# Fate of Mammalian Golgi Sialyltransferases in Yeast

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When we eliminate the impossible, whatever remains, however improbable, must be true.

Sherlock Holmes

**To Karoliina and Jarkko**

# CONTENTS

## ORIGINAL PUBLICATIONS

This thesis is based on the following articles and a manuscript, which are referred to in the text by their Roman numerals I-IV.

I      **Sievi E.**, Suntio T. and Makarow M. (2001). Proteolytic function of GPI-anchored plasma membrane protease Yps1p in the yeast vacuole and Golgi. *Traffic* **2**:896-907.

II      **Sievi E.**, Helin J., Heikinheimo R. and Makarow M. (1998). Glycan engineering of proteins with whole living yeast cells expressing rat liver $\alpha$2,3-sialyltransferase in the porous cell wall. *FEBS Lett.* **441**:177-180.

III      **Sievi E.**, Hänninen AL. and Makarow M. Comparison of the Hsp150$\Delta$ and MF$\alpha$ carriers in expression of rat $\alpha$2,3-sialyltransferase in yeasts. Manuscript.

IV      Kapteyn JC., Van Egmond P., **Sievi E.**, van den Ende H., Makarow M. and Klis FM. (1999). The contribution of the O-glycosylated protein Pir2p/Hsp150 to the construction of the yeast cell wall in wild-type cells and $\beta$1,6-glucan-deficient mutants. *Mol. Microbiol.* **31**:1835-1844.

# ABBREVIATIONS

| | |
|---|---|
| ALP | alkaline phosphatase |
| AP | adaptor protein |
| ARF | adenosine diphosphate-ribosylation factor |
| ATP | adenosine triphosphate |
| BFA | brefeldin A |
| CD | circular dichroism |
| CHX | cycloheximide |
| CMP | cytidine monophosphate |
| COP | coat protein |
| CPS | carboxypeptidase S |
| CPY | carboxypeptidase Y |
| CWP | cell wall protein |
| DAG | diacylglycerol |
| DIG | detergent-insoluble glycolipid-enriched complex |
| Dol-P | dolichol phosphate |
| DTT | dithiotreitol |
| ER | endoplasmic reticulum |
| ERAD | ER associated degradation |
| EtNP | ethanolaminephosphate |
| Gal | galactose |
| GalNAc | N-acetylgalactosamine |
| GAP | GTPase activating protein |
| GDP | guanine diphosphate |
| GEF | guanine nucleotide exchange factor |
| GGA | Golgi-localized, gamma-ear-containing, ARF-binding proteins |
| GTP | guanine triphosphate |
| GlcNAc | N-acetylglucosamine |
| GPI | glycosylphosphatidylinositol |
| GPI-CWP | GPI-dependent cell wall protein |
| GRAS | generally regarded as safe |
| Hsp | heat shock protein |
| KDa | kilodalton |
| LacNAc | N-acetyllactosamine |
| LNT | lacto-N-tetraose |
| MALDI-TOF | matrix-assisted laser desorption/ionization time-of-flight |
| Man | mannose |
| MVB | multivesicular body |
| NeuNAc | sialic acid |
| NSF | N-ethylmaleimide sensitive fusion protein |
| OST | oligosaccharyl transferase complex |
| PDI | protein disulphide isomerase |
| Pir | protein with internal repeats |
| PrA | proteinase A |
| PrB | proteinase B |
| PVC | prevacuolar compartment |
| SDS-PAGE | sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SNAP | soluble NSF attachment protein |
| SNARE | SNAP receptor |
| SRP | signal recognition particle |
| ST3N | Galβ1-3(4)GlcNAc α-2,3-sialyltransferase |
| ST6N | Galβ1-4GlcNAc α2,6-sialyltransferase |
| TCA | trichloroacetic acid |
| TGN | *trans*-Golgi network |
| TM | tunicamycin |
| UPR | unfolded protein responce |
| UPRE | unfolded protein responce element |
| *VPS* | vacuolar protein sorting |
| Wt | wild type |

# SUMMARY

The capability of the yeasts *S. cerevisiae* and *P. pastoris* to produce and secrete enzymatically active rat liver Galβ1-3(4)GlcNAc α-2,3-sialyltransferase (ST3N) and Galβ1-4GlcNAc α2,6-sialyltransferase (ST6N) was explored. The folding and ER exit of the transferases were facilitated by fusing the ectodomains of the enzymes to *S. cerevisiae* derived carrier polypetides, Hsp150Δ and MFα. Expression of Hsp150Δ-ST6Ne in *S. cerevisiae* resulted in a secretion competent and catalytically active recombinant protein. Despite of the apparently correct conformation of the ST6Ne portion, the fusion protein was not externalized, but directed from the Golgi to the vacuole for degradation. However, Hsp150Δ-ST6Ne could be used as a marker for intracellular protein degradation, which revealed a novel relationship between the proteolytic activity of the vacuole and the sterol composition of membranes. Disturbed ergosterol biosynthesis was found to restore the proteolytic function of the vacuole in cells lacking the gene for proteinase A, the central activator of vacuolar hydrolases. The rescue of vacuolar function was found to be due to Yps1p, a GPI-anchored aspartic protease, which normally resides in the yeast plasma membrane, but was targeted to the vacuole in the absence of membrane ergosterol. Furthermore, a novel site for degradation of heterologous proteins was found, as Hsp150Δ-ST6Ne was also degraded in the Golgi by newly synthesized Yps1p molecules, *in transit* to the plasma membrane. In contrast to ST6Ne, α-2,3-sialyltransferase (ST3Ne) had been earlier shown to be stable and transported to the yeast cell wall. Here we studied the capability of high molecular weight, native asialoglycoproteins to penetrate the cell wall of the recombinant yeast by incubating the protein substrates with intact living yeast cells, and the nucleotide sugar donor. The reactions led to sialylation of the N-glycans of the acceptor proteins, indicating that the substrates had free access to cell wall-borne ST3Ne, and diffused then back to the reaction mixture. The sialylation pattern of the different asialoglycoprotein substrates obeyed the acceptor substrate specificity reported for ST3N. The kinetic parameters of Hsp150Δ-ST3Ne were similar to those of the commercially available recombinant ST3N, as well as the authentic rat liver ST3N. To improve the yields, Hsp150Δ-ST3Ne was expressed in *P. pastoris* under the strong *AOX1* promoter. Also in *P. pastoris* Hsp150Δ-ST3Ne was incorporated in the cell wall as an active transferase, but the yields were not significantly higher as compared to *S. cerevisiae*. Furthermore, ST3Ne was cell wall-bound and active when expressed with the aid of the commercial MFα carrier in both *S. cerevisiae* and *P. pastoris*, whereas the yields were lower as compared to the Hsp150Δ carrier. The incorporation of Hsp150Δ-ST3Ne to the cell wall was through non-covalent interactions, and apparently due to the ST3Ne portion. Authentic Hsp150 was shown to bind covalently to cell wall β1,3-glucan, *via* its C-terminal domain.

# INTRODUCTION

The budding yeast *Saccharomyces cerevisiae* is one of the best characterized experimental systems in modern biology. It is a unicellular eukaryote, which shares many genes, organelles and functions with higher eukaryotes. The secretory pathway of *S. cerevisiae* resembles that of the mammalian cell, providing tools for studies aiming at understanding basic cell biology of eukaryotes, protein functions and interactions. Access to the complete genome sequence of *S. cerevisiae*, available since 1996 (Goffeau et al., 1996), has had a great impact on basic yeast research and on the development of new techniques, as well as on the understanding of many human hereditary diseases and genome evolution. A vast amount of information dealing with cell functions and pathologies is still likely to be generated after the systematic functional analysis of novel yeast genes carried out by individual gene disruption has been completed (Oliver, 1996).

While *S. cerevisiae* is an important model organism in basic research, it is also a classical micro-organism exploited in biotechnology. It has been utilized for thousands of years in brewing and baking, and as a result there is confidence that the organism is safe as classified by the GRAS status (generally regarded as safe),

an important aspect for the *S. cerevisiae* utilizing industry. Among the modern biotechnical applications of *S. cerevisiae* is the production of heterologous proteins for research and therapeutic applications. As the majority of the desired products are secretory proteins, post-translational modifications for correct folding and full activity are required, many of which can be provided by the yeast secretory apparatus. Other reasons for considering yeast as the host candidate for heterologous expression include the well-developed fermentation and process technologies for large-scale protein production, and the sophisticated techniques for targeted gene disruption and replacement. Furthermore, yeast cultivation on inexpensive growth media is easy, and there are no ethical concerns when working with it. However, *S. cerevisiae* exhibits some biological restrictions as an expression host, such as a tendency for protein hyperglycosylation, an incapability to synthesize certain mammalian-like glycans, and a moderate capacity to secrete proteins. Thus, while heterologous expression of secretory proteins in yeast was made possible by combining understanding of the yeast secretory apparatus with recombinant-DNA-technology, improvement of the system requires further and deeper understanding of basic cell biology.

# 1 Secretory pathway

The intracellular route of secretory proteins was originally defined in mammals (Palade, 1975), and was shown to be basically the same in *S. cerevisiae* (Novick et al., 1981). The secretory pathway of eukaryotic cells consists of different membrane-bound organelles, each with specialized functions and components, through which secretory proteins move while being modified by glycosyltransferases, glycosidases, proteases, and other enzymes. The movement of proteins from one organelle to another is carried out in transport vesicles, most of which are directed towards the cell surface. The cargo proteins are incorporated in the budding vesicles at the donor membrane and delivered to the subsequent compartment by fusion of the vesicles at the target membrane. Cytosolic coat proteins help in selecting the cargo molecules and form a coat on the budding vesicle, while vesicle fusion is carried out by specialized membrane proteins. The ultimate purpose of the secretory pathway in *S. cerevisiae* is to generate and deliver new membrane and proteins to the growing surface of the bud (Novick and Schekman, 1983; Schekman and Orci, 1992). A schematic picture of the yeast secretory pathway is presented in Figure 1.

The isolation and characterization of temperature sensitive secretory (*sec*) yeast mutants that reversibly accumulate secretory proteins at the restrictive temperature of 37°C, but have no secretory block at the permissive temperature of 25°C (Novick and Schekman, 1979; Novick *et al.*, 1980) has provided tools for investigating the molecular mechanisms that underlie the secretory pathway.
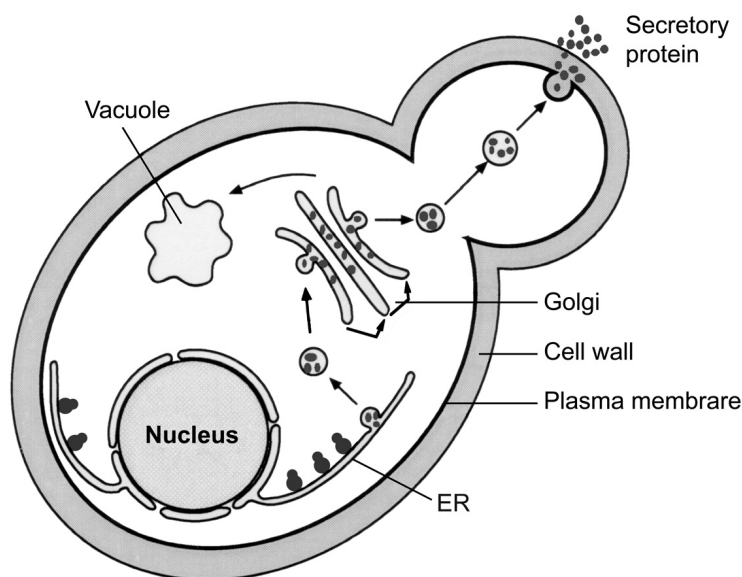


**Figure 1.** The secretory pathway of *S. cerevisiae*. Proteins enter the secretory pathway by translocating into the ER lumen. After folding, exocytic proteins are packaged into transport vesicles, which fuse with the Golgi complex. While being modified in the Golgi, the proteins move through the organelle in *cis*-to-*trans* direction. In the *trans*-Golgi network the cargo is sorted to the vacuole, plasma membrane, cell wall or culture medium.

## 1.1 Translocation into the endoplasmic reticulum (ER)

To enter the secretory pathway, all secretory proteins must cross the ER membrane. Targeting of proteins for translocation into the ER lumen is either co- or post-translational. In co-translational translocation the nascent polypeptide chain enters the ER lumen while being translated and bound to the ribosome. Post-translational translocation takes place after the precursor protein is fully translated and released from the ribosome. In mammalian cells the proteins are targeted to the ER primarily co-translationally, while yeast uses both pathways (Walter and Johnson, 1994). Targeting is determined by the hydrophobicity of the signal peptide located in the N-terminus of the precursor protein. The co-translational translocation-mediating signal recognition particle (SRP) binds signal peptides with hydrophobic cores, whereas less hydrophobic sequences are not bound, and these precursor proteins use the post-translational route (Ng *et al.*, 1996). Signal peptides are not conserved, but usually contain about 20 amino acids which carry a net positive charge, a hydrophobic region of 7-15 residues and a signal peptidase cleavage region of 3-7 polar residues (von Heijne 1985; 1990).

In co-translational translocation SRP binds to the signal peptide of the nascent polypeptide in the cytoplasm, and targets the ribosome-associated complex to the ER membrane by interacting with the SRP-receptor (Walter and Johnson, 1994). Translocation from the cytoplasm to the ER lumen requires the heterotrimeric Sec61p translocation complex (Sec61p, Sss1p, Sbh1p), which forms an aqueous transmembrane channel in the ER membrane, the translocon (Johnson and van Waes, 1999). After binding of the nascent chain-ribosome complex to the Sec61p complex, SRP is released and recycled back to the cytosol. The ribosome interacts tightly with the Sec61p complex and seals the translocon at the cytosolic surface allowing the elongating polypeptide chain to be extended only into the translocation channel (Matlack *et al.*, 1998). Post-translational targeting of fully translated polypeptide chains to the cytosolic surface of the ER membrane is SRP-independent, but requires the heptameric Sec complex of Sec62/63p (Sec62p, Sec63p, Sec71p, Sec72p) and Sec61p. The Sec62/63p complex appears to function as the putative signal peptide receptor, although precursor binding to the ER membrane also requires an intact Sec61p complex (Lyman and Schekman 1997; Matlack *et al.*, 1997; Pilon *et al.*, 1998). While cytosolic Hsp70 protein chaperones have been proposed to function in keeping the fully translated protein in a translocation competent, unfolded state (Chirico *et al.*, 1988), Paunola *et al.* (1998, 2001) demonstrated, that translated polypeptide chains associated with cytoplasmic Hsp70 can fold into native-like structures before post-translational translocation. However, an unfolding step was shown to be required before translocation into the ER lumen. The luminal ER chaperone Kar2p drives the post-translational translocation by using energy from ATP hydrolysis to pull the precursor protein into the ER lumen through the Sec61p translocation channel (Matlack *et al.*, 1998). A similar role has been suggested for Lhs1p, another lumenal Hsp70 family chaperone (Craven *et al.*, 1996). The results so far obtained for the role of Kar2p or other lumenal ER chaperones in co-translational translocation are controversial (Brodsky *et al.*, 1995; Oliver *et*

*al.*, 1995; Wilkinson *et al.*, 1997). However, there is recent evidence that some lumenal ER proteins and the Sec63p are also essential for co-translational translocation (Tyson and Stirling, 2000; Young et al., 2001).

## 1.2 Protein maturation in the ER

In the ER secretory proteins destined for the plasma membrane, cell exterior, secretory or endocytic organelles mature into their transport-competent conformation. Maturation begins as the newly synthesized proteins enter the ER lumen by the removal of the signal peptide, catalyzed by the heterotetrameric signal peptidase. In certain membrane proteins the signal peptide can serve as the membrane anchoring transmembrane domain, and thus remain uncleaved (YaDeau *et al.*, 1991; Schatz and Dobberstein, 1996).

### 1.2.1 Folding

The ER provides an environment optimized for folding, oxidation and oligomeric assembly of proteins translocated into the lumen of the organelle. Folding in the ER is assisted by conventional folding enzymes as well as by molecular chaperones, which recognize and stabilize partially folded structures during polypeptide folding, assembly and disassembly (Gething and Sambrook, 1992). The synthesis of properly folded and secretion-competent proteins is vital for intracellular communication and cellular function, thus the ER quality control system is essential for excerting stringent selection before allowing proteins to exit the ER (Ellgaard and Helenius, 2001).

Formation of covalent disulphide bonds between the correct pairs of cysteine residues is essential for proper folding and ER exit of many secretory proteins. This was exemplified by Jämsä *et al.* (1994), who showed that in the presence of the reducing agent DTT normally disulphide bonded proteins accumulate in the yeast ER. Protein disulphide isomerase (PDI) is the principal catalyst for native disulphide bond formation in the yeast ER lumen (Frand and Kaiser, 1999; Frand *et al.*, 2000). In addition, PDI functions as an isomerase, that reorganizes non-native disulphide bonds in immature protein intermediates during their folding (Laboissière *et al.*, 1995). Deletion of *PDI1* is lethal, but can be suppressed by overexpressing either of the two PDI homologs of *S. cerevisiae*, *EUG1* or *MPD1* (Tachibana and Stevens, 1992; Tachikawa et al., 1995). The activity of PDI is based on the two pairs of reactive cysteine motifs (-Cys-Xaa-Xaa-Cys-) located within its thioredoxin fold (Ferrari and Söling, 1999). These redox-active cysteines are the carriers of the oxidative equivalents delivered to PDI by Ero1p, a novel ER membrane protein required for protein oxidation in the ER (Frand and Kaiser, 1998). Disulphide bonds form on nascent polypeptide chains, as they receive the oxidative equivalents from PDI through a series of direct thiol-disulphide exchange reactions. The former hypothesis of oxidized gluthatione (GSSG) serving as the primary source of oxidizing equivalents has been disapproved by studies in which disulphide bond formation has been shown to proceed with normal kinetics in cells lacking glutathione. Instead, glutathione oxidation competes with protein oxidation and serves as a net reductant in the ER, possibly playing an important role in maintaining the

optimal redox conditions for protein folding (Frand *et al.*, 2000).

Kar2p (BiP) is an abundant Hsp70 family chaperone with multiple roles in the yeast ER lumen, and highly conserved homologues in all eukaryotes examined. The expression of Kar2p is constitutive during normal growth conditions, but is induced by heat shock and other stress conditions, and its deletion is lethal. For ER retention/retrieval Kar2p contains the HDEL signal at its C-terminus (Normington *et al.*, 1989; Gething and Sambrook, 1992; Gething, 1999). Kar2p interacts transiently with newly-synthesized proteins during their folding and assembly by binding to the exposed hydrophobic surface of unfolded proteins, which are normally located in the interior of a fully folded protein (Pelham, 1986; Blond-Elguindi *et al.*, 1993). This prevents premature intra- and intermolecular aggregation, and maintains the proteins in the folding and oligomerization competent forms (Simons *et al.*, 1995). Kar2p is also involved in the salvage of misfolded polypeptides and protein aggregates (Jämsä *et al.*, 1995a), and in the ER quality control system by preventing partially folded or misfolded proteins from escaping the ER (Ellgaard and Helenius, 2001). Accumulation of misfolded proteins in the ER triggers the unfolded protein response (UPR), which leads to increased expression of Kar2p and other ER chaperones that contain the unfolded protein response element (UPRE) in their promoters (Kohno *et al.*, 1993). In addition, Kar2p participates in ER-associated protein degradation (ERAD), and the subsequent retro-translocation of proteins into the cytosol for proteasomal degradation (Plemper *et al.*, 1997; Brodsky *et al.*, 1999). Lhs1p, another Hsp70-related luminal ER chaperone has overlap-

ping functions with Kar2p. Lhs1p is regulated by the UPR pathway, it is involved in processing and refolding of malfolded proteins, and in protein folding under normal circumstances. However, it is not induced by heat (Craven *et al.*, 1996; Saris *et al.*, 1997). Saris *et al.* (1997; 1998) implicated an important role for Lhs1p in the survival of yeast cells after severe heat stress. Lhs1p was shown to be required for refolding of proteins subjected to thermal insult, since in strains lacking Lhs1p the heat-denatured protein aggregates could not be solubilized or reactivated, but were slowly degraded. They suggested, that Lhs1p functions as part of the fundamental survival machinery needed for the yeast cells to cope with extreme heat and other stress conditions. Lhs1p is non-essential for vegetative growth, but combinations of *LHS1* null mutations with certain conditional alleles of KAR2, or with null mutants of Kar2p activating proteins are lethal. This indicates, that the cells cannot tolerate the loss of Lhs1p when Kar2p function is impaired (Craven *et al.*, 1997; Tyson and Stirling, 2000).

In mammalian cells the ER resident lectins calnexin and calreticulin together with the UDP-glucose:glycoprotein glucosyltransferase (UGGTase) constitute the calnexin-calreticulin cycle, which monitors the folding state of glycoproteins and promotes their proper folding. Glucosidase I and II provide substrates for the chaperone cycle by removing glucose residues from the N-glycan core of newly synthesized polypeptides (Trombetta and Helenius, 1998). *S. cerevisiae* does not have a homolog of calreticulin, whereas genes for calnexin (*CNE1*), UGGTase (*KRE5*), glucosidase I (*CWH41*) and glucosidase II (*GLS2*) exist. However, in *S. cere-*

*visiae* these proteins seem to contribute to cell wall synthesis rather than protein folding as they do in mammalian cells. Meaden *et al.* (1990) implicated a role for UGGTase in β1,6-glucan synthesis, an important component of the yeast cell wall. Simons *et al.* (1998) found that yeast glucosidases I and II are not needed for the maturation of several major glycoproteins, but instead they act synergistically with Kar2p in the synthesis of the cell wall β1,6-glucan. Also Cne1p was shown to affect the cell wall structure, although its exact role was not fully established. Simons *et al.* (1998) concluded that if existing in yeast, the putative calnexin cycle is a simplified version of the mammalian chaperone cycle, and the maturation of glycoproteins in the absence of functional chaperones is due to redundancy in the folding machinery.

## 1.2.2 Glycosylation

The covalent addition of glycans to secretory and membrane proteins is one of the major post-translational modifications in eukaryotic cells. Glycoproteins are found in various intracellular compartments and in the plasma membrane. In yeast they are also secreted into the periplasmic space and incorporated into the rigid cell wall, which is rich in mannoproteins. Glycosylation modulates protein structure and function and the glycan chains are involved in a variety of biological processes such as protein folding, stability and targeting. Oligosaccharide chains play a role also in recognition processes and protection from proteases. N-glycosylation and glycosylphosphatidylinositol (GPI)-anchor addition have been highly conserved throughout evolution, whereas O-glycosylation in fungi differs from that observed in other organisms

(Tanner and Lehle, 1987; Herscovics and Orlean, 1993; Lis and Sharon, 1993). GPI-anchored proteins are discussed in section 3.

