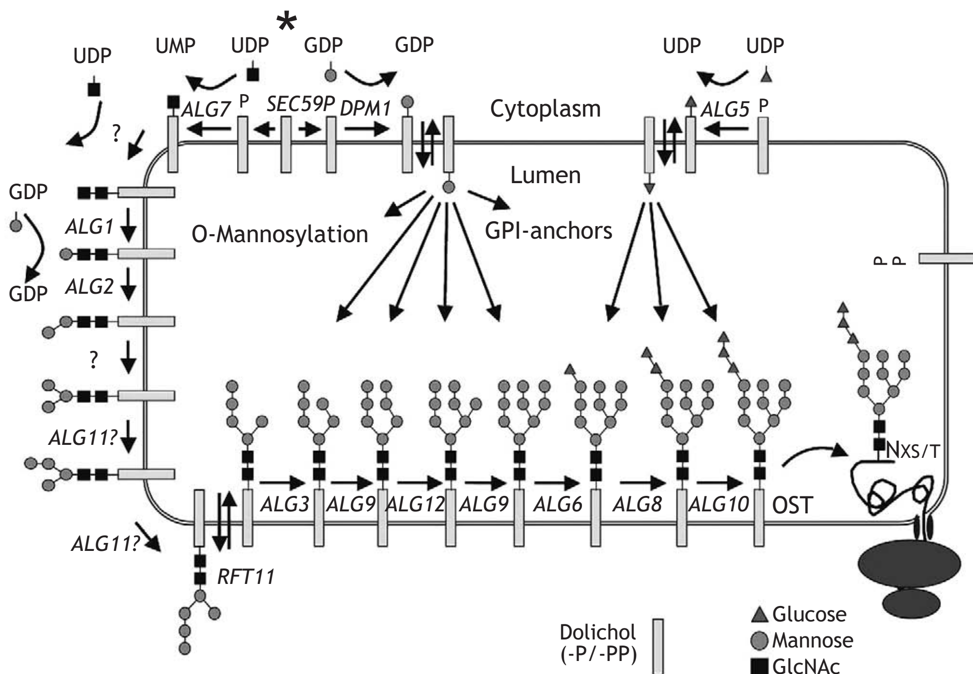

Figure 4. Biosynthesis of the N-glycan core. The yeast loci involved in each step are indicated, where known. Adapted from Helenius and Aebi, 2004. Starting point indicated with an asterisk.

five proteins are essential (reviewed by Yan & Lennarz, 1999). All yeast OST components, except Ost4p and Ost5p, have mammalian homologues (Knauer & Lehle, 1999b).The yeast Stt3p has two homologues in mammalian cells, STT3-A and STT3-B, and the Ost3p/Ost6p are homologous to the mammalian N33 and IAP. These components together with the other mammalian OST components form isoforms of the OST complex that differ in activity, composition and tissue specifity (Kelleher *et al*, 2003). OST transfers a pre-assembeled core oligosaccharide to the amino group of the asparagine residue of the consensus sequence Asp-X-Ser/Thr, where X may be any amino acid except proline. Based on photo-crosslinking of the nascent polypeptide with OST components, the active site of the OST responsible for this action appears to be provided in part, or completely, by the lumenal domain of the STT3 subunit (Nilsson *et al*, 2003).

OST is localised in the vicinity of the Sec61 channel (Görlich *et al*, 1992, Wang & Dobberstein, 1999). The yeast OST subunit Wbp1p has been shown to be in direct contact with Sss1p, a translocon component. This interaction results in anchoring of OST to the translocon, thus enhancing N-glycosylation. The enhanced glycosylation accelerates translocation of proteins containing multiple N-glycosylation sites. Since correct glycosylation is required for the proper folding of a protein, glycosylation and subsequent folding may serve a similar function as BiP in minimizing backwards movement of the polypeptide during translocation (Scheper *et al*, 2003).

As soon as a protein acquires the oligosaccharide precursor, some trimming occurs in the ER (**Figure 3**). First, the terminal α1,2-glucose residue is removed by α-glucosidase I. Then, the remaining two α1,3-glucoses are cleaved off by α-glucosidase II. These steps occur similarly in yeast and mammalian cells. Then, a single mannose residue is cleaved off by α1,2-mannosidase, the product of the *MNS1* gene. In yeast, the α-glucosidases I and II are encoded by *CWH41* and *ROT2*, respectively (reviewed by Herscovics, 1999b). In mammalian cells, there are two mannosidases in the ER, α1,2-mannosidase I and II. Alternatively in some mammalian cells, glucose residues may be removed later on in the Golgi by an endomannosidase that cleaves off the three glucoses and one mannose residue. Mannose trimming is continued in the Golgi in mammalian cells (Herscovics, 1999a). Mannose trimming serves a function in the quality control, at least in mammalian cells, as discussed later.

O-Glycosylation

In yeast, O-glycosylation is known as O-mannosylation, as it refers to the addition of mannoses to the hydroxyl groups of certain serine or threonine residues of the polypeptide. O-mannosylation is initiated in the ER by the protein mannosyl transferase (PMT*)* family proteins Pmt1p-Pmt7p. They transfer a single mannose residue from a Dol-P-Man donor to the O-mannosylation site resulting in an α-D-mannosyl linkage (reviewed by Strahl-Bolsinger *et al*, 1999). The Pmt proteins have different substrate specifities. Analysis of ten different secretory proteins in *pmt1-4* mutants revealed that six of the proteins were mannosylated by Pmt1p or Pmt2p, and the other four by Pmt4p (Gentzsch & Tanner, 1997). Depletion of any one of the *PMT* genes is viable, although cells lacking multiple *PMT* genes exhibit severe defects. Different combinations of *pmt1-4* were assayed by Gentzsch *et al*, and they reported that triple mutants *pmt1/2/4* or *pmt2/3/4* were not viable, and many other double or triple

combinations resulted in abnormal growth (Gentzsch & Tanner, 1996). O- and N-mannosylation compete for the same substrate in the ER. In a study using the covalently linked cell wall protein 5 (Ccw5) as a model, it was shown that the protein could only be N-glycosylated when O-glycosylation of certain sites was abolished, suggesting that O-glycosylation preceeds N-glycosylation in yeast (Ecker *et al*, 2003).

O-linked modifications of mammalian proteins take place exclusively in the Golgi complex. An exception is O-mannosylation that was considered specific for yeast until 1997, when the first reports describing mammalian O-mannosylated proteins were published (Chiba *et al*, 1997, Yuen *et al*, 1997). Little is known about the mammalian O-mannosylation pathway. However, two *PMT* family homologues, POMT1 and POMT2, have been identified as putative mannosylating enzymes (Jurado *et al*, 1999, Willer *et al*, 2002). According to a computer prediction, POMT1 is a putative integral membrane protein localized to the ER (Jurado *et al*, 1999). POMT2 has been reported to be an integral ER membrane protein specifically expressed in sperm (Willer *et al*, 2002). A recent report by Manya *et al* provides the first data suggesting POMT1 and/or POMT2 to have mannosyltransferase activity. This activity required coexpression of POMT1 and POMT2 (Manya *et al*, 2004). Mutations in the *POMT1* gene have been implicated in Walker-Warburg syndrome, a disorder characterized by congenital muscular dystrophy and brain and eye abnormalities (Beltran-Valero de Bernabe *et al*, 2002). These mutations result in defects of O-mannosylation (Akasaka-Manya *et al*, 2004).

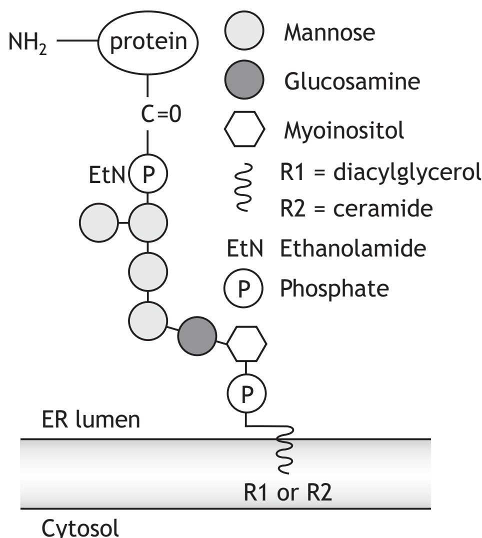

Figure 5. Structure the yeast GPI anchor. Modified from Eisenhaber *et al*, 2003.

Glypiation

Some proteins targeted to the cell surface are modified by glypiation, i.e. addition of a C-terminal glycosyl-phosphatidylinositol (GPI) anchor. Glypiation is carried out by a transamidase enzyme that removes a C-terminal signal peptide from the polypeptide and thereafter transferes a pre-assembled GPI-anchor to the C-terminal amino acid residue exposed by the cleavage. The C-terminal signal peptide consists of 15 - 30 amino acids and it resembles the N-terminal signal sequence required for translocation. The amino acid residue that receives the GPI-anchor is referred to as the ϖ-site. The ϖ-site and the ϖ+2 position have small side chains, but any amino acid except proline or tryptophane may occupy the ϖ+1 site. A hydophilic region of 5 - 7 amino acids and a hydrophobic region of 12 - 20 residues follow the ϖ+2 site (Spiro, 2002). The GPI-anchor consists of three components: a phosphatidyl-inositol moiety that is incorporated into the ER-membrane, a linear tetra-saccharide linker, and a phospho-

ethanolamine group that is linked to the protein. The GPI-anchor structure varies among species. The anchor is composed in a stepwise manner similar to the N-glycan core synthesis. The synthesis starts on the cytosolic side of the ER-membrane and the final steps take place in the ER-lumen, where the anchor is finally transferred to the protein (Suntio *et al,* 2003). The structure of the yeast GPI-anchor is illustrated in **Figure 5**.

1.2.3 Protein folding

Before a protein can exit the ER and proceed along the secretory pathway, it must be properly folded to its native conformation. If folding fails, the protein is captured by the ER quality control machinery and either refolded, or targeted to ER-associated degradation. The ER quality control system is essential, because the organelles, which the proteins are transported to, do not, in most cases, support protein folding. Thus the system ensures that incompletely folded proteins that may be harmful for the cell do not reach their final destinations and are destroyed instead. Cells also use the quality control system to posttranslationally control activation and transport of specific proteins (Ellgaard & Helenius, 2003). The ER provides optimized conditions for folding and for assembly of oligomeric proteins. The ion composition and redox conditions of the ER favour formation of the correct conformation, and the ER resident molecular chaperones and folding enzymes assist the folding by stabilizing partially folded polypeptides during the process of folding or assembly (Gething & Sambrook, 1992). In mammalian cells, folding is initiated cotranslationally and cotranslocationally, i.e. while the polypeptide is still associated with the translocon. Thereafter posttranslational folding takes place, and finally oligomeric proteins are assembled. Chaperones and folding enzymes are involved in all three steps and form part of the quality control system (Ellgaard & Helenius, 2003).

Folding enzymes

Aquiring correct disulfide bonds between cysteine residues is a critical step for a polypeptide to fold to a stable conformation. This is accomplished by the concerted action of the ER-oxidoreductin 1 (Ero1p) and protein disulfide isomerase (PDI) enzymes. The yeast Ero1p is an ER membrane-associated protein, whereas its two mammalian homologues hERO1-Lα and hERO1-Lβ lack the yeast C-terminal membrane anchoring domain that is required for Ero1p to bind to the membrane in yeast. PDI is a soluble protein and it constitutes approximately 2% of the total protein in the ER, being thus the most abundant ER-protein (Tu & Weissman, 2004). Ero1p and PDI function in a protein relay: Ero1p oxidizes PDI *via* disulfide exchange, and PDI then provides the catalysis for disulfide bond formation in proteins. Ero1p is a flavin adenine dinucleotide (FAD) binding protein, and also FAD constitutes a part of the relay. Disulfide bond formation can be reconstituted *in vitro* using these three components (Tu *et al*, 2000). The relay is dependent on oxygen, suggesting that molecular oxygen is the preferred terminal acceptor for the electrons transferred in the reactions. Ero1p thus directly links disulfide bond formation to consumption of oxygen (Tu & Weissman, 2002). PDI that interacts with the protein not only introduces disulfide bonds to the polypeptide, but it also rearranges incorrectly formed disulfides. Formation of disulfide bonds is prone to error early in the folding. Non-native links may be introduced, or native links may be

formed too early inhibiting formation of the correct conformation. Therefore, the PDI isomerase activity is essential. PDI has two active sites containing the sequence CGHC. These active sites may be in an oxidized or a reduced form, i.e. the two cysteines may either form an intermolecular disulfide bridge or exist in non-connected dithiol form, respectively. Depending on the redox state, PDI may either introduce, or remove and rearrange disulfide bonds (Wilkinson & Gilbert, 2004). Formation of disulfide bonds requires that oxidizing conditions are maintained in the ER. The primary redox buffer in the ER is glutathione that may exist in reduced GSH or oxidized GSSG form. The ratio of GSH/GSSG in the ER lumen is 1:1 - 1:3, whereas it is 1:100 in the cytosol (Hwang *et al*, 1992). In yeast, Ero1p oxidizes both glutathione and protein thiol groups and the two substrates compete for the oxidizing machinery. The function of glutathione appears to be to provide net reducing equivalents to the ER thus buffering the ER against transient hyperoxidizing conditions (Cuozzo & Kaiser, 1999). Recent work in mammalian cells shows that cytosolic GSH is the main antagonist of the ER-lumenal Ero1α, and thus limits disulfide bond formation in the ER. Some PDI must be in the reduced form in order to function as the isomerase. Thus, the redox-exchange between the ER and cytosol is important for maintaining conditions that allow isomerization of polypeptides (Molteni *et al*, 2004).

Chaperones

The ER harbours two Hsp70 family member chaperones involved in protein folding, BiP and Lhs1p. BiP is an abundant chaperone of the ER. It is an essential protein that is conserved in eukaryotes and has multiple functions (Gething & Sambrook, 1992, Gething, 1999). In addition to its role in translocation described earlier, BiP is involved in ER quality control (Ellgaard & Helenius, 2003), ER-associated protein degradation (Plemper *et al*, 1997, Brodsky *et al*, 1999), sensing ER stress (Bertolotti *et al*, 2000, Shen *et al*, 2002), and folding and assembly of newly synthesized polypeptides (Gething, 1999). BiP binds to nascent polypeptides transiently, and misfolded proteins more persistantly, but it does not bind to proteins that have acquired native conformation. Thus, by recognizing unfolded proteins and inhibiting their aggregation, BiP maintains the polypeptide in a folding- and oligomerization-competent conformation. BiP has an N-terminal ATPase domain, and a C-terminal substrate biding domain whose affinity for the substrate depends on the nucleotide-binding status of the ATPase domain (Gething, 1999). The polypeptide may be bound and released by BiP, until no more binding motifs for BiP are exposed on the protein. Such motifs are regions of hydrophobic residues that are normally located in the interior of a native protein (Gething, 1999). *In vitro*, BiP binds short peptides of aliphatic residues, that contain a seven residue motif Hy-(W/X)-Hy-X-Hy-X-Hy, where Hy is a bulky hydrophobic residue and X is any amino acid (Flynn *et al*, 1991, Blond-Elguindi *et al*, 1993). The duration of each cycle of binding by BiP depends on the ADP/ATP exchange and ATP hydrolysis rates (Gething, 1999). Lhs1p (also Cer1p, Ssi1p) has overlapping functions with BiP. Similarly to BiP, it acts in folding of nascent polypeptides and refolding and processing of misfolded proteins. It is regulated by the unfolded protein response (UPR) but unlike BiP, not by heat (Craven *et al*, 1996, Saris *et al*, 1997). Recent data indicates that in yeast, Lhs1p and BiP act in coordination. Lhs1p

provides a nucleotide exchange activity resulting in stimulation of the BiP ATPase, and BiP in turn activates the Lhs1p ATPase. The coupling of these two ATPase activities is essential for normal cell function. Such coordinated action might be a mechanism for the two chaperones to bind different regions of the same substrate polypeptide. One chaperone could release the peptide when another binds in its vicinity, thus reducing the possibility that the exposed region would aggregate with other non-native sequences and enhancing native folding (Steel *et al*, 2004).

Addition of glycan moieties to a polypeptide chain affects folding of the polypeptide. Glycoproteins lacking certain glycans may be unable to reach the native conformation and therefore may be targeted to degradation. However, not all glycoproteins are equally dependant on glycans for folding. Some may suffer a less severe loss of secretion efficiency, or remain unaffected. A single glycan may not be essential for folding, but if more glycans are missing, the same glycan may turn out to be important (Helenius & Aebi, 2004). Folding may be affected by the precise location of glycans, or their presence, but not the precise location. Addition of a glycan moiety, a bulky, polar carbohydrate, may directly affect the properties of the polypeptide chain, and thus, folding of the polypeptide. It limits the conformational space accessible for the polypeptide, it may promote and stabilize local folding, and it may affect the solubility of the folding intermediates (Helenius & Aebi, 2004).

In mammalian cells, folding of glycoproteins is assisted by two ER-resident lectin chaperones, calnexin and calreticulin, that compose the calnexin/ calreticulin cycle. Calnexin is a type I transmembrane protein, whereas calreticulin is soluble. Both are monomeric calcium-binding proteins and members of the legume lectin family. After an N-glycan core is added to a polypeptide and the two outermost glucose residues are trimmed by the Glucosidases I and II, the mono-glucosylated core-glycan is sequestered by calnexin and calreticulin. The glycopeptide is thus protected from aggregation as well as premature degradation of the folding intermediate. Binding to calnexin and calreticulin also presents the glycopeptide to ERp57, a PDI homologue that assists disulfide bond formation of glycoproteins. The glycopeptide is released from the lectins by glucosidase II that removes the remaining glucose residue from the core glycan. The protein may then exit the ER, or, if it remains incompletely folded, it will be reglucosylated by the UDP-Glc::glycoprotein transferase (UGGT) that thus provides the folding sensor function in the cycle (Helenius & Aebi, 2004, Schrag *et al*, 2003).

In yeast, a homologue of calnexin, Cln1p, exists. Cln1p is 23% identical to its mammalian homologue, but it lacks the membrane binding domain and calcium binding capacity. Cln1p is not an essential protein (Parlati *et al*, 1995). A yeast homologue for calreticulin has not been found, but other genes coding for homologues of proteins acting in the mammalian calnexin/calreticulin cycle, namely *KRE5* (UGGT), *CWH4* (Glucosidase I) and *CWH41* (Glucosidase II) exist (Meaden *et al*, 1990). A recent study suggests that Cln1p fulfills a similar molecular chaperoning function in the yeast ER as calnexin and calreticulin in the mammalian cells (Xu *et al*, 2004).

Existence of a novel type of yeast chaperones has recently been reported. Shr3p, an integral ER membrane protein, prevents aggregation of amino acid

permeases thus enabling them to fold correctly. The lack of Shr3p results in almost complete aggregation of Gap1p, the general amino acid permease of the plasma membrane, but does not affect folding of other types of proteins (Kota & Ljungdahl, 2005). Similarly, three other Shr3p-like ER membrane proteins, Gsf2p, Pho86p and Chs7p facilitate the proper folding of their substrates, but lack of individual proteins only abolishes ER export of the cognate substrate. These findings suggest that folding of polytopic membrane proteins is mediated by specialized chaperones (Kota & Ljungdahl, 2005).

1.3 Modifications in the Golgi complex

The next compartment in the secretoy pathway is the Golgi complex. Proteins undergo further modifications: glycosyl side chains are added, extended, modified or trimmed, preproteins are proteolytically processed, and proteins are sorted to their final destinations. The Golgi modifications are highly different in yeast and mammalian cells.

