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# Cell Wall Proteins of *Saccharomyces cerevisiae*

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## Function and composition of the cell wall

The cell wall of *Saccharomyces cerevisiae* constitutes approximately 15–25% of the dry weight of the cell. A considerable amount of energy is invested in the formation and the maintenance of the cell wall, which implies an important function for this structure. This is illustrated by the fact that a yeast cell lacking a cell wall will lyse in a hypotonic environment. Furthermore, the cell wall is an essential component in the sexual cycle of yeast and is required for the yeast to survive periods of drought.

The cell wall consists of three components: glucan, mannoproteins and chitin (Cabib *et al.*, 1982; Kollár *et al.*, 1995). The first two, which are present in approximately equal amounts in the cell wall, represent the main components. The glucan fraction gives the cell wall a rigid structure that protects the cell from mechanical stress and determines the cell shape. The mannoproteins determine the porosity of the cell wall and thereby regulate the transport of proteins from the periplasmic space and the entrance of macromolecules from the environment (for review see Klis, 1994a; Cid *et al.*, 1995). Chitin is a major component of the primary septum and is involved in the separation of mother and daughter cells, which makes it an essential component for cell division (Shaw *et al.*, 1991).

Electron microscopic analysis of the cell wall of *Saccharomyces cerevisiae* revealed a two-layered structure: an electron-dense, dark-staining, outer layer and a non-staining inner layer (*Figure 1*). Zlotnik *et al.* (1984) showed that incubation of *S. cerevisiae* cells with Z-protease, a protease found in Zymolyase preparation, resulted in a loss of the electron-dense outer layer of the cell wall. Furthermore, lysis of the cell wall of *Saccharomyces cerevisiae* with Z-glucanase requires a previous incubation with this Z-protease. These results imply that the inner layer of the cell wall is the glucan layer and that the electron-dense outer layer is the mannoprotein layer.

Two subclasses of β-glucans are present. The more abundant β-glucan contains approximately 1500 residues per molecule and is a linear β-1,3-linked polymer of

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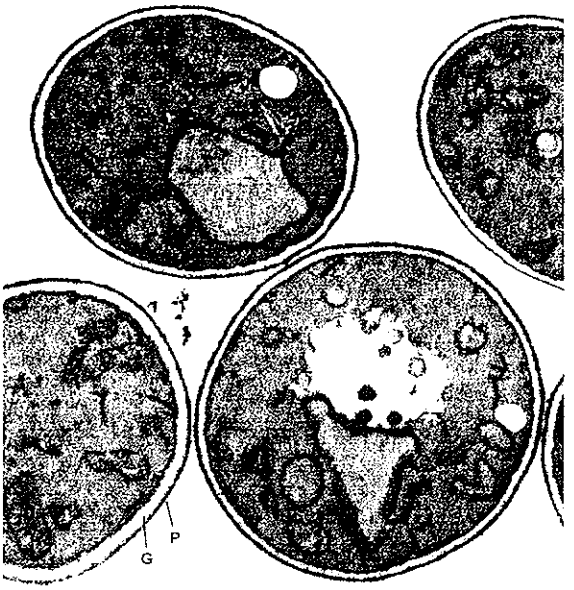


**Figure 1.** Electron micrograph of *Saccharomyces cerevisiae* cells. The outer, electron-dense layer consisting of proteins (P) and the inner-layer, consisting of glucan (G), are indicated. Photograph supplied by Dr M. Veenhuis.

glucose residues containing 3% β-1,6-linkages at branch points (Manners *et al.*, 1973a). The minor β-glucan is highly branched and contains approximately 140 residues per molecule. This is a β-1,6-linked polymer of glucose with a small portion of β-1,3-linked residues (Manners *et al.*, 1973b; Boone *et al.*, 1990). Chitin, which is a β-1,4-linked polymer of N-acetylglucosamine, is present in the glucan layer, close to the plasma membrane and becomes bound to the non-reducing end of β-1,3-glucan (Kollár *et al.*, 1995). This process converts the β-1,3-glucan from an alkali-soluble to an alkali-insoluble form. Thus the alkali-soluble β-1,3-glucan form acts as a precursor for the alkali-insoluble form (Hartland *et al.*, 1994). Both β-1,3-glucan and chitin are synthesized by plasma membrane-bound enzyme complexes (Cabib *et al.*, 1982), whereas β-1,6-glucan synthesis is proposed to start in the endoplasmatic reticulum, to elongate in the Golgi and to have a final extracellular processing step (Bussey *et al.*, 1991).

The mannoprotein fraction of the cell wall can be divided into two populations. One population is extractable with a detergent such as SDS and the remaining proteins are released after incubation of cell walls with a β-glucanase (Valentin *et al.*, 1984). Pastor *et al.* (1984) detected a population of 30 different mannoproteins after an SDS-extraction of cell walls; however, it has become clear that at least part of this population is composed of plasma membrane proteins (Van der Vaart *et al.* 1996a; Molloy *et al.*, 1989). The glucanase-extractable mannoproteins are released after an incubation with a β-1,3- or β-1,6-glucanase which indicates that they are tightly bound to the glucan layer of the cell wall (Fleet and Manners,1977; Pastor *et al.*, 1984; Valentin *et al.*, 1984; Frevert and Ballou, 1985). Furthermore, it has been shown that the glucanase-extractable cell wall proteins carry a carbohydrate side chain containing

β-1,6-linked glucose residues (Van Rinsum *et al.*, 1991; Montijn *et al.*, 1994) and, recently, it was shown that this chain links the mannoprotein to β-1,3-glucan (Kapteyn *et al.* 1996).

β-Glucosylated cell wall proteins are not restricted to *Saccharomyces cerevisiae*. Cell wall proteins containing this type of modification are also found in *Candida albicans* (Kapteyn *et al.* 1994), *Exophiala* (*Wangiella*) *dermatitidis* (Montijn, 1996a), *Hansenula polymorpha* (Ida van der Klei, pers. comm.), *Aspergilus niger*, *Paecilomyces variotii* and *Penicillium roqueforti* (Brul *et al.*, submitted) This indicates that anchoring of cell wall proteins to the cell wall by means of a β-1,6-glucan chain is a general mechanism for cell wall anchorage in Ascomycetes.

## Glucanase-extractable mannoproteins

The glucanase-extractable mannoproteins so far identified have three characteristics in common: they contain a secretion signal at their N-termini, they are rich in serine and/or threonine and they all contain a putative glycosylphosphatidylinositol (GPI)-attachment signal at their C-termini (*Table 1*). Van Rinsum *et al.* (1991) isolated a carbohydrate chain consisting of glucosamine, mannose and β-glycosydically linked glucose from glucanase-extracted cell wall proteins in a molar ratio of 1:17:18. This β-1,6-glucan chain is involved in cell wall anchorage (Kapteyn *et al.*, 1996). Immunological detection of this glucose-containing side chain became possible after the raising of an antiserum directed against a β-1,6-glucan oligomer. This antiserum recognized four protein bands in a glucanase extract of isolated cell walls (Montijn *et al.*, 1994).

N-terminal sequencing of these protein bands led to the identification of two ORFs as the products were identified by systematic sequencing (Van der Vaart *et al.*, 1995). One is the YKL096w gene product, which was sequenced by Pallier *et al.* (1993), and was named Cwp1p (Cell Wall Protein 1). The other was identified as the *TIP1* gene product, which had already been identified by Kondo and Inouye (1991) as a temperature-inducible protein. A third ORF that was identified in this study, *CWP2*, encodes a cell wall protein which was not recognized by the anti β-1,6-glucan

**Table 1.** Glucanase-extractable cell wall proteins in *Saccharomyces cerevisiae*.

| Gene | Protein | β-1,6-glucan[a] | Ser/Thr (%)[b] | *N*-glycosylation[c] | GPI-anchor[d] | Comments |
|---|---|---|---|---|---|---|
| *CWP1* | Cwp1p | + | 44 | + | nd | Deletion of GPI-signal leads to secretion |
| *CWP2* | Cwp2p | + | 48 | – | + | Mature protein consists of 54 aa |
| *TIP1* | Tip1p | + | 50 | – | nd | Temperature-inducible |
| *SED1* | Sed1p | + | 41 | + | nd | Multicopy suppressor of *erd2* |
| *TIR1* | Tir1p | + | 45 | – | + | Anaerobically expressed |
| YCR89w | | + | 47 | + | nd | Homolog of **a**-agglutinin anchor protein |
| *FLO1* | Flo1p | + | 48 | + | nd | Involved in flocculation |
| *AGα1* | α-agglutinin | + | 41 | + | + | Involved in mating, deletion of GPI-signal leads to secretion |
| *AGA1* | **a**-agglutinin anchor-protein | nd | 52 | – | nd | Involved in mating |

[a]proteins are recognized by an anti β-1,6-glucan antiserum. [b](number of Ser-Thr res. / total number of res.)×100% of C-terminal half of protein. [c]presence of potential *N*-glycosylation sites. [d]biochemical evidence for GPI-anchor. nd, no data.

antiserum. This lack of recognition was caused by a blotting artefact of the mature Cwp2 protein. The Cwp2 protein is only immunologically detectable if the fatty acids are present on the GPI-moiety of this protein (Van der Vaart *et al.*, 1996b). By analogy with this property of Cwp2p, more β-glucosylated cell wall proteins might exist in *Saccharomyces cerevisiae*. One example of such a cell wall protein is the *TIR1* (*SRP1*, Marguet *et al.*, 1988) gene product. Mature Tir1p is not detectable by Western analysis but a β-1,6-glucosylated cell wall bound form of this protein does exist (Van der Vaart, 1997).

Furthermore, the creation of fusion constructs consisting of the coding region of the reporter enzyme α-galactosidase and C-terminal parts of several proteins with the characteristics of cell wall proteins confirmed the cell wall localization and β-1,6-glucosylation of several potential cell wall proteins (*Table 2*). The C-terminal regions of Sed1p (Hardwick *et al.*, 1992), Flo1p, which was already identified as a cell surface protein (Teunissen *et al.*, 1993; Bidard *et al.*, 1995) and YCR89w, an **a**-agglutinin homologue, are all capable of immobilizing α-galactosidase on the cell wall and these fusion proteins all contain a β-1,6-glucan chain in their cell wall bound forms (Van der Vaart *et al.*, 1996a, 1997).

Both α-agglutinin (Lipke *et al.*, 1989) and **a**-agglutinin (Roy *et al.*, 1991) are cell surface proteins which are involved in mating. For α-agglutinin it was also shown that it is β-1,6-glucosylated in the cell wall bound form (Lu *et al.*, 1995).

Several mutants depleted for a cell wall protein were created (Van der Vaart *et al.*, 1995). Phenotypical analysis of yeast cells depleted for Cwp2p revealed an increased sensitivity for the cell wall disturbing compounds Congo Red and Calcofluor White, and for Zymolyase (a β-glucanase-containing preparation). These results indicate that Cwp2p is an important constituent of the cell wall and plays an important role in stabilizing the cell wall. Depletion of Cwp1p, Tip1p, Tir1p or YCR89w also caused increased sensitivities to Congo Red and Calcofluor White but the effects were less pronounced than for the *cwp2* deletion mutant.