*N-glycosylation*

N-glycosylation begins while the nascent polypeptide chain is translocating into the ER lumen by the transfer of the preassembled oligosaccharide $Glc_3Man_9GlcNAc_2$ (Figure 2A) *en block* from dolichol pyrophosphate to the amide group of an asparagine residue within a sequence asparagine-x-serine/threonine, where x can be any amino acid except proline (Asn-x-Ser/Thr, x≠Pro). In *S. cerevisiae* the transfer is catalyzed by the hetero-oligomeric membrane bound N-oligosaccharyltransferase (OST) protein complex of nine protein components (Ost1-6p, Stt3p, Wbp1p, Swp1p) (Knauer and Lehle, 1999). The protein-bound $Glc_3Man_9GlcNAc_2$-precursor is processed by the sequential action of glucosidase I (Gls1p) and II (Gls2p), which remove the terminal α1,2-glucose residue, and the two α1,3-glucose residues, respectively (Saunier *et al.*, 1982; Bause *et al.*, 1986). In addition, a single mannose residue is cleaved by the α-mannosidase Mns1p (Byrd *et al.*, 1982; Esmon *et al.*, 1984) leading to the $Man_8GlcNAc_2$-glycan core (Figure 2A). The trimmed oligosaccharide chain is further modified in the Golgi, where Och1p (α1,6-mannosyltransferase) initiates the outer chain synthesis by the addition of a single mannose residue to $Man_8GlcNAc_2$. The elongation of the outer chain backbone is catalyzed by a number of Och1p-related α1,6-mannosyltransferases (*MNN9, ANP1/ MNN8, VAN1* and *MNN10, MNN11* gene families). Specific enzymes modify the α1,6-mannose backbone by branching with α1,3- (Mnn1p) and

7

α1,2-mannoses (Kre2p, Ktr1p, Ktr2p, Yur1p), as well as by the addition of mannosylphosphates (Mnn6p) (Figure 2B; Dean, 1999). The yeast Golgi glycosyltransferases are functionally redundant except for Och1p and Mnn1p, which have unique activities (Dean, 1999). The outer chain of secreted and cell wall mannoproteins may be hypermannosylated and highly branched with up to 200 mannose residues (Herscovics and Orlean, 1993), whereas glycan chain elongation of most intracellular glycoproteins is more limited yielding short oligosaccharide chains of $Man_{9-13}GlcNAc_2$-structures (Ballou et al.,

1990). The initial processing of N-glycans in the ER is similar in all eukaryotic cells, whereas the terminal modifications taking place in the Golgi vary considerably between different organisms and proteins (Varki, 1993). Protein N-glycosylation seems to be critical for the viability of *S. cerevisiae*, because certain mutations interfering with the synthesis of the dolichol pyrophosphate-$Glc_3Man_9GlcNAc_2$ precursor, and those lacking subunits of the OST complex are lethal for the cells (Herscovics and Orlean, 1993; Knauer and Lehle, 1999).
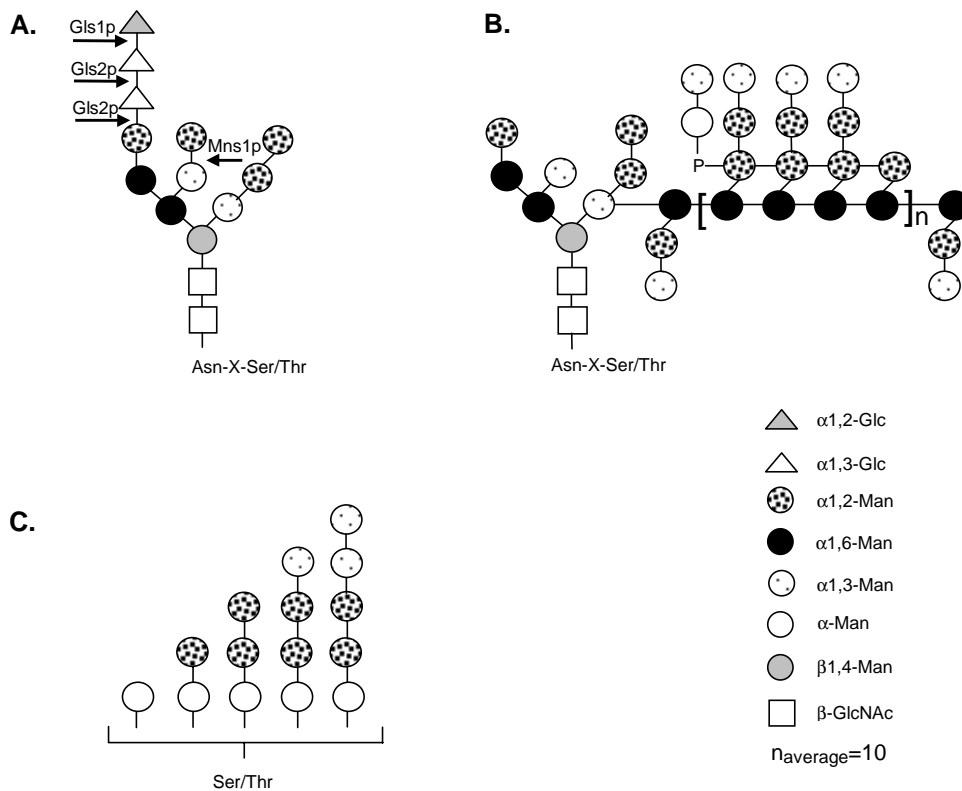


**Figure 2.** N- and O-glycosylation in *S. cerevisiae*. **A.** Structure of the core N-glycan attached to asparagine residues on proteins within the sequence Asn-x-Ser/Thr (x≠Pro). Before the newly glycosylated protein is transported from the ER to the Golgi, the glycan is trimmed by glycosidases at sites marked by arrows. **B.** The Golgi-elongated polymannose type N-glycan. **C.** O-glycosylation starts in the yeast ER by the attachment of the first mannose to Ser/Thr residues on the protein, whereas the elongation takes place in the Golgi. Yeast O-glycans contain one to five mannose residues. The symbols on the right demonstrate the glycosidic linkages created between the monosaccharides.

*O-glycosylation*

In yeast O-glycosylation is initiated in the ER by Dol-P-Man:protein O-mannosyltransferase, Pmt1p, which transfers the first mannose residue from dolichol phosphate mannose (Dol-P-Man) to serine or threonine residues on the nascent polypeptide chain (Figure 2C; Haselbeck and Tannner, 1983; Strahl-Bolsinger *et al.*, 1993). The observation that protein mannosylation was only reduced, but not abolished, in *ptm1* deletion strains, has led to the identification of the *PTM* gene family of O-mannosyltransferases (*PMT1-PMT7*) with differing acceptor specificities. The subsequent mannose residues are added in the Golgi from GDP-mannose leading to the unbranched O-glycan chain of four to five mannose residues (Figure 2C). The Golgi α1,2-mannosyltransferases Ktr1p, Ktr3p and Mnt1p are responsible for the addition of the second mannose residue. The third residue is attached by Mnt1p via α1,2-linkage, and the last one or two mannoses by Mnn1p via α1,3-linkages (Strahl-Bolsinger *et al.*, 1999). O-mannosylation is required for the normal growth of yeast cells and it is essential for maintaining cell wall stability and cell integrity (Strahl-Bolsinger *et al.*, 1999). In higher eukaryotes O-glycosylation takes place in the Golgi and the first monosaccharide most commonly attached to the serine or threonine residue on the protein is N-acetylgalactosamine (van den Steen *et al.*, 1998).

**1.3 Vesicular transport between the ER and the Golgi**

In eukaryotes intracellular protein traffic to the Golgi, vacuole, plasma membrane and cell exterior is carried out in small transport vesicles. Ve-sicular transport between the ER and the Golgi is bidirectional with antero-grade transport guiding the forward traffic of secretory cargo from the ER, and retrograde transport returning material that has escaped the ER, or which needs to be recycled, from the Golgi (Pelham, 1995). In addition to cargo delivery, vesicular transport is needed for maintaining the steady state of the endomembrane system (Schekman and Orci, 1996). For functional vesicle budding, the donor membrane has to attract a specific set of cytosolic coat proteins, include in the vesicle membrane proteins involved in vesicle fusion (v-SNARE), accessory proteins, and the selected cargo.

**1.3.1 Anterograde transport in COPII vesicles**

After proper folding and assembly of secretory proteins in the ER, they are packed into COPII (coat protein complex II) coated anterograde transport vesicles in both yeast and mammals. The COPII components of *S. cerevisiae* were initially identified by genetic approaches and the use of a cell free assay that measured protein transport between the ER and the Golgi (Novick *et al.*, 1980; Baker *et al.*, 1988). The minimum protein requirements for COPII vesicle formation *in vitro* are the small GTPase Sar1p, and the heterodimeric protein complexes Sec23/24p and Sec13/31p (Barlowe *et al.*, 1994; Matsuoka *et al.*, 1998). However, vesicular transport of selected cargo from the ER to the Golgi proceeds *in vivo* also in the absence of functional Sec13p (Fatal *et al.*, submitted). The coat assembly is initiated by the small GTPase Sar1p, which is activated to the GTP-bound form and recruited to the ER membrane by Sec12p, an integral ER

9

membrane protein with guanine nucleotide exchange activity (Barlowe and Schekman, 1993). The sequential binding of Sec23/24p and Sec13/31p to GTP-Sar1p on the donor membrane and the oligomerization of the coat proteins induce deformation of the membrane and the appearance of a coated bud. An additional protein needed for budding *in vivo*, Sec16p, resides on the ER membrane and binds to the coat components, likely participating in organizing their assembly (Espenshade *et al.*, 1995; Campbell and Schekman, 1997). Sar1p-bound GTP is hydrolyzed to GDP by the GAP (GTPase activating protein) activity of Sec23/24p and Sec13/31p assembled on Sar1p, and the coat is disassembled in a few seconds leading to conversion of the pre-budding complex into a completed vesicle (Yoshihisa *et al.*, 1993; Antonny *et al.*, 2001). The docking and fusion of the ER-derived vesicle with the *cis*-Golgi membrane requires specific SNARE proteins on the vesicle (Sec22p, Bos1p, Bet1p) as well as on the target membrane (Sed5p), and the general fusion factors Sec17p and Sec18p (Pelham, 1999).

*Cargo selection*

For incorporation into COPII vesicles, secretory proteins and v-SNAREs must be sorted from immature and misfolded proteins, as well as from ER resident proteins. While the ER quality control system and the ER retention/retrieval signals participate in keeping the ER proteins from entering the budding vesicles (Sato *et al.*, 1996; Ellgaard and Helenius, 2001), there is growing evidence that cargo proteins are actively selected for incorporation into COPII vesicles (Kuehn *et al.*, 1998; Muñiz *et al.*, 2001). Kuehn *et al.* (1998) have demonstrated *in vitro* that soluble and

integral membrane cargo is recruited to COPII vesicles either by direct or indirect interaction with the coat proteins Sar1p and Sec23/24p. Interaction of the coat components with different cargo and vesicle proteins, but not with ER chaperones indicates the presence of variable cargo sorting signals. Known sequences interacting with the COPII coat proteins include the di-acidic Asp-X-Glu (X is any amino acid) motifs (Nishimura and Balch, 1997; Aridor *et al.*, 2001), and the paired phenylalanine (FF) residues found in some transmembrane proteins (Fiedler *et al.*, 1996; Dominguez *et al.*, 1998). Examples of proteins interacting directly with the COPII coat are the yeast ER-to-Golgi v-SNAREs Bet1p and Bos1p (Springer and Schekman, 1998), the *cis*-Golgi syntaxin Sed5p (Peng *et al.*, 1999) and the p24 family members of putative cargo receptors (Dominguez *et al.*, 1998). Shr3p is an example of a specific factor required for packing amino acid permeases into COPII vesicles. Shr3p is an ER resident membrane protein, which is not incorporated in the vesicle, but is suggested to promote the formation of the secretion-competent conformation of the amino acid permeases (Kuehn *et al.*, 1996;1998).

Besides protein-protein interactions, membrane domains such as lipid rafts enriched in sphingolipids and cholesterol, may regulate the packing of cargo into COPII vesicles. Such domains may have specific requirements for incorporation, and their physical properties can affect the packing efficiency of the cargo proteins within these domains. The GPI-anchored Gas1p is known to partition into detergent-insoluble glycolipid-enriched complexes (DIGs) in the yeast ER (Bagnat *et al.*, 2000). Muñiz *et al.* (2001) showed *in vitro* that

10

Gas1p is sorted from other secretory proteins (Gap1p and gpαF) into different vesicles upon ER exit, and efficient packing of Gas1p requires Emp24p, a member of the p24 receptor family. As the ER exit of Gas1p is only retarded, but not inhibited, in a yeast strain lacking all the genes for p24 family proteins, there are alternative mechanisms for Gas1p incorporation into COPII vesicles besides Emp24p (Springer *et al.*, 2000). Another example of raft-associated yeast proteins with specific requirements for ER exit is the transmembrane protein Pma1p. The efficient ER export of Pma1p has been shown to require a specialized subtype of COPII vesicles consisting of a mixture of Sec23/24p and Sec23/Lst1p complexes, where Lst1p represents a Sec24p homologue (Roberg *et al.*, 1999; Shimoni *et al.*, 2000). Vesicles with the mixed coat are larger than standard COPII vesicles. This is, however, likely to reflect the greater surface area occupied by the Pma1p protein oligomer rather than the lipid raft domain, as Pma1p clustering into the rafts was shown to occur not until in the Golgi (Bagnat *et al.*, 2001).

### 1.3.2 Retrograde traffic in COPI vesicles

ER resident proteins that have leaked out of the ER and recycling proteins needed for another round of COPII vesicle budding and fusion, are returned from the Golgi back to the ER by the retrograde transport system in COPI (coat protein complex I) vesicles (Cosson and Letourneur, 1997). In *S. cerevisiae* the minimum protein requirements for COPI vesicle formation *in vitro* are the small GTPase ARF (Arf1p, Arf2p, adenosine diphosphate-ribosylation factor) and the pre-assembled heptameric coatomer

(Ret1p, Sec26p, Sec27p, Sec21p, Ret2p, Sec28p, Ret3p) (Salama and Schekman, 1995; Spang *et al.*, 1998). The initiation of COPI coat formation is dependent on the activation state of ARF, which is regulated by the soluble guanine nucleotide exchange factors (GEFs) Gea1p, Gea2p and Sec7p (Chavrier and Goud, 1999; Jackson and Casanova, 2000). In the active GTP-bound form myristylated ARF associates with the *cis*-Golgi membrane and attracts the preformed coatomer and the putative cargo (Schekman and Orci, 1996; Matsuoka *et al.*, 1998). The coat assembly and oligomerization promote membrane curvature, and the vesicle buds off. The vesicle coat is disassembled by ARF-GTP hydrolysis mediated by the ARF-specific GAPs (GTPase activating protein), Gcs1p and Glo3p (Poon *et al.*, 1999) permitting fusion with the ER membrane. In addition to the general fusion factors Sec17p and Sec18p, the docking/fusion of the Golgi-derived vesicle with the ER membrane requires the ER syntaxin Ufe1p (t-SNARE), which binds to Sec22p (v-SNARE) on the vesicles (Pelham, 1999). The coatomer assembly and retrograde transport, as well as other forms of secretion, are inhibited by Brefeldin A, which stabilizes the Arf-GDP:Sec7 domain complex through inhibition of the GEFs (Peyroche et al., 1999). Examples of mechanisms for cargo selection into COPI vesicles include the Erd2p receptor, the homologue of the mammalian KDEL receptor, which recognizes the C-terminal ER retention/retrieval motif HDEL present in many soluble proteins in the Golgi lumen (Pelham, 1991). Furthermore, many transmembrane proteins display a cytoplasmic C-or N-terminal di-lysine motif (KKXX or KXKXX), which interacts physically with the COPI coatomer components leading to

cargo incorporation in the COPI vesicles (Cosson *et al.*, 1996). Recently, a novel retrograde transport route from as far as the *trans*-Golgi network back to the ER was identified in mammalian cells. This pathway is regulated by Rab6, it is independent of the COPI coat proteins, and has been shown to be used by Golgi glycosyltransferases and some toxins (Girod *et al.*, 1999; White *et al.*, 1999; Storrie *et al.*, 2000). It is not known if a similar COPI-independent retrograde transport route exists in yeast, whereas yeast is capable of transporting selected anterograde cargo in a COPI-independent manner (Gaynor and Emr, 1997; Suntio *et al.*, 1999).

## 1.4 Protein sorting in the Golgi

The Golgi complex, discovered by Camillo Golgi in 1898, is the major site for sorting proteins to their final destinations. Secretory proteins transported from the ER enter the Golgi cisternae at the *cis* face, move through the organelle while being modified by glycosyltransferases and proteolytic enzymes, and exit at the *trans* face. Although the yeast Golgi does not exhibit the clear stacked cisternal morphology seen in higher eukaryotes, it carries out sequential protein modifications in distinct compartments that can be defined by morphological and biochemical techniques (Graham and Emr, 1991; Brigance *et al.*, 2000). Arrival of a protein in the *cis*-Golgi is defined by the acquisition of $\alpha$1,6-mannose residues on its N-glycans transferred by Och1p, while $\alpha$1,2-branching by Mnn2p takes place in the *medial*-Golgi and $\alpha$1,3-branching by Mnn1p in the *trans*-Golgi. The last definable compartment, the *trans*-Golgi network (TGN), is identified by the presence of the proteolytic enzymes Kex1p,

Kex2p and Ste13p (Brigance *et al.*, 2000). The mechanism for protein transport through the Golgi remains controversial. Two models for cargo transport have been proposed, both of which have evidence in favour and against them, and recently they have been suggested to operate simultaneously (Pelham and Rothman, 2000). In the vesicular transport model the secretory proteins are packed into transport vesicles, which move in *cis*-to-*trans* direction through the relatively stable Golgi cisternae. New enzymes are carried to the cisternae by retrograde transport vesicles from an upstream compartment. In the cisternal maturation model the Golgi cisternae are transient and secretory proteins move through the Golgi complex in the entire cisterna. The *cis*-to-*trans* maturation of the cisternae is a continuous process, where enzymes are packed into retrograde vesicles, while new enzymes are received by fusion with retrograde vesicles originating from the upstream compartment (Pelham and Rothman, 2000). From the TGN a variety of transport vesicles with different cargo are directed to the vacuole, the cell surface, or earlier Golgi subcompartments.

### 1.4.1 Targeting to the vacuole

The yeast vacuole is the terminal destination for material targeted for destruction. Its function is critical for many physiological processes including the turnover of normal cellular proteins and disposal of abnormal proteins, and many diseases in higher eukaryotes are related to the impaired function of their degradative organelles (Dell'Angelica et al., 2000). The hydrolases responsible for protein degradation in the yeast vacuole are synthesized as inactive zymogens,

12

which mature into active proteases only after proteolytic processing in the vacuole (van den Hazel *et al.*, 1996). Proteolytic activity of vacuolar hydrolases is largely dependent on functional proteinase A (PrA, *PEP4*) and proteinase B (PrB, *PRB1*), which are central components in inducing the activation cascade of the vacuolar protease precursors. Activation of proPrA is an autocatalytic process, which is further supported by the acidic pH of the vacuole lumen. However, complete maturation of proPrA to PrA requires proteolytic processing by PrB (Woolford *et al.*, 1986; 1993). Maturation of proPrB, in turn, is regulated by both PrA and mature PrB (Moehle *et al.*, 1989). Furthermore, PrA has been reported to stabilize PrB *in vitro*, and the level of PrB activity has been suggested to depend on the extent of *PEP4* expression and the number of mature PrA molecules. Once activated, PrB is capable of maintaining the proteolytic function of the vacuole in PrA depleted cells due to the capability of PrB to activate the PrB-precursor. This phenotype persists over many generations most likely because of vacuolar inheritance (Hirsch *et al.*, 1992). Both PrA and PrB have broad substrate spectrum, and most vacuolar protease precursors can undergo either PrA- or PrB-dependent maturation. However, complete maturation of all precursors requires PrB activity (van den Hazel *et al.*, 1996).

Yeast cells have at least two parallel routes for sorting proteins from the late Golgi to the vacuole (Figure 3). The carboxypeptidase (CPY)-pathway involves protein transit through the prevacuolar compartment (PVC, early and late endosomes), whereas the alkaline phosphatase (ALP)-pathway transports proteins directly to the vacuole bypassing the PVC.
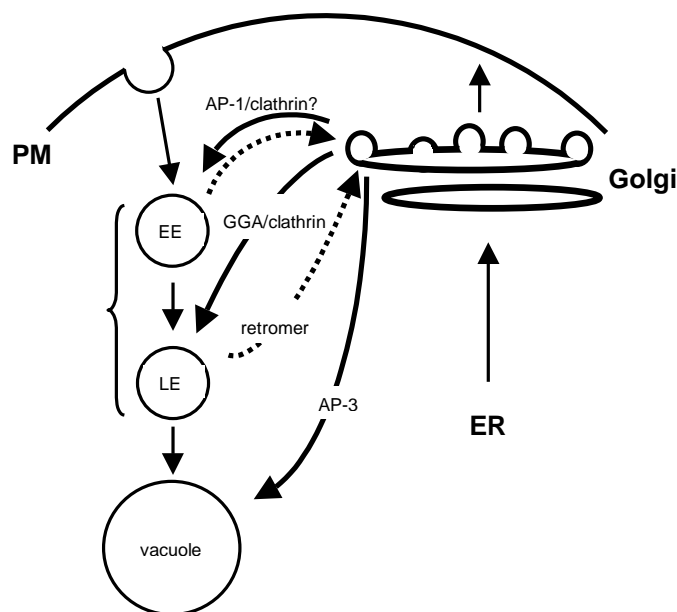


**Figure 3.** Protein transport and recycling between the TGN and the vacuolar compartments. AP-1, adaptor protein complex 1; AP-3, adaptor protein complex 3; EE, early endosome; ER, endoplasmic reticulum; GGA, Golgi-localized gamma-ear-containing ARF-binding protein; LE, late endosome; PM, plasma membrane; PVC, prevacuolar compartment. Modified from Black and Pelham (2000).