1.3.1 Modifications of N-glycans

In yeast, extension of N-glycans is initiated in the *cis*-Golgi compartment by Och1p mannosyltransferase (Romero & Herscovics, 1989). Och1p specifically adds a single mannose to the the 8 mannose-containing core oligosaccharide *via* an α1,6-linkage, and this mannose provides the receptor for addition of further mannoses. Och1p does not participate in the further elongation of the N-glycan (Reason *et al*, 1991, Nakayama *et al*, 1997). The mannose donor for Och1p and for the other Golgi mannosyltransferase-catalysed reactions is GDP-mannose that is synthesized in the cytosol and transferred to the Golgi lumen by the GDP-mannose/GMP antiporter (Hirschberg *et al*, 1998). After Och1p action, the mannose chain may be extended to a large polymannose-type structure, or to a smaller core-type structure (**Figure 6)**. The backbone of the polymannose-type structure is first elongated by two mannan polymerase complexes M-Pol I and M-Pol II. M-Pol I, consisting of Mnn9p and Van1p, extends

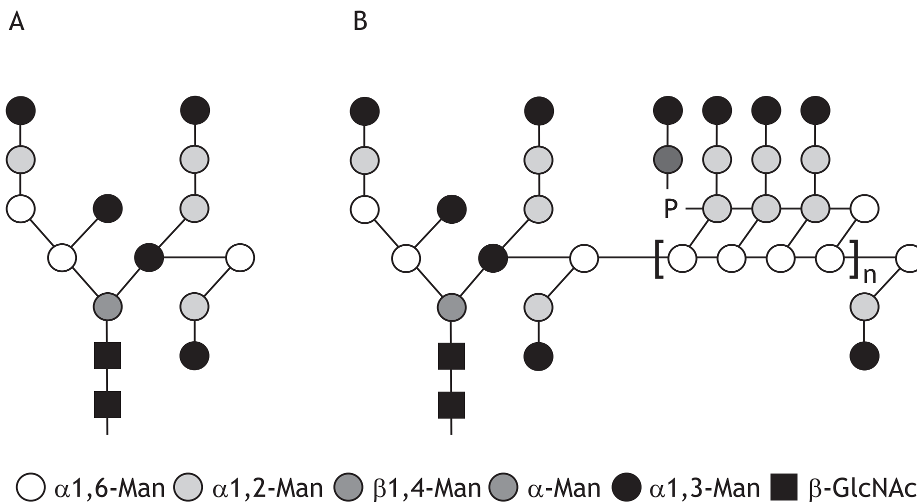

Figure 6. The two N-glycan structures found in yeast. A) The core type. B) The polymannose type. The side chain backbone consists of approximately 50, and the entire structure, of over 200 mannose residues.

the chain of α1,6-linked mannoses by addition of approximately ten mannose moieties. M-Pol II, a complex of Mnn9p, Anp1p, Mnn10p, Mnn11p and Hoc1p, thereafter elongates the chain resulting in a backbone of about fifty α1,6-linked mannoses. The backbone is then branched by Mnn2p and Mnn5p sequentially adding α1,2-linked mannoses, and some branches may receive a phosphomannose added by Mnn4p and Mnn6p acting in co-operation. Finally Mnn1p, localised to the *trans*-Golgi, completes the structure by adding α1,3-linked mannoses (Munro, 2001). Secreted and cell wall glycoproteins typically contain large N-glycans consisting of up to 200 mannose residues. The core-type structures containing 9 - 13 mannoses are found on intracellular proteins, such as the vacuolar carboxypeptidase Y (CPY) (Herscovics & Orlean, 1993). A core type structure is accomplished by the action of an unidentified α1,2-mannosyltransferase and Mnn1p that add α1,2-and α1,3-linked mannoses, respectively (Munro, 2001).

Mammalian N-linked oligosaccharides are highly diverse hybrid and complex structures. Unlike in yeast, the mammalian core oligosaccharide is further trimmed in the Golgi by α1,2-mannosidases IA and IB, and Golgi α-mannosidase II. Action of the α1,2-mannosidases results in a five mannose-containing oligosaccharide that in turn serves as a substrate for N-acetylglucosaminyl transferase I that adds one N-acetylglucosaminyl residue to the core. The α-mannosidase II then removes two more mannoses resulting in a structure that is the substrate for formation of complex N-glycans (Herscovics, 1999a). Elongation of the oligosaccharide is accomplished by N-acetylglucosaminyl-, fucosyl, galactosyl- and sialyltransferases. The resulting structures range from simple bi-antennary structures to highly complicated tetra-antennary structures (Roth, 2002).

1.3.2 Elongation of O-glycans

In yeast, the O-linked mannose residue added in the ER is extended to chains of up to five sugar residues. Similarly to N-mannosylation, GDP-mannose serves as the mannose donor. The second α1,2-linked mannose is transferred by three enzymes, Ktr1p, Ktr3p and Mnt1p. The third α1,2-linked mannose is added by Mnt1p, and the fourth and fifth α1,3-mannoses by Mnn1p, the same enzyme responsible for completing N-mannosylation (reviewed by Strahl-Bolsinger *et al*, 1999).

In mammalian cells, O-linked glycosylation is carried out entirely in the Golgi. The most common O-linked modification is mucin type O-glycosylation, i.e. N-acetylgalactosamine (GalNAc) linked to serine or threonine residues. GalNAc is transferred to the peptide from the UDP-GalNAc donor by a family of UDP-N-acetylgalactos-amine:polypeptide N-acetylgalactos-aminyltransferases (Ten Hagen *et al*, 2003). Up to date, 14 members of this family have been identified (Ten Hagen *et al*, 2003, Wang *et al*, 2003). Addition of further sugar residues to GalNAc results in highly diverse structures. The structural elements of the mucin-type O-glycans are defined as the core, the backbone and the peripheral structure. Eight different core structures have been found so far, that consist of 1 - 2 sugar residues in addition to the GalNAc. The backbone region is formed by large, linear or branched, polylactosamine chains that may consist of up to 20 monosaccharides. The peripheral structures, such as the blood group A and B antigens, are complex, as they contain

a variety of different types of monosaccharides (Hanisch, 2001).

1.3.3 Precursor processing

Some secretory proteins, such as the yeast pheromone α-factor and killer toxin K1 (Julius *et al*, 1984, Bussey, 1991), are initially synthezised as larger precursors that require proteolytic cleavage in order to achieve the mature form. The yeast *trans*-Golgi network (TGN) harbours three enzymes that carry out cleavage of these proproteins, the carboxypeptidase Kex1p, the serine protease Kex2p and the dipeptidyl aminopeptidase Ste13p (Bryant & Boyd, 1993). All three are integral membrane proteins that have a single transmembrane domain. Kex1p and Kex2p are localized to the TGN *via* a C-terminal tail, and Ste13p *via* its N-terminal region. In the absence of these targeting domains, the proteases are transported to the vacuole (Cooper & Bussey, 1992, Wilcox *et al*, 1992, Nothwehr *et al*, 1993). The yeast Kex2p has been shown to process a mammalian pro-hormone precursor pro-opiomelano-cortin, when expressed in mammalian cells (Thomas *et al*, 1988). After this finding, several mammalian proteases homologous to Kex2p have been identified: furin, prohormone convertases (PC) PC1/3, PC2, PC4, PC6A, PC6B, PC7, and paired base amino acid-cleaving enzyme (PACE) 4 (Rockwell & Thorner, 2004). All these enzymes share several common features: they contain an N-terminal signal peptide, a pro-domain that is autocatalytically cleaved off after folding, a catalytic domain and P-domain required for the protease activity. They also harbour C-terminal sorting signals that target the proteases either to the late Golgi compartments of the constitutive secretory pathway, or secretory granules of regulated secretory pathway. Similarly to Kex2p, furin contains a transmembrane domain and a cytosolic tail responsible for its localization to the TGN. These trans-membrane domain-containing proteases form a Kex2/furin subfamily of enzymes that cycle between the TGN and endo-somal compartments (Rockwell & Thorner, 2004). Another subfamily is formed by soluble proteases, such as PC1/3 and PC2 that process proinsulin, prohormones and neuropeptide pre-cursors in neuroendocrine cells. They localise to the regulated secretory path-way and are thus sorted into secretory granules (Rockwell & Thorner, 2004).

2. The molecular mechanisms of vesicular transport between the ER and the Golgi complex

The transport of proteins between the different cellular organelles is accomplished by vesicular transport carriers. Formation of such carriers is driven by different molecular coats. Traffic from the ER to the Golgi complex is mediated by the COPII coat complex (coat protein II), and retrograde traffic from the Golgi to the ER, as well as intra-Golgi transport, is controlled by the COPI coat. In mammalian cells, an ER-Golgi intermediate complex (ERGIC) has been discovered, from where uncoated vesicles migrate to the Golgi complex, and COPI vesicles to the ER. The post-Golgi transport of secretory proteins to their final destinations, as well as recycling from the plasma membrane, is accomplished by clathrin-coated vesicles. The involvement of the different coats in the intracellular transport pathways is illustrated in **Figure 7** (Bonifacino & Glick, 2004). In the following chapters, the molecular

mechanisms in the bi-directional ER-Golgi trafficking will be discussed.

2.1 Anterograde traffic

Two models have been proposed for how cargo is packaged for ER exit. A bulk flow model suggests that non-selected cargo is packaged in transport vesicles at their prevailing concentrations, and due to retrieval of ER- or Golgi-resident proteins, the secretory cargo is concentrated during its passage through the Golgi complex. Thus, lack of retrieval signals would lead to secretion (Wieland *et al*, 1987). A more recent, specific cargo recruitment model suggests that secretory cargo proteins are recruited to COPII-coated vesicles *via* interactions between the cargo and the COPII components, whereas ER-resident proteins are excluded (Kuehn *et al*, 1998). The bulk flow appears to be responsible for ER exit of at least some proteins. Amylase and chymotrypsinogen are soluble proteins secreted by exocrine pancreatic cells. A quantitative immunoelectron microscopic study revealed that these two cargo proteins were found in equal concentrations in the ER, ER exit sites and COPII-coated vesicles, whereas they were concentrated in vesicular-tubular clusters (VTC) prior to arrival at the Golgi. The concentration was suggested to be due to exclusion from COPI-coated retrograde transport vesicles rather than selective recruitment into COPII vesicles (Martinez-Menarguez *et al*, 1999). Another study employing three bulk flow cargo markers, an acyltripeptide, phospholipids and ER-lumenal Green Fluorescent Protein (GFP), showed that in yeast, the soluble secretory glycoprotein pro-α-factor was 20-fold more concentrated than the bulk flow markers in the COPII vesicles. Thus, ER exit in yeast appears to require concentrative and signal-mediated sorting (Malkus *et al*, 2002).

2.1.1 COPII proteins

Export of the secretory proteins from the ER is driven by a set of soluble proteins that compose the COPII coat structure (Barlowe *et al*, 1994). The COPII coat proteins are essential and highly conserved from yeast to mammalian

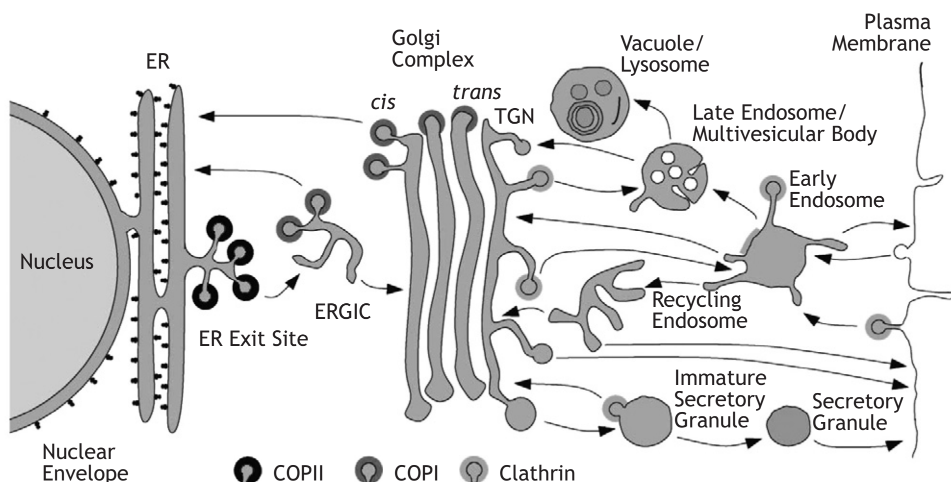

Figure 7. Involvement of different coats in intracellular transport. Modified from Bonifacino and Glick, 2004.

cells. The coat consists of soluble Sec23p/24p and Sec13p/31p complexes, a small GTPase Sar1p and the peripheral ER-associated Sec16p (Kaiser & Schekman, 1990, Pryer *et al*, 1993, Salama *et al*, 1993, Barlowe *et al*, 1993b, Espenshade *et al*, 1995). Formation of the coat also requires the membrane-bound Sec12p (Barlowe & Schekman, 1993). Components of the COPII coat and their functions are listed in **Table 1**.

Sec23p/24p complex
Sec23p/24p complex is a bone-like shaped 195 kDa heterodimer that consists of Sec23p (85 kDa) and Sec24p (104 kDa). The shape of the complex is relatively symmetric, formed of two triangular halves, both containg three globular domains, corresponding to the Sec23p and Sec24p components (Lederkremer *et al*, 2001). Sec23p is a GTPase-activating protein (GAP) specific for Sar1p. This activity appears to be independent of Sec24p (Yoshihisa *et al*, 1993). The yeast Sec23p has two mammalian homologues, hSec23Ap and hSec23Bp. These proteins are 85% identical to each other and 48% to the yeast Sec23p. hSec23Ap is the functional counterpart of Sec23p, but the role of hSec23Bp is unclear (Paccaud *et al*, 1996). Sec24p is the COPII subunit that drives cargo selection into transport vesicles (Miller *et al*, 2002). It has two homologues in yeast, Sfb2p (Iss1p/ Sec24Bp) and Sfb3p (Lst1p/Sec24Cp), which share 56% and 23% similarity with Sec24p, respectively (Kurihara *et al*, 2000, Roberg *et al*, 1999). Both homologues can form a complex with Sec23p and appear to be involved in cargo packaging (Miller *et al*, 2002, Roberg *et al*, 1999, Higashio *et al*, 2000, Peng *et al*, 2000, Shimoni *et al*, 2000). Sec24p also has four mammalian homologues Sec24A - Sec24D. Sec24A/B and Sec24C/D appear to form two subclasses of Sec24, that share 20% identity with each other and with the yeast Sec24p. Epitope-tagged Sec24B, C and D have been shown by immunofluorescence to co-localize in the ER exit sites with the other mammalian COPII components (Tang *et al*, 1999, Pagano *et al*, 1999).

Sec13p/31p complex
Sec13p/31p complex is an asymmetric heterotetramer. It consists of two 33 kDa Sec13p and two 140 kDa Sec31p proteins giving rise for a 380 kDa complex. The complex shape is an elongated and

Yeast protein	***Mammalian homologue***	***Apparent molecular weight***	***Function***
Sec23p	Sec23A, Sec23B	85 kDa	GAP of Sar1p
Sec24p Sfb2p Sfb3p	Sec24A Sec24B Sec24C Sec24D	104 kDa	Cargo selection
Sec13p	Sec13	33 kDa	Outermost layer of the coat Coat polymerization
Sec31p	Sec31A Sec31B	140 kDa	Outermost layer of the coat Coat polymerization
Sar1p	Sar1a Sar1b	21 kDa	GTPase
Sec12p	Sec12	70 kDa	GEF for Sar1p
Sec16p	?	240 kDa	ER exit site scaffold Potentiates coat formation

Table 1. COPII coat components. For references, see text.

flexible side-by-side arrangement, where equivalent regions of two polypeptide chains are associated with each other (Lederkremer *et al*, 2001). Sec13p is a globular protein and it contains seven WD repeats, 40 amino acid repeats that contain a conserved central Trp-Asp motif, that fold as a β-propeller. The N-terminal 400 amino acid sequence of Sec31p also contains WD repeats that may fold as β-propeller (Lederkremer *et al*, 2001). In addition to Sec13p, Sec31p interacts also with Sec23p and Sec24p. Different regions of Sec31p mediate the binding with these components: Sec24p and Sec23 bind to different parts of the central Sec31p region, and Sec13p binds to the N-terminal region, as illustrated in **Figure 8** (Shaywitz *et al*, 1997). Sec13p/31p complex forms the outermost layer of a COPII vesicle (Matsuoka *et al*, 2001). Binding of Sec13p/31p complex initiates the coat polymerization resulting in formation of a vesicle (Barlowe *et al*, 1994). Sec13p has a mammalian homologue mSec13p that is necessary for ER-to-Golgi traffic (Shaywitz *et al*, 1995, Tang *et al*, 1997). Sec31p has two mammalian homologues. Sec31A is ubiquitously and abundantly expressed in different tissues and it is a component of the mammalian COPII, whereas Sec31B is enriched in thymus and testis and its function remains unclear (Tang *et al*, 2000).

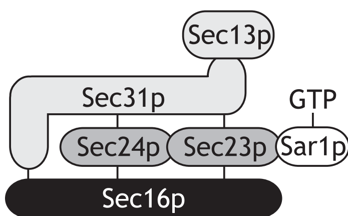

Figure 8. Interactions of the COPII subunits. Sub-complexes are indicated with different shades. Modified from Shaywitz *et al*, 1997.

Sar1p

Sar1p is a 21 kDa GTP-binding protein that belongs to the Ras superfamily (Nakano & Muramatsu, 1989). It has two mammalian homologues, Sar1a and Sar1b, which are implicated in ER-to-Golgi traffic (Kuge *et al*, 1994). The crystal structure of mammalian Sar1p has been studied. As typical for the Ras superfamily proteins, it has a core containing a GDP-binding pocket, and two switch regions, I and II, involved in GDP/GTP exchange and hydrolysis. Sar1p harbours several distinctive features different from the Ras proteins. Sar1p lacks the myristoyl or prenyl group that the Ras GTPases use for membrane association. Instead, the amino-terminus of Sar1p contains a conserved nine amino acid hydrophobic extension, the STAR motif that facilitates membrane recruitment and activation by Sec12p. It also contains an N-terminal amphipathic α1' helix domain that is implicated in interaction with the Sec23p/24p complex (Huang *et al*, 2001). The GTP-bound form of Sar1p is required for formation of ER-derived transport vesicles (Oka & Nakano, 1994). Binding of GTP changes the conformation of Sar1p, resulting in exposure of amino-terminal residues that may be crucial for membrane anchoring. The Sar1p GTPase activity is stimulated by Sec23p that inserts an arginine "finger" in the active site of Sar1p-GTP (Bi *et al*, 2002). The GTPase activity is required for COPII vesicle disassembly (Yoshihisa *et al*, 1993) and according to new data, it is also involved in cargo sorting (Sato & Nakano, 2004, Stephens & Pepperkok, 2004).

Sec12p

Sec12p is the guanine nucleotide exchange factor (GEF) of Sar1p, and it does not promote the Sar1p GTPase activity (Barlowe & Schekman, 1993). It

is a 70 kDa, type II transmembrane protein that has a large cytosolic domain (Nakano *et al*, 1988). The cytosolic domain of Sec12p contains seven WD40 repeats, probably arranged in β-propeller structures, resulting in a compact, seven-bladed β-propeller that comprises most of the cytosolic domain (Chardin & Callebaut, 2002). Sec12p is mostly excluded from the COPII vesicles (Barlowe *et al*, 1994), but some of it is transported to the Golgi complex, perhaps due to its prevailing concentration at the ER, and returned to the ER *via* action of the membrane protein Rer1p (Sato *et al*, 1996). Sec12p has a mammalian homologue, mSec12. As its yeast homologue, mSec12p is the GEF for Sar1p and required for COPII vesicle formation (Weissman *et al*, 2001).

Sec16p

Sec16p is a large, 240 kDa hydrophilic protein. It is peripherally, yet tightly associated with the ER membrane. The C-terminus of Sec16p binds Sec23p (Espenshade *et al*, 1995) and the central region binds Sec24p (see **Figure 8**). Binding of Sec24p is independent of Sec23p, but the presence of Sec23p facilitates it. The C-terminal region of Sec24p binds only Sec16p, whereas the N-terminal region binds both Sec16p and Sec23p (Gimeno *et al*, 1996). Also the Sec31p C-terminus binds Sec16p (Shaywitz *et al*, 1997). Sec16p appears to nucleate the COPII vesicle formation at the ER membrane and stabilize the coat to prevent its premature disassembly (Supek *et al*, 2002). No mammalian homologues of Sec16p have been identified up to date.

