1,6-beta-glucan synthesis in *Saccharomyces cerevisiae*

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Chapter 1

General Introduction

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1. Introduction

The baker's yeast *Saccharomyces cerevisiae* is protected from its environment by its cell wall. The cell wall - which can be found throughout all fungal species - provides the cell with both physical protection and osmotic support. Although the term “wall” suggests this structure to be very solid and rigid, for it to allow growth and mating it must also have dynamic properties. It is a layer composed of about equal amounts of glucan, and mannoproteins and a small amount of chitin (1-2%). The glucan consists mainly of 1,3-β-linked residues, and this so-called 1,3-β-glucan - together with the chitin - can be visualized as an electron-transparent layer by electron microscopy. This layer makes up the inner part of the cell wall, while the mannoproteins appear as an electron-dense layer on the outer side. The proteins which are covalently linked to the cell wall can be divided into two groups: (I) the glycosylphosphatidylinositol-dependent cell wall proteins (GPI-CWPs), which are linked to the 1,3-β-glucan through a 1,6-β-glucan moiety, and (II) the Pir cell wall proteins (Pir-CWPs), which are presumably directly linked to 1,3-β-glucan via an alkali-sensitive binding (for reviews, see Orlean, 1997; Klis et al., 2002). The cell wall proteins are among others involved in interactions of the cell with its surroundings, and processes like mating and flocculation depend on them (Lipke and Kurjan, 1991; Teunissen and Steensma, 1995). Furthermore, they limit the permeability of the wall to macromolecules (Zlotnik et al., 1984; De Nobel et al., 1991) and protect the glucan and the cell itself against external perils.

1,6-β-glucan is an essential component of the cell wall, which interconnects the GPI-CWPs with the 1,3-β-glucan network as mentioned above (Kapteyn et al., 1996; Kollár et al., 1997; Kapteyn et al., 1999). Mutations which result in lowered 1,6-β-glucan levels often lead to decreased viability (Meaden et al., 1990; Roemer et al., 1993; Brown and Bussey, 1993; Vink et al., 2002, Chapter 2). Also in *Candida albicans*, genes involved in 1,6-β-glucan were found to be essential (Mio et al., 1997; Lussier et al., 1998). This stresses the importance of this minor component of the yeast cell wall. However, little is known about its biosynthesis and regulation.

2. Cell wall proteins

2.1. Cell wall proteins are heavily glycosylated

Mannoproteins destined for the cell wall travel through the secretory pathway before they are incorporated into the cell wall. Along this route, they become heavily glycosylated on both N- and O-glycosylation sites. O-chains are short oligomannosyl side chains, which can be added onto either serine or threonine residues. However, the asparagine-linked side chains may contain up to 200 mannose residues, and form large, branched structures. They can make up 90% of the mannoprotein weight (Orlean, 1997). Both N- and O-chains are initiated in the ER, where core structures of the respective chains are added to the protein.
Further extension of these core chains is accomplished in the Golgi, which solely concerns mannosyl additions. Although there is evidence for the presence of an UDP-galactose antiporter in *S. cerevisiae*, no galactosyl modifications have ever been described (Gemmill and Trimble, 1999). Besides the addition of mannose residues, both *N*- and *O*-chains may also be modified by the addition of mannosyl phosphate. The resulting mannosyl phosphodiester bridges give a negative charge to the neutral mannan, the exact function of which remains to be clarified. It has been reported, however, that mannosyl phosphorylation is increased by the addition of KCl to the growth medium and thus might be involved in stress response (Jigami and Odani, 1999).