13

*CPY-pathway*

CPY is the best characterized example of proteins (e.g. protease A, protease B, CPS, Vph1p, Kex2p, Vps10), which enter the Golgi-to-PVC transport route (Conibear and Stevens, 1998). This pathway depends upon the function of over 45 *VPS* (vacuolar protein sorting) gene products, mutations in which result in missorting of the proteins transported along this pathway. Targeting of CPY to the vacuole from the late Golgi is dependent on the QRPL sequence in the N-terminus of pro-CPY, as mutations in the motif lead to secretion of CPY from the cell (Valls *et al.*, 1990). The CPY sorting receptor Vps10p is, however, capable of targeting also QRPL-deficient soluble hydrolases as well as misfolded proteins to the vacuole, indicating that Vps10p is capable of recognizing a broad range of signals lacking obvious sequence similarity. This suggests that the sorting determinant is not a specific motif, but instead a common secondary structure, that is found also in unfolded proteins (Marcusson *et al.*, 1994; Cooper and Stevens, 1996; Hong *et al.*, 1996). Formation of the transport vesicles in the CPY-pathway requires clathrin and the dynamin-like GTPase Vps1p, whose absence results in missorting of the proteins to the cell surface instead of the vacuole (Seeger and Payne, 1992b; Conibear and Stevens, 1995). Two recently identified GGA (Golgi-localized, gamma-ear-containing, ARF-binding) proteins Gga1p and Gga2p have been suggested to function in the formation of clathrin coated vesicles at the TGN, and to contribute to sorting of Golgi resident proteins and those destined to the vacuole (Mullins and Bonifacino, 2001). In the absence of both Gga1p and Gga2p the sorting and/or transport of a number of proteins is defective, and in addition altered protein processing associated with normal transport has been detected (Boman, 2001). Whether the GGA proteins are involved in protein traffic from the late Golgi to the early endosome or the late endosome is not clear. The only adaptor protein complex that binds clathrin in yeast, AP-1, has not been shown to be essential for any sorting process in yeast cells (Yeung *et al.*, 1999; Boman, 2001). However, disruption of AP-1 combined with mutations in the clathrin heavy chain (*CHC1*) cause more pronounced defects in protein sorting at the TGN than either mutation alone, implicating a clathrin dependent function for AP-1 (Stepp *et al.*, 1995; Yeung *et al.*, 1999). Black and Pelham (2000) suggested that two different classes of clathrin coated vesicles exist, those with GGAs and those without (Figure 3). The clathrin-dependent, GGA-independent pathway could be facilitated by AP-1 and constitute the default pathway for membrane proteins carrying no cytoplasmic signals directed to early endosomes, whereas the clathrin- and GGA-dependent route could be signal-mediated and transport cargo to late endosomes.

The receptor-ligand complex of Vps10p and its bound cargo is transported from the late Golgi to the prevacuolar compartment (PVC), where the complex is dissociated. The cargo proteins are delivered further to the vacuole by a number of specific proteins involved in protein sorting, vesicle fusion and formation, whereas Vps10p enters PVC-derived retrograde transport vesicles and returns to the TGN to carry out multiple rounds of sorting (Figure 3; Cooper and Stevens, 1996). The retrieval of recycling proteins from the PVC back to the TGN is carried out by the retromer

14

protein complex formed of the cargo-selecting Vps35p/Vps29p/Vps26p, and the vesicle budding-driving Vps5p/Vps17p subcomplexes (Seaman *et al.*, 1998). The specificity in the retrieval process is suggested to be mediated by additional proteins, such as Grd19p and Grd20p (Voos and Stevens, 1998; Spelbrink and Nothwehr, 1999). Recently a new multimeric complex of Vps52p/Vps53p/Vps54p was found to be required for retrograde transport of a number of resident late Golgi proteins (Conibear and Stevens, 2000). The complex is localized in the TGN membrane and thus it is likely to be involved in the docking and fusion of retrograde transport vesicles with the TGN. Aromatic amino acids in the cytosolic tails of the resident TGN proteins have been shown to be critical for recycling and maintaining their Golgi localization (Bryant and Stevens, 1997).

*ALP-pathway*

The Vps10p-independent transport route, the ALP-pathway, was identified when ALP was found correctly localized to the vacuolar membrane even when the exit from the PVC was blocked (Raymond *et al.*, 1992). The ALP-pathway transports cargo proteins directly from the late Golgi to the vacuole bypassing the prevacuolar compartment (Figure 3). The transport through the pathway requires the vesicular coat of the AP-3 adaptor complex (Apl6p, Apl5p, Apm3p, Aps3p) and the dynamin-like GTPase Vps1p, but is independent of clathrin function. Deletion of any AP-3 subunit delays ALP transport, but has no effect on the vacuolar delivery of CPY. In *vps1* mutants ALP, as well as proteins from the CPY pathway, are targeted to the cell surface, thus Vps1p is required for the formation of both types of transport vesicles (Conibear

and Stevens, 1995; Nothwehr *et al.*, 1995; Odorizzi *et al.*, 1998a). In addition to ALP, the vacuolar t-SNARE Vam3p has been shown to follow this pathway (Stepp *et al.*, 1997). Tyrosine-based sorting signals as well as dileucine-based motifs in the cytoplasmic tails of transmembrane proteins are involved in recruiting the cargo into the AP-3 coated vesicles (Ohno *et al.*, 1998; Rapoport *et al.*, 1998). Vps39 and Vps41 have been proposed to function as recruiting factors for AP-3 vesicle formation. These proteins are, however, probably not specific for the ALP pathway because in *vps39* and *vps41* mutants also the CPY route is disturbed (Nakamura *et al.*, 1997).

### 1.4.2 Secretion to the plasma membrane

The mechanisms by which proteins are sorted into transport vesicles directed to the plasma membrane, as well as the putative vesicle coats, are largely unknown. Soluble proteins are thought to be transported to the plasma membrane by default, whereas membrane proteins lacking appropriate sorting signal are generally directed to the vacuole (Roberts et al., 1992). Two different post-Golgi vesicle populations have been identified by separation on density gradients (Harsay and Bretscher, 1995). The less dense, major vesicle population contained the cell wall endoglucanase Bgl2p, the plasma membrane ATPase Pma1p and the v-SNARE Snc1p. Included in the denser vesicles were the exoglucanase Exg1p, the periplasmic enzymes invertase and acid phosphatase. Roberg and coworkers (1997) showed, that the transport of the regulated amino acid permeases Gap1p and Put4p to the plasma membrane was dependent on

Sec13p, but not on the other COPII coat components. They suggested that Sec13p-dependent transport vesicles constitute the third class of post-Golgi vesicles directed to the plasma membrane, in which Sec13p functions either as a coat component or as a protein involved in loading the permeases into the vesicles. Clathrin is unlikely to be involved in the assembly of the putative coats, because in clathrin mutant yeast strains soluble and integral membrane proteins are transported normally to the plasma membrane (Seeger and Payne, 1992a; 1992b).

The Golgi-derived secretory vesicles are transported along actin cables to exocytic sites at the tips of the developing buds and at mother-daughter necks in dividing cells (Finger and Novick, 1998). Targeting of the vesicles to the appropriate exocytic sites is mediated by the multiprotein exocyst complex (Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p, Exo84p) (TerBush *et al.*, 1996; Guo *et al.*, 1999a). The vesicles budding from the Golgi contain the small GTPase Sec4p, activation of which by the guanine nucleotide exchange factor Sec2p regulates exocytosis, and is a requirement for polarized vesicular transport (Walch-Solimena *et al.*, 1997). The exocyst component Sec15p interacts with the activated Sec4p on the secretory vesicle, and further binding of Sec10p to Sec15p links the subcomplex to the remainder of the exocyst at the plasma membrane (Guo *et al.*, 1999b). The site of exocytosis is defined by Sec3p, which localizes to specific domains of the plasma membrane (Finger and Novick, 1997). After the secretory vesicles are tethered to the plasma membrane, the SNARE complexes (v-SNAREs: Snc1p, Snc2p; t-SNAREs: Sso1p, Sso2p, Sec9p) required for the membrane fusion assemble (Pelham, 2001). Sec1p binds the SNARE complex and assists the fusion of the membranes, whereafter the SNARE complexes are disassembled and recycled for further use (Grote *et al.*, 2000). The exocytosed proteins are targeted to the plasma membrane, the periplasmic space, the cell wall or secreted to the cell exterior.

## 2 Endocytosis

All eukaryotic cells are capable of endocytosis, an energy-, concentration- and temperature-dependent process in which portions of the plasma membrane and extracellular fluid are internalized. Through endocytosis cells absorb nutrients, recycle membrane proteins and lipids, receive chemical and molecular signals and down-regulate membrane receptors in response to ligands and/or environmental changes. The early events of endocytosis involve the invagination of plasma membrane into pits, which pinch off to form vesicles. These primary endosomes fuse with early endosomes, where proteins destined for the late endosome and further to the vacuole are sorted from those that are recycled back to the plasma membrane (Figure 4; Shaw *et al.*, 2001).

Like higher eukaryotes, *S. cerevisiae* cells carry out fluid-phase and receptor-mediated endocytosis. The first reports of endocytosis in *S. cerevisiae* date back to 1985, when Makarow demonstrated that yeast spheroplasts are capable of internalizing enveloped viruses (1985a), and intact yeast cells

16

have the capacity to endocytose soluble macromolecules (1985b). In cell fractionation studies the internalized material was found concentrated in the yeast vacuole. Similar studies were carried out by Riezman (1985), who found that the membrane-impermeant fluorescent dye lucifer yellow carbohydrazide (LY) was internalized and accumulated in the yeast vacuole. The fluid-phase endocytosis is non-saturable, and particles or molecules are internalized at rates dependent on their extracellular concentration. Receptor-mediated endocytosis is saturable, and can be divided into ligand-binding dependent (regulated) or independent (constitutive) endocytosis. The best studied examples of receptor-mediated endocytosis comprise the internalization of the pheromone receptors Ste2p ($\alpha$-factor receptor) and Ste3p (**a**-factor receptor), both of which undergo endocytosis also in the absence of ligands (Rohrer *et al.*, 1993; Tan *et al.*, 1996). In addition to receptors, many plasma membrane proteins, such as nutrient transporters (e.g. Fur4p, Gap1p, Tat2p) and proteins of the multi-drug resistance family (e.g. Pdr5p) undergo endocytic internalization and vacuolar degradation. All the known endocytosed yeast proteins are transmembrane proteins (Munn, 2001).

Actin and a number of actin-associated proteins are directly required to support endocytosis in yeast (Kübler and Riezman, 1993; Munn, 2001). The precise role of actin in endocytosis is not clear, but studies using actin depolymerizing agents, such as latrunculin A, have indicated that a dynamic actin polymerization/depolymerization cycle is necessary for endocytosis to occur (Ayscough *et al.*, 1997). The actin cytoskeleton also participates in endocy-

tosis in mammalian cells (Lanzetti *et al.*, 2001). In contrast to mammalian cells (Kirchhausen, 2000), the endocytic function of clathrin, AP-2 adaptor proteins and the large GTPase dynamin are less clear in *S. cerevisiae.* Clathrin has been shown to be important and involved in receptor-mediated endocytosis in yeast, but its role is not indispensable (Baggett and Wendland, 2001). Furthermore, the clathrin-mediated endocytosis in *S. cerevisiae* is independent of the AP-2 adaptor protein complex (Huang *et al.*, 1999; Yeung *et al.*, 1999), and none of the three dynamin-like proteins (Mgm1p, Dnm1p, Vps1p) appear to act in the internalization process from the plasma membrane in yeast (Baggett and Wendland, 2001).

After internalization, endocytosed proteins are sorted to their correct destinations. The primary sorting event takes place in the early endosome, where the pathways of recycling proteins and those destined to the vacuole separate. In yeast, the recycling proteins travel from the early endosome to the late Golgi, where they are packed into secretory vesicles directed to the plasma membrane. Whether a direct mammalian-like pathway from the early endosome to the plasma membrane exists also in yeast, is not known (Figure 4; Shaw *et al.*, 2001). Endocytosed proteins targeted to the vacuole leave the early endosome to be transported to the late endosome/prevacuolar compartment (LE/PVC), the intersection of the endocytic and biosynthetic pathways. In the LE/PVC proteins directed for vacuolar degradation are sorted from those destined to remain intact in the limiting vacuolar membrane. This sorting event leads to the formation of the multivesicular body (MVB) by the invagination of vesicles from the LE/PVC membrane into the en-

17

dosomal lumen (Odorizzi *et al.*, 1998b). Mature MVBs fuse with the vacuole releasing the internal vesicles into the lumen of the vacuole, where they are degraded by hydrolytic enzymes (Figure 4) (Lemmon and Traub, 2000; Reggiori and Pelham, 2001).
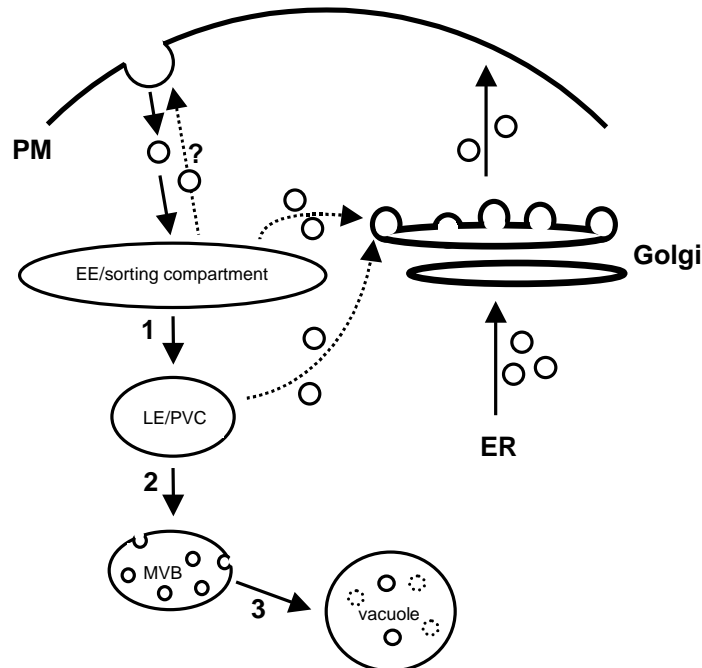


**Figure 4.** Endocytosis and protein recycling between the endocytic compartments and the plasma membrane. Circles indicate vesicular transport between the organelles. (1) It is not known whether the cargo moves from EE to LE/PVC by transport vesicles or as a result of organelle maturation. (2) Organelle maturation. (3) Direct fusion of the organelles. (?) Whether this pathway exists in yeast is unclear. EE, early endosome; ER, endoplasmic reticulum; LE, late endosome; MVB, multivesicular body; PM, plasma membrane; PVC, prevacuolar compartment. Modified from Shaw *et al.* (2001).

## 2.1 Requirements for lipids in endocytosis

Zanolari *et al.* (2000) reported that a sphingoid base, either dihydrosphingosine (DHS) or phytosphingosine (PHS), is required for endocytosis in *S. cerevisiae*. The yeast *end8-1*/*lcb1-100* mutant harbours a temperature-sensitive serine palmitoyltransferase, which cannot catalyze the first step in ceramide synthesis at the restrictive temperature, and results in a rapid defect in endocytosis. Since exogenous DHS could restore endocytosis under conditions where it could not be converted to ceramide or sphingosine, ongoing sphingoid base synthesis was an absolute requirement for endocytosis. The block in internalization caused by the absence of sphingoid base was a general defect, and likely due to the disturbed cytoskeletal organization of actin observed in the mutant cells.

Also the plasma membrane sterol composition affects endocytosis. Yeast *erg* mutants are incapable of synthesizing ergosterol, the major sterol in wild-type yeast plasma membrane (Daum *et al.*, 1998), and

18

accumulate biosynthetic sterol intermediates that differ from ergosterol. Munn *et al.* (1999) studied the internalization of α-factor in *erg2Δ, erg6Δ* and *erg2Δ erg6Δ* mutants, which accumulate sterols with modifications in the B ring saturation (*erg2Δ*, C-8 sterol isomerase) and in the C-24 side chain (*erg6Δ*, C-24 sterol methyltransferase). By correlating the plasma membrane sterol composition of each mutant to their capability to internalize α-factor they concluded, that sterols with two double bonds in the B-ring support endocytosis, whereas C-24 methylation does not play a major role in endocytosis. Consistent with this, the internalization defect was less severe in *erg6Δ* cells, where the amount of sterol intermediates with two desaturations in the B-ring was higher as compared to *erg2Δ*. The strongest defect in endocytosis was seen in the *erg2Δ erg6Δ* mutant, which lacks both the C-24 methyl group and the second double bond in the B-ring.

## 3 Glycosylphosphatidylinositol (GPI)-anchored proteins

Many glycoproteins of lower and higher eukaryotes are attached to the external surface of the plasma membrane through GPI covalently linked to their carboxy terminus (Englund, 1993). In *S. cerevisiae* GPI-anchored proteins are found either attached to the plasma membrane or as structural components of the cell wall. There are 58 open reading frames (ORFs) in the genome of *S. cerevisiae* that encode proteins with a potential GPI-attachment signal. Of these 20 are potential plasma membrane proteins and 38 are likely to be bound to the cell wall (Caro *et al.*, 1997). GPI-anchored proteins play a wide variety of physiological roles including trans-membrane signalling, cell surface protection, cell adhesion and cell wall synthesis. In addition, a role has been proposed for GPI-anchors in protein sorting and intracellular transport (Muñiz and Riezman, 2000). GPI-anchoring is essential for the integrity of the cell wall, and thus for the viability of the yeast cells (de Nobel and Lipke, 1994; Klis, 1994; Leidich *et al.*, 1994).

### 3.1 Biosynthesis of GPI-anchors

The GPI core structure linking the protein to the lipid moiety, protein-CO-NH-$(CH_2)_2$-$PO_4$-6Manα1–2Manα1-6Manα1-4GlcNH$_2$-myo-inositol-PO$_4$-lipid (Figure 5), and its biosynthetic pathway are conserved between organisms, but the attached side chains and lipid tails differ widely among various species (McConville and Ferguson, 1993). GPI-anchors are synthesized by the addition of sugars and phosphoethanolamine to phosphatidylinositol (PI). This multistep process starts on the cytoplasmic side of the ER membrane and continues on the lumenal side of the organelle. In the ER lumen, the completed precursor GPI is transfered *en block* to proteins containing the C-terminal GPI-anchoring signal, and the GPI-anchored protein is transported to the cell surface along the secretory route (Kinoshita and Inoue, 2000).
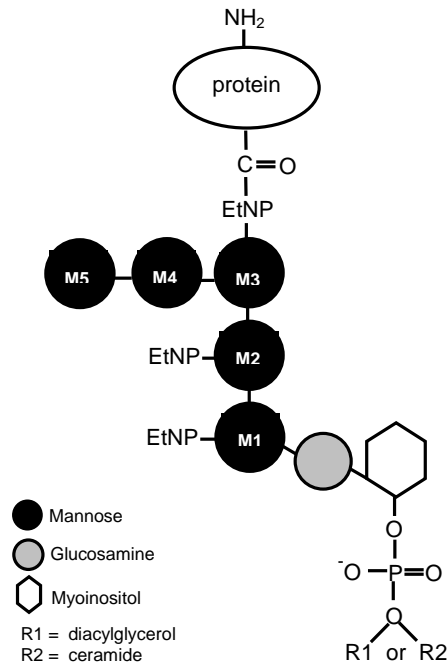
19

**Figure 5.** Structure of GPI-anchored proteins in yeast. The majority of GPI-anchors contain ceramide as the lipid moiety, but also diacylglycerol-based anchors exist. The mannose residues M1-M4 are structural components of all yeast GPI-proteins, whereas some may also contain M5. The presence of M4 is a prerequisite for the addition of the ethanolaminephosphate (EtNP) to M3, and thus for protein GPI-anchoring.

### 3.1.1 Transfer of GPI to proteins

Newly synthesized proteins that are to be GPI-anchored need two signal peptides. The classical N-terminal signal peptide directs ER translocation, whereas the C-terminal signal peptide is needed for the GPI attachment. The GPI-signal consists of three portions: a stretch of three amino acids including the residue to which GPI attaches (the $\omega$ site), a terminal hydrophobic segment of 12-20 amino acids, and a hydrophilic spacer segment of usually less than 10 amino acids between them (Udenfriend and Kodukula, 1995). There is no consensus sequence in the signal, but restrictions for amino acids at certain sites exist. The $\omega$ site can be occupied only by amino acids with small side chains (N,S,G,A,D,C), of which yeast prefers asparagine (Moran *et al.*, 1991; Nuoffer *et al.*,

1993). Also the second residue C-terminal to the $\omega$ site ($\omega$+2) is limited to relatively small amino acids, whereas the $\omega$+1 site tolerates any amino acid except proline and tryptophan (Gerber et al., 1992). During or shortly after translocation into the ER the C-terminal signal of the protein is recognized by the GPI-transamidase, which cleaves the GPI-signal and replaces it with the preformed GPI. The yeast transamidase enzyme complex consists of at least three essential ER-resident proteins encoded by *GAA1*, *GPI8* and *GPI16*, whose exact functions have not been clearly established (Meyer *et al.*, 2000; Fraering *et al.*, 2001). The mouse homolog of Gaa1p was demonstrated to function in the generation of a carbonyl intermediate between the GPI-transamidase and a precursor protein, whereas the conserved cysteine residue of the Gpi8p homolog was shown to be

20

responsible for the catalytic activity of the complex (Ohishi *et al.*, 2000). The yeast Gpi16p was recently isolated as an essential part of the transamidase complex, but its function is not known (Fraering *et al.*, 2001). *GPI16* encodes a functional homolog of mammalian PIG-S, which is a central component of the transamidase complex interacting with the other members of the transamidase, and which is needed for the complex formation (Ohishi *et al.*, 2001). PIG-T, an essential component of the mouse transamidase complex is proposed to function together with Gaa1p. The structural homolog of PIG-T in *S. cerevisiae* is termed *GPI17*, and could encode a fourth member of the yeast transamidase complex (Ohishi *et al.*, 2001).

### 3.1.2 Lipid moieties

In yeast, GPI-anchors with two different types of lipid moieties are found, these are ceramide and diacylglycerol. The primary GPI is synthesized with the diacylglycerol moiety, which is remodeled in virtually all GPI-proteins (Sipos *et al.*, 1997). The lipid remodeling of most GPI-proteins starts in the ER by the replacement of the base-sensitive diacylglycerol moiety with the base-resistant ceramide. Three types of ceramides have been identified in yeast GPI-anchors: dihydrosphingosine-C26:0 and phytosphingosine-C26:0 are incorporated into the anchors in the ER, whereas phytosphingosine-C26:0-OH is found only on proteins that have reached the Golgi (Conzelmann *et al.*, 1992; Reggiori *et al.*, 1997). After replacing the diacylglycerol tail with a ceramide moiety, the ceramide is further exchanged to other ceramides in a long lasting continuous process, which takes place both in the ER and in later

secretory compartments (Reggiori *et al.*, 1997). Those anchors which retain the diacylglycerol moiety, such as the GPI of Gas1p, are modified with very long fatty acids by replacing the original C16 fatty acid with a C26 fatty acid (Sipos *et al.*, 1997). All remodeling events produce anchors containing large lipid moieties with very long fatty acids. This may help GPI-proteins to sort into thicker membrane subdomains and be a prerequisite for the packaging of GPI-proteins into transport vesicles (Doering and Schekman, 1996).