2.1.2 Generation of COPII carriers

Generation of COPII coated vesicles occurs at subdomains of the ER membrane, called ER exit sites or transitional ER sites. In mammalian cells (Hammond & Glick, 2000, Stephens *et al*, 2000) and in the yeast *Pichia Pastoris* (Rossanese *et al*, 1999, Bevis *et al*, 2002) the ER exit sites are stable but dynamic structures. The ER exit sites have been studied as sites of Sec23A-YFP or Sec13p localization in mammalian cells and Sec12p localization in *P. Pastoris*. In fluorescent microscopic studies, the ER exit sites appear as a punctate staining throughout the ER network (Hammond & Glick, 2000, Stephens *et al*, 2000, Rossanese *et al*, 1999). In a similar assay in the yeast *S. cerevisiae,* Sec12p was found evenly distributed in the ER, suggesting that the entire ER network may function in ER exit in this yeast (Rossanese *et al*, 1999). ER exit sites form *de novo*, and also fusion of pre-existing ER export sites and fission of larger structures occurs (Bevis *et al*, 2002, Stephens, 2003). The components responsible for formation of ER exit sites are not known. However, existence of a scaffold located at the cytosolic side of the ER, that may include Sec16p, has been postulated (Soderholm *et al*, 2004). Sec16p has been proposed to nucleate the formation of the COPII coat (Supek *et al*, 2002), thus being a putative candidate for this function. Even though ER exit sites have not been visualized in *S. cerevisiae*, it may be that small domains of Sec12p are formed *via* recruitment of Sar1p and Sec16p to the putative COPII budding sites (Bonifacino & Glick, 2004, Supek *et al*, 2002).

The stepwise formation of a COPII vesicle has been elucidated *in vitro*. Vesicle generation is initiated by Sec12p that activates Sar1p. Thereafter the Sec23p/24p and Sec13p/31p complexes are sequentially added to the coat, culminating in pinching off of the vesicle (Barlowe *et al*, 1994, Matsuoka *et al*, 1998). The sequence of COPII coat

assembly is illustrated in **Figure 9A**. The membrane-bound Sec12p recruits Sar1p to the ER membrane and promotes the exchange of GDP to GTP on Sar1p (Barlowe *et al*, 1993a). Using a liposome budding assay, it has recently been shown that the high GEF activity of Sec12p is required to maintain the activated Sar1p at the vesicle budding sites. The GEF activity of Sec12p is approximately 10-fold higher than the GAP activity of Sec23p. A ratio of more than 1 Sec12p per 15 Sec23p/24p components stabilizes the coat (Futai *et al*, 2004). The Sec12p activation changes the conformation of Sar1p resulting in association of Sar1p with the ER membrane, *via* its STAR motif-mediated contact with Sec12p (Huang *et al*, 2001, Bi *et al*, 2002). The conformational change enhances the affinity of Sar1p towards Sec23p. Consequently, Sar1p recruits the Sec23p/24p complex *via* a direct interaction with Sec23p, mediated by the $\alpha 1'$ helix domain of Sar1p (Yoshihisa *et al*, 1993, Huang *et al*, 2001, Bi *et al*, 2002). The resulting complex composed of Sar1p and Sec23p/24p is referred to as the "pre-budding complex". This complex is able to bind cargo proteins but it can not form a vesicle before the Sec13p/31p complex is bound (Aridor *et al*, 1998, Aridor *et al*, 1999). The pre-budding complex surface that faces the ER membrane is positively charged and concaved, which may be the key to explain how membrane bending is achieved during the COPII coat formation (Bi *et al*, 2002). Lifetime of the pre-

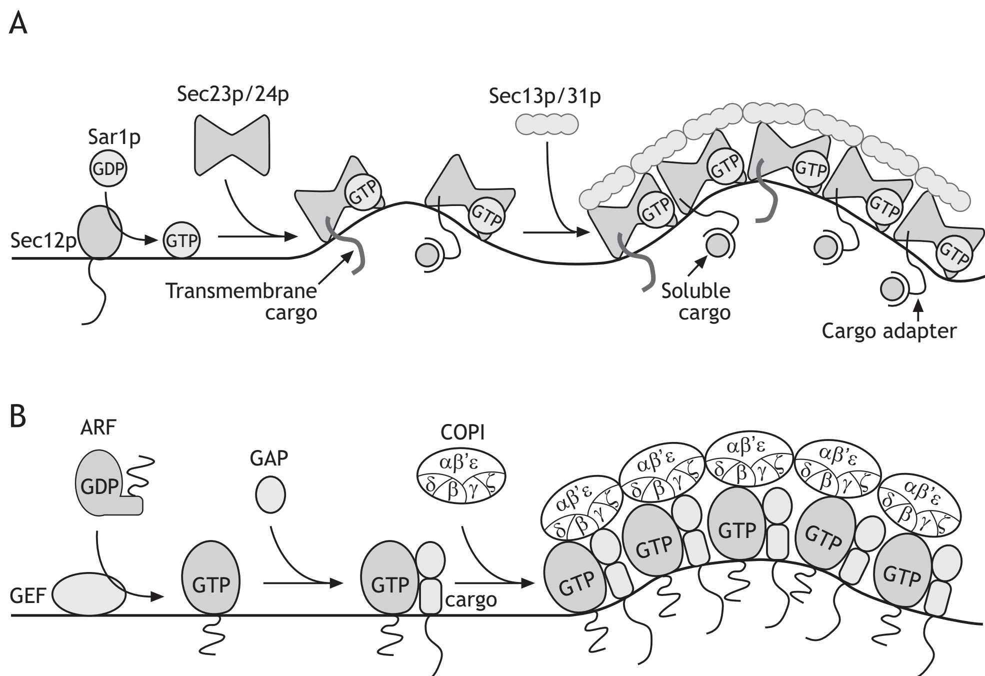

Figure 9. The sequential assembly of the COPII and COPI coats. A) Generation of the COPII coat. Sec12p recruits and activates Sar1p that in turn recruits the Sec23p/24p complex. Cargo is packaged at this stage. Transmembrane cargo directly interacts with the COPII coat, and soluble cargo, *via* adapter proteins. Finally, the Sec13p/31p complex binds to the complex resulting in completion of the coat. B) Generation of the COPI coat. ARFGEF recruits and activates ARF. ARF associates with ARFGAP and cargo proteins, forming a priming complex that recruits COPI. COPI polymerization leads to vesicle formation.

budding complexes appears to be about 30 s. This short time may allow formation and lateral diffusion of cargo-containing complexes that form complete coats through the bridging action of Sec13p/ Sec31p. Binding of Sec13p/31p results in rapid completion of the coat. When the Sec23p/24p and Sec13p/31p complexes are incubated with liposomes pre-loaded with Sar1p-GTP, the COPII coat forms in less than 1 s. Binding of Sec13p/31p enhances the GAP activity of Sec23p 10-fold, which results in fast disassembly of the coat. The disassembly occurs in less than 10 s. Completion of the COPII coat thus accelerates its own disassembly (Antonny *et al*, 2001). A possible model for how vesicles can be formed in the presence of the destabilizing GAP activity has been proposed based on the GEF activity of Sec12p. When COPII subunits cluster on the surface of a growing bud, the ratio of Sec12p to Sec23p diminishes, resulting in destabilization of the coat. However, at the boundary of the ER and the bud, the Sec12p/Sec23p ratio is higher. Sec12p may thus stabilize the boundary region by charging new Sar1p-GTP molecules. The COPII subunits remain transiently bound to the completed vesicles after GTP hydrolysis and dissociation of Sar1p. Thus, the vesicle remains relatively intact until it pinches off from the ER membrane (Futai *et al*, 2004). Coating and disassembly coordinated by Sec12p is illustrated in **Figure 10**.

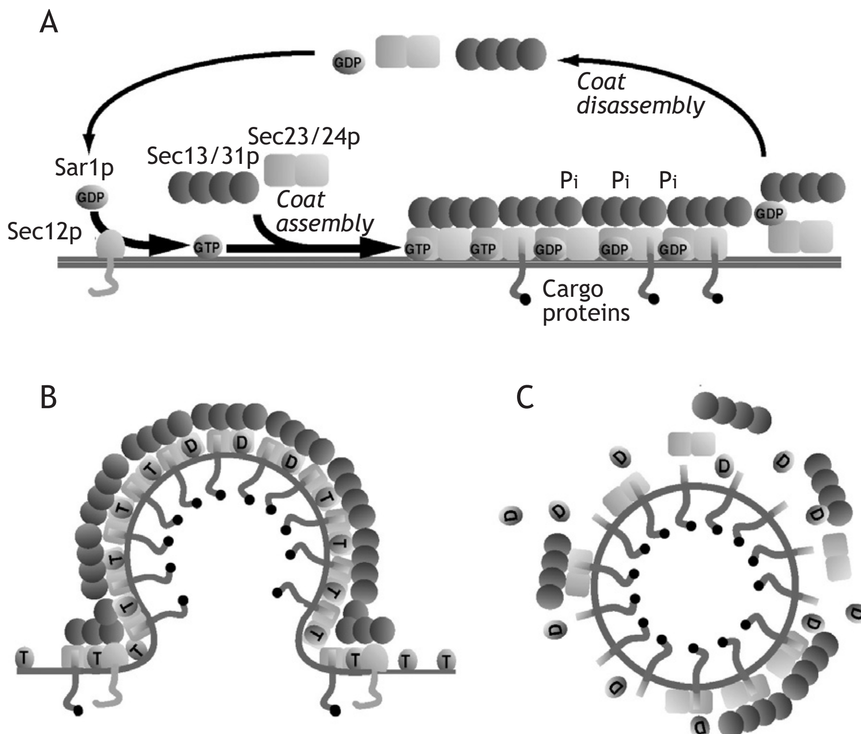

Figure 10. Sec12p-coordinated vesicle budding and disassembly. A) Involvement of Sec12p in assembly and disassembly of the COPII coat. B) Vesicle budding is promoted when more than one Sec12p per 15 Sec23p/24p complexes is present. C) Disassembly of the coat after budding is promoted by more than 15 Sec23p/24p complexes per Sec12p. Modified from Futai *et al*, 2004. D and T symbolize the G<u>D</u>P or G<u>T</u>P binding state of Sar1p.

Sar1p-driven generation of COPII vesicles *in vitro* is greatly enhanced when a high concentration of acidic phospholipids is present on synthetic liposomes (Matsuoka *et al*, 1998). It is likely that also *in vivo*, the physiological conditions have an important effect on coat the formation. In mammalian cells, COPII coat generation requires activation of phospholipase D (PLD) by Sar1p. PLD catalyzes formation of phosphatidic acid, which may result in a transient Sar1p-induced increase of concentration of acidic lipids at the ER exit sites. PLD activation does not alone drive COPII formation, but it supports COPII mediated ER export *in vivo* (Pathre *et al*, 2003).

2.1.3 Cargo sorting

Recruitment of cargo proteins to the COPII vesicles is carried out by the Sec23p/24p/Sar1p-GTP pre-budding complex. Most transmembrane cargo proteins appear to bind directly to the COPII components (Kuehn *et al*, 1998, Aridor *et al*, 1998) whereas lumenal cargo must bind to COPII indirectly *via* transmembrane adapter proteins (Kuehn *et al*, 1998, Appenzeller *et al*, 1999, Muniz *et al*, 2000). *In vitro*, pre-budding complexes have been isolated together with cargo, cargo adapter, and SNARE proteins. In these complexes, the transmembrane proteins Hip1p, Emp24p, Sec22p and Bet1p were found to be specifically bound to the COPII components. A soluble cargo protein, pro-α-factor was also included, possibly *via* a transmembrane adapter protein, but the ER-resident proteins Sec61p, BiP and Shr3p were absent from the complexes (Kuehn *et al*, 1998). Similarly, the transmembrane cargo proteins vesicular stomatitis virus protein VSV-G (Aridor *et al*, 1998) and yeast Sys1p (Votsmeier & Gallwitz, 2001) have been isolated associated with the pre-budding complex. Some transmembrane cargo requires an adapter protein for efficient ER exit. Axl2p, a transmembrane protein necessary for polarized growth in yeast, is not secreted to the cell surface in *Δerv14* cells. *In vitro*, Erv14p binds both Axl2p and COPII, but not the ER-resident BiP or Sec61p. Thus, Erv14p appears to select Axl2p for ER exit (Powers & Barlowe, 1998, Powers & Barlowe, 2002).

Lumenal cargo makes contact with the cytosolic COPII coat *via* membrane spanning adapters. The lectin ERGIC-53, a type I membrane protein that recycles between the ER and ERGIC in mammalian cells, appears to operate as a receptor for soluble glycoproteins. A crosslinking assay has been applied to identify a cathepsin-Z related 40 kDa protein as a ligand for ERGIC-53 (Appenzeller *et al*, 1999). Another lumenal cargo protein, the GPI-anchored Gas1p, requires Emp24p/Erv25p complex for efficient packaging into COPII vesicles in yeast. In an *in vitro* assay, vesicles derived from *emp24* mutant ER membranes contained over 70% less Gas1p than those generated from wild type membranes. Both Emp24p and Erv24p could also be crosslinked to Gas1p in ER-derived vesicles, indicating that the complex was directly involved in Gas1p packaging (Muñiz *et al*, 2000). The soluble yeast pro-α-factor requires Erv29p for ER exit. *In vitro*, Erv29p directly binds pro-α-factor, and vesicles derived from ER membranes lacking Erv29p contain little of this cargo. *In vivo*, according to a pulse-chase analysis, lack of Erv29p results in accumulation of pro-α-factor in the ER (Belden & Barlowe, 2001a).

ER exit motifs on cargo proteins

Binding of cargo proteins to the COPII coat occurs *via* sorting motifs. The transmembrane cargo proteins exhibit

sorting motifs on their cytosolic domains and thus may directly interact with the COPII subunits (Bonifacino & Glick, 2004). Some indications of sorting motifs on soluble cargo have also been described recently (Otte & Barlowe, 2004). Most existing data, however, describes ER exit motifs on transmembrane proteins, and is therefore the focus here. The sorting signals appear diverse: di-acidic, di-basic and hydrophobic motifs have been identified (Bonifacino & Glick, 2004). Examples of ER export signals of transmembrane cargo proteins characterized are presented in **Table 2**.

The di-acidic motif was first identified as the signal required for efficient ER exit of vesicular stomatitis visrus glycoprotein (VSV-G) in mammalian cells. The motif consists of the [DE]X[DE] sequence where X may be any amino acid (Nishimura & Balch, 1997). Also the amino acids surrounding the DXE motif contribute to efficient ER export of VSV-G (Sevier *et al*, 2000). Several other proteins have been found to carry a similar signal. ER exit of two mammalian rectifying potassium channel proteins, Kir2.1 and Kir1.1 depends on signals containing EXE and EXD di-acidic motifs, respectively. The ER exit signal of Kir2.1 is transferable: fusion of the Kir2.1 signal to Kir3.1 that would normally remain in the ER results in ER export of this protein (Ma *et al*, 2001). The di-acidic motif is also used by the cystic fibrosis transmembrane conductance regulator protein CFTR, in mammalian cells (Wang *et al*, 2004). The yeast Sys1p contains a DXE consensus sequence that efficiently binds to the Sec23p/24p complex, thus enhancing ER exit of Sys1p (Votsmeier & Gallwitz, 2001). Another yeast protein Gap1p depends on a C-terminal DXD, a motif highly conserved among yeast amino acid permeases, for recruitment into COPII

	Signal	***Protein***	***Reference***
Di-acidic motifs	DLE	Sys1p	Barlowe, 2003
	DID	Gap1p	Barlowe, 2003
	YTDIE	VSV-G	Barlowe, 2003
	FCYENE	Kir2.1	Barlowe, 2003
	VLSEVDETD	Kir1.1	Bonifacino & Glick, 2004
	YKDAD	CFTR	Wang *et al*, 2004
Hydrophobic motifs	FF	Prm8p	Bonifacino & Glick, 2004,
		ERGIC53	Belden & Barlowe, 2001b,
		p24δ1	Barlowe, 2003
		Erv25p	
		Emp24p	
	FY	Erv46p	Barlowe, 2003
	IL	Erv41p	Barlowe, 2003
	YYMFRINQDIKKVKLL	Emp46p	Sato & Nakano, 2002
	YYTFRIRQEIIKTKLL	Emp47p	Sato & Nakano, 2002
	FNADFQKAF	DRD1	Bermak *et al*, 2001
	FNSHLLPRPL	V3	Robert *et al*, 2004
	V	Kit1	Paulhe *et al*, 2004,
		PRA1	Liang *et al*, 2004
Di-basic motif	RR	GalT2	Bonifacino & Glick, 2004
		GalNAcT	
Other motifs	HLFY	NMDA	Hawkins *et al*, 2004
	YNNSNPF, LMLME	Sed5p	Mossessova *et al*, 2003
	LASLE	Bet1p	Mossessova *et al*, 2003

Table 2. Examples of characterized ER export sorting signals of transmembrane cargo proteins. Underlining indicates known key residues.

vesicles (Malkus *et al*, 2002). Di-basic ER exit motifs have been found in mammalian glycosyltransferases, type II transmembrane proteins that localize to the Golgi complex. The di-basic motif [RK]X[RK] is located in the cytoplasmic tail, in the vicinity of the transmembrane region. Substitution of the C-terminal domain of an ER resident type II membrane protein lip33 with the di-basic motif containing C-terminus of GalT2 resulted in ER exit of this protein. This suggests, that the motif actively mediates ER exit (Giraudo & Maccioni, 2003).

A number of hydrophobic ER exit motifs have been identified. In mammalian cells, ER exit of the cargo receptor ERGIC-53 requires a C-terminal di-phenylalanine (FF) motif. *In vitro,* the FF motif directly binds Sec23p but not Sec13p, indicating that it mediates packaging of ERGIC-53 into the pre-budding complex (Kappeler *et al*, 1997). Also the recruitment of mammalian p24 family protein to COPII vesicles depends on binding to Sec23p *via* an FF motif (Dominguez *et al*, 1998). Similarly, the yeast p24 proteins Emp24p and Erv25p depend on an FF motif for COPII recruitment. However, unlike the mammalian p24 proteins, they appear to bind to Sec13p/31p rather than the pre-budding complex *in vitro* (Belden & Barlowe, 2001b). In a previous study, Emp25p was reported to bind to the COPII coat *via* a motif consisting a leucine and valine residue (LV motif). Replacing LV with other hydrophobic amino acids resulted in reduction of ER exit rate *in vivo,* suggesting that both amino acids of the motif were specifically required (Nakamura *et al*, 1998). However, since *in vitro* mutations of the LV motif did not alter binding to Sec13p/31p, the conclusion remains somewhat controversial (Belden & Barlowe, 2001b). The yeast homologue of ERGIC-53, the putative secretory cargo receptor Emp47p, and its homologue Emp46p use two C-terminal motifs for ER exit. Both contain an LL motif and a tyrosine containing motif that mediate recruitment into COPII vesicles (Sato & Nakano, 2002). Packaging of the yeast Erv41p/46p complex, which cycles between the ER and Golgi, into the COPII vesicles, requires two C-terminal di-hydrophobic signals. Erv41p and Erv46p harbour IL and FY motifs, respectively. The lack of either of the motifs, or exchanging the motifs between the protein components results in ER retention of the complex. Thus, both signals must be presented, and in a specific orientation to mediate ER exit of the complex (Otte & Barlowe, 2002). G protein-coupled receptor (GPCR) family protein dopamine receptor D1 (DRD1) contains a C-terminal $F(X)_3F(X)_3F$ motif that mediates secretion of DRD1 (Bermak *et al*, 2001). Another GPCR protein, human pituitary vasopressin V1b/V3 receptor (V3), contains two C-terminal DXE motifs. However, mutation of both of these does not affect its ER exit. Instead, mutations of a C-terminal di-leucine motif or of certain surrounding hydrophobic residues completely abolishes secretion, indicating that the $FN(X)_2LL(X)_3L$ motif is responsible for the ER exit of the V3 receptor (Robert *et al*, 2005). Most ER export signals appear to contain two or three critical amino acid residues. However, existence of a signal dependent on a single critical amino acid residue has recently been reported. The stem cell factor Kit1 and the prenylated Rab acceptor 1 both have a C-terminal valine residue required for ER exit (Paulhe *et al*, 2004, Liang *et al*, 2004).