2.2. **GPI-dependent cell wall proteins**

GPI-anchors are found in numerous species ranging from fungi to mammals (Ikezawa, 2002). GPI-anchor addition occurs in the ER to newly synthesized proteins, and for this a carboxy-terminal signal peptide is required. Generally, the signal peptide consists of a cleavage/attachment site, a spacer domain of 6 - 14 hydrophilic amino acid residues, and a hydrophobic carboxy-terminal generally ranging from 15 - 30 residues. The attachment site, also named the ω-site, preferentially is a small amino acid such as glycine or asparagine. At the ω+1 and ω+2 positions also small amino acids are found (Udenfriend & Kodukula, 1995; Ikezawa, 2002; De Groot *et al.*, 2003). In general, GPI-anchors result in a permanent attachment of specific proteins to the plasma membrane (Figure 1), but in yeast GPI-anchors can also function in binding cell wall proteins. Then, the GPI-anchor is cleaved to release the cell wall protein from the plasma membrane, after which the GPI-remnant on the protein is cross-linked to 1,6-β-glucan, which in turn is connected to 1,3-β-glucan (Kapteyn *et al.*, 1996; Kollár *et al.*, 1997; Klis *et al.*, 2002).

The above raises the question how GPI-anchored proteins that remain bound to the plasma membrane are distinguished from those destined for incorporation in the cell wall. A dibasic motif upstream of the GPI-attachment site might serve to prevent cell wall incorporation for GPI-anchored proteins (Vossen *et al.*, 1997; Caro *et al.*, 1997; Hamada *et al.*, 1999). In contrast, the amino acid at sites 4 or 5 upstream of the GPI-attachment site (the ω-site) and the amino acid at site 2 upstream of the ω site seem to correlate with cell wall incorporation. This region is also called the ω-minus 5-1 sequence. The presence of valine, isoleucine, or leucine at the ω-5 or ω-4 position and tyrosine or asparagine at the ω-2 position tends to direct a GPI-anchored protein to the cell wall (Hamada *et al.*, 1998; Hamada *et al.*, 1999). The exact mechanism of both negative and positive signals has not yet been resolved. Interestingly, mutations in the ω-minus 5-1 sequence that reduce cell wall incorporation, do not result in the increased release into the medium of fusion proteins (Hamada *et al.*, 1998). This suggests that the ω-minus 5-1 sequence serves as a recognition/target site for the release from the plasma membrane, and thereby facilitates cell wall incorporation. Another mechanism that might help the cell to distinguish between plasma membrane GPI-anchored
proteins and cell wall GPI-anchored proteins, is the clustering of GPI-anchored proteins in sphingolipid-cholesterol microdomains or rafts. These rafts might function as selective platforms to target proteins to specific locations, possibly driven by differential remodelling of the lipid moiety of the GPI-anchors (Sipos et al., 1997; Reggiori et al., 1997; Muñiz and Riezman, 2000).

Figure 1. Schematic representation of a GPI-anchored protein. EtN = ethanolamine, P = phosphate, Man = mannose, GlcN = glucosamine, myo-Ino = myo-inositol, PM = plasma membrane.