## Modifications of cell wall proteins in the secretory pathway and at the cell surface

Secretion and post-translational modifications of proteins are determined by the presence of specific amino acid sequences. Proteins can enter the secretory route when they possess a secretion signal. After entrance into the ER, cell wall proteins can be *N*-glycosylated, *O*-glycosylated and anchored to the membrane by means of a GPI-anchor. An asparagine in an N-X-S/T sequence is a potential site for *N*-glycosylation. This type of glycosylation consists of a core structure of $Man_8GlcNAc_2$, formed in the ER, and is extended with mannose residues in the Golgi. Serine and threonine residues are potential sites for *O*-glycosylation, which consists of a linear glycan of up to five mannose residues. The first residue of this glycan is added in the ER and the following four are presumably added in the Golgi. A hydrophobic C-terminal strech of amino acids is part of the signal required for glycosylphosphatidyl inositol (GPI)-anchor addition. After removal of the carboxyl-terminal hydrophobic region, a preformed GPI-anchor is attached to the newly exposed terminus. The core structure of such an anchor consists of: ethanolamine-P-6Man-α1,2-Man-α1,6-Man-α1,4-GlcN-α1,6-inositol (for review see Englund, 1993; McConville and Ferguson, 1993). Subsequently,

**Table 2.** Cell surface immobilization of fusion proteins in *Saccharomyces cerevisiae*. Cell wall localization of the fusion proteins was shown by immuno-fluorescence

| Fusion protein | | | |
|---|---|---|---|
| N-terminal part | C-terminal part | Comments | References |
| HBs fragments[a] | α-agglutinin | No immune response in mice | Schreuder *et al.*, 1994 |
| lipase | α-agglutinin | Fusion protein displays no enzymatic activity | Schreuder *et al.*, 1996 |
| cutinase | α-agglutinin | Active against small substrate, activity against olive oil not detectable | Schreuder *et al.*, 1996 |
| Single-chain ($V_h$) fragment | α-agglutinin | antibody fragment from *Camelidae* | Schreuder *et al.*, 1996 |
| α-galactosidase | α-agglutinin | active against small and large substrate | Schreuder *et al.*, 1993; Van der Vaart *et al.*, 1997a |
| α-galactosidase | Cwp1p | 30% of fusion protein is immobilized | Van der Vaart *et al.*, 1997a |
| α-galactosidase | Cwp2p | most active against small and large substrate | Van der Vaart *et al.*, 1996a; 1997a |
| α-galactosidase | Tip1p | enzyme activity demonstrated | Van der Vaart *et al.*, 1997a |
| α-galactosidase | Sed1p | active against small and large substrate | Van der Vaart *et al.*, 1996a; 1997a |
| α-galactosidase | Tir1p | 50% of fusion protein is immobilized | Van der Vaart *et al.*, 1997a |
| α-galactosidase | YCR89w | low protein production | Van der Vaart *et al.*, 1997a |
| α-galactosidase | Flo1p | enzyme activity demonstrated | Van der Vaart *et al.*, 1996a; 1997a |
| glucoamylase | α-agglutinin | enzyme activity demonstrated | Murai *et al.*, 1997 |

[a] Parts of Hepatitis B virus surface protein.

at the cell surface, proteins destined for the cell wall receive the β-1,6-glucan chain described above.

### IMPORT INTO THE ENDOPLASMATIC RETICULUM

Secretion of proteins is dependent on the entrance of a protein into the secretory route. A specialized signal, consisting of a positively charged sequence, a hydrophobic region and a polar region is present on proteins destined for the secretory route and is recognized by the Signal Recognition Particle (SRP) after it has emerged from the ribosome. This protein-ribosome-SRP complex subsequently docks on the SRP-receptor on the ER membrane and the protein is translocated into the ER. Such a signal is located at the N-terminus and is generally referred to as a signal sequence. After partial translocation across the ER membrane this sequence is cleaved by a signal peptidase in the polar region (for reviews see Rapoport, 1992; Larriba, 1993).

### *N*-GLYCOSYLATION

Experimentally, it has been shown that *N*-glycosylation is an important modification for cell wall proteins. The cell walls of *mnn9* mutant strains, which lack outer-chain glycosylation of the *N*-linked core structure, contain glucanase-extractable cell wall mannoproteins of lower average molecular weight than the corresponding wild-type cells (Frevert and Ballou, 1985). Furthermore, these mutant cells were shown to have dramatically increased cell wall permeabilities and Zymolyase sensitivities (De Nobel, 1991).

The core oligosaccharide is assembled on a dolichol phosphate carrier lipid partly at the cytosolic and partly at the luminal side of the ER-membrane. At the cytosolic side the donors UDP-GlcNAc and GDP-Man contribute the sugars to form the precursor oligosaccharide, Dol-PP-$Man_5GlcNAc_2$. This core structure is subsequently transferred to the luminal side of the ER. Here the core oligosaccharide is completed. The addition of mannose and glucose from Dol-P-Man and Dol-P-Glc, respectively, results in the formation of Dol-PP-$Glc_3Man_9GlcNAc_2$ (for review see Lehle and Tanner, 1995; Herscovics and Orlean, 1993). Complementation of the temperature sensitive *sec59* mutant (Heller *et al.*, 1992) and of several *alg*-mutants (**a**sparagine **l**inked **g**lycosylation) have identified gene products involved in the formation of the core oligosaccharide (for review see Kukuruzinska *et al.*, 1987).

The mature core oligosaccharide is subsequently transferred from the dolichol phosphate carrier to the asparagine acceptor site of the nascent protein. An asparagine residue is only capable of acting as an acceptor if it is located within the primary sequence N-X-T/S. In this sequence X can be any amino acid residue except proline. Furthermore, it was shown that the N-X-T site is a much better substrate for *N*-glycosylation than N-X-S (for review see Silberstein and Gilmore, 1996). Transfer of the core oligosaccharide from the dolichol phosphate carrier to the acceptor asparagine is catalysed by the oligosaccharide transferase (OTase) complex. This OTase activity was found to co-purify with a protein complex of six major components (Kelleher and Gilmore, 1994). Six components of this complex, Wbp1p, Swp1p, Ost1p, Ost2p, Ost3p and Ost5p, have been identified (Silberstein and Gilmore, 1996). Depletion of Ost4p greatly diminishes OTase activity. This *OST4* gene could encode

for a subunit of the OTase complex, but it is also possible that it is a regulator of the complex (Chi *et al.*, 1996). Another protein important for OTase activity is Stt3p. This protein is suggested to be required for the stability and/or the assembly of the OTase complex but was not detected as a subunit of this complex (Zufferey *et al.*, 1995).

After the core oligosaccharide is transferred to the protein, this oligosaccharide is further modified in the ER and in the Golgi by specific glycosidases and glycosyltransferases. First, two glucosidases remove the glucose residues from the oligosaccharide ($Glc_3Man_9GlcNAc_2 \rightarrow Man_9GlcNAc_2$) and secondly a mannose residue is removed by a specific mannosidase, Mns1p, resulting in a $Man_8GlcNAc_2$ structure. As the *MNS1* gene product was shown to be located in the ER by immuno electron microscopy (Burke *et al.*, 1996), the trimming of the oligosaccharyl structure is performed in the ER.

Further maturation of the $Man_8GlcNAc_2$ structure occurs in the Golgi. Two forms of maturation, core maturation and outer chain formation, can be distinguished. Mannosyltranferases are responsible for the addition of the mannose residues in these processes. Core maturation yields $Man_{8\text{-}13}GlcNAc_2$ and outer chain formation results in the addition of up to 200 mannose residues arranged in a backbone of α-1,6-mannose residues carrying side-chains consisting of α-1,2-mannose residues with terminal α-1,3-linked mannose residues (for review see Lehle and Tanner, 1995; Herscovics and Orlean, 1993). *Mnn* (alterations in cell wall mannan) mutants have been shown to affect the maturation process (Ballou, 1990). *MNN1* has been shown to encode a mannosyltransferase (Graham *et al.*, 1992). Other *MNN* genes could also encode mannosyltransferases, but possibly many of the proteins encoded by these genes influence glycosylation indirectly. Deletion, of and over-expression, of *MNN2* does not, for example, result in a change of the mannosyltransferase activity, indicating that this protein is involved in other aspects of outer-chain addition (Devlin and Ballou, 1990). Genes that do encode mannosyltranferases involved in *N*-glycosylation are: *KRE2/MNT1* (Häusler *et al.*, 1992), *YUR1*, *KTR1* and *KTR2* (Lussier *et al.*, 1996). Based on their homology to *KRE2/MNT1*, both *KTR3* and *KTR4* (Mallet *et al.*, 1994) are potential mannosyltransferases involved in *N*-glycosylation. Recently, Montijn *et al.* (1996b) found that both Kre6p and Skn1p are involved in elongation of *N*-linked sugar chains. It is not known, however, whether these proteins are directly or indirectly involved in this elongation process.

### *O*-GLYCOSYLATION

Proteins containing serine or threonine residues are potential substrates for *O*-glycosylation. All known cell wall proteins are rich in serine and/or threonine (*Table 1*). These residues are located primarily in the C-terminal part of the cell wall proteins except for Cwp2p, which contains only 54 amino acids and is serine/threonine-rich throughout the protein. These serine/threonine-rich regions are probably heavily *O*-glycosylated, which results in steric interactions between carbohydrate and peptide in these regions. Such interactions will result in a stiff and extended conformation (Jentoft, 1990). As already proposed for α-agglutinin (Wojciechowicz *et al.*, 1993), these so-called 'rod-like' structures possibly have spacer functions in exposing functional domains, if present, of cell wall proteins to the cell surface.

*O*-linked sugar chains consist of linear glycans composed of one to five α-linked

mannose residues with the following structure: Man-α1,3-Man-α1,3-Man-α1,2-Man-α1,2-Manα-*O*-Ser/Thr. The addition of the first mannose residue takes place in the ER and the following four are likely to be added in the Golgi, although the addition of the second mannose residue in the ER cannot be ruled out (Lehle and Tanner, 1995; Klis, 1994b). The donor of the first mannose residue is Dol-P-Man, which is encoded for by *DPM1*. This Dol-P-Man donor is also required for *N*-glycosylation and GPI-anchoring (Orlean, 1990). Pmt1p is a mannosyl transferase directly involved in the addition of this first mannose residue to a serine or threonine residue (Strahl-Bolsinger *et al.*, 1993). Depletion of Pmt1p resulted only in reduction and not elimination of *O*-glycosylation. Pmt2p was also shown to be a mannosyl transferase involved in the addition of this first mannose residue, but even depletion of Pmt1p and Pmt2p did not result in the complete abolition of *O*-glycosylation (Lussier *et al.*, 1995). This indicates the existence of additional mannosyl transferases. The proteins encoded by the *PMT3* and *PMT4* genes are significantly homologous to Pmt1p and Pmt2p. Furthermore, depletion of Pmt4p and Pmt3p/Pmt4p resulted in a significant decrease in molecular weight of chitinase due to underglycosylation of this enzyme (Immervoll *et al.*, 1995). Both products are potential mannosyl transferases. Kre2p/Mnt1p is an α-1,2-mannosyltransferase which is responsible for the addition of the third mannose residue (Häusler *et al.*, 1992) and Mnn1p is a α-1,3-mannosyltransferase which is responsible for the addition of the fourth and possibly the fifth mannose residue (Ballou, 1990; Yip *et al.*, 1994). The mannose donor for the second to the fifth mannose residue is GDP-Man.

It is not known which mannosyl transferases are responsible for the attachment of the second mannose residue. Ktr3p and Ktr4p (Mallet *et al.*, 1994) are both potential mannosyl transferases as they are homologous to Kre2p/Mnt1p. Either or both of these proteins could be responsible for the addition of this mannose residue.

### GPI-ANCHORING

Many proteins in yeast are anchored to membranes by means of a glycosylphosphatidyl inositol (GPI) anchor (Conzelmann *et al.*, 1990). This type of anchorage is found in many organisms and, except for the addition of side chains, the structure and biosynthesis of this GPI-moiety is highly conserved (McConville and Ferguson, 1993). In yeast, GPI-anchorage is essential for growth (Leidich *et al.*, 1994). Furthermore, the prior addition of such an anchor onto the cell wall protein α-agglutinin has proved to be necessary for the incorporation of this protein into the cell wall (Lu *et al.*, 1995). The core structure of the highly conserved GPI anchor is: ethanolamine-P-6Man-α1,2-Man-α1,6-Man-α1,4-GlcN-α1,6-inositol-phospholipid (see *Figure 2*).