### 3.1.3 Side chains

In *S. cerevisiae* the conserved GPI-core is modified by side chains consisting of mannose residues and ethanolaminephosphates (EtNPs). All GPI-anchors of *S. cerevisiae* contain a fourth mannose residue (M4) attached to the $\alpha$1,2-linked mannose of the GPI-glycan core. In some anchors a fifth mannose (M5) is attached to M4 either with an $\alpha$1,2- or an $\alpha$1,3-linkage (Fankhauser *et al.*, 1993). The fourth mannose is added as part of the anchor precursor glycolipid in the ER, whereas the fifth mannose is added to the GPI-protein in the Golgi. The glycosidic linkage between the fourth and the fifth mannose residues is determined by the nature of the anchor lipid. Diacylglycerol-based anchors recieve $\alpha$1,3-linked mannose, whereas the ceramide-based ones are preferentially provided with $\alpha$1,2-linked mannose (Sipos *et al.*, 1995). The presence of M4 is a prerequisite for the addition of EtNP to the third mannose, and thus required for the transfer of the GPI moiety to the protein. M4 addition requires Smp3p, a putative dolichol phosphate mannose (Dol-P-Man) utilizing mannosyltransferase encoded by an essential gene

(Grimme *et al.*, 2001). In mammalian cells, GPI-anchors with M4 are found as a species- and tissue-specific modification (Puoti and Conzelmann, 1992). EtNPs make up the other group of the side chains. The first mannose of the GPI is modified with EtNP during anchor assembly in a reaction catalyzed by Mcd4p, an essential yeast protein. In temperature sensitive *mcd4* mutants a strong block in GPI-anchor addition and GPI-protein transport is achieved at the restrictive temperature, indicating that functional Mcd4p is needed for the precursors to mature into anchors that are competent to be added to proteins. The failure to add EtNP in *mcd4* mutants probably reduces the use of the immature GPI-anchor as a substrate for further addition of core mannoses, and this results in the accumulation of a series of aberrant GPI-precursors (Gaynor *et al.*, 1999). YW3548, a natural terpenoid lactone of a fungus, inhibits the addition of EtNP to the first mannose of the GPI, and kills *S. cerevisiae* cells at micromolar concentration (Sütterlin *et al.*, 1997a). The second mannose of the anchor becomes modified with EtNP catalyzed by Gpi7p. This modification is not essential, but in cells lacking the GPI7 gene the extent of anchor lipid remodeling is significantly reduced, the transport of GPI-proteins to the Golgi is delayed, and the cell walls are fragile (Benachour *et al.*, 1999).

## 3.2 Sorting and secretion of GPI-anchored proteins

### 3.2.1 ER exit

GPI-anchors have been proposed to play a role in protein sorting (Takeda and Kinoshita, 1995). In yeast GPI-anchor attachment is a prerequisite

for the incorporation of normally GPI-anchored proteins into COPII transport vesicles (Doering and Schekman, 1996), and their efficient packing requires Emp24p and Erv25p of the p24 family. The Emp24p/Erv25p protein complex is suggested to act as a cargo receptor for GPI-anchored proteins, which lack a cytoplasmic tail that could interact directly with the coat components (Muñiz *et al.*, 2000). In addition, two ER resident transmembrane proteins, Lag1p and Dgt1p, have been identified as proteins specifically involved in the ER-to-Golgi transport of GPI-anchored proteins, and have been suggested to function in collaboration with Emp24p and Erv25p (Barz and Walter, 1999). The transport of GPI-anchored proteins from ER to Golgi is strongly inhibited in mutants of α-COP (*ret1-1*), a COPI coat component (Sütterlin *et al.*, 1997b) involved in retrograde transport from Golgi to the ER (Cosson and Letourneur, 1997). This suggests that COPI vesicles recycle specific factor(s) from the Golgi to the ER that are required for the forward transport of GPI-anchored proteins, such as the cargo receptors (Sütterlin *et al.*, 1997b). GPI-anchored proteins have been shown to exit the ER in different vesicles from other secretory proteins, implicating secretory protein sorting at the level of the ER (Muñiz *et al.*, 2001).

### 3.2.2 Role of lipids

In yeast sphingolipids are essential for the transport of GPI-proteins from the ER to the Golgi (Skrzypek *et al.*, 1997; Sütterlin *et al.*, 1997b). Incubation of the conditional mutant *lcb1-100* defective in ceramide synthesis at the restrictive temperature blocks the ER-to-Golgi transport of GPI-anchored proteins, while other secretory pro-

teins are not affected. The same effect is achieved by treating wild type yeast cells with myriocin, an inhibitor of ceramide synthesis. Although inhibition of ceramide synthesis also blocks the exchange of the GPI-anchor diacylglycerol moiety to ceramide, it cannot explain the block in Gas1p transport, because its GPI-anchor does not contain ceramide (Fankhauser *et al.*, 1993; Sütterlin *et al.*, 1997b).

In animal cells sphingolipids and cholesterol have been proposed to form lipid rafts in the Golgi that function as platforms for the clustering of GPI-anchored and transmembrane proteins, and acylated tyrosine kinases with two saturated fatty acid tails (Brown and Rose, 1992; Simons and Ikonen, 1997; Ikonen, 2001). In the *trans*-Golgi network these proteins are selectively sorted into the newly formed rafts or DIGs (detergent-insoluble glycolipid-enriched domains) on the basis of their physical properties. Protein clustering in the rafts has been assumed to function as the signal for apical targeting in the Golgi, but recent reports suggest that other modifications, such as N-glycosylation, are needed to guarantee apical transport (Simons and Ikonen, 1997; Benting *et al.*, 1999a). Furthermore, the composition of sphingolipids and cholesterol in membranes has been shown to be important for both internalization and recycling of GPI-anchored proteins in animal cells, indicating the involvement of raft-dependent mechanisms (Mayor *et al.*, 1998; Ricci *et al.*, 2000; Chatterjee *et al.*, 2001). Mammalian-like lipid microdomains enriched in sphingolipids and ergosterol have been shown to form in the ER of *S. cerevisiae* by Bagnat *et al.* (2000). Proteins associated within the yeast DIGs were the GPI-anchored Gas1p and a number of other GPI-anchored proteins identified on the bases of [$^3$H]myoinositol incorporation, and the transmembrane proteins Nce2p, Pma1p and Hsp30. The GPI-anchored proteins cluster into the rafts in the ER, whereas Pma1p was shown to become raft-associated in the Golgi (Bagnat *et al.*, 2000; 2001). The DIG-association of these proteins could be disrupted by the depletion of ergosterol and sphingolipids.

The distinct sites for clustering GPI-anchored proteins into the lipid microdomains, the Golgi in animal cells and the ER in yeast cells, could be explained by the differences in the lenghts of their sphingolipid acyl chains and GPI-anchors (McConville and Ferguson, 1993; Dickson, 1998). There is evidence that the acyl- and alkyl-chain composition of the anchor lipids influence raft association (Benting *et al.*, 1999b). Also the thickness of a membrane, which increases progressively towards the cell surface due to the enrichment of ergosterol and sphingolipids, can be a sorting mechanism for membrane proteins (Munro, 1998). In yeast, ceramide contains very long saturated acyl chains (C26), which might favour ceramide clustering and the formation of thick microdomains in the ER (Daum, 1998). The long acyl chains of yeast GPI-anchors could then promote sorting of the GPI-anchored proteins to the ER ceramide microdomains. Since the acyl chains of ceramides and GPI-anchors are shorter in animal cells than in yeast (McConville and Ferguson, 1993; Dickson, 1998), the formation of lipid microdomains promoting clustering of GPI-anchored proteins is more likely to take place in the Golgi, where cholesterol is more abundant and sphingolipids are able to form thicker bilayers in the presence of cholesterol. Furthermore,

23

ceramide fatty acid hydroxylation is common in the yeast ER , and may increase the efficiency of clustering through formation of hydrogen bonds between the ceramides, or ceramides and GPI-anchors (Daum, 1998). In higher eukaryotes the only sphingolipid thought to be present in high quantity in the ER is ceramide, which is rarely hydroxylated in mammalian cells, whereas the complex sphingolipids with polar head groups promoting hydrogen bonding are synthesized in the Golgi. However, the formation of ceramide microdomains in the ER of mammalian cells has not been excluded (Muñiz and Riezman, 2000). Although the existence of lipid rafts in biological membranes is controversial (Jacobson and Dietricht, 1999), this hypothesis could explain certain membrane-sorting and signal-transduction phenomena. The co-sequestration of GPI-anchored proteins and the doubly acylated tyrosine kinases of the Src family into the DIGs could provide an explanation for the capability of GPI-anchored proteins to transduce signals across the plasma membrane (Simons and Ikonen, 1997; Horejsi $et$ $al.$, 1998). Lipid-raft resident GPI-binding transmembrane proteins might also account for the link between the outer and inner leaflets of the plasma membrane (Stockinger, 1997).

## 4  Structure of the yeast cell wall

The yeast cell wall is a rigid but dynamic structure surrounding the cells. It is essential for the viability of the cells and needed for cell integrity and morphogenesis, as well as for protecting the cells against harmful environmental conditions. In addition, the cell wall acts as a permeability barrier by regulating the selective uptake and secretion of macromolecules. The cell wall is a big investment for the cell accounting for up to 30% of the cell dry weight. It is organized into covalently interconnected layers made of $\beta1,3$-glucan (50%), $\beta1,6$-glucan (10%), mannoproteins (40%) and a small amount of chitin (1%) (Figure 6) (Lipke and Ovalle, 1998). $\beta1,3$-glucan and chitin are synthesized intracellularly by plasma membrane-bound enzyme complexes, which act also in transporting the nascent glycan chains from the cytoplasmic side across the membrane to the periplasmic space for the final cell wall assembly (Klis, 1994; Lipke and Ovalle, 1998). Because the gene products required for full levels of cell wall $\beta1,6$-glucan are located throughout the secretory pathway and the cell surface, the synthesis of $\beta1,6$-glucan is suggested to start intracellularly, although the majority of $\beta1,6$-glucan synthesis takes place at the plasma membrane (Montijn $et$ $al.$, 1999; Shahinian and Bussey, 2000). The cell wall mannoproteins are synthesized and modified during their transport to the cell surface via the secretory route. The main purpose of the yeast secretory pathway is to provide new plasma membrane and cell wall material for cell surface growth and bud formation. The cell wall synthesis is regulated by the cell cycle (Smits $et$ $al.$, 2001).
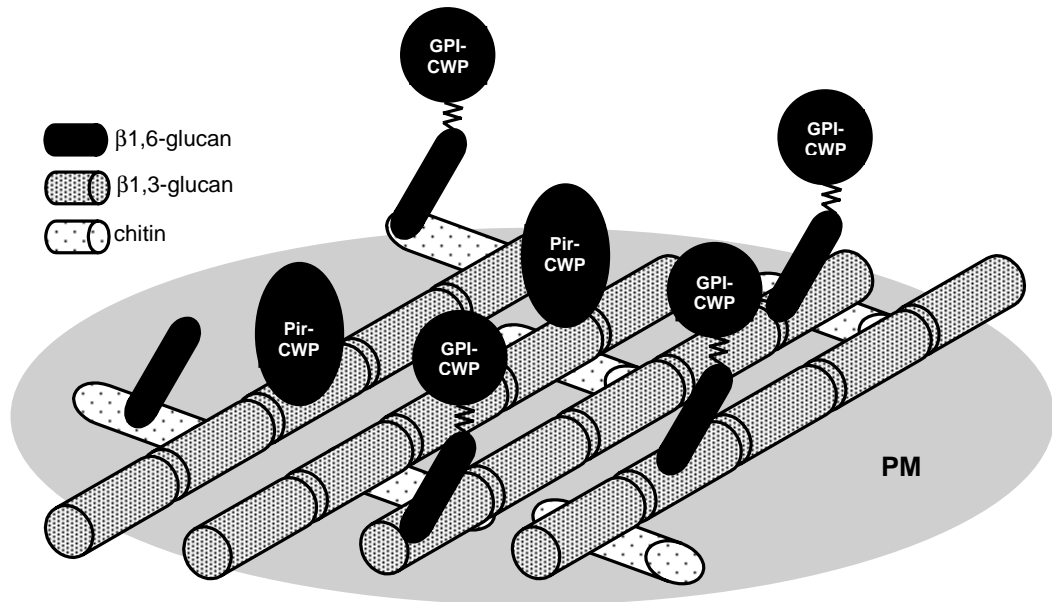
**Figure 6.** Interconnections between cell wall components in *S. cerevisiae*. The covalently bound Pir-CWPs are linked directly to cell wall β1,3-glucan, whereas the binding of GPI-CWPs to β1,3-glucan is mediated by an interconnecting β1,6-glucan moiety. The non-covalently cell wall-associated proteins, as well as those linked to other cell wall proteins through disulphide bonds are not shown for clarity reasons. CWP, cell wall protein; GPI, glycosylphosphatidylinositol; Pir, protein with internal repeats. Modified from Molina *et al.* (2000).

## 4.1 β1,3-Glucan

The main structural component of the cell wall is β1,3-glucan, which forms a fibrous network at the inner surface of the cell wall surrounding the entire cell. Inhibition of β1,3-glucan synthesis by simultaneous disruption of the genes for putative β1,3-glucan synthetase (*FKS1*) and its homolog (*FKS2*) is lethal (Inoue *et al.*, 1995; Mazur *et al.*, 1995). β1,3-glucan is mainly a linear polymer consisting of about 1500 β1,3-linked glucose residues with about 3% of them being branch points (Manners *et al.*, 1973). The branches are formed through the C6-atoms of glucose residues by the various β1,3-glucan-modifying enzymes found in the cell wall and the growth medium. Bgl2p, released from the cell wall under reducing conditions, is the major β-endoglucana-

se/transglucosylase in the yeast cell wall. *In vitro* Bgl2p transfers β1,3-linked oligosaccharides of five or more residues to the non-reducing end of another β1,3-glucan chain containing at least four glucoses, and combines them by β1,6-links (Goldman *et al.*, 1995). Another putative β1,3-glucan-branching enzyme is the GPI-anchored plasma membrane protein Gas1p. *In vitro* Gas1p catalyzes oligosaccharide hydrolysis from β1,3-glucan chains of 10 or more glucose residues, and transfers the reducing end of the released fragment to the non-reducing end of another glucan chain. The existing chain is elongated by β1,3-linkage, thus Gas1p extends newly formed short branches, which may after elongation serve as acceptor sites for cell wall proteins or β1,6-glucan chains (Mouyna *et al.*, 2000). The major

25

β1,3-exoglucanase in the cell wall is encoded by *EXG1*, which hydrolyzes glycosidic bonds between β1,3-linked glucose residues. Exg1p exhibits affinity also for β1,6-linkages (Cid *et al.*, 1995). The multiple nonreducing ends of β1,3-glucan allow it to function as the acceptor for both β1,6-glucan and chitin. The terminal reducing residue of a chitin chain is attached to the nonreducing end of a β1,3-glucan chain by β1,4-linkage (Kollár *et al.*, 1995).

## 4.2 β1,6-Glucan

β1,6-glucan is a highly branched polymer of approximately 140 glucose residues (Klis, 1994), which interconnects the cell wall components by providing attachment sites for both mannoproteins and the β1,3-glucan-chitin complex (Kollár *et al.*, 1997). Some β1,6-glucan is also directly linked to chitin (Kollár *et al.*, 1997). β1,6-glucan is the primary receptor for yeast K1 killer toxin and mutations in genes involved in β1,6-glucan synthesis or assembly lead to killer toxin resistance (KRE) (Bussey, 1991; Schmitt and Compain, 1995). The KRE and KRE-related genes that are required for the full levels of cell wall β1,6-glucan have been localized to the ER (e.g. Kre5p, Cwh41p, Gls2p, Cne1p), the Golgi (e.g. Kre6p) and the cell surface (e.g. Kre1p, Kre9p). This suggests that β1,6-glucan synthesis involves some intracellular key events, although most of the synthesis is likely to take place at the plasma membrane. The function of most KRE gene products remains unknown making their contribution to β1,6-glucan synthesis unclear (Montijn *et al.*, 1999; Shahinian and Bussey, 2000).

## 4.3 Chitin

Chitin is a polymer of β1,4-linked N-acetylglucosamine (GlcNAc) residues, which becomes glycosidically linked to the nonreducing ends of β1,3-glucan and β1,6-glucan branches in the final phase of the bud growth. The glycosidic linkage between β1,3-glucan and chitin has been identified as a β1,4-bond, and the same connection has been suggested for β1,6-glucan and chitin (Kollár *et al.*, 1995; 1997). Chitin coupling to β-glucan takes place mainly at the inner part of the cell wall close to the plasma membrane. It gives the cell wall its rigidity and strength, and determines the cell shape (Klis, 1994).

## 4.4 Mannoproteins

Cell wall mannoproteins are highly glycosylated polypeptides containing often 50 to 95% of carbohydrate by weight. Many of them carry highly branched N-glycans consisting of 50-200 mannose units. N-chain elongation is not essential for wall biogenesis, but the lack of outer chains increases wall permeability and decreases integrity (Lussier *et al.*, 1996). The O-glycans are often clustered and protrude as rigid stalks of one to five mannose residues elevating protein domains from the wall surface (Strahl-Bolsinger *et al.*, 1999). Disruption of O-glycosylation causes aberrant processing of wall mannoproteins and leads to significant reduction of cell wall β1,6-glucan (Häusler *et al.*, 1992; Lussier *et al.*, 1996). The cell wall mannoproteins can be divided into two subgroups, the first of which contains the non-SDS extractable, covalently linked cell wall proteins, whereas the proteins of the second group can be released

from the cell wall by SDS under reducing conditions (Mrša *et al.*, 1997).

Two classes of covalently linked cell wall mannoproteins have been identified. These proteins are not solubilized by SDS under reducing conditions, but can be released from the cell wall by β-glucanase treatment. The GPI-dependent cell wall proteins are serine- and threonine-rich secretory proteins, which are post-translationally modified in the ER by the addition of a GPI-anchor (Kapteyn *et al.*, 1996). As the GPI-anchored proteins targeted to the cell wall reach the plasma membrane, the anchor is cleaved between the M1-mannose and the glucosamine residues (see Figure 5). The protein with the anchor remnant is transferred to β1,6-glucan, which in turn is glycosidically linked to the β1,3-glucan-chitin complex. The linkages of the glycosidic bonds are not known (Lipke and Ovalle, 1998; Kapteyn *et al.*, 1999). Approximately 40 putative GPI-dependent cell wall mannoproteins exist in yeast (Caro *et al.*, 1997). The other class of covalently cell wall-bound proteins consists of the Pir-protein family (proteins with internal repeats, Ccw5p-Ccw8p, Pir2p/Hsp150p). Pir2p/Hsp150p is the only member of the family which is secreted to the culture medium (Russo *et al.*, 1992), and only some of it is retained in the cell wall (Mrša *et al.*, 1997; Mrša and Tanner, 1999). Pir-proteins are highly O-glycosylated proteins with a Kex2 processing site and one or more characteristic repetitive sequences. Unlike the GPI-CWPs, the Pir-proteins do not contain the signal for GPI-anchoring. They can be released from the cell wall by mild alkaline treatment suggesting that O-glycosidic linkages are involved in cross-linking the proteins to other cell wall components. The moieties participating in the linkage are unknown (Mrša *et al.*, 1997; Mrša and Tanner, 1999). Pir-proteins seem to function in protecting the cells under stressful conditions and against harmful agents by maintaining the integrity of the cell wall (Yun *et al.*, 1997; Ezaki *et al.*, 1998; Mrša and Tanner, 1999).

## 5    Yeast as a heterologous host

As heterologous expression often aims at producing secretory proteins of mammalian origin, the chosen host organism must be capable of providing eukaryotic-specific post-translational modifications to confer proper folding and full activity of the expressed protein. In contrast to prokaryotes, yeasts can provide the expressed proteins with many of the required modifications during their transit through the mammalian-like secretory pathway. In addition, secretion of the protein product to the cell exterior avoids the need for cell disruption facilitating down stream processing and purification of the secreted product, because the amount of endogenous proteins in the yeast culture medium is low (Romanos *et al.*, 1992; Eckart and Bussineau, 1996). Other advantages of using yeast as the host organism for recombinant protein expression include the ease of handling, the well-established techniques for genetic manipulation and large-scale protein production, the capability of the organism to grow on inexpensive culture media, and the lack of ethical concerns. Yeasts have been used in the large-scale production of eukary-

otic proteins since 1980. However, yeasts are unable to generate the complex and hybrid type N-glycans found in higher eukaryotes, and consequently the yeast N-glycans contain only high-mannose type oligosaccharides. Also the O-linked oligosaccharides are composed solely of mannose in yeasts, whereas in higher eukaryotes they contain a variety of monosaccharides. Thus, the biological properties of the protein expressed, and the purpose for which it is intended are among the factors determining the expression host for recombinant proteins. The use of yeast as the expression system for heterologous proteins is limited to non-glycosylated proteins, or to applications where authentic mammalian-type glycosylation is not required (Eckart and Bussineau, 1996; Grabenhorst *et al.*, 1999).

Heterologous protein production is desirable especially when the proteins are not available from traditional sources either because they can not be isolated in sufficient quantities from their natural sources, or there is a risk of contaminating agents within the isolated protein. In addition, proteins with specially designed mutations are not found in nature. Whether or not a heterologous protein can be successfully produced in yeast is ultimately dependent on the unique biophysical properties of the individual protein. The most commonly used yeast species for heterologous protein expression are *Saccharomyces cerevisiae* and the methylotrophic yeast *Pichia pastoris*. As the product yields in *S. cerevisiae* are generally low (Buckholz and Gleeson, 1991), other yeasts, such as *Hansenula polymorpha*, *Kluveromyces lactis*, *Schizosaccharomyces pombe* and *Yarrowia lipolytica* may serve as alternative host organisms (Romanos *et al.*, 1992;

Gellissen and Hollenberg, 1997; Reiländer and Weiß, 1998; Lin Cereghino and Cregg, 1999; Gellissen, 2000). Although the suitability of these yeasts for the expression of a broad range of identical heterologous gene products has not been extensively tested and compared, their secretory capacity was found to be higher than that of *S. cerevisiae* among the test proteins in a study by Müller *et al.* (1998). In general, high protein yields can be achieved using methylotrophic yeasts, such as *Hansenula polymorpha* and *Pichia pastoris*, thus they have become the preferred option in cases where the production of the recombinant protein is the only objective (Gellissen, 2000; Lin Cereghino and Cregg, 2000). However, the relative newness of these yeast systems may make the acceptance of recombinant products by regulatory authorities more difficult, whereas *S. cerevisiae* bears the GRAS status. In addition, heterologous expression combined with the complete genome data of *S. cerevisiae* make this system indispensable for applications such as protein functional studies and the development of new methodology (Lin Cereghino and Cregg, 1999).