Detailed studies have been dedicated to address the question of how the cell wall proteins are linked to the 1,6-β-glucan. The GPI-CWP α-agglutinin has been studied most extensively. Upon release from the plasma membrane, part of the GPI-anchor is removed from the protein, including the inositol residue (Lu et al., 1994). However, no 1,6-β-glucan was found to be associated with the periplasmic intermediate form of α-agglutinin. Instead, only α-agglutinin released from the cell wall with laminaranase contained 1,6-β-glucan (Lu et al., 1995). This suggests that cross-linking of α-agglutinin to 1,6-β-glucan results in cell wall attachment. Close examination of another GPI-CWP, Tip1, revealed that the C-terminus of this protein was linked via ethanolamine, a phosphodiester bridge, and mannan to 1,6-β-glucan
Further insight in the linkage between the GPI-remnant and 1,6-β-glucan came from a study by Kollár and co-workers (1997). They digested cell walls with 1,3-β-glucanase, labeled them by reduction with tritiated borohydride, and then further digested them with chitinase. After chromatography on a Bio-Gel P2 column, the void volume was subjected to ConA-chromatography. The ConA\(^+\) fraction was analyzed by NMR, gel chromatography, immunoblotting, chemical and enzymatic degradation. This study confirmed that 1,6-β-glucan is attached to the remnant of the GPI-anchor, through a yet unknown linkage to the mannan core of the GPI-remnant. Furthermore it was established that the terminal glucose residue at the reducing end of 1,6-β-glucan is directly attached to a terminal glucose residue at the non-reducing end of a 1,3-β-glucan polymer (Kollár et al., 1997). Taken together, this suggests that the original GPI-anchor is cleaved between the mannan core and the glucosamine (Figure 1), or at least that glucosamine is lost upon cross-linkage to 1,6-β-glucan. It has been proposed that the non-reducing end of 1,6-β-glucan acts as the accepting group in a transglycosylation reaction linking it to the remnant of the GPI-anchor (Kollár et al., 1997). Although the GPI-CWPs are generally connected via 1,6-β-glucan to 1,3-β-glucan, which in turn is connected to chitin, there are also examples of 1,6-β-glucan directly linked to chitin, which are predominantly seen under cell wall stress conditions (Kollár et al., 1997; Kapteyn et al., 1997; Figure 2).

### 2.3. Pir cell wall proteins

Another class of covalently linked CWPs, Pir-CWPs, does not contain C-terminal GPI-anchor addition signals, but does get covalently linked to the glucan network of the wall. Four PIR (proteins with internal repeats) proteins have been found in *S. cerevisiae*: Pir1, Pir2/Hsp150, Pir3, and Pir4/Cis3 (Toh-e et al., 1993; Yun et al., 1997; Mrša and Tanner, 1999; Moukadir et al., 1999). Two PIR genes were initially identified as genes involved in heat shock tolerance. In addition, a third homolog was identified (Toh-e et al., 1993). Later, it was found that overexpression of PIR1 - PIR3 conferred resistance to tobacco osmotin, and that the gene products were localized to the cell wall (Yun et al., 1997). The PIR proteins are covalently linked to the cell wall, probably by direct attachment to 1,3-β-glucan, through a linkage that is sensitive to mild alkali-treatment (Mrša et al., 1997; Kapteyn et al., 1999; Mrša and Tanner, 1999; Figure 2). All PIR proteins have a similar structure: they consist of an N-terminal signal peptide, a Kex2 protease cleavage site, and a region of one or more repeats with the conserved core sequence Q[IV][STGNH]DGQ[LIV]Q. In addition, the C-terminal domain of the members of the PIR family is highly homologous and contains a conserved four-cysteine motif (Klis et al., 2002). Except for the presence of a Kex2-site in the putative gene product of YJL160c, this gene also appears to encode a protein with an N-terminal signal sequence and 4 PIR-repeats (Apweiler et al., 2001), and thus may represent a fifth member of the PIR-family.
Interestingly, PIR-repeats can also be found in the GPI-CWPs Cwp1, Cwp2, Tir1, and Tir2, where they seem to be present as a single “repeat”. It has been shown that at low pH Cwp1 is both linked via its GPI-remnant to a 1,6-β-glucan - 1,3-β-glucan heteropolymer and via an alkali-sensitive linkage directly to 1,3-β-glucan. In addition, at low pH, Pir2 was incorporated in the cell wall with higher efficiency. This suggests that the incorporation mechanism for Pir-CWPs is functioning more efficiently at low pH, and therefore the alkali-sensitive linkage of Cwp1 to the cell wall might occur more frequently under those conditions (Kapteyn et al., 2001). It is tempting to speculate that the PIR-repeat is necessary and sufficient for this linkage, which could also imply multiple linkages for proteins with multiple PIR-repeats. Furthermore, it would be interesting to study the cell wall linkages of Cwp2, Tir1, and Tir2 at low pH.