The biosynthesis of this structure (see *Figure 3*) begins with the addition of GlcNAc from UDP-GlcNAc to an inositol-containing phospholipid (PI) yielding GlcNAc-PI. At least three gene products, Gpi1p, Gpi2p and Gpi3p/Spt14p/Cwh6p, were shown to be involved in this initial step (Leidich *et al.* 1994; Leidich *et al.* 1995; Vossen *et al.*, 1995). Subsequently, GlcNAc-PI is deacetylated resulting in GlcN-PI followed by the acylation of the inositol residue (Orlean, 1990). Acyl CoA acts as a donor in this process (Costello and Orlean, 1992). It has been shown that the phosphatidylinositol-specific phospholipase C (PI-PLC) enzyme is only capable of digesting a GPI-structure when no fatty acid is attached to the inositol ring (Roberts *et al.*, 1988). As the majority

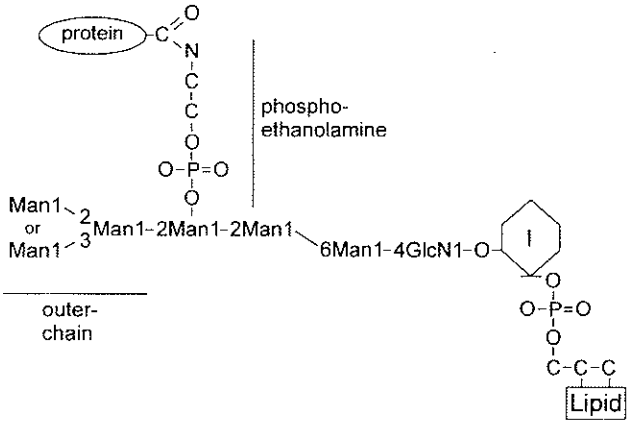


**Figure 2.** Schematic representation of the structure of the GPI anchor from *Saccharomyces cerevisiae*. The glycosylphosphatidylinositol anchor structure was adapted from Sipos *et al.* (1995). P, phosphate; Man, mannose; GlcN, glucosamine; I, inositol. For simplicity, hydrogen atoms are not displayed.

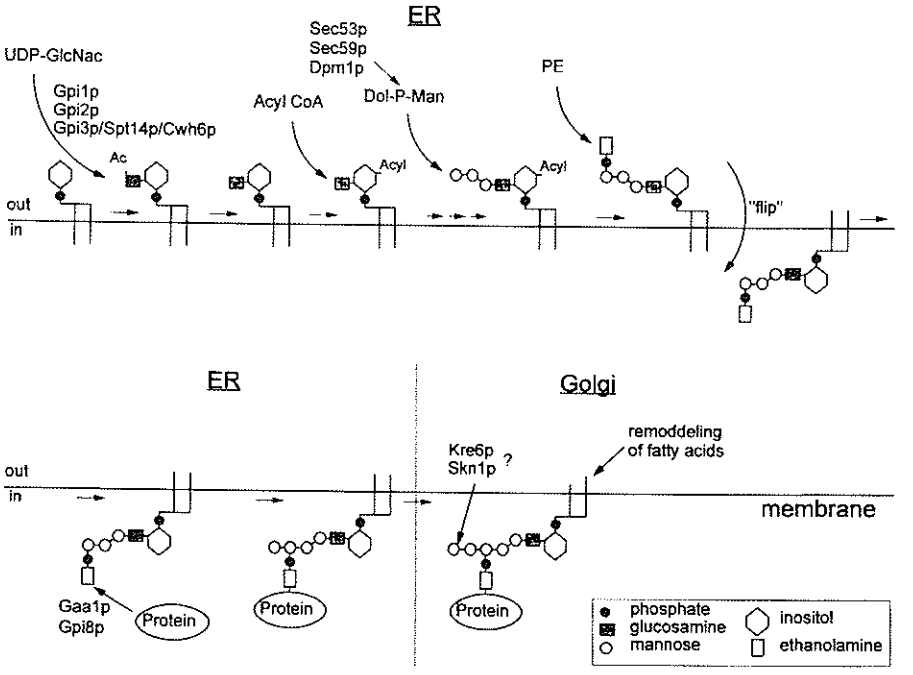


**Figure 3.** Biosynthesis of GPI-anchor. During the development of a GPI-anchor, the inositol ring is acylated. This modification prevents digestion of the premature structure with PI-PLC. During maturation, but before the structure 'flips' to the luminal side of the ER-membrane, the acyl group is removed from the inositol ring. UDP-GlcNac, Acyl CoA, Dol-P-Man and PE are donors for GPI biosynthesis. Remoddeling of fatty acids is not found in *sec18* mutant cells (Conzelmann *et al.*, 1992). Possibly, this reaction takes place in the Golgi but another post *SEC18* compartment could also be involved.

of the GPI-anchored proteins are sensitive to PI-PLC (Conzelmann *et al.*, 1990), the acyl group has to be removed from the inositol residue during maturation of the GPI-anchor. Subsequently, three mannose residues are added to the inositol residue. Mutants blocked in steps leading to the synthesis of Dol-P-Man, *sec53*, *sec59*, and *dpm1*, are disturbed in GPI anchoring (Orlean, 1990; Conzelmann *et al.*, 1990). This indicates that Dol-P-Man is the donor of at least the first of these three mannose residues of the GPI glucan core. The core structure is completed with the addition of phosphoethanolamine (Menon and Stevens, 1992) to yield ethanolamine-P-$Man_3$-GlcN-PI. PI-PLC, added to the cytoplasmic phase, was capable of cleaving GlcNAc-PI and GlcN-PI on microsomes. Therefore, the synthesis of this structure is probably performed at the cytosolic side of the ER membrane. Furthermore, the mannose-specific lectin Concanavalin A bound to the GPI precursor bearing the three mannose residues and to the GPI core structure that includes phosphoethanolamine during incubation with sealed microsomes (Takeda and Kinoshita, 1995). After the synthesis of the GPI core structure, the complete structure is 'flipped' into the ER-lumen where it can be attached to an acceptor protein (McConville and Ferguson, 1993; Englund, 1993). Hydrophobic carboxyl-terminal regions on these proteins are required for this addition (Caras *et al.*, 1987; Caras *et al.*, 1989), but no specific sequence is required (Caras and Weddell, 1989). The cleavage/attachment site (ω) for GPI-anchorage has been investigated and the residues Asn, Ser, Gly, Ala, Asp and Cys were shown to be most effective (Nuoffer *et al.*, 1993; Micanovic *et al.*, 1990). The two adjacent residues on the carboxyl side, ω+1 and ω+2, are also important. They should have relatively short side chains with ω+2 being more important than ω+1 (Nuoffer *et al.*, 1993; Kodukula *et al.*, 1993; Gerber *et al.*, 1992). Replacement of the carboxyl terminal sequence by the GPI-moiety probably occurs by transamidation (Gerber *et al.*, 1992). Gaa1p (**G**PI **a**nchor **a**ttachment) is possibly part of the putative GPI:protein transamidase, as in a *gaa1* mutant yeast the GPI-precursor is still synthesized but not transferred to the protein (Hamburger *et al.*, 1995). Recently, Benghezal *et al.* (1995) identified six mutants, *gpi4* to *gpi9*, involved in the biosynthesis of GPI-anchors. These mutants accumulate GPI glycolipids with either zero, two or four mannose residues or in one case, *gpi8*, a complete GPI anchor that is not transferred on to a protein. Gpi8p is therefore also a potential candidate to be part of the putative GPI:protein transamidase (Benghezal *et al.*, 1996). Interestingly, *GPI8* shows significant homology to a family of vacuolar plant endopeptidases.

Additional sugars are added to the α-1,2-linked mannose of the glucan part of the core GPI structure (see *Figure 2*). The side chains Man-α1,2-, Man-α1,2-Man-α1,2- and Man-α1,3-Man-α1,2- were found. The single α1,2-linked mannose residue is found in every GPI-anchor of *Saccharomyces cerevisiae*, but the addition of a second residue is found in only a proportion of the GPI anchors (Frankhauser *et al.*, 1993). Experiments with *sec7*, *sec12* and *sec18* mutants have revealed that the single α1,2-linked mannose residue is added in the ER. Furthermore, it was found that proteins entrapped in the cis-Golgi contained only mannose residues that were α1,3-linked to the first α1,2-linked residue, whereas proteins that were able to traverse beyond this cis-Golgi compartment contained also α1,2-linked mannose residues on the second position. It is not known which mannosyltransferases are involved in the addition of the mannose residues. Depletion of several α-1,2- and α-1,3-mannosyl transferases or gene products involved in their activity (*Table 3*) did not result in impaired GPI-

**Table 3.** Mannosyl transferases (α-1,2 and α-1,3) and regulators.

| Protein | Comments | Glycosylation[a] |
|---|---|---|
| Mnt1p/Kre2p | α-1,2-Mannosyl transferase | *N, O* |
| Mnn1p | α-1,3-Mannosyl transferase | *N, O* |
| Ktr1p, Ktr2p, Yur1p | Mannosyl transferases | *N* |
| Ktr3p, Ktr4p | Mnt1p-homologues | |
| Mnn2p, Mnn3p, Mnn5p | Control addition of α-1,2-linked mannoses | *N* |
| Mnn9p, Anp1p, Van1p | Involved in maturation of *N*-glucans | *N* |
| Kre6p, Skn1p | Involved in maturation of *N*-glucans | *N* |

[a]Type of glycosylation in which this protein is involved.

anchor synthesis (Sipos *et al.*, 1995). Other potential candidates which could be involved in side chain addition are the gene products of *KRE6* and *SKN1* (Montijn *et al.*, 1996b). The lipid moiety of the majority of GPI-anchors is remodelled when it traverses the secretion-route. During the remodelling reaction diacylglycerol is replaced by a ceramide. In a *sec18* mutant, which is impaired in ER to Golgi transport, this reaction is not detected, indicating that it takes place after the GPI-moiety has left the ER compartment (Conzelmann *et al.*, 1992). This is consistent with the finding of Sipos *et al.* (1995), who found that the remodelling reaction takes place after the GPI-precursor is added to the protein.

### β-1,6-GLUCOSYLATION

The β-1,6-linked polymer of glucose, which was identified as a structural component of the cell wall (Manners *et al.*, 1973b), was only recently recognized as a glycosylated form of cell wall proteins (Van Rinsum *et al.*, 1991). This carbohydrate side chain, isolated from proteins released from cell walls by laminarinase, is composed of equal amounts of β-1,6-glucan and α-1,6-mannan (Montijn *et al.*, 1994). Gene products involved in β-1,6-glucan synthesis have been identified in several ways. Most were identified by making use of K1 killer toxin. This toxin is capable of killing sensitive yeast strains by the formation of lethal cation channels in the plasma membrane (Martinac *et al.*, 1990), after binding to β-1,6-glucan (Bussey *et al.*, 1979; Kasahara *et al.*, 1994). Mutants of K1 killer toxin-sensitive strains that have impaired β-1,6-glucan synthesis are capable of growing in the presence of this toxin. By making use of the K1 Killer toxin REsistance strategy, several *KRE* gene products involved in β-1,6-glucan synthesis were identified (Brown *et al.*, 1994). Additional gene products, involved in β-1,6-glucan synthesis, were identified by multicopy suppressor screens of *kre*-mutants and by complementation of a Calcofluor White hypersensitive mutant (Ram *et al.*, 1994). The identified gene products were localized in the ER, in the Golgi, in the cytosol and on the cell surface. This is consistent with the idea that β-1,6-glucan synthesis is initiated in the secretory route or that secretory proteins are involved in β-1,6-glucan synthesis. However, in contrast to the core structures of *N*-chains, *O*-chains and GPI-anchors, which are linked to a protein moiety in the ER, the β-1,6-glucan chain is coupled to a cell wall protein extracellularly. This is supported by the finding that α-agglutinin (Lu *et al.*, 1995), Cwp2p, Tip1p, Sed1p, Flo1p, Tir1p and YCR89p (Van der Vaart, unpublished results) were only recognized by an antiserum directed against β-1,6-glucan in their cell wall bound forms and not in their precursor forms.