## 5.1 General aspects on recombinant protein expression

For the expression of recombinant proteins in yeast the vector constructs must contain yeast promoter and terminator sequences to guarantee efficient transcription of the foreign gene, as well as selection markers for cloning and selection of the transformants (Hitzeman *et al.*, 1981; Zaret and Sherman, 1984; Romanos *et al.*, 1992). A signal peptide is needed for directing the protein to the secretory pathway. Extracellular production is advanta-

28

geous, because yeasts secrete only low levels of endogenous proteins, which facilitates the subsequent purification steps. Although signal peptides are recognized with low specificity in yeast, it is preferable to choose a sequence of yeast origin to avoid intracellular accumulation of the expressed protein due to the foreign export signal (Kaiser *et al.*, 1987; Hitzeman *et al.*, 1990). Most plasmids used for yeast studies are shuttle vectors, which contain sequences permitting them to be selected and propagated also in *E. coli.*

Usually the secretion of heterologous proteins in yeast requires a carrier protein, which confers correct folding for the foreign protein and smuggles the protein through the ER quality control system aiding ER exit. The classical and most widely used carrier protein in a number of different yeasts is the *S. cerevisiae* α-factor prepropeptide (Romanos *et al.*, 1992; Lin Cereghino and Cregg, 2000; Kjeldsen, 2000). The α-factor preproprotein consists of a 19-amino acid signal (pre) sequence followed by a 66-residue (pro) sequence with three consensus N-linked glycosylation sites, and a dibasic Kex2 endopeptidase processing site (Kurjan and Herskowitz, 1982). For heterologous expression the foreign protein is fused to the C-terminus of the α-factor prepro-carrier, after the Kex2 cleavage site. Upon post-translational translocation into the ER the pre-region of the carrier peptide is removed by the signal peptidase, and in the late Golgi the Kex2 endoprotease cleaves the pro-region on the C-terminal side of the dibasic Kex2 maturation site (Lys-Arg) (Julius *et al.*, 1984; Lyman and Schekman, 1997). Before secretion the rest of the peptide spacer is removed by dipeptidyl aminopeptidase (Ste13p), and the heterologous pro-

tein is released to the extracellular medium (Julius *et al.*, 1983). The pre-pro α-factor leader can be utilized also without the Kex2p cleavage site, which leads to secretion of the heterologous protein in the unprocessed form (Kjeldsen *et al.*, 1998). The gene for another yeast derived carrier protein, Hsp150, has been cloned and characterized in our laboratory (Russo *et al.*, 1992). The Hsp150Δ carrier contains 321 N-terminal amino acids of the endogenous Hsp150 protein. Included in the carrier sequence are the post-translational translocation directing signal peptide (amino acids 1-18), subunit I (amino acids 19-72) and subunit II with 11 repeats of homologous 19-amino acid peptides (amino acids 73-299), followed by 22 amino acids of the unique C-terminus. There are no N-glycosylation sites in the carrier portion, instead it harbours one cysteine residue and is heavily O-glycosylated. The foreign protein to be expressed is fused to the C-terminus of Hsp150Δ, and if desired, the Kex2 cleavage site can be engineered in between the carrier portion and the foreign gene. Several recombinant proteins have been successfully expressed using the Hsp150Δ-carrier system (Holkeri *et al.*, 1996; Mattila *et al.*, 1996; Simonen *et al.*, 1996). Also other carriers have been used. Kjeldsen *et al.* (1997) reported of efficient insulin secretion in *S. cerevisiae*, when fused to synthetic carrier proteins containing potential BiP binding sites. Furthermore, another synthetic carrier with the opportunity for *in vitro* maturation of the product was constructed by Kjeldsen *et al.* (1998). Besides the capability of this expression system to efficiently promote insulin secretion, it harbours the advantage of being independent of the Kex2p endoprotease, whose inefficient capability to process the heterologous protein may lead to reduced

product yields (Kjeldsen *et al.*, 1996). Also the use of prepro-sequences derived from e.g. *Kluveromyces lactis* killer toxin, *Schwanniomyces occidentalis* glucoamylase gene and *Carcinus maenas* hyperglycemic hormone has been reported (Sleep *et al.*, 1990; Weydemann *et al.*, 1995).

## 5.2 Production of recombinant proteins in *S. cerevisiae*

Human interferon was the first recombinant protein produced by *S. cerevisiae* (Hitzeman *et al.*, 1981). Hepatitis B core antigen was produced in *S. cerevisiae* in 1982, and it became the first approved genetically engineered human vaccine (Valenzuela *et al.*, 1982). Also recombinant human insulin has been expressed in *S. cerevisiae* since the 1980s, and today it covers a substantial portion of the insulin needed for the treatment of *diabetes mellitus* throughout the world (Stepien *et al.*, 1983; Kjeldsen, 2000). Despite the successful production of heterologous proteins for diagnostic purposes and human therapeutic agents, and the vast amount of information on the genetics and molecular biology, *S. cerevisiae* exhibits limitations as the host for recombinant protein expression. Its secretory capacity is considered lower when compared to *P. pastoris* and some other yeasts, although this may be due to the unique properties of the individual proteins expressed and could be in some cases improved by modifying the secretory apparatus (Müller *et al.*, 1998; Shusta *et al.*, 1998). Another main disadvantage encountered with *S. cerevisiae* is hyperglycosylation of the secreted heterologous proteins. The elongation of N-linked core glycans in the Golgi leads to outer chains heterogeneous in size, typically 50-150 mannose residues in length. Such hyperglycosylated, non-human structures may cause undesired immunogenic effects, and the long outer chains can potentially interfere with the folding or function of the foreign protein (Romanos *et al.*, 1992; Gemmill and Trimble, 1999).

Introduction of foreign DNA into the genome of *S. cerevisiae* is achieved by using integrative yeast vectors (YIp). YIp vectors integrate into the yeast genome by homologous recombination typically as a single copy. The advantage of chromosomal integration is the stability of the transformed yeast strains, which can be grown in rich rather than selective media to high density without the risk of losing the desired gene. YIp vectors do not contain sequences for autonomous replication (ARS), thus high expression is dependent on the choice of promoters (Hitzeman *et al.*, 1981). Promoters of genes encoding for glycolytic enzymes (eg. *ADH1*, *PKG*, *TPI*, *GAP*) are the most powerful ones of *S. cerevisiae*, but their poor regulation makes them unsuitable for the expression of toxic proteins. The most tightly regulated promoters of *S. cerevisiae* are those of the galactose-regulated genes, *GAL1*, *GAL7* and *GAL10*, involved in galactose metabolism. *PHO5*, the promoter for the acid phosphatase gene regulated by inorganic phosphate concentration, and the glucose-repressible promoter for alcohol dehydrogenase II, *ADH2*, are examples of promoters widely used in foreign gene expression in *S. cerevisiae* (Romanos *et al.*, 1992). The yeast episomal (YEp) plasmids replicate autonomously, because they contain a segment of the yeast endogenous 2μ plasmid that serves as the origin of replication. Plasmids harbouring the 2μ sequence are maintained at 10-40 copies per cell. However, most YEp plasmids are

relatively unstable even under selective conditions (Velmurugan *et al.*, 2000). The yeast centromeric plasmids (YCp) are autonomously replicating vectors carrying a chromosomal centromere important for plasmid association with the cell's mitotic spindle at cell division. YCp vectors mimic the behaviour of chromosomes during mitosis and meiosis by segregating to two of the four ascospores from an ascus. These vectors are typically present at low copy numbers, 1-3 per cell, and are lost in the absence of selective pressure (Mumberg, 1995). Maximal expression is dependent on efficient transcriptional termination (Zaret and Sherman, 1984). For simplicity, terminators corresponding to the chosen yeast promoters are normally used. Terminators from prokaryotes and higher eukaryotes are often non-functional in yeast (Romanos *et al.*, 1992).

## 5.3 *P. pastoris* as the expression system

Since 1984, over 200 foreign proteins have been successfully expressed in *P. pastoris* (Lin Cereghino and Cregg, 2000). Among the factors accounting for the popularity of *P. pastoris* is the utilization of the alcohol oxidase I promoter (*AOX1*), which is one of the strongest and most regulated promoters known (Ellis *et al.*, 1985; Tschopp *et al.*, 1987). *AOX1* is induced by methanol leading to high levels of alcohol oxidase, which can constitute up to 30% of total cell protein. The initial steps of the methanol utilization pathway take place in peroxisomes, where methanol is oxidized by specific oxidases to generate formaldehyde and hydrogen peroxide. Formaldehyde is used for the generation of energy and biomass in the cytoplasm, whereas hydrogen peroxide is de-

composed to water and molecular oxygen by a peroxisomal catalase. Under conditions of high enzyme production the peroxisomes can constitute up to 80% of the cell volume (Gellissen, 2000). However, the use of methanol can be considered as a drawback, since it is a potential fire hazard and may not be appropriate for the production of food products. Recently, another strong *P. pastoris*-derived promoter from the glutathione-dependent formaldehyde dehydrogenase (*FLD1*) gene was described (Shen *et al.*, 1998). *FLD1* can be induced either by methanol or methylamine, a non-toxic nitrogen source. Methylamine induction of the *FLD1* promoter was reported to lead to expression levels comparable to those obtained with the *AOX1* promoter in methanol (Shen *et al.*, 1998). Other advantages of using *P. pastoris* as the expression system include the ability to create stable yeast strains by integrating the expression vectors into the yeast genome either in single or multiple copies, the ability to culture the cells to high density in fermenters, and the commercial availability of the expression system as a kit. The lack of moderately expressed promoters needed in cases where the high expression from the strong promoters causes toxicity problems has been overcome by the characterization of low-level expression promoters derived from the *PEX8* gene involved in peroxisomal biogenesis, and the *YPT1* gene mediating vesicle fusion (Sears *et al.*, 1998; Johnson *et al.*, 1999; Huynh *et al.*, 2001). Also the problem of a limited set of available selectable marker genes was recently alleviated by the construction of new biosynthetic markers (Lin Cereghino *et al.*, 2001).

*P. pastoris* is less prone to hyperglycosylating heterologous proteins as

compared to *S. cerevisiae*. Although some foreign proteins secreted in *P. pastoris* appear to contain long mannan extensions, the most common N-linked oligosaccharide assembled on glycoproteins is $Man_{14}GlcNAc_2$. In addition, *P. pastoris* oligosaccharides do not contain terminal $\alpha$1,3-linked mannose residues, the immunogenicity of which limits the use of many *S. cerevisiae*-produced recombinant glycoproteins as human therapeutics (Bretthauer and Castellino, 1999).

## AIMS OF THE STUDY

The aim of the present study was to explore the potential of the yeasts *S. cerevisiae* and *P. pastoris* for expression and production of catalytically active rat liver Galβ1-3(4)GlcNAc α2,3-sialyltransferase (ST3N) and Galβ1-4GlcNAc α2,6-sialyltransferase (ST6N), which can be used for the synthesis of glycodrugs, such as sialyl Lewis X, and for the sialylation of therapeutic glycoproteins to prolong their half-life in the circulation. To turn the transferases into soluble, secretory proteins, and to promote their folding and ER exit in yeasts, the catalytic ectodomains of the enzymes were fused to yeast-derived carrier polypeptides.

One of the aims was to produce Hsp150Δ-ST6Ne in *S. cerevisiae*. Since the fusion protein was neither stable nor externalized, the work was directed towards studying intracellular protein degradation. This led to unraveling of the Golgi as a novel site of degradation of heterologous proteins, and a membrane sterol-dependent targeting mechanism for the GPI-anchored Yps1p protease.

As Hsp150Δ-ST3Ne was stable and externalized, but bound to the *S. cerevisiae* cell wall, the goal was to investigate whether asialoglycoproteins could penetrate the porous cell wall and be sialylated by the cell wall-borne Hsp150Δ-ST3Ne, and to determine the kinetic parameters of the cell wall-immobilized enzyme. Furthermore, ST3Ne expression was compared in *S. cerevisiae* and *P. pastoris* using the commercial MFα carrier and the Hsp150Δ carrier developed in our laboratory. Finally, the mechanisms of cell wall binding of Hsp150Δ-ST3Ne and authentic Hsp150 were explored.

33

## MATERIALS AND METHODS

Tables 1 and 2 list the yeast strains used in this study. The specific defects of the yeast mutants are described in Table 3. A summary of the experimental methods, with the references to the respective publications for details, is presented in Table 4. A schematic picture of the recombinant proteins used is presented in Figure 7.

**Table 1.** *S. cerevisiae* strains used in the study.

| *S. cerevisiae* strain | Relevant mutation | Fusion protein | Publication | Source |
|---|---|---|---|---|
| H1 | none | | III | R. Schekman |
| H3 | *sec7-1* | | I | R. Schekman |
| H4 | *sec18-1* | | I | R. Schekman |
| H23 | *Δhsp150* | | I, II | Russo *et al.*, 1992 |
| H148 | *Δpep4* | | I | E. Jones |
| H247 | none | | I | J.Thorner |
| H401 | *Δerg6* | | I | Jackson and Kepès, 1994 |
| H430 | *Δhsp150* | Hsp150$_{TRUNC}$ | III | Fatal *et al.* submitted |
| H469 | *sec18-1 Δhsp150* | | I | Mattila *et al.*, 1996 |
| H626 | *Δhsp150* | Hsp150Δ-ST3Ne | II, III | Mattila *et al.*, 1996 |
| H771 | *Δhsp150* | Hsp150$_{SP}$-ST3Ne | II | This study |
| H855 | *sec18-1 Δhsp150* | Hsp150Δ-ST6Ne | I | This study |
| H871 | *Δhsp150* | Hsp150Δ-ST6Ne | I | This study |
| H892 | *sec7-1 Δhsp150* | | I | This study |
| H893 | *sec7-1 Δhsp150* | Hsp150Δ-ST6Ne | I | This study |
| H897 | *Δpep4 Δhsp150* | | I | This study |
| H898 | *Δpep4 Δhsp150* | Hsp150Δ-ST6Ne | II | This study |
| H1016 | Sec61-his6 | | I | R. Schekman |
| H1039 | *Δerg6 Δpep4 Δhsp150* | Hsp150Δ-ST6Ne | I | This study |
| H1070 | *Δyps1* | | I | Zhang *et al.*, 1997 |
| H1120 | *Δhsp150* | MFα-ST3Ne | III | This study |
| H1204 | *sec7-1 Δyps1Δhsp150* | Hsp150Δ-ST6Ne | I | This study |
| H1281 | *Δerg6 Δpep4 Δhsp150* | Hsp150Δ-ST6Ne | I | This study |
| H1283 | *Δerg6 Δpep4 Δhsp150* | Hsp150Δ-ST6Ne | I | This study |
| H1300 | *Δyps1* | | I | This study |
| H1318 | *Δyps2* | | I | Euroscarf |
| H1350 | *Δerg6 Δpep4 Δyps1 Δhsp150* | Hsp150Δ-ST6Ne | I | This study |
| H1355 | *sec7-1 Δyps1 Δhsp150* | Hsp150Δ-ST6Ne | I | This study |
| H1371 | *sec7-1 Δyps1 Δyps2 Δ*hsp150 | Hsp150Δ-ST6Ne | I | This study |
| H1372 | *sec7-1 Δyps2 Δhsp150* | Hsp150Δ-ST6Ne | I | This study |
| DT8-1A | *Δpir2* | | IV | J. Ibeas |
| DT8-1B | *Δpir1* | | IV | J. Ibeas |
| DT8-1C | *Δpir1 Δpir2* | | IV | J. Ibeas |
| DT8-1D | none | | IV | J. Ibeas |
| HAB813 | *Δkre9* | | IV | H. Bussey |
| SEY6210 | none | | IV | H. Bussey |
| TA405 | none | | IV | H. Bussey |
| TR98 | *Δkre6* | | IV | H. Bussey |
| TR99 | none | | IV | H. Bussey |
| TR212 | *Δkre6 Δskn1* | | IV | H. Bussey |
| YDK5 | *Δkre5* | | IV | H. Bussey |

**Table 2.** *P. pastoris* strains used in the study.

| *P. pastoris* strains | Relevant mutation | Fusion protein | Publication | Source |
|---|---|---|---|---|
| P714 | none | | III | InVitrogen |
| P1402 | none | Hsp150Δ-ST3Ne | III | This study |
| P1403 | none | MFα-ST3Ne | III | This study |

**Table 3.** Relevant features of the mutants.

| Mutation | Defect | Reference |
|---|---|---|
| *sec7-1* | protein transport between Golgi cisternae, temperature sensitive | Franzusoff and Schekman, 1989 |
| *sec18-1* | fusion of transport vesicles with target membranes, temperature sensitive | Kaiser and Schekman, 1990 |
| *Δpep4* | vacuolar hydrolases proteolytically inactive due to absence of proteinase A | Jones, 1984 |
| *Δerg6* | altered sterol composition of membranes | Jackson and Képès, 1994 |
| *Δkre5* | reduced synthesis of cell wall β1,6-glucan | Meaden *et al.*, 1990 |
| *Δkre6* | reduced synthesis of cell wall β1,6-glucan | Roemer and Bussey, 1991 |
| *Δkre9* | reduced synthesis of cell wall β1,6-glucan | Brown and Bussey, 1993 |
| *Δskn1* | reduced synthesis of cell wall β1,6-glucan | Roemer *et al.*, 1993 |

**Table 4.** Experimental methods used in publications I-IV.

| Method | Described in publication |
|---|---|
| CD spectroscopy | II |
| Cell wall isolation | III, IV |
| Desialylation of glycoproteins | II |
| DIG isolation | I |
| Immunofluorescent microscopy | I |
| Immunoprecipitation | I, III |
| Ion exchange chromatography | I, II, III |
| MALDI-TOF mass spectrometry | II |
| Metabolic labeling with [$^{35}$S]methionine/cysteine | I, III |
| Metabolic labeling with [$^{14}$C]protein hydrolysate | IV |
| Northern analysis | II, IV |
| Nucleotide sequencing | II, III |
| Plasmid construction | I, II, III |
| SDS-PAGE | I, III, IV |
| Sialyltransferase assay | I, II, III |
| Superdex gel filtration | II |
| Western analysis | I, III, IV |
| Yeast mating and tetrad dissection | I |
| Yeast transformation | I, II, III |
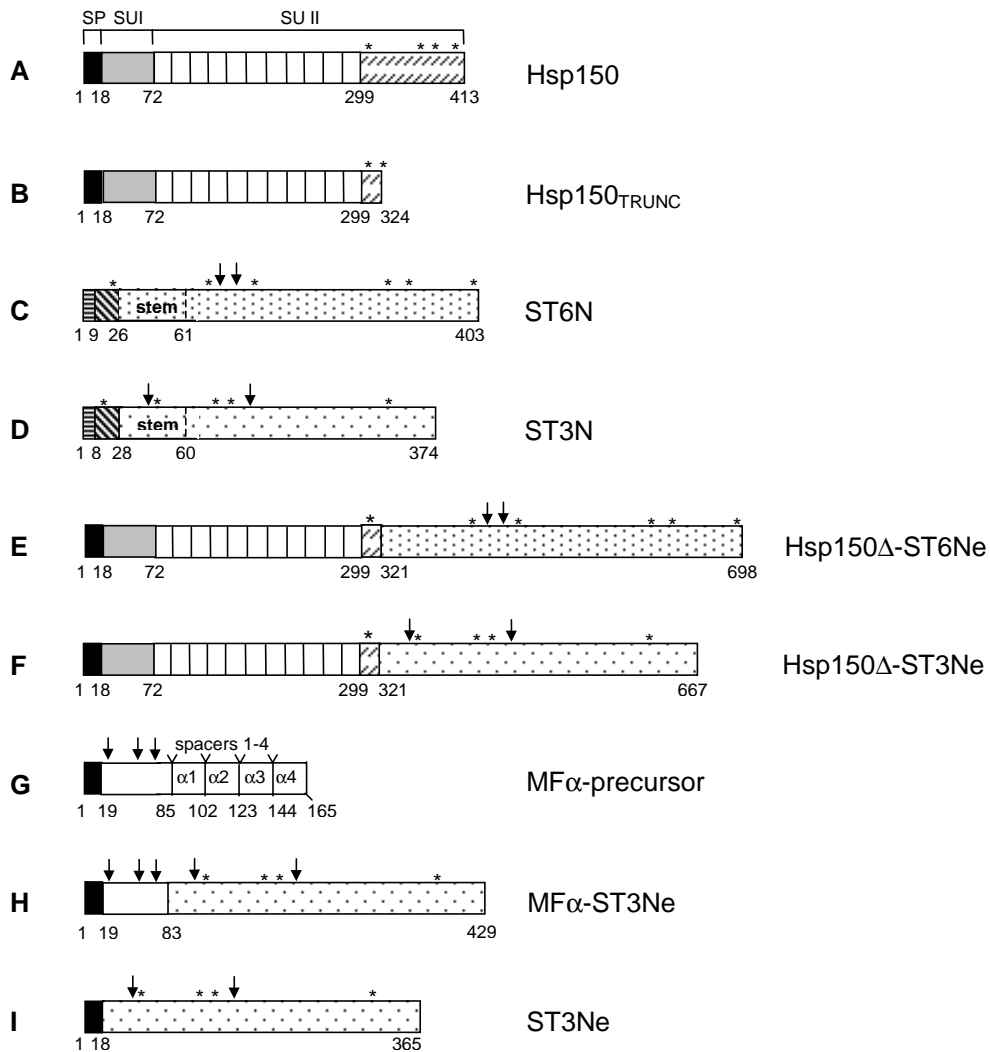
**Figure 7.** The carrier proteins and the recombinant reporter proteins used in the study. **(A)** Hsp150 consists of an 18-amino acid signal peptide and the subunits I (amino acids 19-72) and II (amino acids 73-413). Subunit II consists of 11 tandem repeats of a 19-amino-acid sequence, and the unique C-terminal fragment of 114 amino acids. **(B)** Truncated Hsp150 (Hsp150$_{TRUNC}$) consists of the 319 N-terminal amino acids of Hsp150, and 5 additional amino acids (NLINC) in the C-terminus **(C)** Rat liver Galβ1-4GlcNAc α2,6-sialyltransferase (ST6N) is composed of the N-terminal cytoplasmic tail of 9 amino acids, the transmembrane domain (amino acids 10-26) and the catalytic ectodomain of 377 amino acids. Included in the ectodomain is the stem region (amino acids 27-61) **(D)** The domain order in rat liver Galβ1-3(4)GlcNAc α-2,3-sialyltransferase (ST3N) is identical to that of ST6N, whereas the domain lengths vary as indicated. The recombinant proteins of ST6N ectodomain (ST6Ne) **(E)** and ST3N ectodomain (ST3Ne) **(F)** joined to the C-terminus of the Hsp150Δ carrier. **(G)** The mating factor α (MFα) precursor is composed of the 85-amino acid leader sequence and four 13-amino acid MFα repeats (α1-α4), which are separated from each other by a peptide spacer (sp) of 5-8 amino acids that contains a Kex2p cleavage site. The 85-residue leader sequence is used as the MFα carrier, and it consists of the 19-amino acid signal peptide, followed by a 66-amino acid pro-region and a C-terminal Kex2p processing site. **(H)** ST3Ne fused to the MFα carrier without the Kex2p processing site **(I)** ST3Ne fused directly to the signal peptide of Hsp150. The numbers indicate the last amino acid of each domain. Cysteine residues are designated by stars (∗), and N-glycosylation sites by arrows (↓).