Cargo selection by the Sec24 family proteins

The diversity of ER exit motifs presented in the previous chapter helps to explain how the COPII coat is able to specifically recruit selected cargo into ER exit vesicles. However, in order to interact with such a variety of motifs, COPII coat should have a variety of means to bind to all these signals. Indeed, a family of cargo-binding COPII subunits exists, and multiple cargo binding sites are found in these proteins.

Since most of the ER exit signals appear to bind to Sec24p, cargo selection into COPII vesicles has been proposed to be driven by Sec24p (Miller *et al*, 2002, Miller *et al*, 2003, Mossessova *et al*, 2003). In crystallographic (Mossessova *et al*, 2003), biochemical and genetic (Miller *et al*, 2003) analysis, Sec24p has been shown to contain multiple independent cargo binding sites, which allows capture of a variety of cargo into COPII vesicles. Three such sites have been characterized. The A site binds the YNNSNPF motif present in the yeast SNARE [soluble NSF (N-ethylmaleidimide-sensitive factor) protein attachment protein receptor] Sed5p, the B site binds LXX-M/L-E the motif of Sed5p and another SNARE, Bet1p, and the third, spatially distinct site binds yet another SNARE, Sec22p (Miller *et al*, 2003, Mossessova *et al*, 2003). In addition to SNAREs, other cargo proteins also bind to the B site. A mutant Sec24p with an amino acid substitition in the B site was used in an *in vitro* budding assay to study recruitment of different cargo. In this assay, COPII vesicles were generated with similar efficiency as in the case of a wild type Sec24p. However, cargo recruitment was altered. Some cargo (pro-α-factor, chitin synthase Chs3p, amino acid permeases Hip1p and Can1p, Erv41/46 complex), was packaged equally or even more efficiently with the mutant Sec24p, whereas recruitment of other cargo was impaired (Sed5p, Bet1p, Sys1p, Emp47p, the SNARE Bos1p, Sec22p, and the p24 proteins Erp1p, Erp2p and Emp24). The cargo proteins, the packaging of which was impaired, appear not to share a common ER exit motif (see **Table 2** for the known motifs). Also, cargo with similar motifs was not packaged with same efficiency suggesting that the motifs are recognized in a different manner by the COPII coat (Miller *et al*, 2003).

On synthetic liposomes, the Sec24 homologue Sfb3p can replace Sec24p in the COPII coat and generate vesicles. Such vesicles, however, only contain a limited set of cargo molecules. They lack SNARE proteins and therefore are unable fuse with the target membrane (Miller *et al*, 2002). However, weak binding of Sed5p to Sfb3p has been reported (Peng *et al*, 2000). Sfb3p contains a cargo-binding site equivalent to the B-site of the Sec24p. Despite a high similarity of amino acids between this site and the B-site, the SNARE proteins do not bind to Sfb3p. *In vitro*, Sec23p/Sfb3p is able to package Erp1p, Emp24p, Erv46p and pro-α-factor into COPII vesicles, albeit not as efficiently as Sec23p/24p. As a result of mutating the Sfb3p cargo-binding site, Erp1p and Emp24p were no longer recruited to the vesicles, whereas Erv46p and pro-α-factor were still packaged. *In vivo*, Sfb3p, and also Sfb2p, is able to partially compensate for defects in the Sec24p B-site, suggesting that the site is highly conserved (Miller *et al*, 2003). Sfb3p appears to have a role in packaging specific cargo in cooperation with Sec24p. ER export of the plasma membrane ATPase Pma1p, and another plasma membrane protein, Gas1p, is impaired in *Δsfb3* cells *in vivo* (Roberg *et al*, 1999, Peng *et al*, 2000). *In vitro*,

packaging of Pma1p into COPII vesicles has been best reconstructed using a mixture of Sec23p/24p and Sec23p/Sfb3p complexes, and poorly with only one of them, suggesting that *in vivo* packaging of Pma1p occurs in cooperation of Sec24p and Sfb3p (Shimoni *et al*, 2000).

Also Sfb2p appears to have role in cargo selection. Sec23p/Sfb2p complex forms *in vivo* (Kurihara *et al*, 2000, Peng *et al*, 2000). *In vitro*, it drives the formation of COPII vesicles that contain pro-α-factor and, unlike the Sec23p/ Sfb3p vesicles, also SNARE proteins Sec22p and Bet1p. Overexpression of Sfb2p, but not of Sfb3p, is able to suppress the lethality of *Δsec24* mutation as well as the temperature sensitive phenotype of *sec24-11*, indicating that Sfb2p and Sec24p share some fuctional redundancy (Kurihara *et al*, 2000, Higashio *et al*, 2000, Peng *et al*, 2000).

Involvement of Sar1p in cargo selection
Sar1p has also been proposed to participate in cargo selection (Sato & Nakano, 2004, Aridor *et al*, 2001). The yeast SNAREs Bet1p and Bos1p directly bind Sar1p independently of Sec23p/24p and GTP, *in vitro* (Springer & Schekman, 1998). *In vitro*, the mammalian Sar1 has been found to co-precipitate with the VSV-G cargo protein in the absence of Sec23/24 complex. This interaction was direct and dependent on the cytosolic DXE motif of VSV-G, but independent of GTP. In semi-intact normal rat kidney (NRK) cells, Sar1-GTP, but not Sar1-GDP, initiated formation of tubular ER-dervived domains containing VSV-G and Sec22, but not the ER-resident BiP. Thus, Sar1 appears to initiate formation of ER exit sites and recruitment of cargo (Aridor *et al*, 2001). Another ER exit motif that has been suggested to interact with Sar1 is the di-basic sorting signal of the mammalian glycosyltransferases. Like VSV-G, the galactosyltransferase Gal2T, could be cross-linked to Sar1, independent of GTP (Giraudo & Maccioni, 2003). Sar1p appears also to function in exclusion of improper cargo proteins from the COPII carriers. A recent report shows that a pre-budding complex containing assembled Emp47p oligomer as cargo has a higher rate of COPII coat polymerization than a complex containing unassembled Emp47p monomer, in yeast. The GTPase activity of Sar1p seems to counteract the coat polymerization and thus to impede ER exit of unassembled cargo proteins (Sato & Nakano, 2004). In another recent study, a set of GFP-fusion proteins representing soluble, GPI-anchored and trans-membrane cargo proteins were expressed in mammalian cells. When a GTP-restricted mutant of Sar1p was expressed in these cells, the GPI-anchored GFP, but not the other cargo proteins examined, was observed to accumulate in the ER. GTP hydrolysis by Sar1p may thus be involved in cargo sorting (Stephens & Pepperkok, 2004).

Other factors involved in sorting
GPI-anchored proteins exit the ER in distinct vesicles from the other cargo, in yeast (Muniz *et al*, 2001). Sorting of these proteins requires a set of components required for membrane fusion, the tethering factors Uso1p, Sec34p, Sec35p and Ypt1p (Morsomme & Riezman, 2002), and the SNARE proteins Bos1p, Bet1p and Sec22p (Morsomme *et al*, 2003). Similarly, also in mammalian cells, distinct transport carriers exist. Procollagen is segregated from other cargo at COPII-covered ER exit sites, and transported in distinct carriers. The sorting is dependent on COPI (Stephens & Pepperkok, 2002).

2.2 Retrograde traffic

The COPII vesicle mediated ER-to-Golgi traffic exports cargo receptor proteins and SNAREs from the ER. Also some ER-resident proteins may escape, and the ER membrane is consumed in generating vesicles. COPI coat functions in retrieval of the ER-resident proteins, and recycling cargo adapter proteins and SNAREs back to the ER, and in maintaining a membrane balance between the two organelles.

2.2.1 COPI proteins

Similarly to COPII, COPI components are higly conserved from mammals to yeast. The COPI coat consists of the COPI coatomer, a complex composed of seven different subunits, and the small Rab family GTPase ADP-ribosylation factor 1 (ARF1). In addition, similarly to COPII, two factors that modulate COPI assembly and disassembly are required, the GTP exchange factor ARFGEF and the GTPase-activating protein ARFGAP (Duden, 2003, Gaynor *et al*, 1998).

COPI coatomer is a soluble 700 kDa complex that consists of α-, β-, β′-, γ-, δ-, ε- and ζ-COP subunits, in mammalian cells (see **Table 3** for yeast homologues). The complex self-assembles in the cytoplasm, and binds to membranes as a stable unit. Under certain conditions, the coatomer dissociates into smaller units, α/β′/ε trimer (B-subunit) and β/δ, and γ/ζ dimers (F-subunit), apparently functionally distinct sub-units of the coatomer. Such subcomplexes are not, however, found in cell extracts. Instead, the coatomer appears to exist solely as a completely formed complex *in vivo*. Only ζ-COP has been found as a monomer in cells (Lowe & Kreis, 1998). The structure of COPI has not yet been unraveled to the same detail as that of COPII. However, several features have been discovered. α- and β′-COP contain conserved, N-terminal WD40 domains that comprise six and five WD40 repeats, respectively (Duden *et al*, 1994). The WD40 domain of either subunit may be deleted in yeast, but deletion in both subunits is lethal

Mammalian COPI component	*Yeast protein*	*Apparent molecular weight*	*Function*
AFRGAP	Glo3p Gcs1p	45 kDa	GAP for ARF
ARFGEF	Gea1p Gea2p	160 kDa	GEF for ARF
ARF1	yARF1 yARF2 yARF3 yARL1	21 kDa	GTPase COPI recruitment
α-COP	Ret1p	150 kDa	Coatomer subunit Dilysine motif binding
β-COP	Sec26p	110 kDa	Coatomer subunit
β'-COP	Sec27p	100 kDa	Coatomer subunit Dilysine motif binding
γ-COP	Sec21p	103 kDa	Coatomer subunit Cargo recruitment
δ-COP	Ret2p	60 kDa	Coatomer subunit WXXXW motif binding
ε-COP	Sec28p	35 kDa	Coatomer subunit Stabilizes α-COP
ζ-COP	Ret3p	21 kDa	Coatomer subunit

Table 3. COPI coat components. For references, see text.

(Eugster *et al*, 2004). Unlike all other COPI subunits, the ε-COP is non-essential for growth in yeast. It however has a function in stabilizing α-COP at elevated temperatures (Duden *et al*, 1998). Binding of COPI to the Golgi membrane appears to be mediated by the β/δ subunit of the coatomer (Pavel *et al*, 1998). Some of the COP subunits are structurally related to clathrin coat components (Eugster *et al*, 2000, Hoffman *et al*, 2003, Watson *et al*, 2004). The clathrin coat consists of adapter complexes and clathrin molecules. The membrane-proximal layer of the coat is composed of the adaptor complexes (AP1 or AP2), each of which contains four subunits. The surface is covered by a cage-like structure composed of polymerized clathrin triskelions (Kirchhausen, 2000). β-, δ-, γ- and ζ-COP, are structurally related to the adaptor proteins. The N-terminal regions of β- and γ-COP are homologous to the α- and β-subunits of the adaptor AP2, and crystal structure analysis has revealed that the C-terminal region of γ-COP is similar to the α- and β-AP2 appendage domains (Eugster *et al*, 2000, Hoffman *et al*, 2003, Watson *et al*, 2004). The δ- and ζ-COP are homologous with two other subunits of AP2, μ, and σ, respectively (Cosson *et al*, 1996). Based on these similarities it appears that the B-COP may be functionally similar to clathrin, thus forming the surface layer of the coat, and F-COP, to adaptin, comprising the coat layer proximal to the membrane.

ARF1, or yARF1/2/3 in yeast (**Table 3**), is a 21 kDa protein functionally similar to Sar1p. Unlike Sar1p, ARF1 is myristoylated at its N-terminus, which allows its membrane association (Kirchhausen, 2000). In the GDP-bound form, ARF1 is soluble. Binding of GTP results in a conformational change in the N-terminus of ARF1, resulting in exposure of several hydrophobic residues for membrane association (Jackson & Casanova, 2000). Unlike Sec12p, the GEF involved in COPII vesicle formation, ARFGEFs are soluble proteins. ARFGEFs contain a characteristic motif, a 200 amino acid region with strong homology to the yeast Sec7p, the Sec7 domain, which is essential for the ARFGEF activity. It consists of ten α-helices arranged as an elongated cylinder with a hydrophobic groove and a hydrophilic loop in the central region that comprises the ARF binding site (Jackson & Casanova, 2000). There are two ARFGEFs in yeast, Gea1p and Gea2p that provide overlapping functions in COPI traffic (Spang *et al*, 2001). In yeast also two functionally overlapping ARFGAPs exist, Glo3p and Gcs1p. ARFGAPs do not form a structural part of the coat, unlike Sec23p, the functional homologue of ARFGAP in COPII coat. ARFGAP contains a GAP domain, a 70-residue region with a zinc-finger motif and a conserved arginine residue, which is essential for the ARFGAP activity (Donaldson & Jackson, 2000).

2.2.2 Generation of COPI carriers

Similarly to COPII, COPI coat assembly is initiated by the action of a GEF protein. AFRGEF recruits ARF1 to the *cis*-Golgi membrane and promotes the GDP to GTP exchange on AFR1, resulting in its membrane association. ARF1 binds ARFGAP and a transmembrane receptor protein, and this priming complex then recruits the coatomer, resulting in vesicle formation. The initial formation of the priming complex has been suggested to regulate the coat assembly and cargo recruitment (Kirchhausen, 2000, Spang, 2002). The stepwise assembly of the COPI coat is illustrated in **Figure 9B**. Generation of COPI vesicles has been reproduced *in vitro*. In a liposome

budding assay, ARF, GTP, and coatomer appered to be the minimum requirements for COPI vesicle formation in the presence of acidic phospholipids (Spang *et al*, 1998). In another study, COPI vesicle budding was succesfully reconstituted from neutral liposomes in the presence of either cytoplasmic domains of p23, an abundant p24 family protein of mammalian Golgi membranes, or transmembrane cargo proteins (Bremser *et al*, 1999). In a similar assay, role of ARFGAP was studied. A full cycle of COPI coat formation and disassembly was reproduced using coatomer, ARF-GTP and p23. ARFGAP was not required for vesicle generation in this study, but it was necessary for the disassembly of the coat (Reinhard *et al*, 2003). In another study, however, ARFGAP appeared to be a necessary component for COPI vesicle budding from mammalian Golgi membranes. It was also shown to bind dilysine motifs of cargo proteins and thus probably to form a part of the COPI coat (Yang *et al*, 2002). A recent report demonstrated that one of the yeast ARFGAPs, Glo3p is a component of the COPI coat, but the other, Gcs1p is not. Glo3p was found on COPI vesicles generated *in vitro*, and it also interacted with COPI *in vivo*. Mutant Glo3p with no GAP activity was shown to prevent *in vitro* generation of COPI vesicles, suggesting that the ARFGAP may indeed be required for forming the COPI coat (Lewis *et al*, 2004).

p23 appears to function as the ARF1-GDP receptor at the *cis*-Golgi membrane. In a cross-linking experiment, the cytosolic domain of p23 was found to interact with the GDP-bound ARF1, but not with the GTP-bound form. Upon GTP exchange, p23 dissociated from ARF1 (Gommel *et al*, 2001). Thus, the role of p23 may be to recruit ARF1 to the sites active in COPI vesicle generation. p23 appears also to have a role in driving polymerization of the coat. Interaction of the cytosolic domain of p23 with the coatomer resulted in conformational change in the coatomer and subsequent deformation of membrane and coat polymerization, *in vitro*. Similarly, change of conformation was also observed on authentic isolated COPI vesicles (Reinhard *et al*, 1999). The ARFGAP promoted COPI disassembly appears to be coupled to membrane curvature. In time-resolved assays studying COPI dynamics on liposomes, it was found that the rate of GTP hydrolysis and COPI disassembly increased over two-fold, when the liposome size approached the size of a COPI vesicle. The authors proposed a model where polymerization of the coat by COPI increases membrane curvature that results in penetration of the ARFGAP closer to the membrane and in promoting ARF1 GTPase activity. The negative membrane curvature of the peripheral regions would protect the bud neck from ARFGAP until the coat was completely formed, thus impeding immature coat disassembly (Bigay *et al*, 2003).

2.2.3 Recruitment of cargo to the COPI vesicles

The ARFGAP stimulated GTP hydrolysis by ARF1 appears to be coupled with the recruitment of cargo into COPI vesicles. In a light microscopic study, several cargo markers that are normally packaged into COPI vesicles in mammalian cells, were followed in the presence of a non hydrolysable GTP analog, or a restricted mutant of ARF1. It was found that the COPI vesicles generated under these conditions did not to carry detectable amounts of cargo in living cells (Pepperkok *et al*, 2000). In another investigation, the yeast SNARE proteins have been shown to require the ARFGAPs,

Glo3p and Gcs1p for their recruitment into COPI vesicles (Rein *et al*, 2002). In mammalian cells, ARFGAP1 has been proposed to be involved in sorting p24 proteins into different subpopulations of COPI vesicles. GTP hydrolysis was found necessary for the proper sorting of cargo. The cargo proteins in turn were found to decrease the ARFGAP1 activity, thus allowing COPI coat polymerization (Lanoix *et al*, 2001). Another *in vitro* study showed that the lack of ARFGAP or the presence of a non-hydrolysable GTP resulted in impaired sorting of the KDEL-receptor into COPI vesicles (Yang *et al*, 2002).

Two types of ER-retention signals that mediate recruitment of cargo into COPI vesicles have been characterized to date, K/HDEL motif and the dilysine motif. The soluble ER resident proteins, such as PDI and BiP, containing a C-terminal KDEL-motif (HDEL in yeast), are recruited to COPI vesicles *via* the integral transmembrane protein KDEL-receptor (Erd2p in yeast) (Gaynor *et al*, 1998). Using a coimmunoprecipitation approach, binding of KDEL to ERD2, one of the mammalian KDEL-receptors, has been shown to result in ERD2 oligomerization (Aoe *et al*, 1997). In living cells, increased ligand binding to ERD2 enhances its oligomerization, and subsequently also interaction with ARFGAP, as shown using a fluorescence resonance energy transfer (FRET) analysis. This resulted in increased sorting of ERD2-ligand complex into COPI vesicles (Majoul *et al*, 2001). ERD2 has been shown to recruit ARFGAP to the membrane. The cytoplasmic tail of the receptor binds the non-catalytic domain of ARFGAP. Thus, ERD2 may regulate ARF1 and COPI vesicle generation, *via* the ARFGAP interaction (Yang *et al*, 2002, Aoe *et al*, 1997, Aoe *et al*, 1999). The mechanism of the mammalian KDEL-receptor sorting into COPI vesicles has been suggested to involve a phosphorylation event. The short C-terminal cytoplasmic region of the KDEL receptor appears to undergo phosphorylation by protein kinase A, allowing the receptor to interact with the COPI machinery (Cabrera *et al*, 2003).

Recruitment of transmembrane cargo occurs *via* the dilysine motif, KKXX or KXKXX, that is located at the C-terminus of some ER-resident type I membrane proteins in both mammalian and yeast cells. The dilysine motif is both necessary and sufficient to target the proteins for ER retrieval (Jackson *et al*, 1993, Gaynor *et al*, 1994, Cosson & Letourneur, 1994). The dilysine motif KKXX of the yeast Wbp1p, a component of OST, directly binds to COPI *in vitro*. Specific mutations in the motif result in loss of COPI binding *in vitro*, and also in the lack of ER retrieval *in vivo* (Gaynor *et al*, 1994, Cosson & Letourneur, 1994).