Furthermore, it was found that the sizes of the precursor forms of α-agglutinin were not affected in mutants defective in β-1,6-glucan synthesis (Lu *et al.*, 1995).

Kre5p is a component involved very early in the β-1,6-glucan synthesis pathway, which has extensive sequence homology with an ER glucosyltransferase in *Drosophila* (Parker *et al.*, 1995). As Kre5p contains an N-terminal signal sequence and a carboxyl terminal ER retention consensus sequence (HDEL) this protein is believed to be a soluble ER protein. Depletion of this protein resulted in resistance towards the K1 killer toxin and the lack of a detectable amount of β-1,6-glucan in the cell wall, indicating that this protein is essential for β-1,6-glucan synthesis (Meaden *et al.*, 1990; Boone *et al.*, 1990). Another ER protein involved in β-1,6-glucan synthesis is Cwh41p. This protein is an integral membrane protein and disruption of the *CWH41* gene results in resistance towards the K1 killer toxin and a 50% reduction of β-1,6-glucan level in the cell wall. Disruption of both *KRE5* and *CWH41* resulted in a mutant strain with the same phenotype as the *kre5* single mutant, indicating that *KRE5* is epistatic to *CWH41*. Furthermore, as overexpression of either gene cannot compensate for the defects caused by deletion of the other, the functions of these proteins are not interchangeable (Ram *et al.*, 1994; Jiang *et al.*, 1996).

Kre6p and Skn1p are functional homologues and are both transmembrane proteins, likely to be localized within the Golgi. Disruption of *KRE6* caused slower growth, resistance towards the K1 killer toxin and a reduction of β-1,6-glucan level in the cell wall, whereas depletion of Skn1p gave no detectable phenotype. On the other hand, overexpression of *SKN1* could suppress the *kre6* mutant phenotype. A *kre6-skn1* double mutant displayed a more severe phenotype than the *kre6* single mutant and in contrast to the single mutant, the double mutant had β-1,6-glucan chains of smaller size and with altered structure. It has been postulated that Kre6p and Skn1p function independently, in parallel, in β-1,6-glucan biosynthesis (Roemer and Bussey, 1991; Roemer *et al.*, 1993; Roemer *et al.*, 1994).

A number of proteins involved in β-1,6-glucan chain synthesis are localized extracellularly. Epitope tagging of Kre1p, which has a C-terminal GPI-attachment signal, has revealed that this protein is localized at the cell surface (Roemer and Bussey, 1995). Gene disruption of *KRE1* leads to K1 killer toxin resistance and a 40% reduction of the β-1,6-glucan level in the cell wall. This reduction is caused by a decrease of the average polymer size of the β-1,6-glucan chains (Boone *et al.*, 1990). This indicates that Kre1p plays a role in extending β-1,6-glucan chains at the cell surface and that this chain is assembled in a sequential fashion. Kre9p is also involved in β-1,6-glucan synthesis and, as overexpression of *KRE9* leads to the detection of the corresponding gene product in the extracelluar medium, this is probably a soluble secretory-pathway protein. Depletion of Kre9p leads to a similar, but more severe, phenotype as depletion of Kre1p. In a *kre9* deletion mutant the β-1,6-glucan level in the cell wall is reduced by 80% and there is also a decrease in the average polymer size of β-1,6-glucan chains. The growth defect of this mutant was most severe if cells were grown on glucose and less severe if galactose was added to the growth-medium (Brown *et al.*, 1993; Brown and Bussey, 1993a; Brown *et al.*, 1994). Knh1p (*KRE9* nine homolog) was identified based on its similarity to Kre9p and both were shown to be functional homologues. Depletion of Knh1p gave no detectable defects, but overexpression of *KNH1* could suppress the *kre9* mutant phenotype. The *kre9-knh1* double mutant is non-viable. Both Kre9p and Knh1p seem to have specialized

functions in β-1,6-glucan synthesis and as expression of *KNH1* is carbon source dependent (increased expression on galactose medium) these products possibly function in parallel in β-1,6-glucan biosynthesis, under different environmental conditions (Dijkgraaf *et al.*, 1996).

Kre11p was also identified as being involved in β-1,6-glucan synthesis and is a putative cytosolic protein. Depletion of Kre11p leads to a 50% reduction of the β-1,6-glucan level in the cell wall. Analysis of the β-1,6-glucan chains from cell walls of *kre11* mutants revealed that the size of the polymer was smaller than those of wild-type strains but contained similar proportions of β-1,6- and β-1,3-linkages. Since a *kre6-kre11* deletion mutant is lethal, Kre11p is possibly involved in the regulation of Kre6p.

The anchorage of cell wall proteins to the cell wall has been investigated in several mutants with impaired β-1,6-glucan synthesis. First, Lu *et al.* (1995) found that more than 80% of α-agglutinin, produced in a *kre5* mutant, is secreted into the growth medium, whereas in the parent strain this was only 7%. Secondly, Montijn *et al.* (1996a) found a reduction of incorporation of Cwp1p in the cell wall and a corresponding increase in the amount of this protein in the growth medium in a *kre6-skn1* double mutant. Thirdly, secretion of Cwp1p was also found in a mutant strain containing deletions in *KRE1* and *CWH41* (Jiang *et al.*, 1996). These results emphasize the importance of β-1,6-glucan chains in the process of cell wall anchorage of cell wall proteins in yeast.

## The incorporation of proteins in the cell wall

The identified cell wall proteins of yeast share several characteristic features. They are β-1,6-glucosylated in their cell wall-bound forms, they contain a (putative) N-terminal signal sequence, are rich in serine and/or threonine and contain a (putative) C-terminal GPI-attachment signal. Van Berkel *et al.* (1994) showed that a fusion protein consisting of the invertase signal sequence, the guar α-galactosidase coding sequence, and the C-terminal thirty amino acids of α-agglutinin, which includes the GPI-attachment signal, is incorporated into the cell wall and becomes β-1,6-glucosylated. This result indicates that, while the serine/threonine-rich region of this protein is not involved in cell wall anchorage, GPI-anchoring is required. A similar result was also obtained if the 30 C-terminal amino acids of Tir1p were used as an anchor (Van der Vaart, 1997b). The addition of only 16 C-terminal amino acids of this protein, containing only part of the GPI-attachment signal, was not enough for cell wall incorporation. Further proof for the involvement of GPI-anchorage in cell wall incorporation of cell wall proteins was presented by Vossen *et al.* (1995). They showed that a temperature-sensitive mutant of *CWH6* (*GPI3*, *SPT14*), a gene involved in GPI-synthesis, displays a large decrease of the amount of mannan in the cell wall, which indicates a lower amount of incorporated cell wall proteins in this mutant. Furthermore, Lu *et al.* (1995) found that the prior addition of such a GPI anchor to the cell wall protein α-agglutinin was necessary for the incorporation of this protein into the cell wall and for β-glucosylation of this protein. Wojciechowicz *et al.* (1993) showed that removal of the C-terminal hydrophobic sequence of α-agglutinin resulted in secretion of active α-agglutinin into the medium. Similar results were also obtained for Cwp1p (Shimoi *et al.*, 1995). Chimeric proteins consisting of the reporter enzyme α-galactosidase fused to the C-terminal part of several cell wall proteins were secreted

into the growth medium in a *pmt1* deletion strain, indicating the importance of GPI-anchoring in cell wall incorporation (Van der Vaart., 1997). Recently, the cleavage/attachment residue for GPI-anchorage of Cwp2p, asparagine was replaced with a serine residue. This replacement resulted in a dramatic decrease of GPI-anchorage of Cwp2p and secretion of this protein into the growth medium (Van der Vaart, unpublished results).

Not all GPI-anchored proteins become incorporated into the cell wall. Gas1p (Nuoffer *et al.*, 1993; Popolo *et al.*, 1993) and Yap3p (Ash *et al.*, 1995) are GPI-anchored proteins and have the plasma membrane as their final destination. The mechanism by which a cell discriminates between plasma membrane and cell wall localization is unknown. A dibasic motif, N-terminal of the GPI-attachment site, is found in plasma membrane proteins but not in cell wall proteins (Vossen *et al.*, 1997). This motif could act as a plasma membrane residence signal or could facilitate cleavage of plasma membrane proteins resulting in the complete loss of their GPI-moieties. Such cleavage reactions may therefore cause the loss of the acceptor sites of these proteins for the β-1,6-glucan chain, preventing cell wall incorporation. This would imply rapid turnover of plasma membrane proteins as loss of the GPI-anchor would free them from the plasma membrane. It is also possible that plasma membrane proteins are cleaved after mistargeting to the cell wall. The insertion of a dibasic motif at position ω-3 and ω-4 of Cwp2p did not convert this cell wall protein into a resident plasma membrane protein (Van der Vaart, unpublished results). This modified form of Cwp2p was correctly GPI-anchored intracellularly and β-glucosylated extracellularly, which indicates that the dibasic motif is not acting as a retention signal for plasma membrane localization. The mechanism of cleavage of plasma membrane proteins to prevent cell wall incorporation cannot be excluded. The peptide sequences surrounding the dibasic motif inserted in Cwp2p could have negative influence on the cleavage efficiency of the responsible protease. It is not known which enzymes are involved in the release of cell wall proteins from the plasma membrane and in the subsequent β-1,6-glucosylation of these proteins. Potential enzymes and possible mechanisms will be discussed below.

## The attachment site of the β-1,6-glucan chain to cell wall proteins

Cell wall proteins are β-glucosylated in their cell wall bound forms. Recently, it was shown that the β-1,6-glucan chain links cell wall mannoproteins to β-1,3-glucan in the cell wall (Kapteyn *et al.* 1996). The exact attachment site of this chain on to a cell wall protein is not known. Potential attachment sites are: amino acid residues, *N*-linked carbohydrate side chains, *O*-linked carbohydrate side chains, or the GPI-moiety. Two amino acid residues, asparagine and glutamine, could potentially act as attachment site for the β-1,6-glucan chain. In analogy to the existence of a β-linkage between glucose and asparagine in the mammalian protein laminin (Schreiner *et al.*, 1994), the ω asparagine residues of cell wall proteins are potential attachment sites for the β-1,6-glucan chain. To test whether an asparagine residue is responsible for this linkage, the ω asparagine residue of Cwp2p was replaced by a serine residue. Although GPI-anchorage of Cwp2p was dramatically reduced by this replacement, a β-glucosylated cell wall bound form of Cwp2p was detectable. This shows that the removal of this asparagine residue in Cwp2p does not impair β-1,6-glucan chain attachment. It has

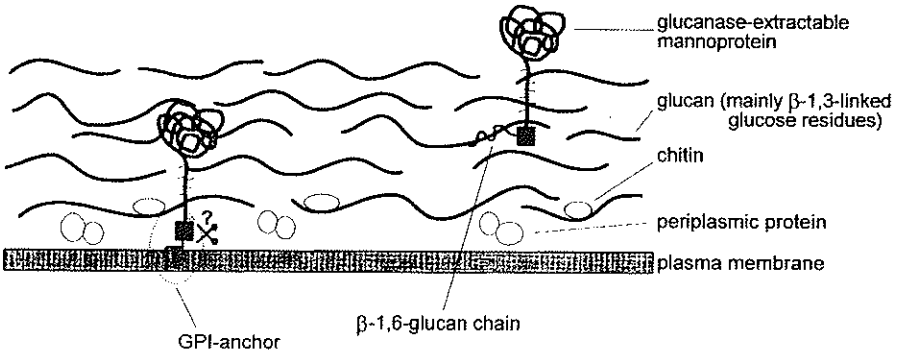


**Figure 4.** Schematic representation of the cell wall of *Saccharomyces cerevisiae*. The movement of a glucanase-extractable cell wall protein from the plasma membrane form to the cell wall bound form is displayed. The exact mechanism by which this movement takes place is unknown but the presented attachment of the β-1,6-glucan chain to the remnant of the GPI-moiety is essential for cell wall anchorage.

been proposed for *Candida albicans* that a transglutaminase is involved in the formation of covalent cross-links in the cell wall (Ruiz-Herrera *et al.*, 1995). This enzyme is capable of forming interpeptidic links in which a glutamine residue is involved. In the *S. cerevisiae* cell wall proteins: Cwp1p, Cwp2p, Tip1p, Tir1p, Tir2p, Aga1p, Agα1p and Flo1p, glutamine residues are present N-terminally of the GPI-attachment site. To test whether glutamine residues are involved in the covalent attachment of cell wall proteins to the cell wall, the two glutamine residues present on position ω-3 and ω-4 in Cwp2p were replaced by a serine and a threonine residue (Van der Vaart, unpublished results). This replacement did not affect β-glucosylation and cell wall localization of the modified Cwp2p, showing that a transglutaminase reaction is not responsible for cell wall anchorage of cell wall proteins in *S. cerevisiae*.