![Diagram](image_url)

**Figure 2.** Overview of existing CWP-polysaccharide interconnections in *S. cerevisiae*. For details, see text.

### 3. Chitin

#### 3.1. Chitin in the cell wall

Chitin, which consists of 1,4-β-linked N-acetylglucosamine (GlcNAc) residues, is predominantly present in the septal region of vegetatively growing cells of *S. cerevisiae* (Orlean, 1997; Cabib et al., 2001). It is routinely visualized with the fluorescent compound calcofluor white. There is also some chitin dispersed throughout the lateral wall, but this is a minor part compared to the septal region. However, under stress conditions this can be considerably increased, and can account for up to 20% of the cell wall dry weight (Popolo et al., 1997; Dallies et al., 1998; Kapteyn et al., 1998; Ram et al., 1998). The increase in chitin levels in the lateral wall upon activation of the cell wall salvage mechanism is accomplished by Chitin Synthase 3 (CSIII), one of the three chitin synthases in *S. cerevisiae* (Valdivieso et al., 2000).
3.2. The three chitin synthases

Three chitin synthase activities have been identified in *S. cerevisiae*, named CSI, CSII, and CSIII. The catalytic part of these synthases is supposed to be encoded by *CHS1*, *CHS2*, and *CHS3*, respectively (Cabib *et al.*, 2001). All three synthases share the QRRRWW motif, which is essential for activity (Cos *et al.*, 1998). Chitin synthases are processive enzymes, which catalyze the transfer of GlcNAc from UDP-GlcNAc to a growing chitin chain. In addition, a number of genes (*i.e.* CHS4 - CHS7) have been identified whose gene products regulate chitin synthesis (Cabib *et al.*, 2001).

Although sharing a common biochemical function, the three chitin synthases play a distinct physiological function. CSIII is responsible for the synthesis of the chitin ring at the base of emerging buds and the chitin in the lateral wall, which together represent the bulk of the chitin in *S. cerevisiae* (Shaw *et al.*, 1991; Bulawa, 1992). The synthesis of this chitin ring at the bud neck is the first step in the synthesis of the septum, a cross-wall separating the mother and daughter cell. The second step encompasses the synthesis of the primary septum, a chitin disc perpendicular to the mother-daughter axis, which is accomplished by CSI (Silverman *et al.*, 1988; Shaw *et al.*, 1991). The third and last step in septum formation comprises the deposition of secondary septa on both mother and daughter sides of the primary septum. Cell separation is finally completed by means of a chitinase that partially hydrolyzes the primary septum (Kuranda and Robbins, 1991). During the separation, CSI seems to function as a repair mechanism to protect cell wall integrity from excessive chitinase activity (Cabib *et al.*, 1989; Cabib *et al.*, 1992).

3.3. Zymogenic nature of chitin synthases

The activity of all three chitin synthases is significantly increased after partial proteolysis by trypsin. CSI and CSII are predominantly found in an inactive state. This state has been designated as zymogenic (Cabib *et al.*, 1996). In contrast, CSIII can be isolated in an active state, but in *chs4* mutants or after extractions with detergents, CSIII also appeared to have a zymogenic state (Choi *et al.*, 1994a; Trilla *et al.*, 1997). For Chs1 and Chs3 activity, posttranslational regulation seems to be the major mechanism during vegetative growth in rich medium. Chs2 also appears to be regulated on the posttranslational level, yet Chs2 levels oscillate during the cell cycle whereas Chs1 and Chs3 levels remain constant (Choi *et al.*, 1994b; Chuang and Schekman, 1996).