Using a mutant screen approach, mutations in α-, δ-, γ-and ζ-COP were found to result in defects in retrieval of the pheromone receptor Ste2p-dilysine motif chimera to the ER. A particularly severe defect was observed when α-COP was mutated, suggesting that the α-subunit was the dilysine motif binding component (Letourneur *et al*, 1994). In accordance with this, the KKXX motif has been shown to bind to α-COP in a yeast two-hybrid assay. The binding affinity appears to be influenced by the amino acids occupying the X positions (Zerangue *et al*, 2001). The WD40 domain of α-COP appears to be responsible for dilysine motif binding. Analysis of a set of *ret1* mutants defective for KKXX-mediated Wbp1p retrieval revealed that the mutations were located in the fifth or sixth WD40 motif of α-COP (Schröder-Köhne *et al*, 1998). In another study using truncated α-COP proteins, it was shown

that deletion of the WD40 domain resulted in specific defects in KKXX-mediated Golgi-to-ER trafficking, as well as in loss of binding to the KKXX motif *in vitro* (Eugster *et al*, 2000). A recent report demonstrated that the WD40 domain of β′-COP also mediates dilysine motif binding. In a two-hybrid study, the naturally occuring dilysine motifs, KKTN of Wbp1p, and KTKLL of Emp47p were tested for binding the WD40 domains of α- and β′-COP. It was found that KKTN preferably interacted with the α-COP domain, whereas KTKLL only bound to the β′-COP. Modifications of the amino acids surrounding the lysine residues resulted in binding of the two motifs to both COP-proteins (Eugster *et al*, 2004). The WD40 domains of α-COP and β′-COP, thus appear to bind to distinct and possibly overlapping sets of KKXX signals, also *in vivo.* Whereas the dilysine motif of Wbp1p fails to target a protein for ER-retireval in *ret1-1* cells (Letourneur *et al*, 1994), Emp47p has been found to localize normally in the *ret1-1* cells, but to mislocalise to the vacuole in mutants with defective β′, γ-, δ- and ζ-COP subunits (Schröder-Köhne *et al*, 1998, Schröder *et al*, 1995). Binding of dilysine-motif has also been proposed to be mediated by γ-COP, in mammalian cells. In a study using photo-cross-linking approach, the intact COPI was found to bind the KKXX motif containing cytoplasmic tail of p23 exclusively *via* its γ-COP subunit. The same site on γ-COP appeared to bind also other KKXX-containing cargo. When dissociated components of COPI were analysed, also α- and β-COP were found to bind the p23 peptide (Harter & Wieland, 1998). In an *in vitro* study, a GTP-binding protein Cdc42 has also been shown to bind directly to γ-COP, *via* its dilysine motif (Wu *et al*, 2000).

A di-basic motif similar to the dilysine, mediates ER retention of type II transmembrane proteins. The motif consists of two arginine residues that are either successive (RR), or separated by one amino acid (RXR), located at the N-terminus of the protein (Schutze *et al*, 1994). The RR-motif has been shown to be sufficient to actively mediate targeting of a normally plasma membrane-located transferrin receptor to the ER. The RR-tagged transferrin receptor was found to partially localize to ER-Golgi intermediate compartment, suggesting that the RR-motif was a retrieval signal for the retrograde traffic (Schutze *et al*, 1994). Indeed, the RR-motif has been found to interact with the β-COB subunit of COPI (O'Kelly *et al*, 2002). Interestingly, a similar dibasic signal mediates ER exit of glycosyltransferases. Location of the RR-motif within the cytoplasmic domain appears to be critical to distinguish wheter it will function as an ER exit or ER retention signal (Giraudo & Maccioni, 2003).

Another mechanism of sorting transmembrane cargo into COPI vesicles involves Rer1p, a yeast Golgi membrane protein with four membrane spanning regions (Sato *et al*, 1995, Sato *et al*, 1997, Boehm *et al*, 1997). Rer1p appears to function in retrieval of topologically different proteins, such as the type II and III transmembrane proteins Sec12p and Mns1p, and Sec71p, respectively, and Sec63p that contains three membrane spanning regions. None of these proteins harbour a dilysine or HDEL motif (Sato *et al*, 1996, Sato *et al*, 1997, Massaad *et al*, 1999). The ER retrieval signal of Sec12p is located in its transmembrane domain that directly interacts with Rer1p. Transfering the transmembrane domain of Sec12p to a reporter protein is

sufficient to cause ER retrieval of the reporter (Sato *et al*, 1996, Sato *et al*, 2001). The Rer1p mechanism may be involved in ER quality control. The iron transporter subunit Fet3p is retained in the ER when it remains in unassembeled form. This requires Rer1p that interacts with the transmembrane domain of Fet3p. This suggests that the Rer1p-mediated retrieval of unassembeled membrane proteins may be a mechansim of quality control (Sato *et al*, 2004).

2.2.4 Role of COPI in anterogradre traffic

In addition to its ER retrieval function, COPI also appears to have a role in anterograde transport from the ER to the Golgi, in mammalian cells. A model has been proposed, where COPII and COPI act sequentially in this route. Following the GFP-tagged cargo-marker protein ts-045-G, the tempreature sensitive variant of VSV-G, it was found that COPII colocalized with the cargo close to the ER, whereas COPI was associated with the transport complexes, devoid of COPII, migrating along microtubules towards the Golgi complex. Transport complexes were formed when COPI was dysfunctional, but cargo could not be delivered to the Golgi (Scales *et al*, 1997). While moving to the Golgi, the anterograde cargo and COPI have been shown to segregate to different domains of the transport complex, suggesting that the role of COPI may be in sorting of cargo for retrieval to the ER (Shima *et al*, 1999). Further evidence supporting the sequential model has been provided using *in vivo* time lapse imaging of living cells to study the dynamics of COPI, COPII and cargo markers. COPII was found mostly associated with the ER-proximal area, whereas COPI associated with transport complexes in the vicinity of the COPII sites, and migrated towards the Golgi together with the cargo in carriers lacking COPII (Stephens *et al*, 2000).

2.3 Delivery of cargo to the target organelle

In order to maintain the directionality of the vesicular traffic, vesicles generated by the coat complexes must find their correct targets and fuse with the acceptor membrane in order to release their cargo. This is mediated by the action of SNARE proteins present both on the transport vesicles (v-SNARE) and the target membranes (t-SNARE). The vesicles must also avoid back-fusion with the donor membrane. Prior to the SNARE-mediated release of cargo to the acceptor compartment, the transport vesicle is targeted to the acceptor membrane by tethering complexes. Assembly of these complexes is mediated by Rab (Ypt in yeast) GTPases. Then, SNARE proteins of the vesicle and the acceptor membrane interact and dock the vesicle to the target membrane, form a tight complex drawing the membranes close together, thus enabling membrane fusion. This cooperation of tethers, Rab proteins and SNAREs contributes to the accurate targeting of the transport vesicles (Bonifacino & Glick, 2004).

2.3.1 Vesicle tethering to target membrane

Rab proteins, the small Ras-like GTPases initiate tethering of vesicles to their target membranes. They recruit tethering molecules to both vesicle and target membranes resulting in formation of bridges between the two, thus enhancing SNARE-mediated membrane fusion. Most tethers are long, coiled coil structures, or large multimeric protein complexes. The tethering step is independent of the SNARE proteins (Pfeffer, 1999, Waters & Pfeffer, 1999). In yeast, ER-derived vesicles are initially

anchored to the Golgi by three tethers, Uso1p, Sec34p/35p complex, and TRAPP I (Cao *et al*, 1998, Van Rheenen *et al*, 1998, Van Rheenen *et al*, 1999, Barrowman *et al*, 2000). The large, Golgi-associated TRAPP I consists of seven subunits and it appears to be the first component to recognize and attract the COPII vesicle (Barrowman *et al*, 2000, Sacher *et al*, 2001). It probably then activates the Rab protein Ypt1p, which in turn recruits Uso1p, both necessary components for efficient tethering (Cao *et al*, 1998). Uso1p is a large, cytosolic protein dimer that consists of a long coiled coil region and two globular head domains (Sapperstein *et al*, 1996, Yamakawa *et al*, 1996). Such a long and flexible structure may be the key for linking vesicles to the target membranes. The function of Uso1p appears to be to facilitate SNARE complex assembly (Pfeffer, 1999, Waters & Pfeffer, 1999). Less is known about tethering of retrograde vesicles. Dsl1p, a peripheral ER membrane protein may be involved in this step. It is an essential protein that is implicated in retrograde transport. It interacts with COPI, and with proteins of the ER target site, Tip20p and Sec20p, which in turn interact with the t-SNARE Ufe1p (Reilly *et al*, 2001, Andag *et al*, 2001).

2.3.2 Fusion with the target

Vesicle fusion with the target membrane is mediated by SNARE proteins. The name SNARE comes from ***s**oluble **N**-ethylmaleidimide-sensitive factor **attach**ment protein **re**ceptor*. Initially, the N-ethylmaleidimide-sensitive factor (NSF, Sec18p in yeast) was identified as a factor required for membrane fusion. Then, a partner protein that binds NSF to membranes, the soluble NSF attachment protein (α-SNAP, Sec17p in yeast) was found and it became evident that NSF and α-SNAP formed a complex with additional components that were finally identified as SNAP receptors (SNAREs) (Bonifacino & Glick, 2004).

The majority of the SNARE proteins are C-tail-anchored transmembrane proteins. The cytosolic N-terminus of a SNARE protein contains a SNARE-motif, a heptad repeat of 60-70 amino acids that participates in formation of a coiled coil structure in the SNARE complex. The SNARE complex is composed of a v-SNARE and an oligomeric t-SNARE, and the components provide one and three α-helices, respectively, to form a four-helix, coiled coil bundle (Bonifacino & Glick, 2004). This complex may be formed between SNAREs of separate membranes resulting in a *trans*-SNARE complex, or of the same membrane, resulting in a *cis*-SNARE complex. In the process of vesicle fusion, a *trans*-SNARE complex is initially formed and it becomes a *cis*-SNARE complex after completion of the fusion (Bonifacino & Glick, 2004). Dissociation of the complex is mediated by NSF and α-SNAP. The latter binds to the SNARE complex and recruits NSF that is an ATPase. NSF-driven ATP hydrolysis results in disassembly of the complex, possibly due to rotational force provided by NSF (Bonifacino & Glick, 2004). Membrane fusion appears to be a direct consequence of SNARE complex formation. *In vitro*, liposomes that present purified recombinant v- or t-SNAREs are able to form SNARE-complexes and concomitantly spontaneously fuse, suggesting that the SNAREs are the minimum requirement for membrane fusion to occur (Weber *et al*, 1998). Also biological membranes have been shown to fuse spontaneously as a result of SNARE complex formation. In an elegant experimental setup, flipped v- and t-SNAREs were expressed on the cell surface. The coiled coil regions facing the

outside of the cell were found to be sufficient to mediate fusion of two cells (Hu *et al*, 2003). SNAREs appear to facilitate membrane fusion by bringing two membranes in close contact and providing energy for the fusion to occur. It has been proposed, that the assembly of the helical bundle-containing rod of the SNARE complex results in exerting force on the anchors by pulling on the linkers, and thus promoting simultaneous inward movement of the lipids from the two membranes (McNew *et al*, 1999, McNew *et al*, 2000).

In yeast, fusion of ER-derived vesicles with the Golgi membrane is mediated by the SNAREs Sed5p, Bos1p, Sec22p and Bet1p (Pelham, 1999). All of the four SNAREs are found in transport vesicles and the *cis*-Golgi membranes. However, they are not similarly required on both membranes for vesicle fusion to occur. In an *in vitro* budding and fusion assay, Sed5p appeared to be required on the Golgi membrane and Bet1p and Bos1p on the vesicle (Cao & Barlowe, 2000). Another assay using a liposome fusion approach, however, demonstrated that only the combination of Sed5p, Bos1p and Sec22p as the t-SNARE and Bet1p as the v-SNARE, resulted in liposome fusion (Parlati *et al*, 2000). Similarly to anterograde traffic, four SNAREs are implicated in membrane fusion of retrograde vesicles. Ufe1p, Sec22p, Sec20p (Lewis *et al*, 1997) and Use1p/Slt1p (Dilcher *et al*, 2003, Burri *et al*, 2003) have been characterized by genetic and biochemical approaches. How these components are assembled into v- and t-SNAREs, remains to be elucidated in liposome fusion assays.

In addition to SNAREs, a number of accessory proteins that modulate the SNAREs have been discovered. In yeast, Sly1p, an essential protein and member of Sec1/Munc18 family proteins, has been reported to form a complex with Sed5p with very high affinity, and thus probably to mediate SNARE complex generation (Peng & Gallwitz, 2002). However, a recent report reveals that despite the high affinity, Sly1p/Sed5p interaction is dispensable for *trans*-SNARE complex formation. Sly1p was also found to bind Bet1p and Bos1p. Thus, the role of Sly1p in SNARE complex generation might be assisting the assembly through stepwise interactions with different SNARE proteins (Peng & Gallwitz, 2004).

2.3.3 Coordinating directionality of the traffic

Specificity of membrane fusion of ER-derived vesicles with the Golgi membrane appears to be coupled to formation of the COPII vesicle. According to a detailed biochemical analysis of the SNARE-COPII interactions, the assembly state of the SNAREs is a determinant of recognition by the COPII. COPII coat appears to selectively recruit the fusogenic forms, the t-SNARE Bos1p/Sec22p/Sed5p, and v-SNARE Bet1p. The ER exit motif of Sed5p is occluded in the monomeric form, but assembly of the t-SNARE exposes the YNNSNPF motif of Sed5p for COPII binding. Similarly, the ER exit motif of Bet1p is not available for COPII binding in the non-fusogenic v-/t-SNARE complex, but free Bet1p may be recruited to the vesicle. Thus, selection of SNAREs appears to contribute to directionality of vesicular traffic (Mossessova *et al*, 2003). Also tethering may be coupled to formation of vesicles. Uso1p, Sec34p/35p and Ypt1p, but not the TRAPP complex, are involved in sorting of GPI-anhored proteins at the ER (Morsomme & Riezman, 2002). The same set of proteins is also required for tethering the vesicles, but despite the requirement for common factors, the two functions are independent. Coupling of

the two events may be a general mechanism necessary to maintain the specifity of vesicular traffic (Morsomme & Riezman, 2002). A recent report describes a novel mechanism that actively prohibits fusion of COPII vesicles with the ER membrane in yeast. The peripheral ER membrane protein Tip20p appears to act as a sensor for vesicles. It does not interfere with COPII assembly, but it inhibits fusion of the COPII vesicles to the ER membrane, suggesting that at least in part, directionality of the transport is accomplished by impeding back-fusion to the donor membrane (Kamena & Spang, 2004).

AIMS OF THE STUDY

1) To study whether Golgi-specific mannosyltransferases recycle between the ER and the Golgi complex, and whether they can exert their activity in the ER.

2) To search for alternative ER exit routes differing in the composition of the COPII coat. Specifically, to elucidate the role of the COPII component Sec24p and its two homologues Sfb2p and Sfb3p in ER exit of the yeast secretory glycoprotein Hsp150.

3) To identify the signature that guides Hsp150 for ER exit in the absence of functional Sec24p.

4) To develop an HRP-based method to identify secretory compartments in yeast at the electron microscopic level.

MATERIALS AND METHODS

The experimental methods employed in this study are summarized in **Table 4** with references to the publications in which they have been applied. The *S. cerevisiae* yeast strains used are listed in **Table 5** and the publications in which they have been used are indicated. **Table 6** presents the relevant features of the key mutations.

Table 4. The methods used in this study.

Method	*Publication*
Calcofluor staining of bud scars	III
Immunofluorescence microscopy	I
Immunoprecipitation	I, II, III
Invertase activity staining in non-denaturating gel	II
Metabolic labeling with [^{35}S]-Methionine-Cysteine	I, II, II
Metabolic labeling with [^{5}H]-Mannose	I
Nucleotide sequencing	III
Plasmid construction	I, II, III
Recombinant HRP method applied for yeast electron microscopy	II
Scanning electron microscopy	III
SDS-PAGE	I, II, III
Transmission electron microscopy	II, III
Yeast mating and tetrad dissection	III
Yeast strain construction	I, II, III
Yeast transformation	I, II, III
Western blot analysis	III

Table 5. The *S. cerevisiae* strains used in this study.

Strain	*Relevant mutant genotype*	*Publication*	*Source or reference*
H1	none	I, II, III	R.Schekman
H3	*sec7-1*	II	R.Schekman
H4	*sec18-1*	I, II, III	R.Schekman
H23	*Δhsp150*	II	Russo *et al*, 1992
H230	*sec13-1*	I	Novick *et al*, 1980
H238	*sec23-1*	I	Novick *et al*, 1980
H245	none	I,III	K. Kuchler and J. Thorner
H247	none	III	K. Kuchler and J. Thorner
H335	*URA3::HSP150Δ-β-lactamase*	I	Simonen *et al*, 1994
H430	*Δhsp150 LEU2::HSP150Δ*	II	Fatal *et al*, 2002
H440	*LEU2::HSP150Δ*	II	Fatal *et al*, 2002
H480	*sec23-1*	I	R.Schekman
H481	*sec23-1*	I, II	R.Schekman
H606	*URA3::HSP150Δ-β-lactamase-HDEL*	I	This study
H610	*sec18-1 URA3::HSP150Δ-β-lactamase-HDEL*	I	This study
H830	*sec21-1*	I	H. Riezman
H1065	*sec13-1 LEU2::HSP150Δ-β-lactamase*	I	Fatal *et al*, 2002
H1101	*sec24-1*	II, III	C. Kaiser
H1141	*Δsec24b*	II, III	J.P.Paccaud
H1142	*Δsec24c*	II, III	J.P.Paccaud
H1143	*Δsec24b Δsec24c*	II	J.P.Paccaud
H1233	*Δhsp150*	II	Fatal *et al*, 2002
H1236	*sec13-1*	II	Fatal *et al*, 2002
H1237	*sec24-1*	II	This study
H1429	*sec13-1 TRP1::SUI-Cterm*	II	Fatal *et al*, 2002
H1455	*sec7-1 LEU2::HSP105Δ-HRP*	II	This study
H1458	*sec24-1 LEU2::HSP105Δ-HRP*	II	This study
H1459	*sec23-1 LEU2::HSP105Δ-HRP*	II	This study

Table 5. Continued

Strain	*Relevant mutant genotype*	*Publication*	*Source or reference*
H1488	*URA3::OCH1-HA*	I	This study
H1489	*sec7-1 URA3::OCH1-HA*	I	This study
H1490	*sec23-1 URA3::OCH1-HA*	I	This study
H1495	*LEU2::SCW4-HIS$_6$*	I	This study
H1496	*sec23-1 LEU2::SCW4-HIS$_6$*	I	This study
H1497	*sec21-1 LEU2::SCW4-HIS$_6$*	I	This study
H1499	*sec24-1 Δhsp150 LEU2::HSP150Δ*	II	This study
H1500	*sec24-1 TRP1::SUI-Cterm*	II	This study
H1508	*Δhsp150 TRP1::SUI-CTERM*	II	Fatal *et al*, 2002
H1540	*Δhsp150 TRP1::SUI-CTERM-INVERTASE*	II	Fatal *et al*, 2002
H1542	*sec18-1 TRP1::SUI-CTERM-INVERTASE*	II	Fatal *et al*, 2002
H1544	*sec24-1 TRP1::SUI-CTERM-INVERTASE*	II	This study
H1555	*sec24-1 Δsfb2*	II	This study
H1575	*sec18-1 TRP1::SUI-INVERTASE-CTERM*	II	This study
H1577	*Δhsp150 TRP1::SUI-INVERTASE-CTERM*	II	This study
H1578	*sec13-1 TRP1::SUI-INVERTASE-CTERM*	II	This study
H1579	*sec24-1 TRP1::SUI-INVERTASE-CTERM*	II	This study
H1628	*sec18-1 LEU2::SCW4-His$_6$*	I	Euroscarf
H1691	*Δygl038c*	I	This study
H1791	*sec23-1 URA3::OCH1-HA LEU::cytb(5)-opsin*	I	This study
H1866	*Δyil109c/YIL109C*	III	Euroscarf
H1895	*sec24-1 Δsfb3*	III	This study
H1914	*sec24-1/SEC24 Δsfb3/SFB3 Δsfb2/SFB2*	III	This study
H1927	*Δsec24 URA::SEC24-HIS$_6$ LEU::pCM244 CEN*	III	This study
H1930	*sec24-1 Δsfb3 Δsfb2*	III	This study
H1996	*Δsec24 URA:: SEC24-HIS$_6$ LEU::pCM244 CEN Δsfb3*	III	This study
H2006	*Δsfb3 Δsfb2*	III	This study
H2023	*Δsec24 URA::SEC24-HIS$_6$ LEU::pCM244 CEN Δsfb3 Δsfb2*	III	This study
H2025	*Δsec24 URA::SEC24-HIS$_6$ LEU::pCM244 CEN Δsfb2*	III	This study

Table 6. The relevant mutations of strains used in this study.