36

# RESULTS

## 1 Expression of recombinant sialyltransferases in yeast

Rat liver Galβ1-4GlcNAc α2,6-sialyl-transferase (ST6N or ST6Gal I) and Galβ1-3(4)GlcNAc α2,3-sialyltransferase (ST3N or ST3Gal III) were expressed in the yeasts *S. cerevisiae* and *P. pastoris*, and the fate of the transferases studied. Authentic ST6N and ST3N are type II transmembrane proteins of the Golgi complex of mammalian cells. To direct the transferases for secretion as soluble proteins, and to facilitate their folding and ER exit in yeast cells, the cytosolic and transmembrane domains of the enzymes were omitted and the soluble catalytic ectodomains were fused to yeast-derived carrier polypeptides. By directing the transferases to the yeast secretory pathway, the enzymes were provided with an opportunity for acquisition of post-translational modifications, like disulphide formation and N-glycosylation, which are required for proper folding, secretion competence and catalytic activity (Weinstein *et al.*, 1987; Wen *et al.*, 1992; Mattila *et al.*, 1996; Datta *et al.*, 2001). Furthermore, extracellular expression of foreign proteins is desirable in general for reducing the efforts and costs of down stream processing.

ST6N and ST3N catalyze the transfer of sialic acid (NeuNAc) from the activated nucleotide sugar (CMP-NeuNAc) to terminal galactose residues in oligosaccharides of specific sequence. ST6N transfers sialic acid to accceptor substrates containing terminal oligosaccharides of the type II structure (Galβ1-4GlcNAc), and introduces an α2,6-glycosidic linkage between the sialic acid and the galactose residue. The oligosaccharide acceptors of ST3N are either of type I (Galβ1-3GlcNAc) or type II (Galβ1-4GlcNAc), but the preference is for the type I substrates (Weinstein *et al.*, 1982b), and the glycosidic bond created is an α2,3-linkage. α2,3- and α2,6-linked sialic acids are important determinants *in vivo*. They are found e.g. as terminal modifications among many serum glycoproteins (Spik *et al.*, 1975; Townsend *et al.*, 1982; Hokke *et al.*, 1995), and function in determining their clearance time (Lodish, 1991). Furthermore, α2,3-linked sialic acid is a structural component of the tetrasaccharide sialyl Lewis X, which is recognized by selectins in the blood vessels during the early steps of inflammation. Thus, large-scale *in vitro* α2,3- and α2,6-sialylation of glycoproteins and oligosaccharides has potential applications e.g. in prolonging the half life of recombinant proteins in the circulation, and in enzyme-assisted synthesis of glycodrugs for anti-inflammatory therapy. While purification of the sialyltransferases from their natural sources is not feasible (Sadler *et al.*, 1979; Weinstein *et al.*, 1982a), expression of desired recombinant transferases could provide the means for obtaining sufficient amounts of pure enzymes for *in vitro* sialylation reactions. Yeast as a heterologous host for ST6N and ST3N expression should meet the requirements of the transferases. However, successful production of a recombinant protein is ultimately dependent on the unique properties of the protein itself.

## 2  The role of Yps1p, a GPI-anchored plasma membrane protease, in intracellular degradation of active Hsp150Δ-ST6Ne in *S. cerevisiae* (I)

### 2.1 Enzymatic activity and intracellular transport of Hsp150Δ-ST6Ne (I)

ST6N is a type II transmembrane glycoprotein with a short N-terminal segment in the cytoplasm, a transmembrane domain spanning the Golgi membrane, and an extended stem region, followed by a large catalytic ectodomain facing the Golgi lumen (Fig. 7C, Field and Wainwright, 1995). To transform ST6N into a soluble secretory protein for extracellular expression in *S. cerevisiae*, the N-terminal amino acids coding for the cytoplasmic tail and the transmembrane domain were omitted, and the catalytic ectodomain of the enzyme (ST6Ne, amino acids 27-403) was fused to the C-terminus of the yeast derived Hsp150Δ carrier polypeptide (hereafter Hsp150Δ-ST6Ne, Fig. 7E). The Hsp150Δ carrier consists of the 321 N-terminal amino acids of Hsp150, which is a secretory glycoprotein of yeast (Russo *et al.*, 1992). Included in the carrier are the N-terminal signal peptide, the subunit I and the repetetive region of subunit II, followed by a 22-amino-acid fragment of the unique C-terminus (Fig. 7A). There are no N-glycosylation sites in the Hsp150Δ carrier, whereas it contains 99 potential sites for O-glycosylation. The Hsp150Δ carrier has been shown to promote proper folding and ER exit of many recombinant proteins expressed in *S. cerevisiae* (Holkeri *et al.*, 1996; Mattila *et al.*, 1996; Simonen *et al.*, 1996).

The Hsp150Δ-ST6Ne fusion protein was found to exhibit ST6N activity, which could be measured by incubating yeast cell lysates with CMP-[$^{14}$C]NeuNAc and N-acetyllactosamine (LacNAc, Galβ1-4GlcNAc), the respective donor and acceptor substrates of ST6N. The [$^{14}$C]NeuNAcα2-6Galβ1-4GlcNAc product synthesized in the reaction was separated from the substrates by ion-exchange chromatography, and the radioactivity of the product counted. The catalytic activity of Hsp150Δ-ST6Ne suggested, that the transferase portion was correctly folded. Further support for this was provided by the kinetic parameters, determined according to Lineweaver-Burk, which were comparable to those with isolated authentic ST6N (Weinstein *et al.*, 1982b). However, Hsp150Δ-ST6N$_e$ was not secreted, as no newly synthetized molecules could be detected in the medium.

To study the cellular localization of Hsp150Δ-ST6Ne, the cells were labeled with [$^{35}$S]methionine/cysteine, chased for different time periods in the presence of cycloheximide (CHX) to inhibit *de novo* protein synthesis, lyzed and immunoprecipitated with Hsp150-antiserum. SDS-PAGE followed by autoradiography revealed different biosynthetic forms of Hsp150Δ-ST6Ne. The 84 kDa protein was likely to represent the cytosolic form of the fusion protein, and the 126-144 kDa protein its ER forms (I, Fig. 5A). The 160 kDa protein was the mature Golgi-modified Hsp150Δ-ST6Ne, as after immunoprecipitation with Hsp150-antiserum the protein could be represipitated with antiserum against α1,6-linked mannoses, which represent a Golgi-specific modification carried out by the *cis*-Golgi α1,6-mannosyltransferase Och1p (I, Fig. 5A, 6). After a prolonged chase period no Hsp150Δ-ST6Ne could be detected in the cell lysates, neither was

it secreted to the medium or cell wall, indicating intracellular degradation. According to immunofluorescent staining, the final destination of the fusion protein was the vacuole (I, Fig. 4F). Thus, Hsp150Δ-ST6Ne acquired a secretion-competent conformation in the yeast ER, whereafter it entered the Golgi and was then sorted for vacuolar degradation, for an unknown reason.

## 2.2 Intracellular degradation of Hsp150Δ-ST6Ne (I)

When the fate of Hsp150Δ-ST6Ne was studied in a yeast mutant lacking the *PEP4* gene (*Δpep4*) coding for proteinase A (PrA), a central activator of vacuolar proteases (van den Hazel *et al.*, 1996), the degradation of the fusion protein was slowed down markedly (I, Fig. 3Ac, 5C). This suggested a major role for the vacuolar hydrolases in Hsp150Δ-ST6Ne degradation. However, in the *Δpep4* mutant still about half of ST6N activity was lost during a two hour chase, indicating the presence of an additional degradative mechanism for the recombinant protein. Besides the vacuole, the yeast proteasome is known to degrade proteins. Misfolded, abnormal proteins are recognized by the molecular chaperones of the yeast ER quality control system, and retro-translocated back to the cytosol for destruction by the proteasome machinery (Brodsky and McCracken, 1999; Nishikawa et al., 2001). To explore the putative role of the proteasome in the residual degradation of Hsp150Δ-ST6Ne, the plasma membrane of the *Δpep4* cells was permeabilized to enable uptake of the proteasome inhibitor MG132 (Lee and Goldberg, 1996; Loayza *et al.*, 1998). This was achieved by deleting the gene for *ERG6* (Gaber *et al.*, 1989;

Graham *et al.*, 1993; Jackson and Képès, 1994), which encodes the C-24 sterol methyltransferase required for the synthesis of ergosterol, the major sterol of the yeast membranes (Daum *et al.*, 1998). Instead of ergosterol, *Δerg6* cells accumulate the sterol intermediates zymosterol and cholesta-5,7,24-trienol in the membranes (Munn *et al.*, 1999). MG132 did not increase the stability of Hsp150Δ-ST6Ne in the *Δerg6 Δpep4* strain. Neither was the degradation rate slowed down in a yeast mutant with nonfunctional proteasomal proteins (unpublished results). Furthermore, when blocked in the ER and ER-derived vesicles at the restrictive temperature of 38°C in the *sec18-1* mutant (I, Fig. 3Bb, 5B), no decrease in the amount of $^{35}$S-labeled Hsp150Δ-ST6Ne, nor in ST6N activity could be detected, although the proteasomal pathway was available under these conditions. Thus, the proteasome was not responsible for the residual degradation of Hsp150Δ-ST6Ne. Indeed, ST6N activity was slightly increased in the *sec18-1* strain during the chase in the presence of CHX. This can be explained by the signal peptide of Hsp150, which targets the newly synthesized polypeptides for post-translational translocation. Therefore it is likely that at the moment of CHX addition there were still untranslocated proteins in the cytoplasm, which entered the ER after CHX addition and became active.

## 2.3 Localization and activity of the GPI-anchored aspartic protease Yps1p in the absence of membrane ergosterol (I)

Interestingly, the *ERG6* disruption itself was found to have a profound effect on the degradation kinetics of

Hsp150Δ-ST6Ne (I, Fig. 3Ad, 5D). The degradation rate of the fusion protein was significantly increased in the Δerg6 Δpep4 strain, and resembled that found in the wild type cells (I, Fig. 3Aa vs. 3Ad and 5A vs. 5D). As raft-like domains enriched in sphingolipids, ergosterol and GPI-anchored proteins have been shown to exist in yeast (Bagnat et al., 2000), the effect of disturbed ergosterol biosynthesis on Hsp150Δ-ST6Ne degradation in the Δerg6 Δpep4 strain suggested that putative raft-associated proteases could be involved in degrading Hsp150Δ-ST6Ne. Indeed, when the localization of Yps1p, an aspartic protease with a GPI-tail, was studied by immunofluorescent microscopy, its staining pattern in Δerg6 Δpep4 cells was altered as compared to Δpep4 cells (I, Fig. 1). In the absence of membrane ergosterol (Δerg6 Δpep4), Yps1p was found in the vacuole and vacuolar membranes (I, Fig. 1C), whereas in cells with normal ergosterol content (Δpep4) Yps1p remained associated with the plasma membrane (I, Fig. 1A) like in wild type cells (Ash et al., 1995).

Yps1p has a low pH optimum for activity (Azaryan et al., 1993; Cawley et al., 1998), and it is homologous to proteinase A, which activates pro-CPY and other vacuolar zymogens (van den Hazel et al., 1996; Olsen et al., 1999). However, the biological functions of Yps1p and the other proteases of the yapsin family are not known. To study whether Yps1p localized to the vacuole in the Δerg6 Δpep4 cells was proteolytically active, we performed pulse-chase experiments to follow the maturation of $^{35}$S-labeled carboxypeptidase Y (CPY). The biosynthetic forms of CPY are readily identifiable in SDS-PAGE. The core N-glycosylated ER form, pro-CPY, migrates as a 67 kDa band (p1),

the glycan extensions in the Golgi lead to the 69 kDa form (p2), whereas maturation to the 61 kDa protein (m) requires proteolytic removal of the pro-region in the vacuole by proteinase A (I, Fig. 2A). The pulse-chase experiment of $^{35}$S-labeled CPY revealed that, in contrast to the Δpep4 strain where the p2 form of pro-CPY persisted due to the absense of proteinase A (I, Fig. 2A), CPY acquired its mature form after a 20 min chase in the Δerg6 Δpep4 strain (I, Fig. 2C). This, together with the immunofluorescent data on vacuolar localization of Yps1p (I, Fig. 1), strongly suggested that Yps1p was capable of activating vacuolar proteases in the Δerg6 Δpep4 cells.

To directly explore whether vacuolar Yps1p was involved in the maturation of CPY, we deleted the YPS1 gene from the Δerg6 Δpep4 strain. In the Δerg6 Δpep4 Δyps1 strain, only a little CPY could be detected in its mature form (I, Fig. 2D), whereas in the presence of Yps1p (I, Fig. 2C) all CPY was in its mature form at the end of the chase. This indicates that Yps1p, targeted to the vacuole in the absence of membrane ergosterol, was capable of rescuing the proteolytic function of the vacuole in cells lacking proteinase A. Proteolytic activity of vacuolar Yps1p explained partly also the increased degradation rate of Hsp150Δ-ST6Ne in the Δerg6 Δpep4 strain (I, Fig. 3Ad), since ST6N activity was lost more slowly after YPS1 deletion (I, Fig. 3Ae). The localization of Hsp150Δ-ST6Ne in the vacuole was confirmed by immunofluorescent microscopy, where the staining of the fusion protein coincided with the vacuoles visualized by differential interference contrast (DIC) images (I, Fig. 4F, 4G).

### 2.3.1 Raft-association of Yps1p (I)

Bagnat *et al.* (2000) demonstrated the clustering of Gas1p and other GPI-anchored proteins in the sphingolipid and ergosterol enriched lipid rafts in yeast. To study whether Yps1p was raft-associated under normal conditions, and if the vacuolar targeting of Yps1p in the $\Delta erg6$ $\Delta pep4$ strain was due to disruption of the rafts in the absence of membrane ergosterol, we performed density gradient runs of cell lysates on Optiprep to isolate the rafts according to Bagnat *et al.* (2000). Both Yps1p (I, Fig. 7C, 7D) and Gas1p (I, Fig. 7A, 7B), the positive control for raft-associated proteins (Bagnat *et al.*, 2000), were enriched in the detergent-insoluble complexes in normal and $\Delta erg6$ cells, since the proteins floated in the top fractions of the gradient. Sec61p, a non-raft protein of the ER membrane, was used as the negative control. In contrast to Yps1p and Gas1p, Sec61p concentrated in the bottom fractions of the gradient. Thus, vacuolar targeting of Yps1p in the $\Delta erg6$ $\Delta pep4$ cells was not due to exclusion from rafts, and Yps1p was apparently raft-associated in the vacuolar membranes.

### 2.4 Golgi-associated degradation of Hsp150Δ-ST6Ne (I)

The stability of Hsp150Δ-ST6Ne in the Golgi was studied by using a *sec7-1* mutant, which blocks secretory proteins in the Golgi at the restrictive temperature. As there was no known degradative machinery in the yeast Golgi, it was a surprise that ST6N activity disappeared in the *sec7-1* strain during chase at 38°C (I, Fig. 3Ba) with kinetics similar to those in the $\Delta pep4$ cells (I, Fig. 3Ac). To find out whether Yps1p or Yps2p, another

member of the yeast yapsin family, were involved in Golgi-associated degradation of Hsp150Δ-ST6Ne, yeast strains with the respective gene deletions were constructed. In the *sec7-1 yps1* strain ST6N activity was found to diminish with slower kinetics, as compared to *sec7-1* strain (I, Fig. 3Bb vs. 3Ba, 3C). This is in agreement with the increased stability of [$^{35}$S]Hsp150Δ-ST6Ne in the strain lacking Yps1p (I, Fig. 5E vs. 5F). In *sec7-1 yps2* cells ST6N activity was not altered as compared to *sec7-1* cells (I, Fig. 3Bc vs. 3Ba). Since the degradation of Hsp150Δ-ST6Ne in the Golgi was slowed down after *YPS1* deletion, newly synthetisized Golgi-blocked Yps1p molecules, *en route* to the plasma membrane, were apparently partly responsible for the disappearance of the fusion protein. However, Hsp150Δ-ST6Ne was still subject to other unknown degradative mechanisms in the Golgi, as deletion of Yps1p did not abolish the degradation of the Golgi-localized fusion protein completely, and Yps2p disruption had no effect on Hsp150Δ-ST6Ne stability. Neutralization of the Golgi lumen by the addition of $NH_4Cl$ to the incubation mixture totally abolished the Golgi-associated degradation of Hsp150Δ-ST6Ne (I, Fig. 3Bd). This suggests that the pH of the Golgi lumen, which is lower as compared to the ER, promotes the activation of Yps1p and possibly other Golgi-blocked proteases with low pH optimum. The Golgi localization of Hsp150Δ-ST6Ne was confirmed by the colocalization of the fusion protein with the Golgi marker Kex2p in immunofluorescent staining assays (I, Fig. 4A, 4C, 4D).

In summary (I, Fig. 8), the GPI-anchored aspartic protease Yps1p was shown to be a raft-associated protein, whose cellular destination

was largely determined by the sterol composition of the membranes. Under normal ergosterol biosynthesis Yps1p resided raft-associated in the plasma membrane (I, Fig. 8A), whereas replacement of membrane ergosterol by zymosterol and cholesta-5,7,24-trienol led to vacuolar targeting of raft-associated Yps1p (I, Fig. 8B). The vacuole proved to be a novel site for Yps1p function. Vacuolar Yps1p was capable of rescuing the proteolytic activity of the vacuole in *Δerg6 Δpep4* cells, thus bypassing the need for proteinase A in the activation cascade of vacuolar protease precursors. Another novel site for the degradative activity of Yps1p was found to be the Golgi complex. When se-

cretory proteins *in transit* to the plasma membrane were captured in the Golgi, Yps1p was shown to participate in the degradation of Hsp150Δ-ST6Ne (I, Fig. 8C). The biological function of Yps1p is not known, but the autoactivation of the Yps1p zymogen is dependent on low pH (Cawley *et al.*, 1998). Since the lumen of the vacuole is acidic, it was likely to promote Yps1p activation. Furthermore, the pH of the Golgi lumen supported Yps1p activation, since neutralization of the organelle abolished Golgi-associated degradation of Hsp150Δ-ST6Ne. No proteolytic activity could be assigned to Yps1p in the ER, the lumen of which is neutral.

## 3   Yeast as a source for α2,3-sialyltransferase activity (II, III)

### 3.1 α2,3-Sialylation of protein substrates by Hsp150Δ-ST3Ne immobilized in the cell wall (II)

Hsp150Δ-ST3Ne has been earlier shown to acquire a secretion-competent conformation in *S. cerevisiae* (Mattila *et al.*, 1996). However, the fusion protein was not secreted to the medium, but remained bound to the yeast cell wall in a catalytically active conformation. This was demonstrated by incubating whole intact recombinant yeast cells with CMP-[$^{14}$C]NeuNAc and LacNAc (Galβ1-4GlcNAc), the respective sugar donor and acceptor substrates of ST3N, whereafter [$^{14}$C]NeuNAcα2-3Galβ1-4GlcNAc product could be harvested from the reaction mixture. Thus, the substrates could penetrate the cell wall, LacNAc was α2,3-sialylated by the cell wall-borne ST3Ne, whereafter the sialylated product diffused back to the reaction buffer. ST3Ne contains two potential N-glycosylation sites and four cysteine residues. N-

glycosylation was found to be essential for the catalytic activity of ST3Ne, whereas it had no effect on the intracellular transport of the fusion protein. On the other hand, disulphide bond formation was required for the ER exit of Hsp150Δ-ST3Ne (Mattila *et al.*, 1996). ST3N shares the common domain structure of type II transmembrane proteins with ST6N (Field and Wainwright, 1995). Thus, like in the case of ST6N (I), ST3N was transformed into a soluble secretory protein by fusing its catalytic ectodomain (amino acids 29-374) to the Hsp150Δ carrier, creating the Hsp150Δ-ST3Ne chimera (Mattila *et al.*, 1996).

Here we studied whether also glycoproteins with native conformation, high molecular weight substrates as compared to oligosaccharides, could access Hsp150Δ-ST3Ne in the cell wall and become α2,3-sialylated. Among the applications for such reactions is the sialylation of recombinant pharmaceutical proteins, that are

often undersialylated due to the incapability of the host cell (e.g. CHO cells) to perform extensive sialylation of N-glycans (Curling *et al.*, 1990; James *et al.*, 1995; Jenkins *et al.*, 1996). Complete sialylation of glycoproteins is important. The lack of terminal NeuNAc-residues exposes the penultimate galactose residues, which are detected by the hepatic asialoglycoprotein receptor, leading to rapid removal of the protein from the circulation. Although the lack of sialic acids does not necessarily affect the biological activity of the protein, the increased clearance rate severely compromizes the effect of the pharmaceutical protein (Lodish, 1991; Weikert *et al.*, 1999).

To study whether glycoproteins in their native conformation, could penetrate the cell wall and be sialylated by the cell wall-borne ST3N activity, bovine plasma asialofetuin was first used as the acceptor substrate. Asialofetuin is a 48 kDa glycoprotein with three branched N-glycans, to which altogether eight NeuNAc residues can be linked by ST3N (Spiro, 1973). In addition, there are three O-glycans with terminal Galβ1-3GalNAc structures (Spiro and Bhoyroo, 1974), which should not act as acceptor substrates for ST3N. Asialofetuin was incubated with live whole yeast cells expressing cell wall-immobilized Hsp150Δ-ST3Ne, in the presence of CMP-[$^{14}$C]NeuNAc. Sodium azide was included in the reaction mixture to stop metabolic activity of the cells. After removal of the yeast cells by centrifugation, the protein product was TCA-precipitated from the reaction mixture for scintillation counting. The amount of protein-bound radioactivity was found to increase linearly with time, indicating that the substrates could freely penetrate the cell wall for the acquisition of α2,3-linked [$^{14}$C]NeuNAc, whereafter

they diffused back to the reaction buffer. Furthermore, when completely sialylated fetuin was incubated with the yeast cells, or when asialofetuin was incubated with the parental yeast cells lacking the *HSP150Δ-ST3Ne* gene, no radioactivity could be TCA-precipitated. The transfer of [$^{14}$C]NeuNAc to the acceptor substrate was accurate, as no radioactivity was transferred to desialylated bovine submaxillary mucin (II, Fig. 1A), which carries only O-glycans devoid of terminal Galβ1-3/4GlcNAc structures (Tsuji and Osawa, 1986; Savage *et al.*, 1990). When the porosity of the cell wall was increased by reducing disulphide bonds between cell wall proteins with DTT (de Nobel *et al.*, 1989), the sialylation of asialofetuin was enhanced, apparently due to the facilitated entry of the substrate into the cell wall. Reduction of the disulphide bonds in asialofetuin (Dziegielewska *et al.*, 1990) was also likely to support its cell wall entry. Further increase in the amount of sialylated product was obtained, when Hsp150Δ-ST3Ne synthesis and transport were allowed to continue during the four hour assay by omitting sodium azide from the incubation mixture and adding glucose instead. With both glucose and DTT present, the sialylation of asialofetuin was improved 2.5-3 fold (II, Fig. 1B). To use bovine prothrombin and human transferrin as the acceptor substrates in the α2,3-sialylation reactions, the proteins were first desialylated by mild acid hydrolysis (Warren, 1959; Kaplan *et al.*, 1983), whereafter they were subjected for CD spectroscopy to confirm that they had retained their native conformations. Both prothrombin and transferrin served as substrates for the cell wall-born ST3Ne, but the transfer of [$^{14}$C]NeuNAc to them was less efficient than to asialofetuin (II, Table 1).