3.4. Localization of chitin synthases

The chitin synthases must be located in the plasma membrane to perform their biological activities. Best documented is the migration of Chs3, which requires Chs7 to exit the endoplasmic reticulum (ER) (Trilla *et al.*, 1999). In the Golgi, Chs3 is transferred to so-called chitosomes, a process for which Chs5 and Chs6 are necessary (Santos and Snyder, 1997; Ziman *et al.*, 1998). Finally, the chitosomes transport Chs3 to the plasma membrane (Chuang and Schekman, 1996; Ziman *et al.*, 1998). The translocation of Chs1 and Chs2 to
the plasma membrane is poorly documented. The bulk of these proteins is located at the plasma membrane (Cabib et al., 1996), but also a small amount can be detected in chitosomes (Chuang and Schekman, 1996; Ziman et al., 1996).

4. 1,3-β-Glucan

4.1. 1,3-β-Glucan in the cell wall

1,3-β-Glucan represents the major class of β-glucans in the cell wall of S. cerevisiae, and is responsible for the mechanical strength of the cell wall. The elasticity is exemplified by the observation that cells exposed to hypertonic pressure show dramatic shrinkage, a process that is reversed when they are placed back in an isotonic environment (Morris et al., 1986). This suggests that the cell wall is in an extended state under normal growth conditions, which is supported by the observation that isolated walls are far less permeable to macromolecules than the stretched walls of living cells (Scherrer et al., 1974; De Nobel et al., 1990a; De Nobel et al., 1990b; De Nobel et al., 1991). The elastic nature of the cell wall can be explained by the 3-D structure of 1,3-β-glucan chains, which can assume the shape of a spring (Klis et al., 2002). Of the carbohydrate polymers in the yeast cell wall, 1,3-β-glucan has the highest degree of polymerization with an average of 1,500 glucose residues per chain in stationary phase cells (Fleet, 1991).

4.2. Enzymatic properties of the 1,3-β-glucan synthase

The development of an in vitro assay for 1,3-β-glucan synthesis has been of great value to cell wall research. After important groundwork (Sentandreu et al., 1975; Balint et al., 1976; Lopez-Romero and Ruiz-Herrera, 1977), an efficient assay for in vitro biosynthesis of this polymer was devised (Shematek et al., 1980). The activity is associated with the plasma membrane, and requires the presence of GTP or ATP (Shematek et al., 1980; Shematek and Cabib, 1980). UDP-glucose is used as sugar donor for this assay, and the in vitro reaction product is a linear 1,3-β-glucan of 60 - 80 sugar residues long. This polysaccharide is synthesized de novo, since the terminal reducing group was found to contain $^{14}$C from the UDP-$[^{14}$C]glucose (Shematek et al., 1980). However, 1,3-β-glucan occurs in the cell wall as a large linear polymer, branched with some 1,6-β residues (Fleet, 1991). It is unlikely that in vivo 1,3-β-glucan is directly synthesized into such a large polymer, and there are candidate genes for extracellular modification (Popolo and Vai, 1999).

4.3. GTP is required for 1,3-β-glucan synthase

The activation of 1,3-β-glucan synthesis by nucleotide triphosphates, in particular GTP, suggested that these nucleotides are involved in a regulatory mechanism. Evidence came from reconstitution experiments, in which 1,3-β-glucan synthase was dissociated by detergent and NaCl into a soluble- and a membrane fraction. Either fraction was practically inactive, while activity was reconstituted by mixing both fractions. The catalytic activity appeared to reside in the membrane fraction, whereas the soluble fraction contained a
putative regulatory GTP-binding component (Kang and Cabib, 1986). A small GTP-binding protein was purified from the soluble fraction, which no longer required the addition of GTP for activity. Its ability to stimulate 1,3-β-glucan synthesis depended on the association with GTP, as hydrolysis of the bound GTP to GDP lead to the recurring need for GTP addition (Mol et al., 1994).