*N*-glycosylation as attachment site for such a chain is very unlikely. First, *N*-chain-digesting enzymes, such as endo-H and PNGase-F, cannot remove the β-1,6-glucan chain from cell wall proteins (Van Rinsum *et al.*, 1991; Montijn *et al.*, 1994). Secondly, α-agglutinin is efficiently anchored to the cell wall in the presence of tunicamycin, which is an inhibitor of *N*-glycosylation (Hasegawa and Yanagishima, 1984). Thirdly, the cell wall proteins Cwp2p, Tip1p and Tir1p are β-glucosylated in their cell wall bound forms while these proteins do not contain any potential *N*-glycosylation sites.

Recent findings provided evidence for the existence of a phosphodiester-linkage in *O*-chains (Dr Y. Jigami, pers. com.). The results obtained by Kapteyn *et al.* (1996), who showed that the β-1,6-glucan chain could be removed from the cell wall proteins α-agglutinin and Cwp1p by phosphodiesterases or aqueous hydrofluoric acid, could not exclude *O*-linked carbohydrate side chains from being the attachment site for the β-1,6-glucan chain. Cleavage of an IgA protease recognition site introduced immediately N-terminal to the ω site in Cwp2p resulted in the loss of the β-1,6-glucan chain from the remaining protein (Van der Vaart *et al.*, 1996b). As no serine or threonine residues were lost from the protein by this cleavage reaction, *O*-glycosylation cannot be an attachment site for the β-1,6-glucan chain.

The above described results of Kapteyn *et al.* (1996), combined with the fact that *O*-glycosylation is not an attachment site for the β-1,6-glucan chain, indicate that the

GPI-moiety is the attachment site of the β-1,6-glucan chain. The finding that the β-1,6-glucan chain of several cell wall proteins can be removed by phosphodiesterases indicates that a phosphate group is present between the protein and the β-1,6-glucan chain. This is consistent with the analysis of cell wall bound Tip1p. Fujii *et al.* (1996) showed the presence of ethanolamine and the loss of glucosamine from the GPI-moiety of the cell wall bound form of Tip1p. These results indicate that the GPI-anchor of the plasma membrane bound form of a cell wall protein is processed in such a way that the β-1,6-glucan chain becomes bound to the glycan-core of the GPI-anchor (*Figure 4*).

**Potential anchoring mechanisms of cell wall proteins**

The precise cell wall anchoring mechanism of cell wall proteins in *Saccharomyces cerevisiae* is not known. As discussed above, GPI-anchorage of cell wall proteins is necessary for the subsequent incorporation of these proteins into the cell wall. For a subset of cell wall proteins, processing of the GPI-moiety of the plasma membrane bound form of a cell wall protein by a PI-PLC is probably the initial step in the release of a cell wall protein from the plasma membrane. Müller *et al.* (1996) found that the GPI-anchored cAMP-binding ectoprotein, Gce1p, was processed twice before cell wall incorporation. First, the GPI-moiety of this protein was cleaved by PI-PLC and secondly in such a way that the complete GPI-moiety was lost from this protein. This is in contrast to the above described results which indicate that only part of the GPI-moiety is lost before cell wall incorporation. It is possible that more cell wall proteins are initially processed by PI-PLC. Western analysis of cell wall extracts with an anti CRD antiserum, which recognizes PI-PLC-cleaved proteins, has revealed the existence of low amounts of several PI-PLC processed proteins (Brul *et al.*, 1997). However, Western analysis of transformants expressing one of the described fusion proteins, consisting of α-galactosidase and the carboxyl terminal part of Cwp1p, Cwp2p, Tip1p, Tir1p, Sed1p, Flo1p or YCR89w did not result in the detection of the expressed fusion protein with an anti CRD antiserum in the corresponding cell wall fraction (J.M. Van der Vaart, unpublished observations). These results suggest that these cell wall proteins are not initially processed by PI-PLC. Alternatively, it is also possible that the concentrations of these intermediate fusion proteins were below the detection level of the Western analysis. According to these results, two mechanisms for cell wall protein anchorage seem to exist: one mechanism by which the complete GPI-moiety is lost from the incorporated cell wall protein and one by which only part of the GPI-moiety is lost. The mechanism of cell wall protein anchoring by means of the attachment of a β-1,6-glucan chain to the remnants of the GPI-anchor will be discussed in more detail (*Figure 5*).

In analogy with Gce1p, the identified cell wall proteins could be transferred from the plasma membrane form to the cell wall form by means of two processing steps, but it is also possible that these proteins are only processed once. As described above, it is unlikely that, if two processing steps exist, the initial processing step is performed by PI-PLC. The involvement of a phospholipase D in this initial step is also not likely as cell wall incorporation of Cwp2p was not impaired in the presence of 1,10-o-phenantroline, a PLD inhibitor. Perhaps a yet unidentified phospholipase is present extracelluarly and mediates this initial processing step. After this potential first

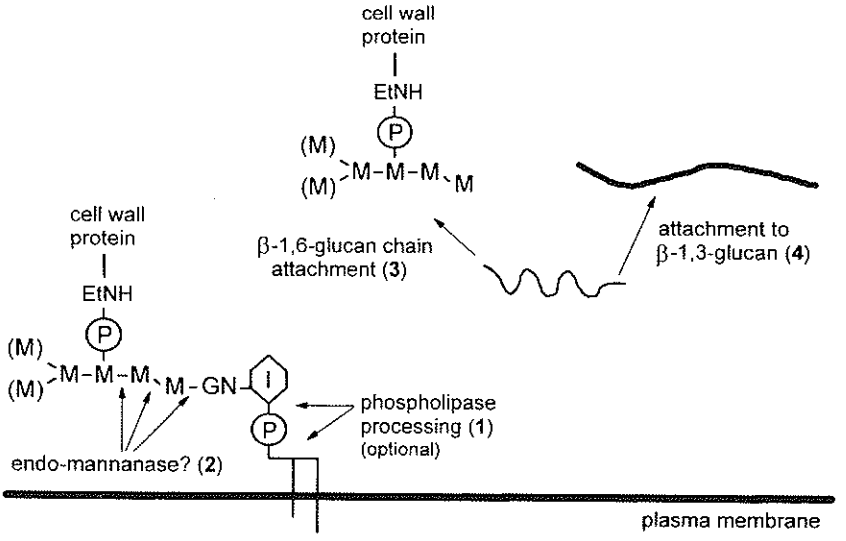


**Figure 5.** Potential cell wall anchoring mechanism for cell wall proteins in *Saccharomyces cerevisiae*. In this model the plasma membrane bound form of a cell wall protein is first cleaved by a phospholipase (1, optional) and secondly by a mannanase (2). The resulting terminal mannose residue of the GPI-moiety will subsequently act as an acceptor of the β-1,6-glucan chain (3), which will be bound to the cell wall glucan layer (4). Step 2 and 3 might be carried out by the same transglycosydase.

processing step, the remnant of the GPI-moiety has to be cleaved in such a way that glucosamine is lost from this structure, but the ethanolamine is still present. A cleavage between one of the mannose residues of the GPI-glycan core by a specific endo-mannanase seems most likely. The two identified mannosidases in *Saccharomyces cerevisiae* are probably not involved in this processing step. The first, Mns1p, is a α-1,2-mannosidase which is localized in the ER (Burke *et al.*, 1996) and is involved in *N*-glycosylation (Camirand *et al.*, 1991). The second, Ams1p, is located in the vacuole (Yoshihisa and Anraku, 1989). After the cleavage of the glycan core of the GPI anchor, the resulting terminal mannose residue would then be available as a substrate for the β-1,6-glucan chain addition by a transglycosylation reaction as proposed by De Nobel and Lipke (1994). Both, the cleavage of the glycan core and the attachment of the β-1,6-glucan chain might be carried out by the same transglycosylase. Subsequently, this β-1,6-glucan chain is probably linked to a β-1,3-glucan polymer in the cell wall.

## Immobilization of proteins on the cell surface of *Saccharomyces cerevisiae*

Enzymic conversions are very important for the production of chemicals, detergents, personal care and food products. Such conversions based on free enzymes are expensive as much of the enzyme will be lost in the process, or expensive equipment, like ultramembrane systems or chemically immobilized enzymes, are required to entrap the enzyme. Enzymes immobilized on the surface of yeast cells provide a way to develop a new generation of enzyme reactors. Such an enzymic catalyst can be simply removed from the reaction medium by filtration or centrifugation and, if necessary, regenerated easily.

Proteins can be immobilized on the cell surface of yeast cells by the expression of a fusion of the protein with the carboxyl terminal part of a cell wall protein. This was first tested with a construct consisting of the invertase signal sequence, the coding sequence of the α-galactosidase enzyme and the DNA sequence encoding the 320 carboxyl terminal amino acids of the cell wall protein α-agglutinin (Schreuder *et al.*, 1993). Immuno-fluorescence of cells expressing this fusion construct showed that the fusion protein was localized on the cell surface and extractions of isolated cell walls showed that it was covalently linked to the cell wall.

Many applications can be thought of in which immobilized enzymes are of interest. For example, the use of immobilized lipases for the quality upgrade of natural raw materials used in food processes. Lipase from *Humicola lanuginosa* and cutinase from *Fusarium solani* sp. *pisi*, are two of those lipases. Both were immobilized by the anchoring domain of α-agglutinin and proved to be immunologically detectable on the surface of intact cells. However, the immobilized lipase from *Humicola lanuginosa* displayed a very low enzymatic activity towards the small substrate *p*-nitro-phenyl butyrate and no enzymatic activity activity towards an emulsion of olive oil. The immobilized cutinase from *Fusarium solani* sp. *pisi* displayed a good enzymatic activity towards the small *p*-nitro-phenyl butyrate substrate but, like the immobilized lipase from *Humicola lanuginosa*, no enzymatic activity activity towards an emulsion of olive oil. The lack of enzymatic activity of the immobilized lipase from *Humicola lanuginosa* is probably caused by hinderance of the lipid-binding ability by the C-terminal extension and the lack of activity of cutinase from *Fusarium solani* sp. *pisi* towards the large olive oil substrate can be explained by the low specific activity of cutinase on this substrate (Schreuder *et al.*, 1996).