43

MALDI-TOF mass spectrometry was used to determine the degree of asialoglycoprotein sialylation (II, Fig. 3). The N-glycans were released from the protein backbones by N-glycosidase F, and prepared for the analysis according to Nyman *et al.* (1998). After a four hour sialylation reaction, 31.7% of the exposed galactose residues of asialofetuin were found to contain NeuNAc (Fig. 3A vs. 3B), whereas after 16 hours the respective percent for asialofetuin was 61.3% and for asialotransferrin 41.5% (not shown). In comparison to asialofetuin, the sialylation of asialoprothrombin and asialotransferrin detected by [14C]NeuNAc incorporation was less efficient (II, Table 1), in agreement with the MALDI-TOF analysis of asialotransferrin. The different degree of asialoglycoprotein sialylation between the test proteins can be explained by their diverse N-glycan branching pattern. ST3N exhibits higher affinity for the triantennary N-glycans found in asialofetuin than for the biantennary N-glycans of asialotransferrin and asialoprothrombin (Yeh and Cummings, 1997).

## 3.2 Kinetic properties of cell wall immobilized Hsp150Δ-ST3Ne (II)

To explore the effects of cell wall immobilization and the presence of the Hsp150Δ carrier on the enzymatic properties of ST3Ne, its kinetic parameters were compared to those of the commercially available recombinant ST3N produced by the recombinant baculovirus system in insect cells. The assays for Km determinations were performed by keeping one substrate under saturating conditions, while varying the other for kinetic assessment. To achieve the high concentrations of CMP-NeuNAc needed, CMP-[14C]NeuNAc was mixed with unlabelled CMP-NeuNAc. The amount of CMP-[14C]NeuNAc was kept constant in all samples. The values for relative Km were determined from intercepts of double reciprocal plots (Lineweaver-Burk). The Km values for asialofetuin, lacto-N-tetraose (LNT) and N-acetyllactosamine (LacNAc) were found to be nearly identical to those defined for the commercial ST3N (II, Table 2A). ST3N prefers type I oligosaccharides (Galβ1-3GlcNAc, LNT) over type II oligosaccharides (Galβ1-4GlcNAc, LacNAc) (Weinstein *et al.*, 1982b), which was demonstrated here by the relative ratios of $V_{max}/K_m$ (II, parenthesis in Table 2A). Also the $V_{max}/K_m$ value determined for asialofetuin agrees with this, as its terminal N-glycan structures are mostly of type II (Spiro, 1973; Rice *et al.*, 1990). Furthermore, the $K_m$ value of yeast cell wall-borne ST3Ne for CMP-NeuNAc was found to be similar to those reported by others for recombinant ST3N from insect cells, and authentic isolated rat liver ST3N (II, Table 2B). For determining the amount of cell-associated ST3N activity, yeast cells ($50 \times 10^6$) were incubated with LNT (0.4mM) and CMP-NeuNAc (10mM), whereafter the sample was subjected to ion-exchange chromatography and concentration. Gel filtration and UV-detection were used to quantitate the NeuNAcα2-3Galβ1-4GlcNAc product against external LNT. The yield of active enzyme in one litre recombinant yeast cells grown to optical density of four ($10^8$ cells/ml) was 117 mU.

## 3.3 ST3Ne expression in *S. cerevisiae* with the aid of the MFα carrier as compared to the Hsp150Δ carrier (III)

The *S. cerevisiae* mating factor α (MFα) is synthesized as a precursor

44

of 165 amino acids, which contains an 85-amino-acid prepro leader and four tandem repeats of the mature 13-amino-acid α-factor sequence. Each repeat is preceded by a short peptide spacer of 5-8 amino acids with a site for Kex2p processing (Kurjan and Herskowitz, 1982; Julius *et al.*, 1983). In the TGN, the Kex2p endoprotease cleaves the precursor after the leader sequence, and between the α-factor repeats. Kex2p processing occurs on the C-terminal side of the dibasic KR sequence. The N- and C- termini of the released α-factor peptides undergo further proteolytic processing by dipeptidyl aminopeptidase A (*STE13*) and Kex1p (*KEX1*), whereafter the mature α-factor pheromones are secreted to the cell exterior (Kjeldsen, 2000). The 85-residue prepro leader of the MFα precursor (MFα carrier) has been used for expression of foreign proteins in *S. cerevisiae* and other yeasts. Already in the 1980's the MFα carrier was shown to confer secretion competence to foreign proteins expressed in *S. cerevisiae* (Bitter *et al.*, 1984; Brake *et al.*, 1984), and since then a number of heterologous proteins have been successfully produced using the MFα carrier expression system (Bitter *et al.*, 1984; Brake *et al.*, 1984; Romanos *et al.*, 1992; Lin Cereghino and Cregg, 2000; Kjeldsen, 2000). The MFα carrier consists of the 19-amino-acid signal peptide (pre), followed by a 66-amino-acid pro-sequence containing three potential N-glycosylation sites and a C-terminal site for Kex2p endopeptidase processing (Kurjan and Herskowitz, 1982). If the foreign protein is fused to the MFα carrier after the Kex2p cleavage site, the fusion protein undergoes Kex2p processing in the Golgi, the MFα carrier is released and the foreign protein secreted to the culture medium. However, the Kex2p site can be excluded from the constructs, which leads to secretion of the full lenght fusion protein to the medium. In this work, none of the constructs contained the Kex2p cleavage site due to the unavailability of ST3N-antiserum needed for detection of the transferase after Kex2p processing.

To compare the carriers, the Hsp150Δ fragment of Hsp150Δ-ST3Ne was replaced by the MFα carrier, and the construct (hereafter MFα-ST3Ne) was expressed in *S. cerevisiae* under the control of the *HSP150* promoter. The intracellular transport of MFα-ST3Ne was studied by pulse-chase experiments. The cells were treated with tunicamycin before labeling to prevent the formation of smears in SDS-PAGE due to heterogenous protein N-glycosylation. Cell lysates immunoprecipitated with prepro α-factor antiserum revealed that the fusion protein remained within the cell still after a two hour chase, whereas no newly synthesized protein could be detected in the culture medium. Of the two bands in gels after pulse, the upper one (46kDa) was likely to represent the cytosolic form of the fusion protein as it disappeared during the chase, whereas the 44 kDa protein persisted, and was apparently the mature MFα-ST3Ne. Furthermore, the size difference of 2 kDa between the bands corresponds to the size of the signal peptide (III, Fig. 2). Expression of the fusion protein was up-regulated at 37°C as compared to 24°C due to the heat-inducible *HSP150* promoter (Russo *et al.*, 1993).

The catalytic activity of MFα-ST3Ne was measured from cell lysates, cell wall and culture medium, and compared to the activity distribution of Hsp150Δ-ST3Ne. The ST3N activity measured indicated the amount of enzyme accumulated in the cells and

the medium during the overnight cultivation. The total ST3N activity was 1.7 times higher in the yeast strain expressing Hsp150Δ-ST3Ne, as compared to the activity of MFα-ST3Ne. This correlates with the metabolic labeling experiments, where the respective difference in the protein amounts was 2.3 fold, according to fosfoimager quantitation (data not shown). Thus, the higher ST3N activity of Hsp150Δ-ST3Ne was due to its elevated expression levels, and not to different catalytic properties of ST3Ne excerted by the carriers. With Hsp150Δ as the carrier, about 50% of the ST3N activity was bound to the yeast cell wall, and with MFα the respective proportion was 40% (III, Fig. 3). As heterologous proteins fused to the MFα carrier sequence in the absence of the Kex2p processing site are normally secreted to the yeast culture medium (Kjeldsen *et al.*, 1998), the cell wall association of the ST3N-fusion proteins was most likely largely mediated by the ST3Ne portion. About 60% of the MFα-ST3Ne activity was secreted to the culture medium, and hardly any remained intracellular. Of Hsp150Δ-ST3Ne activity 23% was detected in the culture medium and 27% remained intracellular (III, Fig. 3).

Thus, both the MFα and the Hsp150Δ carriers provided secretion competence and promoted proper folding of ST3Ne in *S. cerevisiae*. The importance of the carrier was demonstrated by directing ST3N to the secretory pathway of *S. cerevisiae* with the Hsp150 signal peptide in the absence of a carrier polypeptide. As a result, 90% of ST3N activity was lost, whereas the expression levels of ST3Ne and *HSP150Δ-ST3Ne* were comparable (II, Fig. 2). Furthermore, in the absence of the carrier, none of the detected ST3N activity was exter-

nalized. These data suggest that a carrier polypeptide was required for the acquisition of a secretion competent and biologically active conformation of ST3Ne in *S. cerevisiae*. The intracellular transport of ST3Ne could not be studied due to the unavailability of specific ST3N-antiserum.

### 3.4 Expression of ST3N in *P. pastoris* (III)

The possibilities for scaling up ST3N production were investigated by expressing both Hsp150Δ-ST3Ne and MFα-ST3Ne in the methylotrophic yeast *P. pastoris* under the strong methanol-inducible *AOX1* promoter. The *AOX1* promoter allows high expression of the desired gene, and numerous foreign proteins have been produced in high yields in *P. pastoris* since 1984 (Lin Cereghino and Cregg, 2000).

To study the induction and secretion of Hsp150Δ-ST3Ne in *P. pastoris*, shake flask cultures of the recombinant and the parental yeast strains were grown overnight in glycerol, control samples were collected, whereafter the methanol induction was started. After three days of induction, samples were harvested and analyzed together with non-induced samples by Western blotting using Hsp150-antiserum. An induction of Hsp150Δ-ST3Ne expression was detected both in the cell lysate and culture medium samples of the methanol-treated recombinant yeast strain (data not shown). While no protein could be visualized in the lysate of the parental strain, a background band was detected in the supernatants of both the recombinant and the parental strains independently of induction (not shown). This is likely to represent the Hsp150 homologue of *P. pastoris*,

46

due to cross-reaction with the Hsp150-antiserum. Homologs of Hsp150 have been found in *T. delbrueckii*, *K. marxianus* and *Sch. pombe* (Russo *et al.*, 1992).

Samples from both the Hsp150Δ-ST3Ne and the MFα-ST3Ne expressing *P. pastoris* strains were harvested for activity assays after 3 days of shake flask cultivation in 0.5% methanol. The assays for ST3N activity were performed as described for *S. cerevisiae* using LNT as the acceptor substrate and CMP-[$^{14}$C]NeuNAc as the donor substrate. In *P. pastoris*, there were no significant differences between the carriers in the distribution of ST3N activity within the cell and the cell exterior, whereas the amount of catalytic activity was about 3 times higher in each sample for the strain carrying Hsp150Δ-ST3Ne, as compared to that of the MFα-ST3Ne (III, Fig. 3). In comparison with the catalytic activity of Hsp150Δ-ST3N expressed in *S. cerevisiae*, about twice the amount of ST3N activity was produced by Hsp150Δ-ST3Ne in *P. pastoris*, while no increase could be detected with the MFα carrier. This demonstrates that *P. pastoris* can be used as the expresson host for ST3N, whereas its potential in respect to the enzyme yields remains to be studied under optimal growth conditions in fermenters.

## 4 Cell wall anchoring of endogenous Pir2p/Hsp150, truncated Hsp150 and ST3Ne fusion proteins (III, IV)

The two known groups of mannoproteins covalently bound to cell wall β-glucan in *S. cerevisiae* are the Pir-proteins (Pir-CWPs) and the GPI-dependent cell wall proteins (GPI-CWPs) (Kapteyn *et al.*, 1996; Mrša *et al.*, 1997). While the structural unit linking the GPI-CWPs to the cell wall has been known for some years (see section 4 in introduction, Kapteyn *et al.*, 1996; Lipke and Ovalle, 1998), the mechanism of cross-linking Pir-proteins to other cell wall components was studied here. Hsp150 (also called Pir2p) is the only member of the Pir-protein family, which is secreted to the yeast culture medium (Russo *et al.*, 1992). However, as some Pir2p/Hsp150 has been shown to be retained in the cell wall (Mrša *et al.*, 1997; Yun *et al.*, 1997) it could be used as the model protein for studying the cell wall anchoring of Pir-CWPs. Besides gaining more information on the structure of the yeast cell wall, this was of interest in respect to the cell wall immobilization mechanisms of Hsp150Δ-ST3Ne, since the Hsp150Δ carrier is a derivative of Hsp150.

### 4.1 Pir2p/Hsp150 is bound to cell wall β1,3-glucan (IV)

Approximately 98% of all covalently cell wall associated proteins, including Pir-CWPs, are β1,3-glucanase extractable (Kapteyn *et al.*, 1997). Furthermore, Pir-CWPs are released from the cell wall by mild alkali treatment, and are thus likely to be linked to the cell wall through an O-glycosidic linkage (Mrša *et al.*, 1997; Mrša and Tanner, 1999). However, the cell wall component ultimately responsible for binding Pir-CWPs was not known. To study whether β1,6-glucan, which connects GPI-CWPs to the cell wall, participated in the an-

47

choring of Pir-proteins to the cell wall, isolated yeast cell walls were digested with β1,6-glucanase followed by blotting and immunodetection with Hsp150-antiserum. No immunoreactive material could be detected in the β1,6-glucanase extracts, suggesting that β1,6-glucan was not linking Pir2p/Hsp150 to the cell wall (IV, data not shown). Instead, immunoreactive Pir2p/Hsp150 was released when the 1,6-glucanase-digested cell walls were further treated with β1,3-glucanase, or subjected to mild alkaline hydrolysis. The altered electrophoretic mobility of Pir2p/Hsp150 after the hydrolysis was due to loss of the β1,3-glucan epitopes as the consequence of the alkali treatment (IV, Fig. 2A). The band detected in the *pir2Δ* strain was due to cross-reaction of the Hsp150-antiserum with Pir1p, a protein highly homologous to Pir2p (Toh-E *et al.*, 1993), as no band could be visualized in the strain lacking genes for both Pir1p and Pir2p (IV, Fig. 2A). Further staining of the β1,6-glucanase-resistant, β1,3-glucanase-soluble cell wall proteins with concanavalin A, a lectin for α-mannosyl residues, revealed a number of proteins (IV, Fig. 2B). These were likely to resemble Pir1p, Pir2p/Hsp150 and other Pir-CWPs, as the total number of the detected proteins was similar to that reported for the putative Pir-CWPs by Mrša *et al.* (1997). The β1,6-glucanase-resistant, β1,3-glucanase-soluble cell wall proteins reacted strongly with β1,3-glucan antiserum (IV, Fig. 2C). Staining with β1,6-glucan antiserum resulted in a faint signal only after a long exposure indicating that hardly any β1,6-glucan epitopes were present in the released proteins (IV, Fig. 2D). Thus, unlike GPI-CWPs, the Pir-proteins were anchored directly to the yeast cell wall β1,3-glucan without the interconnecting β1,6-glucan moiety. Ccw1p, a GPI-CWP normally solubilized by β1,6-glucanase, was used as a marker for studying the presence of GPI-CWPs in the β1,6-glucanase-resistant, β1,3-glucanase-soluble extract. A faint band representing Ccw1p could be detected in the β1,6-glucanase resistant cell wall extract of wild type cells, whereas its amount was increased in the *PIR1* and *PIR2* deletion strains (IV, Fig. 2E). Whether this fraction of Ccw1p indicates binding of the protein to both β1,6-and β1,3-glucan, or to short β1,6-glucan chains resistant to β1,6-glucanase, is not known.

## 4.2 Activation of rescue mechanisms in responce to cell wall weakening (IV)

Mutant yeast strains deficient of normal β1,6-glucan synthesis (*Δkre*) were used to further confirm that the binding of Pir2p/Hsp150 to the yeast cell wall was β1,6-glucan-independent. Defective cell wall β1,6-glucan synthesis in the *Δkre* mutants is known to result in secretion of GPI-CWPs to the culture medium, due to reduced number of the attachment sites in the cell wall (Lu *et al.*, 1995; Jiang *et al.*, 1996; Kapteyn *et al.*, 1996). To study the effect of deficient β1,6-glucan synthesis on Pir2p secretion, *Δkre6* and *Δkre9* mutants, with 50% and 80% reduced β1,6-glucan synthesis, respectively, were used (Roemer and Bussey, 1991; Brown and Bussey, 1993). The altered β1,6-glucan levels in the *Δkre* strains did not lead to increased Pir2p/Hsp150 secretion as compared to wild type cells (IV, Fig. 3A), whereas the amount of the normally β1,6-glucan bound Ccw1p was significantly greater in the supernatants of the *Δkre* mutants as compared to the wild type cells (IV, Fig. 3B). On the other hand, in *Δfks1* and

*Δgas1* strains, which are deficient in β1,3-glucan synthesis and assembly, respectively (Inoue *et al.*, 1995; Popolo *et al.*, 1997), Pir2p/Hsp150 secretion was increased to some extent (IV, Fig. 3A). This further supports the role for β1,3-glucan as the structural unit for Pir2p/Hsp150 binding.

Cell wall incorporation of Pir2p/Hsp150 was found to be greatly enhanced in the yeast mutants with deficient β1,6-glucan synthesis (IV, Fig. 4). According to Northern analysis the mRNA levels of Pir2p/Hsp150, Pir3p and another Pir-protein, Ccw5p (Ccw11p), increased as the function of the severity of the β1,6-glucan defect (IV, Table 2). Also the expression of *FKS2*, which encodes for one of the putative catalytic subunits of β1,3-glucan synthetase (Mazur *et al.*, 1995), was greatly induced (IV, Table 2). Furthermore, the more severe the defect in β1,6-glucan synthesis, the fewer proteins could be solubilized by β1,6-glucanase digestion, whereas the amount of proteins released by β1,3-glucanase treatment was increased (IV, Table 1). Increased chitin levels in the lateral cell walls of the *Δkre* strains were also observed. These data suggest a rescue mechanism for the cell to maintain the integrity of the cell wall through increasing the synthesis of chitin, β1,3-glucan and Pir-proteins in response to reduced β1,6-glucan synthesis.

## 4.3 Cell wall immobilization of Hsp150Δ-ST3Ne and Hsp150$_{TRUNC}$ (III)

Pir2p/Hsp150 was shown to bind covalently to cell wall β1,3-glucan (IV), whereas the determinant responsible for the association remained unclear. Furthermore, nothing was known about the mechanism of cell wall binding of Hsp150Δ-ST3Ne, which is a Pir2p/Hsp150 derivative. Thus, to compare the cell wall binding of Pir2p/Hsp150 to that of Hsp150Δ-ST3Ne, the cells were labeled with [$^{35}$S]methionine/cysteine, chased in the presence of CHX and subjected for cell wall isolation as described in IV. The SDS extract containing the non-covalently associated cell wall material, as well as the β1,3-glucanase-digested proteins attached to the cell wall *via* covalent linkages, were subjected to immunoprecipitation with Hsp150-antiserum. As expected, no endogenous Hsp150 was solubilized by SDS, but it was quantitatively released by β1,3-glucanase (III, Fig. 4). In contrast, all Hsp150Δ-ST3Ne was found in the SDS-soluble fraction, indicating non-covalent cell wall association (III, Fig. 4). The cell wall immobilization of Hsp150Δ-ST3Ne was apparently due to the ST3Ne portion, since also MFα-ST3N was found to be non-covalently cell wall associated (data not shown).

The non-covalent cell wall binding of Hsp150Δ-ST3Ne could have resulted from the absence of the 92 C-terminal amino acids of Pir2p/Hsp150, which were replaced by ST3Ne in the fusion protein. To explore this more directly, a truncated version of Hsp150 (Hsp150$_{TRUNC}$) lacking the 94 C-terminal residues of Pir2p/Hsp150 was used. Most of Hsp150$_{TRUNC}$ was found to be released by SDS, and a minor part could be solubilized only after β1,3-glucanase digestion. This suggests that the C-terminal amino acids of Pir2p/Hsp150 are largely determining its covalent binding to the cell wall. The release of Pir2p/Hsp150 and other Pir-CWPs from the cell wall by mild alkali treatment indicates that they are linked to the cell wall β1,3-glucan through O-glycosidic linkages (IV, Fig. 2, 4; Mrša *et al.*, 1997). Such

49

linkages could form between the cell wall $\beta$1,3-glucan and the Ser/Thr residue of the protein, or the O-glycans of the protein. Pir2p/Hsp150 contains altogether 106 Ser/Thr residues, of which most are glycosylated (Jämsä *et al.*, 1995b; Suntio *et al.*, 1999). However, of all the potential O-glycosylation sites only 8 reside in the 94 C-terminal amino acids of the protein, the removal of which abolished most of the covalent binding (III, Fig 4). This suggests that either the 8 Ser/Thr residues absent in Hsp150$_{TRUNC}$ are crucial for forming the glycosidic bonds to the cell wall $\beta$1,3-glucan, or that the C-terminus is required for covalent cell wall binding due to conformational reasons.

## DISCUSSION

While cloning and expression in heterologous hosts can provide means to obtain proteins that cannot be purified from their natural sources, unexpected biological difficulties may arise when working with living organisms. When the catalytic ectodomain of rat liver ST6N was expressed in *S. cerevisiae* with the aid of the Hsp150Δ carrier, the fusion protein was targeted for vacuolar degradation regardless of its apparently correct conformation. However, when ST6Ne was replaced by the catalytic ectodomain of rat liver ST3N, another member of the sialyltransferase family, the expression of the fusion protein yielded an active cell wall-bound enzyme with kinetic properties resembling those of the authentic ST3N. Thus, even if the expression system is chosen to meet the requirements of the heterologous protein, the successful production of the desired protein is ultimately dependent on the unique biological properties of the protein itself.

### 1 Vacuolar targeting and membrane sterol-dependent degradation of Hsp150Δ-ST6Ne

Expression of the catalytic ectodomain of ST6N as an Hsp150Δ-fusion in *S. cerevisiae* resulted in secretion competent, catalytically active ST6Ne with kinetic parameters resembling those of the authentic ST6N. Since ST6Ne exited the ER and reached the Golgi, the carrier polypeptide apparently completed its function in facilitating protein folding and smuggling the foreign protein through the ER quality control. Previous experiments by Krezdorn *et al.* (1994) demonstrated that expression of full-length rat liver ST6N in *S. cerevisiae* resulted in ER retention and relatively rapid degradation of the transferase. However, not even as an Hsp150Δ-fusion was ST6Ne externalized, but instead, sorted from the Golgi to the vacuole for degradation. As Hsp150, Hsp150Δ and Hsp150Δ-ST3N are secreted to the yeast cell wall or culture medium (Russo *et al.*, 1992; Jämsä *et al.*, 1995b; Mattila *et al.*, 1996) the signal, which targets Hsp150Δ-ST6Ne to the vacuolar pathway must reside in the ST6Ne portion. The expressed ST6Ne sequence was devoid of the 26 N-terminal amino acids coding for the short cytoplasmic tail and the transmembrane domain of the authentic transferase, whereas included in the recombinant protein were the lumenal stem region and the catalytic ectodomain (see Fig. 7C, E). The stem domain of ST6N has been proposed to have a role as an independent Golgi retention signal (Colley, 1997), and to determine the catalytic properties of the enzyme (Legaigneur *et al.*, 2001). ST6N lacking the transmembrane and stem domains has been shown to be secreted in CHO cells (Colley *et al.*, 1989), whereas in the absence of the stem, chimeric proteins containing the ST6N transmembrane domain were targeted to the lysosome (Tang et al., 1995). To study if the stem region contained signals determining the destination of Hsp150Δ-ST6Ne in yeast, the amino acids encoding the stem were excluded from the construct. However, this did not rescue secretion or stabilization of the fusion protein. Instead, it was degraded like in the presence of the stem (unpub-

lished data). Whether targeting of Hsp150Δ-ST6Ne to the vacuole was due to specific sorting signals in the ST6N sequence, or a putative quality control function at the Golgi complex (Hong *et al.*, 1996; Holkeri and Makarow, 1998), is not known.