4.4. Rho1 is the GTP-binding protein

In search for the identity of the small GTP-binding protein that regulates 1,3-β-glucan synthase, the known small GTP-binding proteins were explored. Conditional mutations in RHO1 - RHO4 result in the lysis of cells with a small bud (Madaule et al., 1987; Matsui and Toh-e, 1992; Yamochi et al., 1994), which is a stage in the cell cycle that clearly requires 1,3-β-glucan synthesis. Thus, these genes were candidates for the regulatory G-protein involved in 1,3-β-glucan synthesis. Rho1 turned out to be the GTP-binding regulatory component of 1,3-β-glucan synthase, as extracts of rho1 mutants were defective in 1,3-β-glucan synthase activity. Also, the addition of recombinant Rho1 could reconstitute the 1,3-β-glucan synthase activity of the membrane fraction of rho1 mutants (Drgonová et al., 1996; Qadota et al., 1996). Furthermore, Rho1 copurified with the 1,3-β-glucan synthase complex, and co-precipitated when Fks1 - another component of the 1,3-β-glucan synthase - was “pulled down” with antibodies (Qadota et al., 1996; Mazur et al., 1996).

4.5. Fks1 and Gsc2 are essential components of 1,3-β-glucan synthase

FKS1 was first identified in a screen for mutants hypersensitive for the immunosuppressants FK506 and Cyclosporin A (Parent et al., 1993). Also in other studies this gene was discovered, which, when mutated caused resistance to 1,3-β-glucan synthase inhibitors (etg1; Douglas et al., 1994a), or displayed hypersensitivity to the cell wall perturbing compound calcofluor white (cwh53; Ram et al., 1994). Mutants of fks1 showed decreased in vitro 1,3-β-glucan synthase activity (Douglas et al., 1994b). The Fks1 protein was highly enriched in 1,3-β-glucan synthase purified by product entrapment (Inoue et al., 1995), and when immunoprecipitated, the synthase activity was copurified (Mazur et al., 1996). In S. cerevisiae, also a close homolog of FKS1 is present, named GSC2 or FKS2 (Inoue et al., 1995; Mazur et al., 1995). Since GSC2 was used in the first publication that described this gene, this name will be used here. According to their sequence, both FKS1 and GSC2 encode large proteins with 16 putative membrane-spanning domains (Douglas et al., 1994; Mazur et al., 1995).

Although deletion of either FKS1 or GSC2 is not lethal, cells in which both genes are deleted simultaneously are inviable (Inoue et al., 1995; Mazur et al., 1995). FKS1 and GSC2 are considered to encode alternative subunits of the 1,3-β-glucan synthase complex, since they are differentially expressed. FKS1 is expressed in vegetatively growing cells, cultured in rich glucose-containing medium, whereas GSC2 is expressed in medium without glucose. Expression of GSC2 in glucose-containing medium is induced by calcium or mating

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pheromones, in a calcineurin-dependent manner. FK506 inhibits the expression of \( GSC2 \), explaining the hypersensitivity of \( fks1 \) mutants for this compound (Mazur et al., 1995). Both Fks1 and Gsc2 are clearly essential components of 1,3-\( \beta \)-glucan synthase, and they are generally proposed to be the catalytic subunit of this complex. However, no unequivocal evidence has been presented yet to confirm this. Also, these proteins lack the nucleotide-glucose binding consensus site (K/R-X-G-G), as present in \textit{Escherichia coli} glycogen synthase (Furukawa et al., 1993), so it cannot be excluded that there are more - yet unknown - components of the 1,3-\( \beta \)-glucan synthase.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Schematic representation of Rho1 activating the 1,3-\( \beta \)-glucan synthase. Rho1 can be converted to the GTP-bound form, facilitated by Rom2. This active form can activate the 1,3-\( \beta \)-glucan synthase. The activity of the synthase can be down regulated by Lrg1.}
\end{figure}
4.6. More on the regulation of 1,3-β-glucan synthase

Small G-proteins have two forms: (1) an inactive form that binds GDP, and (2) a GTP-bound active form (Figure 3). Inactive GDP-bound G-proteins, upon receiving the correct stimulation, can be converted into the GTP-bound form. This process is facilitated by so-called guanine nucleotide exchange factors (GEFs), and is inhibited by GDP dissociation inhibitors (GDIs). Conversion of GTP to GDP occurs by the intrinsic GTPase activity of the G-protein, which is promoted by GTPase activating proteins (GAPs), because the default GTPase activity is very low (Matozaki et al., 2000).