Mutation	*Description of phenotype*	*Reference*
sec7-1	Temperature-sensitive mutation, defect in fusion of ER-derived vesicles to Golgi and in transport between the Golgi cisternae	Franzusoff & Schekman, 1989
sec13-1	Temperature-sensitive mutation, defect in formation of COPII coat	Pryer *et al*, 1993
sec18-1	Temperature-sensitive mutation, inhibits vesicle fusion with target membrane	Kaiser & Schekman, 1990
sec23-1	Temperature-sensitive mutation, defect in formation of COPII coat	Hicke & Schekman, 1989
sec24-1	Temperature-sensitive mutation, defect in formation of COPII coat	Hicke *et al*, 1992
Δsfb2/ Iss1/ sec24b	Mutant phenotype found only in combination with other defects, altered ER-Golgi transport	Kurihara *et al*, 2000, Peng *et al*, 2000
Δsfb3/ lst1/ sec24c	At elevated temperatures defect in growth and in Pma1p secretion, altered ER-Golgi transport	Roberg *et al*, 1999
Δsec24/ yil109c/ anu1	Lethal mutation, defect in formation of COPII coat	Giaever *et al*, 2002
Δoch1/ ygl038c	Defect in N-glycosylation	Nakayama *et al*, 1992

RESULTS AND DISCUSSION

1. Glycosylation of proteins trapped in the yeast ER (I)

1.1 N-glycans are extended in the ER when ER-to-Golgi traffic is blocked

Addition of an α1,6-linked mannose residue to a secretory protein has been taken for evidence of the protein having reached the Golgi complex. The mannose residue is added by the α1,6-mannosyltransferase Och1p that normally localizes to the *cis*-Golgi and it is a prerequisite for addition of further mannose residues to the N-glycan. Loss of the *OCH1* gene results in lack of N-glycosylation (Munro, 2001, Nakayama *et al*, 1992). We have observed in metabolic labelling experiments, that impeding ER exit of secretory proteins results in increasing of the apparent molecular weights of the proteins accumulated in the ER. Others have shown that components of the Golgi localised mannosylpolymerases MPolI and MPolII (Mnn9p, Anp1p, Van1p) are actively recycled through the ER (Todorow *et al*, 2000). Thus, it appeared possible that the observed increase of the apparent molecular weight could be due to Golgi-specific glycosylation that occurred in the ER.

In order to study whether Och1p functions in the ER, a pulse-chase experiment was carried out to follow the glycosylation of CPY. CPY is synthesized as a 59 kDa protein precursor (pre) that receives core N-glycans when translocated into the ER lumen, resulting in a 67 kDa "ER form" (p1). Upon arrival to the Golgi, Och1p adds the mannose residue to the core glycan giving rise for the 69 kDa "Golgi form" (p2) that is proteolytically cleaved once it arrives in the vacuole, yielding the mature 62 kDa form (m) (Stevens *et al*, 1982). Since the α1,6-mannose decoration can be immunologically detected, CPY could be used as a reporter for Och1p activity *in vivo*. Using temperature sensitive yeast mutants where anterograde transport from the ER is blocked upon shift to the non-permissive temperature 37°C, newly synthesized ^{35}S-labelled CPY was accumulated in the ER. After the chase, cell lysates were subjected to immunoprecipitation with antiserum against CPY. As expected, the ER-specific form p1 was detected in *sec13-1* and *sec23-1* cells with defective COPII subunits, as well as in *sec18-1* cells, defective for NSF and thus membrane fusion (**Fig. 1A**, lanes 1, 4 and 3, respectively). The mature form m was detected in *sec13-1* control cells incubated at the permissive temperature (lane 2). A set of parallel samples was reprecipitated with α1,6-mannose antiserum after CPY immuno-precipitation, in order to find out if the p1 forms of CPY had been decorated with the α1,6-mannose residue. As seen in **Fig. 1B**, in both COPII mutant strains, the p1 form was indeed decorated by the α1,6-mannose (lanes 1 and 4). The mature form (lane 2) was also recognized by the antiserum, as expected, but the p1 form in the NSF mutant cells (lane 3) was not. Thus, when CPY was blocked inside the ER lumen, in the absence of COPII-mediated traffic, it acquired the Golgi-specific α1,6-mannose decoration.

Since newly synthesized mannosyl-transferases also accumulated in the ER together with CPY, it appeared possible that they could be responsible for the modification. However, the α1,6-mannose modification was not detected in NSF deficient cells. In these cells, CPY and the mannosyltransferases could be

packaged into COPII vesicles, but could not fuse with the Golgi membranes due to deficient NSF. If the newly synthesized enzymes were active prior to arrival to the Golgi, the α1,6-mannose decoration should have been found in this pool of CPY. Since this was not the case, it was concluded, that the newly synthesized Och1p could probably not be responsible for the α1,6-mannose modification.

To rule out the possibility that other enzymes would replace the Och1p action, the same experiment was repeated with a strain lacking the *OCH1* gene. It was found, that the cleaved form of CPY contained no α1,6-linked mannoses, indicating that Och1p was indispensable for the modification. Thus, when CPY could not leave the ER, it was decorated with α1,6-linked mannose residues. These were added by Och1p, the normally Golgi-resident mannosyltransferase that was recycled and trapped in the ER together with the substrate protein. *De novo* synthesized Och1p was not active in the ER.

To study whether proteins other than CPY trapped in the ER would also be glycosylated by Och1p, a pulse-labelling experiment was performed. *Sec13-1*, *sec23-1* and *Δoch1* cells were labelled for 30 minutes at the restrictive temperature, and the newly synthesized N-glycosylated proteins were precipitated from the cell lysates with the lectin concanavalin A (ConA). A set of parallel samples were thereafter reprecipitated with α1,6-mannose antiserum. In both COPII mutant strains, a similar subset of proteins precipitated with ConA (**Fig. 2**, lanes 1 and 3) was recognized by the α1,6-mannose antiserum (lanes 2 and 4) whereas none were found in the *Δoch1* strain (lane 6). Thus, the Golgi-specific modification, addition of the α1,6-linked mannose residue by Och1p, was found not only on CPY but on a set of newly synthesized substrate proteins that were retained in the ER.

1.2 Och1p relocates to the ER when COPII-mediated traffic is impaired

Components of the mannosylpolymerases MPolI and MPolII have been shown in an immunofluorescence assay to recycle between the ER and the Golgi complex. In the same study, Och1p, however, was found not to relocalize to the ER in the temperature-sensitive *sec12-4* COPII mutant (Todorow *et al*, 2000). Since this is in contradiction with the biochemical data presented above, the issue of Och1p localization was revisited. An epitope-tagged Och1p variant (Och1p-HA) was expressed in the *sec23-1* strain that was also used for the pulse-chase analysis above. Opsin-tagged cytochrome B5 (Cyt-B5) that localizes to the ER (Yabal *et al*, 2003) was co-expressed in the same cells. An immunofluorescence microscopy experiment was carried out in similar conditions as in Todorow *et al*, 2000. In order to stop protein synthesis, cells were chased in the presence of cycloheximide for 1 hour prior to shift to the restrictive temperature, and then incubated for 30 minutes at 37°C, to impose the COPII block. Prior to the temperature shift, a punctate staining was observed suggesting that Och1p localized to the Golgi (**Fig. 7B**). After imposing the anterograde transport block, Och1p colocalized with the ER marker Cyt-B5 (**Fig. 6C** and **D**). Ring-like structures typical of the yeast ER-staining (**Fig. 6E**) lining the nucleus (**6F**), but not vacuole (**6A** and **B**), were also observed. The ring-like ER-staining observed appeared to consist of small dots suggesting that Och1p perhaps accumulated in sub-regions of the ER. Small dots also appered throughout the cytoplasm, but no intense dots typical of

Golgi-staining (**6D**) were observed. Thus, Och1p-HA did indeed recycle to the ER in the *sec23-1* cells, in the absence of COPII traffic.

1.3 Recycling of Och1p is mediated by COPI

Since blocking of anterograde traffic resulted in Och1p accumulating in the ER and adding α1,6-linked mannose residues on substrate proteins, a block in retrograde traffic should result in the lack of this modification in the ER. In order to study if this was the case, another reporter protein, the His_6-epitope-tagged cell wall protein Scw4p was expressed in the temperature-sensitive *sec21-1* cells having a defective COPI subunit. In *sec21-1* cells, also anterograde traffic is blocked. Thus, at the restrictive temperature in these cells, newly synthesized proteins can not exit the ER, and recycling from the Golgi is blocked.

A pulse-chase analysis and immunoprecipitation with anti-His_5 antibody was carried out either in the presence or absence of tunicamycin, a drug that blocks N-glycosylation. The analysis revealed that after the chase, the apparent molecular weight of Scw4p-His_6 was similar, whether or not tunicamycin was present (**Fig. 5**, lanes 12 and 10, respectively). In addition, Scw4p migrated faster in *sec21-1* cells than in *sec23-1* cells (lanes 10 and 6, respectively). These data suggest that Scw4p-His_6 did not acquire N-glycans when trapped in the ER in the absence of COPI-mediated retrograde traffic, whereas N-glycans were added to it when COPI was functional. In order to confirm that also here the differences observed in the apparent molecular weights originated from the Och1p-specific α1,6-mannose modification, a set of chase samples precipitated with the anti-His_5 antibody were reprecipitated with the α1,6-mannose antiserum. The antiserum detected Scw4p-His_6 in *sec23-1* (lane 14) and normal (lane13) cells, but not in *sec21-1* (lane 15) cells. These observations indicate that relocalization of Och1p to the ER was mediated by the COPI coat.

1.4 The recycled, but not *de novo* synthesized Och1p is active in the ER

The Scw4p pulse-chase experiment provided means to further investigate whether the Golgi-specific α1,6-mannose modification was carried out by the recycled or the *de novo* synthesized Och1p. In both *sec21-1* and *sec23-1* cells, newly synthesized mannosyltransferases and Scw4p accumulated in the ER at the restrictive temperature. In the *sec23-1* cells, COPI-mediated retrograde transport from the Golgi was functioning, but in *sec21-1,* it was blocked. In these conditions, N-glycans of Scw4p in *sec23-1* cells were extended. They received an α1,6-linked mannose (**Fig.5**, lane 14), whereas no mannose was added on Scw4p in the *sec21-1* cells (lane 15). In addition, the presence of tunicamycin reduced the apparent molecular weight of Scw4p in *sec23-1* cells (lanes 6 and 8), whereas Scw4p in *sec21-1* cells was of the same apparent molecular weight whether tunicamycin was present or not (lanes 10 and 12). Since Scw4p and the *de novo* synthesized enzymes could interact in both types of cells, the difference in the apparent molecular weights of Scw4p could not be due to *de novo* synthesized enzymes. Thus, *de novo* synthesized Och1p was not active in the ER. Instead, Och1p, recycled from the Golgi and trapped in the ER, could perform its function on the newly synthesized proteins in the ER.

1.5 O-glycans are extended in the ER when ER-to-Golgi traffic is blocked

O-glycosylation is initiated in the ER by the Pmt family proteins that add a single mannose residue to serine or threonine residues of a newly synthesized polypeptide. Further extension of the O-glycans occurs in the Golgi complex (Strahl-Bolsinger *et al*, 1999). Since the N-glycosylating enzymes were found to recycle and to be functional in the ER, we assumed that the mannosyltransferases involved in O-glycosylation could be localized in a similar, dynamic manner. To study if O-glycans were extended in the ER, a fusion protein consisting of the N-terminal fragment of the yeast glycoprotein Hsp150 and the mature portion of the *E.coli* β-lactamase (Hsp150Δ-β-lactamase) was expressed in the COPII mutant *sec13-1*. This reporter remains in the ER when the cells are incubated at the restrictive temperature (Fatal *et al*, 2002). The Hsp150 portion of the fusion protein is heavily O-glycosylated, whereas the β-lactamase portion has no or only few O-glycans. Neither component is N-glycosylated (Russo *et al*, 1992). A pulse-chase analysis as described above was performed and cell lysates and culture medium samples were subjected to immunoprecipitation with β-lactamase antiserum. After the pulse, a 110 kDa form (**Fig. 3**, lane 1) corresponding to the ER form of Hsp150Δ-β-lactamase was detected. During one hour of chase at 37°C, the apparent molecular weight of the ER-trapped reporter protein increased to reach the size of a mature, 145 kDa protein (lanes 2 - 3). In order to confirm that this was due to glycosylation, the pulse-chase was repeated using [^{3}H]-mannose label. The analysis revealed that the increase in size indeed was due to addition of mannose residues. The proteins precipitated from the [^{3}H]-mannose labelled samples (lanes 10 - 12) comigrated with those from the ^{35}S-labelled ones (lanes 2 - 4). The 66 kDa form in lane 1 corresponds to the cytosolic fusion protein, and as expected, it was not labelled with the [^{3}H]-mannose (lane 9). Thus, similarly as N-glycans, also O-glycans are extended in the ER, in the absence of anterograde transport.

To investigate whether the O-glycan extension in the ER was due to *de novo* synthesized enzymes or to recycling ones, the pulse-chase experiment using ^{35}S-label was repeated with *sec18-1* cells expressing Hsp150Δ-β-lactamase. The reporter protein acquired some glycans in these cells, developing from a 110 kDa form into a 120 kDa form during one hour of chase (lanes 13 - 16). Since in both cell types the substrate protein and the *de novo* synthesized transferases could accumulate in the ER similarly, the glycans should have been extended equally if the newly synthesized transferases were active. A clear difference was, however, observed, and it was therefore deduced that the *de novo* synthesized enzymes did not carry out the glycosylation in the ER. Instead, also the mannosyltransferases involved in O-glycosylation appeared to be recycled from the Golgi, and accumulated in the ER in the absence of anterograde transport.

1.6 Activity of the recycling mannosyltransferases is only detectable when normal anterograde transport is blocked

In this study, both N- and O-glycans of exocytic proteins were found to be extended in the ER, when retained there due to a block in the COPII traffic. The extension was found to be dependent on the normally Golgi-resident proteins that recycle between the ER and the Golgi complex. In normal conditions where anterograde transport is not inhibited,

the immunofluorescence staining of mannosyltransferases was typical of the yeast Golgi. Thus, the cycling through the ER is probably rapid and could therefore not be observed in normal conditions. The Golgi-specific modifications of exocytic proteins are not likely to take place in the ER under normal conditions. The substrate and the enzymes do probably not encounter each other frequently in the ER. Thus, the glycosylation carried out by the recycling mannosyltransferases in the ER is most likely not a frequent nor biologically relevant event.

1.7 Biological function of recycling Golgi enzymes

The Golgi complex appears to be a highly dynamic organelle. Glycosyltransferases are constantly recycled through the ER. In addition, Och1p has also been shown to be retrieved to the *cis*-Golgi from the TGN (Harris & Waters, 1996). Glycosyltransferases of not only yeast, but also of mammalian cells have been shown to recycle. In a light microscopic analysis of live cells, the Golgi enzyme N-acetylgalactosaminyltransferase-2 was found to slowly cycle to the ER, when COPII was blocked by expressing the dominant negative Sar1p in HeLa cells (Storrie *et al*, 1998). Also other Golgi-resident proteins are recycled. In a similar light microscopic assay as above, fate of three different Golgi proteins was followed after expressing the dominant negative Sar1p mutant in HeLa cells. The integral Golgi membrane protein giantin, the putative *cis*-Golgi matrix protein GM130 and the tethering protein p115 (homologous to the yeast Uso1p), were found to relocalize to the ER when COPII was blocked, suggesting that the entire Golgi complex may be subject to recycling (Miles *et al*, 2001).

What could be the purpose of relocating resident Golgi enzymes to the ER? A simple answer could be the recovery of escaped Golgi proteins from the ER. Similarly as ER-resident proteins leak from the ER and must be retrieved from Golgi, Golgi residents may leak to the ER. However, a block in retrograde traffic has been shown to result in mislocalization of the MPolI and MPolII components Van1p and Anp1p, respectively, to the vacuole. This suggests that recycling may have a more active role in maintaining the steady state levels of Golgi-resident proteins in and organelle integrity of the dynamic Golgi apparatus (Todorow *et al*, 2000).

It has also been proposed, that the recycling pathway could be involved in quality control. The Golgi proteins could be screened for damages as they circulate through the ER (Storrie *et al*, 1998). Recycling of Golgi enzymes might even be linked with inheritance of the Golgi complex. Golgi components accumulated in the ER under experimental conditions have been shown to promote formation of a *de novo* Golgi originating from the ER, in mammalian cells (Puri & Linstedt, 2003). In a recent report, inheritance of Golgi was shown to be linked with inheritance of ER, in yeast. The authors presented a general model for inheritance of Golgi in eukaryotic cells, in which COPII-dependent transport from the ER would result in formation of a platform, to which Golgi-derived vesicles then fuse, giving rise to new Golgi cisternae (Reinke *et al*, 2004). Thus, the active recycling of the Golgi enzymes might be a part of this mechanism of *de novo* Golgi formation. Future experiments should provide more information concerning the physiological significance of the recycling of Golgi enzymes.

2. Role of the Sec24p family members in ER exit of the secretory glycoprotein Hsp150 (II, III)

Hsp150 (also known as Pir2p) is a constitutively expressed protein coded by the non-essential *HSP150* gene. Hsp150 is efficiently secreted to the culture medium and its expression is upregulated seven-fold upon a shift of cells to 37°C (Russo *et al*, 1992, Russo *et al*, 1993). The half time of Hsp150 secretion is about 2 minutes (Jämsä *et al*, 1994). Hsp150 is initially synthesized as a 413 amino acid polypeptide that loses an N-terminal 18 amino acid signal peptide upon posttranslational translocation into the ER (Paunola *et al*, 1998, Russo *et al*, 1992). The remaining peptide is cleaved by the Kex2p protease to yield 54 and 341 amino acid subunits (SUI and II, respectively) that remain non-covalently associated. SUII contains a repetitive region consisting of 11 tandem repeats of mostly 19 amino acids (see **II**, **Fig.1A** for a schematic of Hsp150). Hsp150 lacks N-glycans, but it is extensively O-glycosylated (Russo *et al*, 1992). The O-glycans of SUII consist of di-, tri-, tetra- and pentamannosides that occur in the ratio of 4:1:1:1 (Jämsä *et al*, 1995). An average of 65 of the 85 potential O-glycosylation sites of SUII, appear to be occupied by O-glycans, as assessed by MALDI-TOF mass spectrometry. SUI has 21 potential O-glycosylation sites, many of which also carry O-mannoses (Suntio *et al*, 1999).

Hsp150 is selectively secreted to the culture medium in cells with the deficient COPII subunit Sec13p (Fatal *et al*, 2002) or COPI component Sec21p (Suntio *et al*, 1999, Gaynor & Emr, 1997). ER exit of Hsp150 variants lacking the C-terminal domain of SUII have been found to depend on Sec13p for ER exit. In addition, fusion of the C-terminal domain to another reporter protein, dependent on the authentic form of Sec13p for ER exit, revealed that the domain actively mediated Sec13p-independent ER exit. The C-terminal domain thus appears to carry a molecular signature guiding Hsp150 for secretion when Sec13p is deficient (Fatal *et al*, 2002). A molecular signature responsible for COPI-independent ER exit has also been mapped. This was found to be in the repetitive region of SUII (Suntio *et al*, 1999). The COPII components Sec23p, Sec31p and the GEF Sec12p have been found necessary for ER exit of Hsp150 (Fatal *et al*, 2002). The role of Sec24p and its two homologues Sfb2p and Sfb3p was investigated in this study.