Recently, Murai *et al* (1997) improved the metabolic ability of yeast cells by the immobilization of glucoamylase on their cell surfaces. α-Agglutinin was used as a cell wall anchoring domain and the correct localization of the fusion protein was confirmed by cell fractionation, immunofluorescence microscopy and immuno electron microscopy. The observation that a transformant expressing the glucoamylase-α-agglutinin fusion protein was capable of growing on media with starch as the sole carbon source shows that the immobilized glucoamylase was active.

Interesting applications in the field of purification and detection of chemical and biological compounds are possible if proteins with specific binding properties are immobilized on the yeast cell surface. In this respect, the immobilization of variable domains of *Camelidae* antibodies by means of the C-terminal part of α-agglutinin has been reported (Schreuder *et al.*, 1996). These immobilized antibody fragments were detectable by immunofluorescence and were tightly linked to the cell wall glucan.

Another potential type of application tested is the creation of a live oral vaccine by the cell surface immobilization of parts of a Hepatitis B virus surface protein. Again, the carboxyl terminal half of α-agglutinin was used as a cell wall anchoring domain, and expression of this fusion protein resulted in the detection of the immobilized antigen at the cell surface by immuno-fluorescence (Schreuder, 1994). However, a specific immune response against the immobilized antigen in mice was very low. This could be the result of a low amount of immobilized molecules in the yeast cell wall or of the lack of extracellular exposure of these molecules. Perhaps the use of the anchoring domain of α-agglutinin results in immobilized proteins which are embed-

ded in the glycoprotein outer-layer, only accessible to smaller substrates which are capable of penetrating the cell wall.

The accessibility of α-galactosidase, immobilized by several anchoring domains, to the large guar-gum substrate from *Cyamopsis tetragonoloba* has been described (Van der Vaart *et al.*, 1997a). The α-galactosidase enzyme is capable of removing the side chain (1-6)-α-linked D-galactosyl residue from the (1-4)-β-D mannan backbone (Meier and Reid, 1982) which is an important conversion in food industry. The α-galactosidase enzyme, immobilized by α-agglutinin, was capable of hydrolysing guar-gum, but the efficiency was relatively low. Immobilization of this enzyme with the carboxyl terminal 67 amino acids of Cwp2p resulted in a much higher efficiency towards guar gum, but the efficiency of guar-gum hydrolysis of the free α-galactosidase enzyme was always higher. It is possible that a fraction of the immobilized α-galactosidase is not exposed on the outside of the cell wall, but located at the inner part of the protein layer. The enzyme activity of this fraction of the fusion protein is presumably not available for the large guar-gum substrate.

Although the anchoring-part of Cwp2p is only 67 amino acids long (including the GPI-attachment signal), the α-galactosidase immobilized with this anchor is best accessible to the large substrate guar-gum. A spacer region between the anchoring part and the enzyme does therefore not seem necessary to make the enzyme extracellularly available. An alternative explanation might therefore be that the small α-Gal-Cwp2p fusion protein is capable of penetrating the glucan layer of the cell wall more easily, whereas the larger fusion proteins may be entrapped in the internal part of the glucan layer.

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## References

ASH J., DOMINGUEZ, M., BERGERON, J.J.M., THOMAS, D.Y. AND BOURBONNAIS, Y. (1995). The yeast proprotein convertase encoded by *YAP3* is a glycosylphosphatidyl inositol-anchored protein that localizes to the plasma membrane. *Journal of Biol.ogical Chemistry* **270**, 20847–20854.

BALLOU, C.E. (1990). Isolation, characterisation and properties of *Saccharomyces cerevisiae mnn* mutants with non-conditional protein glycosylation defects. *Methods in Enzymo*logy **185**, 440–470.

BENGHEZAL, M., LIPKE, P.N. AND CONZELMANN, A. (1995). Identification of six complmentation classes involved in the biosynthesis of glycosylphosphatidylinositol anchors in *Saccharomyces cerevisiae*. *Journal of Cell Biology* **130**, 1333–1244.

BENGHEZAL, M., BENACHOUR, A., RUSCONI, S., AEBI, M. AND CONZELMANN, A. (1996). Yeast Gpi8p is essential for GPI anchor attachment onto proteins. *EMBO Journal* **15**, 6575–6583.

BIDARD, F., BONY, M., BLONDIN, B., DEQUIN, S. AND BARRE, P. (1995). The *Saccharomyces cerevisiae FLO1* flocculation gene encodes for a cell surface protein. *Yeast* **11**, 809–822.

BOONE, C., SOMMERS, S.S., HENSEL, A. AND BUSSEY, H. (1990). Yeast *KRE* genes provide evidence for a pathway of cell wall beta-glucan assembly. *Journal of Cell Biology* **110**, 1833–1843.

BROWN, J.L., KOSSACZKA, Z., JIANG, B. AND BUSSEY, H. (1993). A mutational analysis of killer toxin resistance in *Saccharomyces cerevisiae* identifies new genes involved in cell wall (1-6)-β-glucan synthesis. *Genetics* **133**, 837–849.

BROWN, J.L. AND BUSSEY, H. (1993a). The yeast *KRE9* gene encodes an *O*-glycoprotein involved in cell surface β-glucan assembly. *Molecular and Cellular Biology* **13**, 6346–6356.

BROWN, J L., NORTH, S. AND BUSSEY, H. (1993b). *SKN7*, a yeast multicopy suppressor of a mutation affecting cell wall β-glucan assembly, encodes a product with domains homologous to prokaryotic two-component regulators and to heat shock. *Journal of Bacteriology* **175**, 6908–6915.

BROWN, J.L., ROEMER, T., LUSSIER, M., SDICU, A -M. AND BUSSEY, H. (1994). The K1 killer toxin: molecular and genetics applications to secretion and cell surface assembly. In *Molecular Genetics of Yeast: A Practical Approach*, ed. J.R. Johnston, p. 217–232. Oxford: IRL Press, Oxford University Press.

BRUL, S., KING, A., VAN DER VAART, J.M., CHAPMAN, J.W., KLIS, F. M. AND VERRIPS, C. T. (1997). The incorporation of mannoproteins in the cell wall of *Saccharomyces cerevisiae* and filamentous *Ascomycetes*. *Antonie van Leeuwenhoek* **72**, 229–237.

BURKE, J., LIPARI, F., IGDOURA, S. AND HERSCOVICS, A. (1996). The *Saccharomyces cerevisiae* processing α1,2-mannosidase is located in the endoplasmatic reticulum, independently of known retrieval motifs. *European Journal of Cell Biology* **70**, 298–305.

BUSSEY, H., D. SAVILLE, H. HUTCHINS, AND R. G. E. PALFREE. (1979). Binding of yeast killer toxin to a cell wall receptor on sensitive *Saccharomyces cerevisiae*. J. Bacteriol. **140**, 888–892.

BUSSEY, H. (1991). K1 killer toxin, a pore-forming protein from yeast. Mol. Microbiol. **5**, 2339–2343.

CABIB, E. AND ROBBERTS, R. (1982). Synthesis of the yeast cell wall and its regulation. *Annual Reviews in Biochemistry* **51**, 763–793.

CAMIRAND, A., HEYSEN, A., GRONDIN, B. AND HERSCOVICS, A. (1991). Glycoprotein biosynthesis in *Saccharomyces cerevisiae*. Isolation and characterization of the gene encoding a specific processing alpha-mannosidase. *Journal of Biological Chemistry* **266**, 15120–15127.

CARAS, I.W., WEDDELL, G.N., DAVIDZ, M.A., NUSSENZWEIG, V. AND MARTIN, D.W., JR. (1987). Signal for attachment of a phospholipid membrane anchor in decay accelerating factor. *Science* **238**, 1280–1293.

CARAS, I.W. AND WEDDELL, G.N. (1989). Signal peptide for protein secretion directing glycophospholipid membrane anchor attachment. *Science* **243**, 1196–1198.

CARAS, I.W., WEDDELL, G.N. AND WILLIAMS, S.R. (1989). Analysis of the signal for attachment of a glycophospholipid membrane anchor. *journal Cellular Biology* **108**, 1387–1396.

CHI, J.H., ROOS, J. AND DEAN, N. (1996). The *OST4* gene of *Saccharomyces cerevisiae* encodes an unusually small protein required for normal levels of oligosaccharyltransferase activity. *Journal of Biological Chemistry* **271**, 3132–3140.

CID, V.J., DURÁN, A.F., DEL REY, SNYDER, M.P., NOMBELA, C. AND SÁNCHEZ, M. (1995). Molecular basis of cell integrity and morphogenesis in *Saccharomyces cerevisiae*. *Microbial Reviews* **59**, 345–386.

CONZELMANN, A., FANKHAUSER, C. AND DESPONDS, C. (1990). Myoinositol gets incorporated into numerous membrane glycoproteins of *Saccharomyces cerevisiae*; incorporation is dependent on phosphomannomutase (*SEC53*). *EMBO Journal* **9**, 653–661.

CONZELMANN, A., PUOTI, A.,LESTER, R.L. AND DESPONDS, C. (1992). Two different types of lipid moieties are present in glycosylphosphatidylinositol-anchored membrane proteins of *Saccharomyces cerevisiae*. *EMBO Journal* **11**, 457–466.

COSTELLO, L.C. AND ORLEAN, P. (1992). Inositol acylation of a potential glycosyl phosphoinositol anchor precursor from yeast requires acyl coenzyme A. *Journal of Biological Chemistry* **267**, 8599–8603.

DE NOBEL, J.G.(1991). Isolation and partial characterisation of mutants with increased cell wall permeability in *Saccharomyces cerevisiae*. by T. Munnik, J. G. de Nobel, R. C. Montijn, J. van Rinsum, and F. M. Klis. In Permeability of the cell wall in *Saccharomyces cerevisiae*. Thesis, University of Amsterdam.

DE NOBEL, H. AND LIPKE, P.N. (1994). Is there a role for GPIs in yeast cell-wall assembly. *Trends in Cell Biology* **4**, 42–45.

DEVLIN, C. AND BALLOU, C.E. (1990). Identification and characterization of gene and protein required for glycosylation in the yeast golgi. *Molecular Microbiology* **4**, 1993–2001.

DIJKGRAAF, G.J.P., BROWN, J.L. AND BUSSEY, H. (1996). The *KNH1* gene of *Saccharomyces cerevisiae* is a functional homologue of *KRE9*. *Yeast* **12**, 683–692.

ENGLUND, P.T. (1993). The structure and biosynthesis of glycosylphosphatidylinositol protein anchors. *Annual Reviews in Biochemistry* **62**, 121–138.

FANKHAUSER, C., HOMANS, S.W., THOMAS-OATES, J.E., MACCONVILLE, M.J., DESPONDS, C., CONZELMANN, A. AND FERGUSON, M.A.J. (1993). Structures of glycosylphosphatidylinositol membrane anchors from *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **268**, 26365–26374.

FLEET, G.H. AND MANNERS, D.J. (1977). The enzymatis degradation of an alkali-soluble glucan from the cell walls of *Saccharomyces cerevisiae*. *Journal of General Microbiology* **98**, 315–327.

FREVERT, J. AND BALLOU, C.E. (1985). *Saccharomyces cerevisiae* structural cell wall mannoproteins. *Biochemistry* **24**, 753–759.

FUJII, T., SHIMOI, H. AND IMURA, Y. (1996). Structure of glycan binding region of Tip1p, a cell wall protein of *Saccharomyces cerevisiae*. Yeast genetics and molecular biology meeting 1996, August 6–11, p. 76.

GERBER, L D., KODUKULA, K. AND UDENFRIEND, S. (1992). Phosphatidylinositol glycan (PI-G) anchored membrane proteins. *Journal of Biological Chemstry* **267**, 12168–12173.