In *S. cerevisiae* the activity of Rho1 is controlled by several GEFs and GAPs. With respect to the function of Rho1 in regulating 1,3-β-glucan synthesis, the Rho1 GEF encoded by *ROM2* (Ozaki et al., 1996) activates Rho1 upon cell wall alterations (Bickle et al., 1998) and stimulates 1,3-β-glucan synthase in the *fks1-1154 fks2Δ* mutant when overexpressed. This mutant has a temperature-sensitive defect in 1,3-β-glucan synthase activity (Sekiya-Kawasaki et al., 2002). Furthermore, cells deleted for *ROM2* show decreased incorporation of glucose into 1,3-β-glucan, a phenomenon which is also seen in cells deleted for *WSC1* (also known as *SLG1/HCS77*). The WSC family of genes is thought to encode membrane-located sensors to control cell integrity (Gray et al., 1997; Verna et al., 1997; Jacoby et al., 1998). Overexpression of *WSC1* or *WSC3* was found to stimulate 1,3-β-glucan synthase in the *fks1-1154 fks2Δ* mutant (Sekiya-Kawasaki et al., 2002). The Wsc1 protein is known to directly interact with Rom2 to convert Rho1 to the active GTP-bound form (Philip and Levin, 2001), so this signal cascade seems to provide a mechanism for regulation of 1,3-β-glucan synthesis. The GAP Lrg1 is a negative regulator of Rho1 activity in 1,3-β-glucan synthesis. When deleted, 1,3-β-glucan synthesis is restored in the *fks1-1154 fks2Δ* mutant. This is also seen in a *rho1-2* mutant in which *LRG1* has been deleted, indicating an inhibitory function for Lrg1 in 1,3-β-glucan synthase activity (Watanabe et al., 2001). Altogether, based on the currently available knowledge, 1,3-β-glucan synthase activity seems to be tightly regulated.

4.7. Localization of 1,3-β-glucan synthase

As a complex that manufactures a major cell wall component, one would expect the 1,3-β-glucan synthase to localize to sites of growth. Indeed, the synthase is present at the plasma membrane (Shematek et al., 1980). For Rho1 to activate the 1,3-β-glucan synthase, it must also localize to the plasma membrane. Small G-proteins are known to be prenylated, *i.e.* they are modified with a lipid moiety that promotes binding to the membrane (Zhang and Casey, 1996). Rho1 is also known to be prenylated, and this modification is critical for its capability to activate 1,3-β-glucan synthase (Inoue et al., 1999). Both Rho1 and Fks1 localize to the bud site at bud emergence, and to the bud tip of small buds. Subsequently, Rho1 and Fks1 disperse throughout the bud during isotropic growth of the bud, in order to finally repolarize to the bud-neck region before cell separation (Yamochi et al., 1994; Qadota et al.,
In conclusion, the dispersion of the Rho1 - Fks1 complex during the cell cycle coincides perfectly with the occurrence of cell wall synthesis in the growing bud.

5. 1,6-β-Glucan

5.1. 1,6-β-Glucan in the cell wall

1,6-β-Glucan isolated from S. cerevisiae cell walls is a highly branched, water-soluble polymer, which is reported to consist on average of 130 - 350 glucose residues (Manners et al., 1973; Kollár et al., 1997). It only accounts for 5 - 10 % of the cell wall dry weight (Manners et al., 1973), yet it plays a crucial role in the cell wall of S. cerevisiae and C. albicans. Mutations in genes involved in 1,6-β-glucan synthesis often result in cells with severely decreased viability (Meaden et al., 1990; Roemer et al., 1993; Brown and Bussey, 1993; Mio et al., 1997; Lussier et al., 1998; Sullivan et al., 1998; Vink et al., 2002, Chapter 2). At the molecular level, 1,6-β-glucan functions as a linker between cell wall proteins and 1,3-β-glucan (Kapteyn et al., 1996; Kollár et al., 1997). Under stress conditions, it might also be connected directly to chitin (Kollár et al., 1997; Kapteyn et al., 1997).