2.1 Hsp150 is secreted in the absence of functional Sec24p (II)

2.1.1 Hsp150 is secreted in sec24-1 cells

Incubation of the temperature sensitive *sec24-1* cells at 37°C renders the Sec24p component of COPII dysfunctional. In order to study if Hsp150 was able to exit the ER despite this defect, a pulse-chase experiment was performed. ^{35}S-labelled cells were chased in the presence of cycloheximide for up to 90 minutes, and cell lysates and culture media samples were analysed by immunoprecipitation with Hsp150 antiserum. It was found that already after 30 minutes of chase, Hsp150 appeared in the culture medium (**Fig.2A**, lane 4). After 90 minutes, most of the labelled Hsp150 was secreted (lanes 7 and 8). In the *sec18-1* cells where vesicle fusion is blocked, Hsp150 remained in the ER (not shown).

ER exit of two other reporter proteins was assessed in the same *sec24-1* cells. First, a pulse-chase experiment was

carried out and cell lysates were analyzed by immunoprecipitation with CPY-antiserum. The analysis revealed that even after 1 hour of chase, CPY remained in the ER-specific p1 form (**Fig.2B**, lane 4), at the restrictive temperature. When the experiment was repeated at 24°C, the p1 form observed after the pulse (lane 5) disappeared already after 10 minutes (lane 6), and CPY appeared to be in the mature form after 20 minutes (lane 7). Thus, ER exit of CPY required functional Sec24p. In the second experiment, *sec24-1* cells were incubated at the restrictive temperature for 15 minutes and then shifted to low glucose culture medium to derepress the synthesis of invertase. A cell lysate sample was then analysed in non-denaturating gel electroforesis followed by an invertase activity staining procedure. The assay revealed that invertase remained in the ER in *sec24-1* cells (**Fig. 2C**, lane 3), as in the similarly treated *sec18-1* cells (lane 2). In normal cells (lane 1), as well as in *sec24-1* cells incubated at the permissive temperature 24°C (lane 4), invertase was found in the mature form. Thus, like CPY, also invertase required Sec24p for ER exit. Taken together, Hsp150 was selectively exported from the ER under conditions that resulted in accumulation of CPY and invertase in the ER.

2.1.2 The sorting signal resides in the C-terminal domain of Hsp150

Since different regions of Hsp150 had previously been assigned to be responsible for ER exit of Hsp150 in the absence of functional COPI or COPII components, a molecular signature for Sec24p-independent ER exit was next searched for. Variants of Hsp150 lacking either the C-terminal domain (Hsp150Δ; **Fig.1B**) the entire SUII (SUI-β-lactamase; **Fig. 1D**), or the repetitive region of SUII (SUI-Cterm; **Fig. 1C**), were expressed in *sec24-1* cells. A pulse-chase analysis of the SUI-Cterm variant was first carried out. This variant consisted of the signal peptide and SUI with the Kex2p site, fused to the C-terminal 114 amino acid domain of Hsp150. Since Kex2p is located in the late Golgi, cleavage of the peptide could be taken as evidence for ER exit of the fusion protein. Immunoprecipitation of cell lysates and the corresponding culture media samples with Hsp150 antiserum, revealed that uncleaved, cell-associated SUI-Cterm was found in the pulse-labelled sample (**Fig. 3A**, lane 1), but not in the chased samples (lanes 2 - 4). In culture media, a double band of 14 and 16.5 kDa proteins corresponding to the Kex2p-cleaved Cterm fragment derived from SUI-Cterm, appeared after 15 minutes of chase (lane 6) and appeared to be maximally secreted after 30 minutes (lane 7). The occurrence of two bands may have been the result of further processing by the other late-Golgi proteases, after the Kex2p cleavage. Similarly as in *sec24-1* cells, cell-associated forms of SUI-Cterm were not found in normal cells (**Fig. 3C**, lanes 1 - 4), but the released Cterm double band appeared in the culture medium already after the pulse (lane 5), and was fully secreted after 15 minutes of chase (lane 6). Thus, lack of the repetitive region, which is the sorting signal for Sec21p-independent ER exit of Hsp150, did not slow down secretion of Hsp150 in *sec24-1* cells.

Subsequently, it appeared likely, that the C-terminal domain contains the sorting signal for Sec24p-independent ER exit. To test this, SUI-β-lactamase was expressed in *sec24-1* cells, and a pulse-chase experiment as above was performed. The assay demonstrated that SUI-β-lactamase remained intracellular in the *sec24-1* cells (data not shown). From

these findings it was deduced, that the sorting signal of Hsp150 was indeed located in the C-terminal domain, and not in the SUI or repetitive region. To directly asses this, the Hsp150Δ variant lacking the last 89 amino acids of Hsp150 was finally tested. Again, a pulse-chase analysis as described above was carried out. The assay demonstrated that no Hsp150Δ was found in the culture medium of *sec24-1* cells even after 1 hour of chase at 37°C (**Fig.4**, lane 8). The cell-associated form migrated similarly as the form detected in *sec18-1* cells (lane 21), indicating that the cell-associated form in *sec24-1* cells remained in the ER. At 24°C, Hsp150Δ was secreted to the culture medium already after 15 minutes of chase (lane 12) suggesting that the C-terminal domain was dispensable for ER exit of Hsp150 in *sec24-1* cells. The C-terminal domain thus indeed appeared to contain the determinant guiding Hsp150 for ER exit in the absence of functional Sec24p.

2.1.3 Hsp150Δ-HRP fusion protein localizes to the ER

In order to further confirm that the C-terminal domain was required for ER exit of Hsp150 in the absence of Sec24p function, a morphological approach was developed. A highly sensitive staining method based on enzymatic activity of HRP was applied (see **II**, Materials and methods). HRP reacts with the substrate diaminobenzidine (DAB) in the presence of hydrogen peroxide and yields an insoluble precipitate that becomes electron-dense upon reaction with reduced osmium tetroxide. The precipitate formed inside of membrane structures may then be visualized in TEM as a dark staining. This method was first develped for animal cells and tissues, using HRP-conjugated antibodies (Brown & Farquhar, 1989). A recombinant HRP, directly linked to a protein of interest has previously been used in mammalian cells (Connolly *et al*, 1994), and was adapted here for the first time for yeast.

A construct where the C-terminus of Hsp150 was replaced by horse radish peroxidase (Hsp150Δ-HRP; **Fig. 1E**) was expressed in *sec24-1* cells. The cells were incubated for one hour at 37°C, fixed and processed for transmission electron microscopy (TEM). In *sec24-1* cells incubated at 37°C (**Fig. 5A**), membrane structures that contained the reaction product, as well as some unstained membranes were observed. The stained membrane appeared to be continuous with the cortical ER, suggesting that the fusion protein was indeed located in the ER. In *sec23-1* cells (**Fig. 5C**), proliferated ER was observed, that contained the dark reaction product. Stained stacked structures were observed in the Golgi-specific *sec7-1* mutant cells (**Fig. 5D**). From these data it was concluded, that Hsp150Δ-HRP did remain in the ER in the absence of functional Sec24p.

2.1.4 The Hsp150 sorting signal actively mediates ER exit of invertase

The next question addressed was if the sorting determinant in the Hsp150 C-terminal domain was sufficient to recruit other reporter proteins for ER exit in *sec24-1* cells. To this end, fusion proteins containing invertase that in authentic form requires functional Sec24p for ER exit were constructed. Invertase was fused either between SUI and Cterm (SUI-invertase-Cterm; **Fig. 1F**), or to the C-terminus of SUI-Cterm (**Fig. 1G**). Both of the constructs contained the Kex2p cleavage site. Thus, cleavage at this site would indicate that the protein had been exported from the ER to the late Golgi. The experiments were carried out in normal 2% glucose-containing medium, in

which synthesis of endogenous invertase is repressed. Thus, any invertase activity would to be due to the fusion protein expressed under *HSP150* promoter.

SUI-invertase-Cterm was first expressed in *sec24-1* and in a set of control cells, and folding of invertase within the fusion protein was assessed in an activity assay. At 24°C, high levels of invertase activity were detected in the cell wall (data not shown), suggesting that the invertase portion of the fusion protein was folded into a catalytically functional and transport-competent form in *sec24-1,* and in control cells. Next, the fate of the fusion protein was studied in a pulse-chase analysis. ^{35}S-labelled cells were immunoprecipitated with Hsp150 antiserum followed by an endo-glycosidase H treatment to remove the heterogenous N-glycans of the invertase portion. After these treatments, in normal cells chased for 40 minutes, a protein migrating at 73 kDa (**Fig. 6A**, lane 4), and in *sec18-1* cells, at 81 kDa (lane 6), were observed in SDS-PAGE analysis. Since the O-glycosylated SUI is approximately 9 kDa, corresponding to the size difference of these two forms, it was deduced that the 81 kDa protein is the uncleaved ER form of SUI-invertase-Cterm, whereas the 73 kDa form corresponded to the invertase-Cterm released in the late Golgi. The same experiment repeated with the *sec24-1* cells revealed both forms in the chased sample (lane 5). Approximately half of SUI-invertase-Cterm appeared to be cleaved after the 40 minutes chase. Thus, the C-terminal domain actively recruited invertase for ER exit in the absence of functional Sec24p.

In order to elucidate if the signal as such was sufficient, or if the C-terminal domain was required to be located in the tail of the fusion protein, the experiment was repeated with the SUI-Cterm-invertase construct. The same results (lanes 1-3) as described above were obtained, indicating that the function of the sorting signal was independent of the location of C-terminal domain in the fusion protein.

2.2 Role of the Sec24p homologues, Sfb2p and Sfb3p in the absence of functional Sec24p (II, III)

2.2.1 Sfb2p is dispensable for ER exit of Hsp150 (II)

Since a functional Sec24p appeared to be dispensable for ER exit of Hsp150, we investigated the possibility that one or both of the two homologues of Sec24p, Sfb2p or Sfb3p, could replace Sec24p in a COPII coat. *In vitro*, both of the homologues have been shown to form COPII vesicles in the absence of Sec24p. However, they differ from the vesicles derived by Sec24p in size and cargo content (Miller *et al*, 2002, Higashio *et al*, 2000). Cells lacking either or both of the homologues are viable and have no detectable phenotype. In order to study, whether ER exit of Hsp150 was normal in these cells, a pulse-chase analysis was performed, and it revealed that lack of the homologues had no effect on Hsp150 secretion in the presence of a normal Sec24p (data not shown).

Whether or not the homoloues become necessary in the absence of a functional Sec24p was next studied. A *sec24-1* mutant strain lacking the *SFB2* gene was constructed, and the fate of Hsp150 was analysed by a pulse-chase experiment. Immunoprecipitation with Hsp150 antiserum revealed that Hsp150 was secreted in the double mutant strain (**Fig. 7B**). The kinetics of Hsp150 secretion appeared similar in the double mutant and the *sec24-1* strain (**Fig. 7A**). Thus, Sfb2p was found to be dispensable for ER exit of Hsp150 not only in normal

cells, but also in cells containing a mutant Sec24p, unable to function at 37°C. The only normal Sec24p family member present in the *sec24-1 Δsfb2* cells was Sfb3p. Thus, we studied next whether Sfb3p replaced Sec24p in the COPII coat.

2.2.2 Sec24-1 cells lacking SFB are viable (III)

In order to study if Sfb3p indeed was able to function as a component of the COPII coated vesicles carrying Hsp150 *in vivo*, construction of a double mutant *sec24-1 Δsfb3* was considered. However, others had reported that the combination of *sec24-1* or *sec24-11* mutations with *Δsfb3* was lethal (Roberg *et al*, 1999, Peng *et al*, 2000). Therefore, construction of a conditional mutant, in which expression of *SFB3* could be regulated, was attempted. Surprisingly, it turned out during the work, that a *sec24-1 Δsfb3* strain was viable. After tetrad dissection, the double mutant haploid spores germinated slowly. However, the generation time of these cells was only slightly longer than that of the parental *sec24-1* strain.

Structural abnormalities were observed in the *sec24-1 Δsfb3* cells in scanning electron microscopic (SEM) analysis. The SEM analysis revealed that the double mutant cells were irregularly shaped when compared to the parental *sec24-1* strain (**Fig. 1A**, 2 and 1, respectively). In addition, bud scars seemed to be located on opposite sides of the cells (**Fig. 1A**, 2, white arrows), when they would be expected to be adjacent to each other, as in normal cells. To study if the budding pattern indeed was abnormal, a calcofluor staining procedure was applied. Confocal imaging revealed that bud scars were randomly distributed around the cell in the double mutant (**Fig. 1B**, 2), whereas they were located in one end of the cell, adjacent to each other in the parental *sec24-1* cells (**Fig. 1B**, 1). Cells lacking *SFB3, SFB2* or both, but with a normal *SEC24*, had a regular unipolar budding pattern (data not shown). From these data it was concluded that the combination of the *sec24-1* mutation and *SFB3* deletion, but neither defect alone, resulted in the random budding pattern.

A TEM study to elucidate the ultrastructure of the *sec24-1 Δsfb3* cells was next carried out. The cells were incubated for 1 hour at 24 or 37°C prior to fixing and processing for TEM (see **III**, Materials and methods). It was discovered, that already at 24°C, the double mutant *sec24-1 Δsfb3* cells contained some proliferated ER (**Fig. 1C**, 3), whereas none was observed in the parental cells (**Fig. 1C**, 1). 1 hour incubation at 37°C resulted in appearance of ring-like structures consisting of 1 - 3 layers of membrane (**Fig. 1C**, 4, open arrows), some of which appeared to be continuous with cortical ER (arrow). These structures were not found in the parental cells (**Fig. 1C**, 2). The ring-like structures were most likely proliferated ER, suggesting that the double defect resulted in a strong block of anterograde traffic from the ER to the Golgi.

2.2.3 Hsp150 is secreted in sec24-1 Δsfb3 cells (III)

Since constructing a double mutant strain was succesful, it was possible to test next if complete lack of Sfb3p molecules would impede secretion of Hsp150 in *sec24-1* cells. A pulse-chase analysis was performed and cell lysates and culture media samples were assayed by immunoprecipitation with Hsp150 antiserum. Autoradiograms were quantitated by phosphoimager, and the relative amounts of mature and ER forms

of Hsp150 were plotted *versus* time. Surprisingly, deletion of *SFB3* appeared not to have any influence in the rate of Hsp150 secretion. After approximately 50 minutes, 50% of the labelled Hsp150 was found to have exited the ER, and after 2 hours of chase, 70% was in the mature form, in both cases (**Fig. 2C** and **2D**). Sfb3p appears thus to be dispensable for ER exit of Hsp150, in the absence of a functional Sec24p.

Even though no difference was detected in the kinetics of secretion, the ER-associated form of Hsp150 migrated differently in the *sec24-1* and *sec24-1 Δsfb3* of cells. In the parental cells, the Hsp150 migrated as a 97 kDa protein after pulse (**Fig. 2A**, lane 1), whereas the corresponding protein in the double mutant cells migrated at 86 kDa (**Fig. 2B**, lane 1). After the 2 hours chase, the ER forms were 121 kDa and 107 kDa, respectively (**Fig. 2A** and **2B**, lane 9). The difference between the apparent molecular weights was most likely due to Golgi-specific glycosylation that took place in the ER (**I**). It did not, however, influence the size of the mature protein that was secreted to the culture medium. Thus, it appears that immediately after the protein was exported from the ER, it received the complete Golgi-specific glycans resulting in the mature Hsp150. Thereafter Hsp150 was secreted with a similar rate after leaving the ER, regardless of the state of glycosylation upon arrival at the Golgi complex.

In order to control that the secretion of Hsp150 was due to active recruitment for ER exit rather than to bulk flow or a failure of the experimental setup, fate of a protein known to depend on a functional Sec24p for ER exit, CPY, was assayed. A pulse-chase analysis of cell lysates showed that after up to two hours of chase, CPY persisted in the ER in the double mutant (**Fig. 3B**, lane 12) as well as in the parental cells (lane 9). No Golgi-specific p2 or mature form could be observed. However, a 59 kDa form appeared during the chase, that could not represent the cytosolic pre form of the same apparent molecular weight, because cycloheximide that impedes protein synthesis was present (Stevens *et al*, 1982). No such protein was observed in the pulse samples (lanes 7 and 10), nor in the chased *sec18-1* cells, in which membrane fusion was impeded (lane 3). The 59 kDa form therefore probably represented a previously non-described partially degraded form of CPY. Thus, CPY remained in the ER in the same conditions where Hsp150 was secreted. To confirm the result, maturation of Gas1p in the double mutant and the parental cells was studied. Again, a pulse-chase analysis was performed, and cells were subjected to immunoprecipitation with anti-Gas1p antiserum. The analysis revealed, that during the 2-hour chase, the apparent molecular weight of Gas1p increased in both *sec24-1* (**Fig. 3A**, lanes 2 - 4) and the double mutant cells (lanes 5 - 7), suggesting that Gas1p acquired glycans, but remained in the ER, at 37°C. Thus, Hsp150 was secreted in the absence of Sfb3p molecules and the functional Sec24p in conditions in which two other secretory proteins, CPY and Gas1p, remained in the ER. ER export of Hsp150 appeared thus to be specific and active, rather than due to bulk flow.

Interestingly, similarly as for Hsp150, a slight difference in the glycosylation of the Gas1p ER form was observed. Pulse-forms in both cells migrated as 86 kDa proteins in SDS-PAGE (lanes 2 and 5), but after two hours of chase a sharp 88 kDa band was observed in the double mutant *sec24-1 Δsfb3* cells (lane 7), whereas in the parental strain, a 88 kDa band plus a smear reaching 93 kDa was observed

(lane 4). For some reason, Gas1p thus appeared to be heterogenously glycosylated in the *sec24-1* cells, but not in the cells lacking also *SFB3*. Lack of Sfb3p alone has been previously reported to slow down maturation of Gas1p (Peng *et al*, 2000), suggesting that Sfb3p might be involved in sorting of Gas1p into ER-derived transport carriers. Thus, perhaps in the double mutant cells, sorting of Gas1p into COPII-coated ER exit sites is slower than in the parental cells, whereas glycosylating enzymes targeted to the Golgi are more efficiently recruited to such sites. The enzymes and the substrate would thus encounter each other less frequently in the double mutant cells. Alternatively, Sfb3p might have a role in the recycling of the Golgi mannosyltransferases.

2.2.4 A triple mutant sec24-1Δsfb3 Δsfb2 is viable but exhibits a severe phenotype (III)

Since Hsp150 was secreted in the *sec24-1* double mutant cells lacking either *SFB2* or *SFB3*, it appeared likely that either Sfb2p or Sfb3p could replace Sec24p in the COPII coat. To test this hypothesis, a *sec24-1* mutant strain lacking both of the two homologous genes was constructed. The strain was viable, but it had a very long generation time (6.5 h) compared to the *sec24-1* and *sec24-1 Δsfb3* strains (~3 h and 4 h, respectively). A SEM analysis revealed further defects in the triple mutant cells. Cells grown at 24°C, to the early logarithmic phase, were found to be irregularly shaped (**Fig. 4A**). The cell wall was collapsed forming depressions (white arrows) similar to those observed on normal cells grown to the stationary phase (not shown). Since the depressions appeared in normal aged, dying cells, it was deduced that the triple mutant cells were short-lived. Several cells with two undetatched buds were also observed (**Fig. 4A**, insert), indicating that the cells suffered some defects in cell separation. A calcofluor staining showed that the triple mutant had a random budding pattern (**Fig. 4B**). The TEM study confirmed the abnormalities in cell shape and budding. The cell in **Figure 4C-1** exhibits a strech of cell wall that protrudes into the cell (double headed arrow), suggesting that budding of a daughter cell was aborted. In addition, there appeared to be multiple nuclei in the cell (N). This was perhaps due to abortive budding of daughter cells. Alternatively, the shape of the nucleus in the triple mutant cell may have been abnormal resulting in the nucleus appearing divided in the thin section. In addition to the defects in shape and cell division, extensive accumulation of ER was observed in these cells already at the permissive temperature (**Fig. 4C-2**, arrows). Incubation at 37°C resulted in further ER proliferation, and carmellae-like structures consisting of several layers of membrane were observed (**Fig. 4C-3**, open arrows). Since lack of Sfb2p and Sfb3p resulted in an exaggerated accumulation of ER in the *sec24-1* mutant cells, it appears that the homologues of Sec24p indeed are involved in ER-to-Golgi traffic, and that these Sec24p family members have overlapping functions.