GRAHAM, T.R., VEROSTEK, M.F., MACKAY, V., TRIMBLE, R. AND EMR, S. D. (1992). Characterisation of the *Saccharomyces cerevisiae* α-1,3 mannosyltransferase. *Yeast* **8**, S458.

HAMBURGER, D., EGERTON, M. AND RIEZMAN, H. (1995). Yeast Gaa1p is required for attachment of a completed GPI anchor onto proteins. *Journal of Cell Biology* **129**, 629–639.

HARDWICK, K.G., BOOTHROYD, J.C., RUDNER, A.D. AND PELHAM, R.B. (1992). Genes that allow yeast cells to grow in the absence of the HDEL receptor. *EMBO Journal* **11**, 4187–4195.

HARTLAND, R. P., C. A. VERMEULEN, F. M. KLIS, J. H. SIETSMA, AND J. G. H. WESSELS. (1994). The linkage of (1,3)-β-glucan to chitin during cell wall assembly in *Saccharomyces cerevisiae*. *Yeast* **10**, 1591–1599.

HASEGAWA, S. AND YANAGISHIMA, N. (1984). Alpha mating type-specific suppression and a mating type specific enhancement of sexual agglutinability in *Saccharomyces cerevisiae*. *Archives in Microbiology* **138**, 310–314.

HÄUSLER, A., BALLOU, L., BALLOU, C.E. AND ROBBINS, P.W. (1992). Yeast glycoprotein biosynthesis: *MNT1* encodes an α-1,2-mannosyltransferase involved in O-glycosylation. *Proceedings of the National Academy of Sciences, USA* **89**, 6846–6850.

HELLER, L., ORLEAN, P. AND ADAIR, W.L. (1992). *Saccharomyces cerevisiae sec59* cells are deficient in dolichol kinase activity. *Proceedings of the National Academy of Sciences, USA* **89**, 7013–7016.

HERSCOVICS, A. AND ORLEAN, P. (1993). Glycoprotein biosynthesis in yeast. *FASEB Journal* **7**, 540–550.

IMMERVOLL, T., GENTZSCH, M. AND TANNER, W. (1995). *PMT3* and *PMT4*, two new members of the protein-*O*-mannosyltransferase gene family of *Saccharomyces cerevisiae*. *Yeast* **11**, 1345–1351.

JENTOFT, N. (1990). Why are proteins O-glycosylated? *Trends in Biological Science* **15**, 291–294.

JIANG, B., SHERATON, J., RAM, A.F.J., DIJKGRAAF, G.J.P., KLIS, F M. AND BUSSEY, H. (1996). *CWH41* encodes a novel endoplasmatic reticulum membrane *N*-glycoprotein involved in β-1,6-glucan assembly. *Journal of Bacteriology* **178**, 1162–1171.

KAPTEYN, J.C., MONTIJN, R.C., DIJKGRAAF, G.J P. AND KLIS, F.M. (1994). Identification of β-1,6-glucosylated cell wall proteins in yeast and hyphal forms of *Candida albicans*. *European Journal of Cell Biology* **65**, 402–407.

KAPTEYN, J.C., MONTIJN, R.C., VINK, E., DE LA CRUZ, J., LLOBELL, A., DOUWES, J.E., SHIMOI, H., LIPKE, P.N. AND KLIS, F.M. (1996). Retention of *Saccharomyces cerevisiae* cell wall

proteins through a phosphodiester-linked β-1,3-/β-1,6-glucan heteropolymer. *Glycobiology* **6**, 337–345.

KASAHARA, S., INOUE, S.B., MIO, T., YAMADA, T., NAKAJIMA, T., ICHISHIMA, E., FURUICHI, Y. AND YAMADA, H. (1994). Involvement of cell wall β-glucan in the action of HM-1 killer toxin. *FEBS Letters* **348**, 27–32.

KELLEHER, D.J. AND GILMORE, R. (1994). The *Saccharomyces cerevisiae* oligosaccharyltransferase is a protein complex composed of Wbp1p, Swp1p and four additional polypeptides. *Journal of Biological Chemistry* **269**, 12908–12917.

KLIS, F.M. (1994a). Review: cell wall assembly in yeast. *Yeast* **10**, 851–869.

KLIS, F.M. (1994b). Protein secretion in yeast. In *The Mycota I. Growth, Differentiation and Sexuality*, ed. Wessels and Meinhard, Berlin Heidelberg: Springer-Verlag.

KODUKALA, K., GERBER, L.D., AMTHAUER, R., BRINK, L. AND UDENFRIEND, S. (1993). Biosynthesis of glycosylphosphatidylinositol (GPI)-anchored membrane proteins in intact cells: specific amino acids requirements adjacent to the site of cleavage and GPI attachment. *Journal of Cell Biology* **120**, 657–664.

KOLLÁR, R., PETRÁKOVÁ, E., ASHWELL, G., ROBBINS, P.W. AND CABIB, E. (1995). Architecture of the yeast cell wall: the linkage between chitin and β(1,3)-glucan. *Journal of Biological Chemistry* **270**, 1170–1178.

KONDO, K. AND INOUYE, M. (1991). *TIP1*, a cold shock-inducible gene of *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **266**, 17537–17544.

KUKURUZINSKA, M.A., BERGH, M.L.E. AND JACKSON, B.J. (1987). Protein glycosylation in yeast. *Annual Reviews in Biochemistry* **56**, 915–944.

LARRIBA, G. (1993). Translocation of proteins across the membrane of the endoplasmatic reticulum: a place for *Saccharomyces cerevisiae*. *Yeast* **9**, 441–463.

LEHLE, L. AND TANNER, W. (1995). Protein glycosylation in yeast. In *Glycoproteins*, ed. J. Montreuil, H. Schachter and J.F.G. Vliegenthart, pp. 475–509.

LEIDICH, S. D., D. A. DRAPP, AND P. ORLEAN. (1994). A conditionally lethal yeast mutant blocked at the first step in glycosyl phosphatidylinositol anchor synthesis. *Journal of Biological Chemistry* **269**, 10193–10196.

LEIDICH, S.D., KOSTOVA, Z., LATEK, R.R., COSTELLO, L.C., DRAPP, D.A., GRAY, W., FASSLER, J.S. AND ORLEAN, P. (1995). Temperature-sensitive yeast GPI anchoring mutants *gpi2* and *gpi3* are defective in the synthesis of N-acetylglucosaminyl phosphatidylinositol. Cloning of the *GPI2* gene. *Journal of Biological Chemistry* **270**, 13029–13035.

LIPKE, P.N., WOJCIECHOWICZ, D. AND KURJAN, J. (1989). *AGα1* is the structural gene for the *Saccharomyces cerevisiae* α-agglutinin, a cell surface glycoprotein involved in cell–cell interactions during mating. *Molecular and Cellular Biology* **9**, 3155–3165.

LU, C-F., MONTIJN, R.C., BROWN, J.L., KLIS, F., KURJAN, J., BUSSEY, H. AND LIPKE, P.N. (1995). Glycosyl phosphatidylinositol-dependent cross-linking of α-agglutinin and β-1,6-glucan in the *Saccharomyces cerevisiae* cell wall. *Journal of Cell Biology* **128**, 333–340.

LUSSIER, M., GENTZSCH, M., SDICU, A-M., BUSSEY, H. AND TANNER, W. (1995). Protein *O*-glycosylation in yeast. The *PMT2* gene specifies a second protein *O*-mannosyltransferase that functions in addition to the *PMT1*-encoded activity. *Journal of Biological Chemistry* **270**, 2770–2775.

LUSSIER, M., SDICU, A., CAMIRAND, A. AND BUSSEY, H. (1996). Functional characterisation of the *YUR1*, *KTR1* and *KTR2* genes as members of the yeast *KRE2/MNT1* mannosyltranferase gene family. *Journal of Biological Chemistry* **271**, 11001–11008.

MCCONVILLE, M.J. AND FERGUSON, M.A. (1993). The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protazoa and higher eukaryotes. *Biochemical Journal* **294**, 305–324.

MALLET, L., BUSSEREAU, F. AND JACQUET, M. (1994). Nucleotide sequence analysis of an 11.7 kb fragment of yeast chromosome II including *BEM1*, a new gene of the WD-40 repeat family and a new member of the *KRE2/MNT1* family. *Yeast* **10**, 819–831.

MANNERS, D.J., MASSON, A.J. AND PATTERSON, J.C. (1973a). The stucture of a β-(1,3)-D-glucan from yeast cell walls. *Biochemical Journal* **135**, 19–30.

MANNERS, D.J., MASSON, A.J. AND PATTERSON, J.C. (1973b). The stucture of a β-(1,6)-D-glucan from yeast cell walls. *Biochemical Journal* **135**, 31–36.

MARGUET, D., GUO, X.J. AND LAUQUIN, G.J.M. (1988). Intragenic repeat structure and

identification of a family of *SRP1*-related DNA sequences. *Journal of Molecular Biology* **202**, 455–470.

MARTINAC, B., ZHU, H., KUBALSKI, A., ZHOU, X.L., CULBERTSON, M., BUSSEY, H. AND KUNG, C. (1990). Yeast K1 killer toxin forms ion channels in sensitive yeast spheroplasts and in artificial liposomes. *Proceedings of the National Academy of Sciences, USA* **87**, 6228–6232.

MEADEN, P., HILL, K., WAGNER, J., SLIPETZ, D., SOMMER, S.S. AND BUSSEY, H. (1990). The yeast *KRE5* encodes a probable endoplasmatic reticulum protein required for (1-6)-β-D-glucan synthesis and normal cell growth. *Molecular and Cellular Biology* **10**, 3013–3019.

MEIER, H. AND REID, J.S.G. (1982). Reserve polysaccharides other than starch in higher plants, pp. 418–471. In *Encyclopedia of Plant Physiology*, New Series 13A, ed. F.A. Loewus and W. Tanner. New York: Springer-Verlag.

MENON, A.K. AND STEVENS, V.L. (1992). Phosphatidylethanolamine is the donor of the ethanolamine residue linking a glycosylphosphatidylinositol anchor to protein. *Journal of Biological Chemistry* **267**, 15277–15280.

MICANIVIC, R., GERBER, L.D., BERGER, J., KODUKULA K., AND UDENFRIEND, S. (1990). Selectivity of the cleavage/attachment site of phosphatidylinositol-glycan-anchored membrane proteins determined by site-specific mutagenesis at Asp-484 of placental alkaline phosphatase. *Proceedings of the National Academy of Sciences, USA* **87**, 157–161.

MOLLOY, C., SHEPHERD, M.G. AND SULLIVAN, P.A. (1989). Identification of envelope proteins of *Candida albicans* by vectorial iodination. *MICROBIOS* **57**, 73–84.

MONTIJN, R.C. (1996a). Involvement of Kre6p and Skn1p in the biogenesis of cell wall proteins in yeast. In Biogenesis of cell wall proteins in fungi. Thesis, University of Amsterdam.

MONTIJN, R.C. (1996b). β-Glucosylated proteins in the cell wall of *Exophiala* (*Wangiella*) *dermatitidis*. In Biogenesis of cell wall proteins in fungi. Thesis, University of Amsterdam.

MONTIJN, R.C., VAN RINSUM, J., VAN SCHAGEN, F.A. AND KLIS, F.M (1994). Glucomannoproteins in the cell wall of *Saccharomyces cerevisiae* contain a novel type of carbohydrate side-chain. *Journal of Biological Chemistry* **269**, 19338–19342.

MÜLLER, G., GROß, E., WIED, S. AND BANDLOW, W. (1996). Glucose-induced sequential processing of a glycosylphosphatidylinositol-anchored ectoprotein in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **16**, 442–456.