In wild type yeast cells the majority of Hsp150Δ-ST6Ne degradation was due to the proteolytic activity of vacuolar hydrolases, since deletion of the *PEP4* gene coding for protease A, the activator of pro-CPY and other vacuolar proteases (van den Hazel *et al.*, 1996), significantly increased the stability of the fusion protein. Interestingly, the proteolytic activity of the vacuole in the *Δpep4* strain was restored when the biosynthetic pathway of ergosterol, the major sterol in yeast membranes, was disrupted (*Δerg6*). The rescue of vacuolar function in the *Δerg6 Δpep4* strain was found to be due to altered intracellular localization of Yps1p, a GPI-anchored aspartic protease of the yeast yapsin family (Ash *et al.*, 1995; Olsen *et al.*, 1999) with specificity for paired or single basic amino acid residues (Copley *et al.*, 1998; Komano *et al.*, 1999; Olsen *et al.*, 1999). In wild type yeast cells with normal ergosterol biosynthesis Yps1p resides in the yeast plasma membrane (Ash *et al.*, 1995), whereas in the absence of membrane ergosterol in the *Δerg6* strain we found part of Yps1p targeted to the vacuole. Yps1p was proteolytically active in the vacuole, since it rescued the processing of pro-CPY to mature CPY in the absence of the *PEP4* gene, and the deletion of *YPS1* significantly reduced CPY maturation. Also the degradation of Hsp150Δ-ST6N proceeded with lower kinetics in the *Δerg6 Δpep4 Δyps1* strain as compared to the *Δerg6 Δpep4* strain. In other words, Yps1p localized to the vacuole in the absence of membrane

ergosterol was able to activate the vacuolar hydrolases in the absence of proteinase A, the central activator of vacuolar proteases. Yps1p and proteinase A are homologous aspartic proteases with 27% identical amino acids and low pH optimum (Cawley *et al.*, 1998; Olsen *et al.*, 1999). These data implicate a novel site of function for Yps1p in the vacuole. Since membrane sterol composition can vary depending on growth conditions and the stage of cellular growth (Leber et al., 1995), the membrane-sterol dependent localization of Yps1p suggests that GPI-anchored yeast yapsins, whose biological roles are unknown, may reside and function in different subcellular locations under different physiological conditions. An example of the regulatory role of nutrients on intracellular protein targeting is provided by the plasma membrane amino acid permeases, Gap1p and Tat2p, which are directed to the vacuole from the plasma membrane, or directly from the Golgi, upon nutrient deprivation (Roberg *et al.*, 1997; Beck *et al.*, 1999).

We found Yps1p clustered in the detergent-insoluble microdomains of membranes both in the presence and absence of ergosterol. Since according to immunostaining, part of Yps1p resided in the vacuolar membrane in the *Δerg6* cells, Yps1p was likely to be raft-associated also in the vacuolar membranes. This indicates that the vacuolar targeting of Yps1p was not due to exclusion of the protein from the rafts, and that rafts can exist also in the vacuolar membranes, which are generally poor in sterols (Zinser *et al.*, 1993; Schneiter *et al.*, 1999). The *Δerg6* strain is devoid of membrane ergosterol, whereas the cells accumulate sterol intermediates of zymosterol and cholesta-5,7,24-trienol (Munn *et al.*, 1999), which thus seem

to be sufficient for the formation of the raft clusters. Raft disruption by using yeast mutants incapable of sphingolipid synthesis was recently shown to result in vacuolar targeting of Pma1p, a raft-associated transmembrane protein of the yeast plasma membrane (Bagnat *et al.*, 2001). Yeast GPI-anchored proteins become raft-associated in the ER, whereas Pma1p was shown to cluster in the lipid rafts not until in the Golgi (Bagnat *et al.*, 2000; 2001). As membrane proteins lacking appropriate sorting signals are generally directed from the Golgi to the vacuole (Roberts *et al.*, 1992), and raft disruption caused Pma1p to follow this route, the lipid raft apparently functioned as the plasma membrane sorting signal for Pma1p (Bagnat *et al.*, 2001).

Besides raft association, also N-glycans have been proposed an important role in sorting GPI-anchored proteins to the plasma membrane (Simons and Ikonen, 1997; Benting *et al.*, 1999a). We did not explore whether Yps1p was directed to the vacuole from the Golgi or the plasma membrane. However, as Yps1p was heavily N-glycosylated (data not shown) and the rafts assembled in the *Δerg6* strain promoting Yps1p clustering into the platforms, the putative plasma membrane sorting signals of Yps1p were not affected in the *Δerg6* cells. Consequently, there were no obvious reasons for targeting Yps1p to the vacuole from the Golgi. If Yps1p was first transported to the plasma membrane, it must have undergone endocytosis to reach the vacuole. In mammalian cells GPI-anchored proteins are known to undergo raft-dependent endocytosis and recycling back to the cell surface (Mayor *et al.*, 1998; Verkade *et al*, 2000; Chatterjee *et al*, 2001), whereas in yeast all known endocytosed

proteins are transmembrane proteins, and therefore no data on the lipid requirements for endocytosis of GPI-anchored proteins in yeast exist. However, endocytosis of the yeast α-factor receptor-ligand complex has been reported to depend on the composition of membrane sterols (Munn *et al.*, 1999). Thus, it will be interesting to elucidate the route of the GPI-anchored Yps1p to the vacuole in the ergosterol deficient yeast cells. Nevertheless, the membrane sterol-dependent targeting of Yps1p identifies a novel sorting pathway for GPI-anchored proteins in yeast.

Hsp150Δ-ST6Ne was degraded not only in the vacuole, but also in the Golgi under conditions where secretory proteins could not exit the organelle. Yps1p was found to be involved in this process, while Yps2p, another yeast yapsin, was not. However, in addition to Yps1p, other degradative mechanisms existed in the Golgi, since the fusion protein was still degraded to same extent as in the absence of *YPS1*. Since neutralization of the pH of the Golgi lumen totally abolished the Golgi-associated degradation of Hsp150Δ-ST6N, the pH of the organelle seemed to play an important role in the process. The finding that the fusion protein was stable when blocked in the ER further supports this, since the ER lumen is more neutral as compared to the Golgi (Wu *et al.*, 2000). Thus, it is likely that the pH of the Golgi apparatus could promote the activation of the Golgi-blocked Yps1p and other proteases with low pH optimum. Our findings indicate, for the first time, a role for Yps1p in Golgi-associated proteolytic degradation.

Besides our findings of Yps1p acting as an activator of vacuolar proteases, and the report of Yps1p-dependent α-

factor processing by Egel-Mitani *et al.* (1990), no other endogenous substrates of Yps1p are known. Instead, Yps1p has been shown to degrade many secreted recombinant mammalian prohormones at the exterior of *S. cerevisiae* cells (Copley *et al.*, 1998; Kang *et al.*, 1998; Kerry-Williams *et al.*, 1998; Bourbonnais *et al.*, 2000), but intracellular Yps1p activity towards heterologous proteins has been suggested and studied only in a few cases before (Bourbonnais *et al.*, 1993;

Zhang *et al.*, 1997). While the function of Yps1p in yeast is not known, it has been reported to exhibit α-secretase-like activity towards human β-amyloid peptide precursor (APP) (Zhang *et al.*, 1997; Komano *et al.*, 1998). In mammals, proteolytic processing of APP by α-secretase precludes the formation of β-amyloid peptide, which is involved in Alzheimer's disease and produced as the consequence of alternative APP cleavage by β- and γ-secretases (Haass and Selkoe, 1993).

## 2 Expression of the catalytic ectodomain of rat liver ST3N in yeasts

Whole living yeast cells could be used as a source of ST3N activity, since the porous structure of the yeast cell wall allowed the penetration of both the nucleotide sugar donor and the acceptor substrates to the cell wall, whereafter the α2,3-sialylated products freely diffused back to the reaction mixture. Besides oligosaccharides, the cell wall-borne Hsp150Δ-ST3Ne was found to be capable of utilizing completely and incompletely desialylated glycoproteins of high molecular weight and native conformation as acceptor substrates. The differences in the molecular weights and the number of disulphide bonds between asialofetuin (Spiro, 1973), asialoprothrombin (MacGillivray and Davie, 1984) and asialotransferrin (MacGillivray *et al.*, 1982) used as the asialoglycoprotein acceptor substrates in the study, could partly explain their different degree of sialylation by the cell wall immobilized ST3N, since facilitated cell wall entry under reducing conditions was shown to enhance the sialylation efficiency. However, ST3N is known to differentiate between glycoprotein acceptors according to the degree of their N-glycan branching. ST3N has been reported to prefer

asialoglycoproteins with highly branched N-glycans to those with biantennary N-glycans (Yeh and Cummings, 1997). Bovine asialofetuin contains one biantennary and two triantennary N-glycans with terminal structures of Galβ1-3GlcNAc and Galβ1-4GlcNAc (Spiro, 1973; Rice *et al.*, 1990). The three N-glycans of bovine asialoprothrombin are biantennary and terminate with Galβ1-3GlcNAc and Galβ1-4GlcNAc structures (Mizuochi *et al.*, 1979), whereas the terminal structures of the two biantennary N-glycans of human asialotransferrin end with Galβ1-4GlcNAc (Spik *et al.*, 1975). Thus, besides the small size of asialofetuin, its branched N-glycans as well as the presence of Galβ1-3GlcNAc oligosaccharide sequences are likely to promote its sialylation by the cell wall-borne Hsp150Δ-ST3Ne. As the molecular weight and the N-glycan branching pattern are similar in asialoprothrombin and asialotransferrin, the preference for asialoprothrombin as the acceptor substrate can be explained by the presence of terminal Galβ1-3GlcNAc structures, for which ST3N has higher affinity as compared to the Galβ1-4GlcNAc structures in asialotransferrin (Weinstein *et al.*, 1982b).

54

Since sialylated carbohydrates are involved in many different biological phenomena, e.g. selectin-mediated processes (Foxall *et al.*, 1992; McEver, 1997), host-pathogen recognition (Miller-Podraza *et al.*, 1999; Hughes *et al.*, 2001) and lectin-mediated endocytosis (Lodish, 1991; Flesher *et al.*, 1995), these interactions could serve as therapeutic targets (Turunen *et al.*, 1994; Tojo *et al.*, 1996; Seppo *et al.*, 1996), and the *in vitro* synthesis of sialylated glycocompounds by an economical and simple procedure would have many applications. The advantages of using an enzymatic approach as compared to chemical synthesis are the stereoselectivity of the enzymes and their applicability to different structures (Kameyama *et al.*, 1991; de Vries *et al.*, 1993). For obtaining large amounts of pure glycosyltransferases, cloning and heterologous expression are the method of choice, as the isolation of transferases is difficult, and they cannot be obtained in sufficient quantities due to their low expression levels (Weinstein *et al.*, 1982b; Tsuji, 1996). The recombinant yeast strain expressing the cell wall-immobilized Hsp150Δ-ST3Ne provides an inexpensive and endless, self-perpetuating source of the transferase. The potential applications include e.g. the synthesis of sialyl Lewis X epitopes for anti-inflammatory therapy, and the sialylation of undersialylated N-glycans of recombinant pharmaceutical proteins to reduce their clearance rate from the circulation.

To be exploited in large-scale reactions, enhanced production of ST3Ne is required. For this *P. pastoris* provides an attractive alternative, although ST3Ne production might be improved also in *S. cerevisiae* by the use of stronger promoters (Martegani *et al.*, 1992), or by overexpressing certain proteins of the secretory apparatus (Robinson *et al.*, 1994; Ruohonen *et al.*, 1997). Among the factors accounting for the popularity of *P. pastoris* is the exploitation of the strong alcohol oxidase I promoter (*AOX1*), that allows high expression levels of the foreign gene, and the availability of the expression system as a commercial kit. Since 1984, over 200 foreign proteins have been successfully expressed in *P. pastoris*, many of which in high yields (Lin Cereghino and Cregg, 2000). The expression of ST3Ne fusion proteins in *P. pastoris* under the control of the methanol inducible *AOX1* promoter was successful in terms of protein secretion and catalytic activity, whereas the yield of active ST3Ne was not significantly increased as compared to *S. cerevisiae*. However, the growth conditions for the *P. pastoris* strains were not optimal as they were cultured in shake flasks without any extra oxygen supplementation while inducing the transcription of the heterologous gene with methanol. For optimal growth on methanol, the oxygen supply should not be limiting, as the alcohol becomes toxic to the cells if it cannot be oxidized to formaldehyde, which is further used for the generation of energy and biomass (Gellissen, 2000). Thus, the yield of active ST3Ne expressed in *P. pastoris* may be significantly increased as the cultivation is carried out in fermenters under controlled growth conditions.

To study the effect of carrier polypeptides on the catalytic activity of ST3Ne, the transferase was expressed in *S. cerevisiae* and in *P. pastoris* with the aid of the commercial MFα carrier and the Hsp150Δ carrier developed in our laboratory. The classical MFα carrier was shown to confer secretion competence to foreign proteins already in the 1980's

(Bitter *et al.*, 1984; Brake *et al.*, 1984), and since then it has been exploited in the production of numerous heterologous proteins in *S. cerevisiae* and in *P. pastoris* (Romanos *et al.*, 1992; Lin Cereghino and Cregg, 2000; Kjeldsen, 2000). Furthermore, a substantial portion of the recombinant insulin used for treating *diabetes mellitus* is produced in *S. cerevisiae* using the MFα carrier expression system. Russo *et al.* (1992) cloned the gene for the secretory glycoprotein Hsp150, the N-terminal fragment of which constitutes the Hsp150Δ carrier, that has been shown to confer secretion competence to several recombinant proteins expressed in *S. cerevisiae* (Holkeri *et al.*, 1996; Mattila *et al.*, 1996; Simonen *et al.*, 1996). Expression of ST3Ne with either the Hsp150Δ carrier or the MFα carrier resulted in catalytically active, cell wall-bound ST3Ne in both yeasts. In *S. cerevisiae* most of Hsp150Δ-ST3Ne was incorporated to the yeast cell wall, whereas the ratio of secreted vs. cell wall bound MFα-ST3N was about 1:1. The overall ST3Ne yield was higher when expressed as an Hsp150Δ-fusion. However, as this was due to the difference in the expression levels of the fusion proteins, both carriers were equally suitable for ST3Ne expression in *S. cerevisiae.* In *P. pastoris*, regardless of the carrier, most of the ST3N activity remained immobilized to the cell wall, whereas the activity levels were higher when expressed with Hsp150Δ. Thus, either of the carriers could be used for the expression of active ST3Ne in both *S. cerevisiae* and *P. pastoris.* However, as demonstrated in *S. cerevisiae*, a carrier polypeptide was required for ST3Ne to acquire a secretion competent and catalytically active conformation. The mechanism by which carrier proteins mediate folding and secretion of heterologous proteins is not known. They have been proposed to provide additional time for correct folding, which would ensure the escape from the ER quality control, and thus from degradation (Kjeldsen *et al.*, 1997; Ellgaard and Helenius, 2001). The Hsp150Δ carrier lacks regular secondary structure and persists as a random coil, which is likely to account for its capability to assist protein folding and secretion (Jämsä *et al.*, 1995b) .

## 3 Cell wall anchoring of Pir2p/Hsp150 and its derivatives

The chemical components of the *S. cerevisiae* cell wall are known, but information on the assembly process and the interactions between the various macromolecules has begun to emerge only in recent years (Kapteyn *et al.*, 1999; Smits *et al.*, 2001). The major components of the cell wall are β1,3- and β1,6-glucan, mannoproteins and chitin. β1,3-glucan and chitin provide the strength to this layered structure, whereas mannoproteins are important in determining the porosity of the wall, in addition to their eventual other functions. β1,6-glucan provides attachment sites for all cell wall components, thus interconnecting the macromolecules.

GPI-dependent cell wall proteins (GPI-CWP) and Pir-proteins (Pir-CWP) form the two groups of covalently cell wall bound mannoproteins identified so far. These proteins are not solubilized by SDS under reducing conditions, but can be released from the cell wall by β-glucanase treatment (Kapteyn *et al.*, 1996; Mrša

*et al.*, 1997). The GPI-CWPs are known to bind to β1,6-glucan through the GPI-remnant, which forms after the cleavage of the GPI-anchor at the plasma membrane (Kapteyn *et al.*, 1996). Previously this was the proposed anchoring mechanism of all β1,3-glucanase-extractable cell wall proteins. However, as the Pir-CWPs do not contain the C-terminal sequence required for GPI-anchoring (Russo *et al.*, 1992), their mechanism of binding to the cell wall was likely to be different from that of the GPI-CWPs. Furthermore, alternative anchoring mechanisms were likely to exist, since part of all covalently cell wall-bound proteins were found to remain insoluble after digestion with β1,6-glucanase, which releases GPI-CWPs.

Hsp150 (also called Pir2p) is the only member of the Pir-protein family, which is secreted to the yeast culture medium (Russo *et al.*, 1992). However, as some Pir2p/Hsp150 remains bound to the cell wall (Mrša *et al.*, 1997; Yun *et al.*, 1997), it could be used as a model protein for exploring the covalent cell wall association of Pir-CWPs. Several lines of evidence were found, which indicated covalent binding of Pir2p/Hsp150 directly to cell wall β1,3-glucan, without the interconnecting β1,6-glucan moiety found in GPI-CWPs. Pir2p/Hsp150 could not be extracted from isolated cell walls by β1,6-glucanase digestion, but it was released by β1,3-glucanase. Furthermore, the β1,3-glucanase released Pir2p/Hsp150 contained a vast amount of β1,3-glucan epitopes, whereas hardly any β1,6-glucan moieties could be detected. These β1,6-glucan epitopes were likely to represent β1,6-remnants bound to the β1,3-glucan moiety rather than interconnecting β1,6-glucan, since no increase in Pir2p/Hsp150 secretion was observed

in cells with deficient β1,6-glucan synthesis, whereas elevated levels of normally β1,6-glucan-bound proteins were detected in the medium. On the other hand, enhanced secretion of Pir2p/Hsp150 was observed in cells, which were defective in synthesizing cell wall β1,3-glucan.

In yeast mutants with low β1,6-glucan levels, the amount of cell wall bound Pir2p/Hsp150 and β1,3-glucan-linked proteins in general was remarkably increased as compared to wild type cells. Furthermore, the expression levels of three Pir-proteins, as well as *FKS2*, the other subunit of the putative β1,3-glucan synthetase (Ram *et al.*, 1998), were highly elevated in response to the decreased β1,6-glucan synthesis. Also chitin deposition on the lateral cell walls was enhanced. Thus, reduced β1,6-glucan synthesis seemed to induce a salvage pathway to maintain the integrity of the cell wall by increasing the synthesis of other cell wall components and using alternative mechanisms for incorporation of cell wall proteins. Respective rescue mechanisms to prevent cell wall weakening have been reported in cells with defective β1,3-glucan synthesis. In these cells chitin synthesis is increased, and more proteins are incorporated to the cell wall through chitin-bound β1,6-glucan (Kapteyn *et al.*, 1997). The yeast cell wall is not a fixed structure, but it undergoes continous remodelling during growth and development, and in response to environmental conditions. The compensatory changes providing cell wall strenght as a response to stress signals are essential for the cell survival (Smits *et al.*, 2001).

Since Pir-proteins can be released from the cell wall by mild alkaline hydrolysis, their O-glycans are likely to be involved in forming the glycosidic

linkage to the cell wall (Mrša *et al.*, 1997). The heavily O-glycosylated domains of Pir2p/Hsp150 are the subunit I and the repetitive region of the subunit II, whereas the unique C-terminus contains only about 10% of the putative O-glycosylation sites (Jämsä *et al.*, 1995b; Suntio *et al.*, 1999). However, after removal of the C-terminal domain from Pir2p/Hsp150, most of the protein was non-covalently associated with the cell wall. Thus, inspite of the low number of O-glycans, the C-terminal domain was largely responsible for covalent binding of Pir2p/Hsp150 to cell wall $\beta$1,3-glucan.

Hsp150$\Delta$-ST3Ne, a derivative of the covalently cell wall bound Hsp150, was found to bind to the cell wall through non-covalent interactions. The binding was apparently mediated by the ST3Ne portion, since a large part of MF$\alpha$-ST3Ne was non-covalently cell wall bound, as well. Immobilization of enzymes in the yeast cell wall can be considered as an advantage in different applications, as no purification of the enzyme is needed and the enzyme is easily removed from the reaction (Schreuder *et al.*, 1996). Under most optimal circumstances the expressed heterologous protein would bind to the cell wall covalently, thus minimizing the loss of the protein to the culture medium. As covalent cell wall binding of Pir2p/Hsp150 was largely mediated by its C-terminal domain, the exploitation of this sequence in heterologous constructs might support and strenghten cell wall binding of the desired proteins. However, additional amino acids in the C-terminus of a protein can lead to loss of catalytic activity, as with ST3Ne (unpublished data). Fusion of foreign proteins to the C-terminus of authentic Hsp150, in turn, has been shown to result in inefficient secretion of the recombinant protein (Jämsä *et al.*,1994). Alternatively, the Pir2p/Hsp150 homologs Pir1 and Pir3, which are covalently bound to the yeast cell wall and not secreted to the culture medium as most of Pir2p/Hsp150 (Russo *et al.*, 1992; Toh-E *et al.*, 1993; Mrša *et al.*, 1997), could be tested as carrier proteins for their potential to provide secretion competence to heterologous proteins and their capability to covalently immobilize fusion proteins in the yeast cell wall. Sequences responsible for covalent cell wall binding of $\alpha$-agglutinin and flocculin have been successfully utilized in anchoring heterologous proteins to the yeast cell wall (Schreuder *et al.*, 1996). Whereas the cell wall of *S. cerevisiae* is the best studied among yeasts, there are indications that the structure is not unique among the different yeast species (Kapteyn *et al.*, 1995; Montijn *et al.*, 1997; Lipke and Ovalle, 1998). Thus, approaches aiming at cell wall immobilization of heterologous proteins are likely to be applicable to a range of different yeasts. Cell wall anchoring could be exploited e.g. by expressing multiple cell wall immobilized glycan engineering enzymes. By coupling the action of the enzymes within a single cell, the sugar intermediates could be recycled from one enzyme to another making the purification of the intermediates unnecessary, and the reaction more economic.

# ACKNOWLEDGEMENTS

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60

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71

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