2.2.5 Hsp150 is secreted in the triple mutant sec24-1Δsfb3 Δsfb2 (III)

Secretion of Hsp150 in the triple mutant was studied next. A pulse-chase analysis followed by immunoprecipitation and quantitation of the autoradiogram was performed as described before. It was found that Hsp150 could exit the ER even in the triple mutant. A small amount of mature Hsp150 appeared in the culture medium after 15 minutes of chase (**Fig. 5A**, lane 6), and after the two hours chase, approximately half of Hsp150 had

been exported from the ER (**Fig. 5B**). The ER forms (**Fig. 5A**, lanes 1 - 5) migrated similarly as in the *sec24-1 Δsfb3* cells (**Fig. 2B**, lanes 1 - 5). The rate of secretion was clearly slower in the triple mutant than in the *sec24-1 Δsfb3* or *sec24-1* cells. Also ER proliferation was more pronounced in the triple mutant, indicating that anterograde traffic was severly impaired. Since retrograde traffic in these cells was not impeded, and Golgi proteins are known to recycle through the ER, it could be that the Golgi complex was rapidly consumed by the active COPI-mediated recycling after imposing the temperature block. Thus, only remnants of the late Golgi compartment containing the protease Kex2p that does not recycle to the ER, would remain. The remaining Golgi elements could not process the Hsp150 polypeptide to the mature form as rapidly in the triple mutant as in the double mutant cells. Hsp150 found in the culture medium was in the mature form, indicating that it had been cleaved by Kex2p. Thus, Hsp150 did not bypass the Golgi. In conclusion, Hsp150 was able to exit the ER in cells lacking a functional Sec24p and both of the Sec24p homologues.

2.3 Sec24p family proteins appear to be dispensable for ER exit of Hsp150 (III)

Based on the studies using temperature sensitive *sec24-1* mutant cells, a functional Sec24p was dispensable for Hsp150 ER exit. However, in these cells, mutant Sec24p molecules are present, and they could be involved in mediating ER exit of Hsp150. Sequencing of the *sec24-1* locus showed that the mutation in the *SEC24* gene results in replacement of the last 35 C-terminal amino acids of Sec24p with 8 different amino acids. Thus, the mutation affects a small region of Sec24p, and the mutant protein might still function as a semi-functional COPII component, at 37°C. In order to rule out the possibility that the mutant Sec24p mediated Hsp150 secretion, a strain lacking the *SEC24* gene was constructed.

2.3.1 Construction of the Δsec24 strain

Deletion of *SEC24* is lethal. Therefore, a mutant strain carrying an epitope-taggeg *SEC24* under a controllable promoter, and lacking the original *SEC24* gene, was constructed (see **III**, Materials and methods, for details). A tetracycline-regulated direct dual system allowing tightly switching off of a gene, was used (Belli *et al*, 1998). The *SEC24-HA* gene was expressed under the tetracycline-regulated *tetO* promoter, and a tetracycline-activable repressor element plus a tetracycline-inactivable activator component were expressed in the same cells. Thus, in the absence of tetracycline, *SEC24-HA* was expressed, and upon addition of tetracycline (or its derivative, doxycycline), expression of *SEC24-HA* was turned off.

In order to find optimal conditions for carrying out the assesment of Hsp150 secretion, depletion of Sec24p-HA was first studied. To this end, cells were incubated in the presence of doxycycline for up to 36 hours, and cell lysate samples were analysated in a Western blot assay with an anti-HA antibody. The assay revealed that 24 hours after doxycycline addition Sec24p-HA could no longer be detected in the cells (**Fig. 6**, lane 3). Next, the protein synthesis under repressing conditions was assayed. Cells were ^{35}S-labelled after 24 - 48 hours of incubation in the presence of doxycycline, and Hsp150 was immunoprecipitated. The assay revealed, that 24 hours after doxycycline addition Hsp150 was still efficiently synthesized. After 32 hours of incubation with doxycycline, efficiency of labelling had decreased to

30% of that observed after 24 hours. 24 hour repression thus appeared to be sufficient for Sec24p-HA to be degraded from the cells, but still allowed a metabolic labelling experiment.

2.3.2 Hsp150 is secreted in the absence of Sec24p

A pulse-chase experiment was performed to analyse secretion of Hsp150. Prior to labelling, cells were incubated in the presence of doxycycline to turn down *SEC24-HA* expression. For control, the same cells were grown in the absence of the antibiotic. The labelling was carried out at 37°C, in order to upregulate the *HSP150* promoter (Russo *et al*, 1993). The pulse-chase analysis demonstrated that Hsp150 could be secreted in the absence of Sec24p. After 15 minutes of chase, some Hsp150 already appeared in the culture medium (**Fig. 7C**, lane 6), and approximately 40% was secreted after 1 hour (lane 8). In the control cells, no ER form could be detected in the cell lysates (**Fig. 7C**, lanes 1 - 5), and maximal secretion was already observed in the first culture medium sample taken after 15 minutes of chase (**Fig. 7B**). Thus, in the *Δsec24* mutant incubated without doxycycline, secretion of Hsp150 was similar as in normal cells, where the half time of secretion is 2 minutes (Jämsä *et al*, 1994). Kinetics of Hsp150 secretion was slower in the absence of Sec24p than in the *sec24-1* cells (**Fig. 7D** and **2C**, respectively). This difference could be an indication that the mutant Sec24p was involved in Hsp150 secretion in the temperature-sensitive mutant cells. However, the experimental conditions were very different. The temperature-sensitive cells were incubated at 37°C for 15 minutes prior to labelling, whereas the *Δsec24* cells were incubated for 24 hours at 24°C after switching off of the *SEC24-HA* gene. The kinetics measured using the two approaches can therefore not be directly compared.

In order to control the experimental setup, maturation of CPY was assayed in the very same ^{35}S-labelled cells, as above. In the presence of doxycycline, CPY remained in the ER after 2 hours chase (**Fig. 8A**, lane 4). Maturation of invertase was also assayed. Samples of the same cells used for metabolic labelling were incubated in a low glucose medium to derepress the expression of invertase, and cell lysates were analysed by the activity staining procedure. In the presence of the antibiotic, invertase was observed to remain in the ER form (**Fig. 8B**, lane 2). Finally, a Western blot analysis as described previously was also carried out, and no Sec24p-HA was detected (data not shown). Taken together, Hsp150 was secreted to the medium in cells lacking Sec24p, whereas two other secretory proteins, invertase and CPY, remained in the ER.

2.3.3 Hsp150 is slowly secreted in the absence of all Sec24p family members

Finally, in order to investigate if the Sec24p homologues were needed for ER exit of Hsp150 in cells lacking Sec24p, strains lacking either or both of the homologues in the *Δsec24* background (*Δsec24 Δsfb2, Δsec24 Δsfb3, Δsec24 Δsfb2 Δsfb3*; see **Table 5** for details), were constructed. Secretion of Hsp150 was then assessed in all of the three strains, in similar conditions as described above. The same controls, maturation of CPY and invertase, and Western blot analysis, were repeated for each experiment. Pulse-chase analyses were performed in all three strains. In the cells lacking either Sfb2p or Sfb3p, in addition to Sec24p, Hsp150 secretion was similar as in the parental *Δsec24* cells (data not shown). Surprisingly, even in the triple deletion mutant, some Hsp150 was found

in the culture medium already after 15 minutes of chase (**Fig. 7E**, lane 6), and approximately 30% was secreted after 2 hours (**Fig. 7F**). The ER forms migrated faster in the triple mutant (**Fig.7E**, lanes 1 - 5) than in the *Δsec24* cells (**Fig.7C**, lanes 1 - 5), similarly as in the corresponding temperature-sensitive mutants described in section 2.2.3.

The rate of secretion of Hsp150 was very slow in the triple deletion mutant compared to the *Δsec24* cells. However, CPY and invertase were quantitatively found in the ER in the same conditions (data not shown), suggesting that albeit slow, the secretion of Hsp150 was significant. In addition, as discussed earlier, the rate of Hsp150 secretion may have been reduced due to the diminished Golgi complex resulting from the COPI mediated recycling from the Golgi to the ER. In conclusion, all Sec24 family proteins appear to be dispensable for ER exit of Hsp150.

2.4 Selective recruitment of Hsp150 for ER exit (II, III)

In this study it has been shown that the soluble secretory glycoprotein Hsp150 is secreted under conditions in which other soluble secretory cargo proteins remain in the ER (**II**, **III**). The sorting of Hsp150 for ER exit thus appears to be selective, rather than due to bulk flow. A recent report shows that soluble cargo proteins also contain ER exit motifs. A hydrophobic ILV motif has been found to mediate binding of pro-α-factor to Erv29p and packaging to COPII vesicles. The ILV motif is located in the pro-region of pro-α-factor. The motif promotes secretion of the normally ER-resident protein BiP, suggesting that ER exit motifs predominate over ER retention mechanisms (Otte & Barlowe, 2004). Here, the sorting signal responsible for the selective ER exit of Hsp150 in the absence of a functional Sec24p component was mapped and found to be located in the C-terminal domain of Hsp150 (**II**, **Fig.1A**, amino acids 299-413). In previous work, the C-terminal domain has also been shown to mediate Sec13p-independent ER exit (Fatal *et al*, 2002). Another domain of Hsp150, the repetitive region (**II**, **Fig.1A**, RR), mediates sorting of Hsp150 for ER exit in the absence of a functional Sec21p component of COPI (Suntio *et al*, 1999).

The coat components are cytosolic proteins. Hsp150 can therefore not directly interact with the COPII components. Instead, a transmembrane adaptor protein that associates with COPII components and provides a receptor domain for the lumenal Hsp150 cargo probably exists. Such adaptors have been found to mediate ER exit of other lumenal cargo. For instance, the GPI-anchored Gas1p requires the transmembrane protein complex Emp24p/Erv25p for efficient ER exit. *In vitro*, efficiency of packaging of Gas1p into COPII vesicles is reduced in the absence of Emp24, and Gas1p can be chemically cross-linked to Emp24p in *in vitro* generated ER-derived vesicles (Muñiz *et al*, 2000). In addition, Emp24p contains a cytosolic ER exit motif that interacts with the COPII coat, and Erv25p has a dilysine-motif that binds to COPI (Belden & Barlowe, 2001b). These findings showing, that Emp24p/Erv25p complex cycles between the ER and the Golgi complex, and that it binds both coat proteins and the cargo molecules, indicate that the complex indeed functions as a cargo adapter. Erv29p has been reported to be the adapter for packaging pro-α-factor. Efficient packaging of pro-α-factor requires Erv29p *in vitro*, and *in vivo* deletion of *ERV29* results in accumulation of pro-α-factor and CPY in the ER whereas invertase and

Gas1p are exported normally (Belden & Barlowe, 2001a). Since Hsp150 contains at least two different signatures for ER exit, perhaps more than one cargo receptor exist for Hsp150. Alternatively, the putative receptor or receptor complex may interact with Hsp150 *via* multiple domains. In ongoing work, candidates for an Hsp150 receptor are being searched for in our laboratory.

Hsp150 is able to exit the ER under conditions where other cargo proteins remain in the ER. Secretion of Hsp150 is rapid and the *HSP150* gene is strongly expressed at 37°C. One could thus assume that Hsp150 performes an important function in the secretory pathway under heat shock conditions. The deletion of *HSP150* however has no obvious phenotype (Russo *et al*, 1992). Three genes coding for covalently linked cell wall proteins, homologous to Hsp150, exist in yeast, *PIR1/CCW6*, *PIR3/CCW8* and *PIR4/CCW5*. None of these genes is upregulated at 37°C, nor is any of them essential. Even a quadruple mutant lacking *HSP150* and the three homologous genes is viable, but exhibits defects in the cell wall. The quadruple mutant cells are irregularly shaped and larger than wild type cells, and they are highly sensitive to cell wall synthesis inhibitors. The mating ability of these cells is decreased, but the thermal stability is not significantly altered (Mrsa *et al*, 1997, Mrsa & Tanner, 1999). Thus, function of the *PIR* family proteins, including Hsp150, appears to be in stabilizing the cell wall. However it seemes unlikely, that this is the only function of Hsp150. The efficiency of secretion and the ability of Hsp150 to escape from the ER when COPII components are deficient, or even lacking, may indicate a more sophisticated role. Hsp150 might perform some function inside the cell. Perhaps the ability of Hsp150 to exit the ER when other cargo proteins remain inside is an indication of a chaperoning role: Hsp150 might escort other proteins to exit the ER under stress conditions. Indeed, further investigation is required to elucidate the function of Hsp150 in the secretory pathway.

2.5 Formation of anterograde transport carriers in the absence of Sec24p (II, III)

The finding that Hsp150 could exit the ER in cells lacking Sec24p is puzzling. How can ER-derived carrier vesicles be formed in these cells? *In vitro*, the minimal components that the COPII vesicle budding machinery requires, have been shown to be Sar1p, Sec23p/24p complex and Sec13p/31p complex (Matsuoka *et al*, 1998). However, Sec24p is not absolutely indispensable, since both of its homologues are able to drive COPII vesicle formation in the absence of Sec24p, and overexpression of *SFB2* has been found to suppress the lethality caused by deletion of *SEC24* (Miller *et al*, 2002, Roberg *et al*, 1999, Higashio *et al*, 2000, Peng *et al*, 2000, Shimoni *et al*, 2000). Suppressors have also been discovered for deletion of the essential *SEC13* gene. Mutations in *BST1, BST2/ EMP24* or *BST3* rescue the lethal phenotype caused by *Δsec13,* and ER-to-Golgi transport of CPY and invertase were shown to function normally in these cells. Interestingly, in these mutants, an increased amount of the resident ER proteins BiP and Pdi1p was found to escape from the ER. The authors proposed that the *BST* genes code for proteins that impede budding of COPII vesicles with defective or missing subunits, thus performing a negative regulator function (Elrod-Erickson & Kaiser, 1996). Normal Sec13p function has been found to be dispensable for ER exit of Hsp150 in cells with normal *BST*

genes (Fatal *et al*, 2002). Thus, it appears that ER-derived transport vesicles may be covered with varying compositions of COPII proteins, or even lack essential components.

Vesicles generated by Sfb2p or Sfb3p only contain a subset of the cargo proteins packaged into Sec24p containing vesicles. Importantly, Sfb3p generated vesicles lack SNARE proteins and can therefore not fuse with the Golgi membrane (Miller *et al*, 2002, Roberg *et al*, 1999, Higashio *et al*, 2000, Peng *et al*, 2000, Shimoni *et al*, 2000). One could anyway speculate that in the absence of Sec24p, the homologues could generate transport carriers that would mediate Hsp150 transport *in vivo*. In normal cells, the homologues may co-exist with Sec24p in COPII vesicles, thus extending the repertoire of the cargo sorting machinery.

But what could be the mechanism mediating Hsp150 ER export in the absence of the entire family of Sec24p proteins? Perhaps *in vivo*, aberrant transport vesicles are formed in such conditions. Since the Sec24p family members serve an important role in cargo selection, vesicles lacking the Sec24p component would probably be unable to recruit cargo, except for Hsp150. Some possible models for how ER export might be accomplished in the absence of Sec24p family proteins are presented in **Figure 11**. First (A), an aberrant COPII coat might form in the absence of a binding partner for Sec23p. Such coats might be stabilized by Sec16p that interacts with Sec23p and Sec31p (Shaywitz *et al*, 1997), or perhaps the putative adaptor of Hsp150, for long enough to support recruitment of Hsp150 and budding of the vesicle. However, since vesicles generated with Sfb3p instead of Sec24p do not recruit SNARE proteins (Miller *et al*, 2002), a vesicle lacking a Sec24 family member could probably not recruit SNARE proteins needed for delivery of the cargo, making the model unlikely. In the second model (B), a component X could form a complex with Sec23p and support COPII formation. Sec23p and Sec24p are structurally similar. They only share 14% sequence homology, but a crystal structure analysis has revealed that the polypeptides fold to highly similar subunits (Bi *et al*, 2002). Thus, the component X in the second model might be Sec23p itself (B-1). However, as in model A, packaging of the SNAREs would be a problem in this model as well. The component X could rather be a novel, previously uncharacterized protein that interacts with Sec23p, and is able to promote recuitment of SNAREs. However,

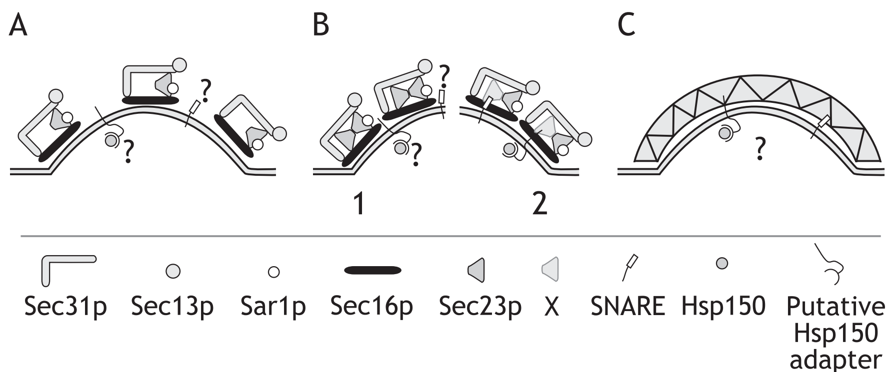

Figure 11. Models for generating carriers to mediate ER exit in the absence of Sec24p family proteins. See text for descriptions.

no indications of existence of such a component have been reported as yet. In the third model (C), Hsp150 uses an ER exit route completely independent of COPII. This, however, also appears unlikely, since Hsp150 has been shown to require functional Sec31p, Sec23p and Sec12p components for ER exit (Fatal *et al*, 2002).

All of these models are completely speculative and a lot of work is still required to answer all the questions. For instance, an *in vitro* budding assay should be performed to analyse if generation of vesicles containing Hsp150 in the absence of Sec24p can be reproduced in a cell-free system. Also a thorough TEM assay might help to elucidate, whether COPII vesicles are indeed formed in cells lacking all Sec24 family members, and whether Hsp150 can be found inside of ER derived vesicles *in vivo*.

3. Concluding remarks

The present study shows that the normally Golgi-localized N- and O-glycosylating mannosyltransferases recycle through the ER in yeast. We found that the mannosyltransferases excert their activity in the ER, but recycling in normal cells is rapid and the enzymes do not frequently encounter their substrates in the ER. The Golgi complex appears to be a highly dynamic organelle and the recycling may be an important means of maintaining its organelle integrity. However, the biological function of recycling of Golgi-proteins remains to be elucidated.

This study provides evidence that *in vivo*, COPII vesicles covered by different compositions of COPII components are formed. The Sec24p component is shown to be dispensable for ER exit of the secretory yeast glycoprotein Hsp150. Under normal conditions, Sec24p probably co-exists with its homologues Sfb2p and Sfb3p in COPII coats. Since Sec24 family proteins are involved in cargo sorting into COPII vesicles, the COPII coats composed of different sets of components may be specialized in packaging of different cargo proteins, thus contributing to recognition of the variety of ER exit motifs on cargo proteins. In the absence of Sec24p, Sfb2p or Sfb3p may replace Sec24p in the COPII coat, or perhaps an aberrant coat lacking a binding partner for Sec23p is formed.

Hsp150 is specifically recruited for ER exit in the absence of Sec24 function, whereas other exocytic proteins remain in the ER. A signature guiding Hsp150 for ER exit is located in the C-terminal domain of the protein. A chimeric protein consisting of a reporter portion that requires the authentic Sec24p for ER exit, and the signature containing domain of Hsp150, is also recruited for secretion, indicating that the signature function is active and specific. Previously, also function of another COPII component, Sec13p, has been been found dispensable for ER exit of Hsp150. Even though Hsp150 is not an essential protein, the exceptional ability of Hsp150 to exit the ER when other proteins are trapped suggests that it could have an important role in the secretory pathway. Elucidating the biological function for Hsp150 indeed is a challenge for further investigation.

Hsp150 is a soluble protein and can therefore not directly interact with the cytosolic COPII coat. An adapter protein, whose lumenal part is a receptor for Hsp150, and which can interact with the COPII coat components with a cytosolic portion, must exist. Identifying the putative receptor is one of the main aims of current research in our laboratory.

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