MURAI, T., UEDA, M., YAMAMURA, M., ATOMI, H., SHIBASAKA, Y., KAWASAWA, N., OSUMI, M., AMACHI, T. AND TANAKA, A. (1997). Construction of a starch-utilizing yeast by cell surface engineering. *Applied Environmental Microbiology* **63**, 1362–1366.

NUOFFER, C., HORVATH, A. AND RIEZMAN, H. (1993). Analysis of the sequence requirements for glycosylphosphatidylinositol anchoring of *Saccharomyces cerevisiae* Gas1 protein. *Journal of Biological Chemistry* **268**, 10558–10563.

ORLEAN, P. (1990). Dolichol phosphate mannose synthase is required *in vivo* for glycosyl phosphatidylinositol membrane anchoring, *O*-mannosylation and *N*-glycosylation of proteins in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **10**, 5796–5805.

PALLIER, C., VALENS, M., PUZOS, V., FUKUHARA, H., CHÉRET, G., G. SOR, G. AND BOLOTIN-FUKUHARA, M. (1993). DNA sequence analysis of a 17 kb fragment of yeast chromosome XI physically localizes the *MRB1* gene and reveals eight new open reading frames including a homologue of the KIN1/KIN2 and SNF1 protein kinases. *Yeast* **9**, 1149–1155.

PARKER, C.G., FESSLER, L.I., NELSON, R.E. AND FESSLER, J.H. (1995). Drosophila UDP-glucose:glycoprotein glucosyltransferase: sequence and characterization of an enzyme that distinguishes between denaturated and native proteins. *EMBO Journal* **14**, 1294–1303.

PASTOR, F.I.J, VALENTIN, E., HERRERO, E. AND SENTANDREU, R. (1984). Structure of the *Saccharomyces cerevisiae* cell wall. Mannoproteins released by Zymolyase and their contribution to wall architecture. *Biochimica et Biophysica Acta* **802**, 292–300.

POPOLO, L., VAI, M., GATTI, E., PORELLO, S., BONFANTE, P., BALESTRINI, R. AND ALBERGHINA, L. (1993). Physiological analysis of mutants indicates involvement of the *Saccharomyces cerevisiae* GPI-anchored protein gp115 in morphogenesis and cell separation. *Journal of Bacteriology* **175**, 1879–1885.

RAM, A.F.J., WOLTERS, A., TEN HOPEN, R. AND KLIS, F.M. (1994). A new approach for

isolating cell wall mutants in *Saccharomyces cerevisiae* by screening for hypersensitivity to Calcofluor white. *Yeast* **10**, 1019–1030.

RAPOPORT, T.A. (1992). Transport of proteins across the endoplasmatic reticulum membrane. *Science* **258**, 931–936.

ROBERTS, W.L., MYHER, J.J., KUKSIS, A., LOW, M.G. AND ROSENBERRY, T.L. (1988). Lipid analysis of the glycoinositol phospholipid membrane anchor of human erythrocyte acetylcholinesterase. *Journal of Biological Chemistry* **263**, 18766–18775.

ROEMER, T. AND BUSSEY, H. (1991). Yeast β-glucan synthesis: *KRE6* encodes a predicted type II membrane protein required for glucan synthesis in *vivo* and for glucan synthase activity in *vitro*. *Proceedings of the National Academy of Sciences, USA* **88**, 11295–11299.

ROEMER, T. AND BUSSEY, H. (1995). Yeast Kre1p is a cell surface *O*-glycoprotein. *Molecular and General Genetics* **249**, 209–216.

ROEMER, T., DELANEY, S. AND BUSSEY, H. (1993). *SKN1* and *KRE6* define a pair of functional homologs encoding putative membrane proteins involved in β-glucan synthesis. *Molecular and Cellular Biology* **13**, 4039–4048.

ROEMER, T., PARAVICINI, G., PAYTON, M.A. AND BUSSEY, H. (1994). Characterization of the yeast 1- 6-β-glucan biosynthetic components, Kre6p and Skn1p, and genetic interactions between the *PKC1* pathway and extracelluar matrix assembly. *Journal of Cell Biology* **127**, 567–579.

ROY, A., LU, C.F., MARYKWAS, D.L., LIPKE, P.N. AND KURJAN, J. (1991). The *AGA1* product is involved in cell surface attachment of the *Saccharomyces cerevisiae* cell adhesion glycoprotein a-agglutinin. *Molecular and Cellular Biology* **11**, 4196–4206.

RUIZ-HERRERA, J., IRANZO, M., ELORZA, M.V., SENTANDREU, R. AND MORMENEO, S. (1995). Involvement of transglutaminase in the formation of covalent cross-links in the cell wall of *Candida albicans*. *Archives in Microbiology* **164**, 186–193.

SCHREINER, R., SCHNABEL, E. AND WIELAND, F. (1994). Novel *N*-glycosylation in eukaryotes: laminin contains the linkage unit β-glucosylasparagine. *Journal of Cell Biology* **124**, 1071–1081.

SCHREUDER, M.P. (1994). Yeast expressing hepatitis B virus surface antigen determinants on its surface, implications for a possible oral vaccine. In Targeting of proteins to the cell wall of yeast and possible applications. Thesis, University of Amsterdam.

SCHREUDER, M.P., BREKELMANS, S., VAN DEN ENDE, H. AND KLIS, F.M. (1993). Targeting of a heterologous protein to the cell wall of *Saccharomyces cerevisiae*. *Yeast* **9**, 399–409.

SCHREUDER, M.P., MOOREN, A.T.A., TOSCHKA, H.Y., VERRIPS, C.T. AND KLIS, F.M. (1996). Immobilizing proteins on the surface of yeast cells. *TIBTECH* **14**, 115–120.

SHAW, J.A., MOL, P.C., BOWERS, B., SILVERMAN, S.J., VALDIVIESO, M.H., DURAN, A. AND CABIB, E. (1991). The function of chitin synthases 2 and 3 in the *Saccharomyces cerevisiae* cell cycle. *Journal of Cell Biology* **114**, 111–23.

SHIMOI, H., IMURA, Y. AND OBATA, T. (1995). Molecular cloning of *CWP1*: a gene encoding a *Saccharomyces cerevisiae* cell wall protein solubilized with *Rarobacter faecitabidus* protease I. *Journal of Biochemistry* **118**, 302–311.

SILBERSTEIN, S. AND GILMORE, R. (1996). Biochemistry, molecular biology, and genetics of the oligosaccharyltransferase. *FASEB Journal* **10**, 849–858.

SIPOS, G., PUOTI, A. AND CONZELMANN, A. (1995). Biosynthesis of the side chain of yeast glycosylphosphatidylinositol anchors is operated by novel mannosyltransferases located in the endoplasmic reticulum and the Golgi apparatus. *Journal of Biological Chemistry* **270**, 19709–19715.

STRAHL-BOLSINGER, S., IMMERVOLL, T., DEUTZMANN, R. AND TANNER, W. (1993). *PMT1*, the gene for a key enzyme of protein O-glycosylation in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences, USA* **90**, 8164–8168.

TAKEDA, J. AND KINOSHITA, T. (1995). GPI-anchor biosynthesis. *TIBS* **20**, 367–371.

TEUNISSEN, A.W.R.H., HOLUB, E., VAN DER HUCHT, J., VAN DEN BERG, J.A. AND STEENSMA, H.Y. (1993). Sequence of the open reading frame of the *FLO1* gene from *Saccharomyces cerevisiae*. *Yeast* **9**, 423–427.

VALENTIN, E., HERRERO, E., PASTOR, J.F.I. AND SENTANDREU, R. (1984). Solubilization and analysis of mannoprotein molecules from the cell wall of *Saccharomyces cerevisiae*.

*Archives in Microbiology* **130**, 1419–1428.

VAN BERKEL, M.A A., CARO, L.H.P., MONTIJN, R.C. AND KLIS, F.M. (1994). Glucosylation of chimeric proteins in the cell wall of *Saccharomyces cerevisiae*. *FEBS Letters* **349**, 135–138.

VAN DER VAART, J.M. (1997b). Identification and characterization of cell wall proteins of *Saccharomyces cerevisiae*. Thesis, Utrecht University.

VAN DER VAART, J.M., CARO, L.H.P., CHAPMAN, J.W., KLIS, F.M. AND VERRIPS, C.T. (1995). Identification of three mannoproteins in the cell wall of *Saccharomyces cerevisiae*. *Journal of Bacteriology* **177**, 3104–3110.

VAN DER VAART, J.M., VAN SCHAGEN, F.S., MOOREN, A.T.A., CHAPMAN, J.W., KLIS, F.M. AND VERRIPS, C.T. (1996a). The retention mechanism of cell wall proteins in *Saccharomyces cerevisiae*. Wall-bound Cwp2p is β-1,6-glucosylated. *Biochimica et Biophysica Acta* **1291**, 206–214.

VAN DER VAART, J.M., TE BIESEBEKE, R., CHAPMAN, J.W., KLIS, F.M. AND VERRIPS, C.T. (1996b). The β-1,6-glucan containing side-chain of cell wall proteins of *Saccharomyces cerevisiae* is bound to the glycan core of the GPI moiety. *FEMS Letters* **145**, 401–407.

VAN DER VAART, J.M., TE BIESEBEKE, R., CHAPMAN, J.W., TOSCHKA, H.Y., KLIS, F.M. AND VERRIPS, C.T. (1997a). Comparison of cell wall proteins of *Saccharomyces cerevisiae* as anchors for cell surface expression of heterologous proteins. *Applied Environmental Microbiology* **63**, 615–620.

VAN RINSUM, J., KLIS, F.M. AND VAN DEN ENDE, H. (1991). Cell wall glucomannoproteins of *Saccharomyces cerevisiae mnn9*. *Yeast* **7**, 717–726.

VOSSEN, J.H., RAM, A.F.J. AND KLIS, F.M. (1995). Identification of *SPT14/CWH6* as the yeast homologue of hPIG-A, a gene involved in the biosynthesis of GPI anchors. *Biochimica et Biophysica Acta* **1243**, 549–551.

VOSSEN, J. H., MÜLLER, W.H., LIPKE, P N. AND KLIS, F.M. (1997). Restrictive glycosylphosphatidylinositol anchor synthesis in *cwh6/gpi3* yeast cells cause aberant biogenesis of cell wall proteins. *Journal of Bacteriology* **179**, 2202–2209.

WOJCIECHOWICZ, D., LU, C.F., KURJAN, J. AND LIPKE, P.N. (1993). Cell surface anchoring and ligand-binding domains of the *Saccharomyces cerevisiae* cell adhesion protein α-agglutinin, a member of the immunoglobulin superfamily. *Molecular and Cellular Biology* **13**, 2554–2563.

YIP, C.L., WELCH, S.K., KLEBL, F., GILBERT, T., SEIDEL, P. GRANT, F.J., O'HARA, P.J. AND MACKAY, V.L. (1994). Cloning and analysis of the *Saccharomyces cerevisiae MNN9* and *MNN1* genes required for complex glycosylation of secreted proteins. *Proceedings of the National Academy of Sciences, USA* **91**, 2723–2727.

YOSHIHISA, T., ANRAKU, Y. (1989). Nucleotide sequence of AMS1, the structure gene of vacuolar alpha-mannosidase of *Saccharomyces cerevisiae*. *Biochemical Biophysical Research Communications* **163**, 908–915.

ZLOTNIK, H., FERNANDEZ, M.P., BOWERS, B. AND CABIB, E. (1984). *Saccharomyces cerevisiae* mannoproteins form an external cell wall layer that determines wall porosity. *Journal of Bacteriology* **159**, 1018–1026.

ZUFFEREY, R., KNAUER, R., BURDA, P., SAGLJAR, I., TE HEESEN, S., LEHLE, L, AND AEBI, M. (1995). *STT3*, a highly conserved protein required for yeast oligosaccharyl transferase activity *in vivo*. *EMBO Journal* **14**, 4949–4960.