5.2. Identification of genes involved in 1,6-β-glucan synthesis

Genetic screens have been most valuable in identifying genes involved in 1,6-β-glucan synthesis. One approach exploited the cell wall perturbing compound calcofluor white to identify genes involved in cell wall biogenesis. Mutants with increased sensitivity to this compound were expected to have a weakened cell wall, and the corresponding mutations might therefore identify genes involved in cell wall synthesis. This approach resulted in the identification of a large number of genes involved in many aspects of cell wall synthesis (Ram et al., 1994; Lussier et al., 1997). A method for identifying genes more specifically involved in 1,6-β-glucan synthesis was developed by Bussey and co-workers, taking advantage of the fact that mutants with reduced levels of 1,6-β-glucan display a resistance to K1 killer toxin, which is a pore-forming protein that binds 1,6-β-glucan and kills cells (Al-Aidroos and Bussey, 1978; Boone et al., 1990; Bussey, 1991; Brown et al., 1993a; Pagé et al., 2003). These screens have resulted in a large number of genes either involved in cell wall biogenesis in general, or, in 1,6-β-glucan biogenesis in particular. These genes are named KRE (for Killer REsistant), CWH (for Calcofluor White Hypersensitive), or ECM (for ExtraCellular Matrix).

Since the synthesis of both chitin and 1,3-β-glucan takes place at the plasma membrane, it came as a surprise that the gene products involved in 1,6-β-glucan synthesis, identified in the genetic screens mentioned above, were localized along the secretory pathway. Although this might suggest that 1,6-β-glucan is synthesized throughout the secretory pathway, there are at least two other explanations for this observation: 1,6-β-glucan synthesis may take place at the plasma membrane, but it requires some intracellular key
events such as the synthesis of a primer, or, alternatively, the synthesis takes place at the plasma membrane, but this process is very sensitive to certain intracellular defects.

5.3. Endoplasmic Reticulum (ER)

The KRE5 gene encodes a large, soluble secretory glycoprotein with a carboxy-terminal HDEL sequence (Meaden et al., 1990), which is an ER-retention signal for soluble proteins. Deletion of KRE5 results in extremely compromised growth, and an aberrant cell wall (Meaden et al., 1990) which is entirely void of the electron dense outer layer (Simons et al., 1998; Levinson et al., 2002). Furthermore, kre5 mutants have a substantial defect in the incorporation of the GPI-CWP α-agglutinin (Lu et al., 1995), which might be a consequence of their lack of 1,6-β-glucan (Meaden et al., 1990). Also, the percentage of Pir-CWPs which are linked directly to 1,3-β-glucan is dramatically increased in the kre5Δ mutant. The transcript levels of PIR2 and other Pir-family members are upregulated, which probably is the result of a compensatory mechanism to ensure cell wall integrity under compromised conditions (Kapteyn et al., 1999). The Kre5 protein has weak, though significant similarity to UDP-glucose:glycoprotein glucosyltransferase (GT) of Drosophila (Parker et al., 1995) and S. pombe (Fernández et al., 1996). GT is part of the ER quality control mechanism for protein folding. Glycoproteins that are N-glycosylated receive a core oligosaccharide (Glc3Man9GlcNAc2) in the ER, of which the glucose residues subsequently are removed by glucosidase I and II. However, if a protein is not yet properly folded, GT attaches one glucose to the core which allows recognition of the protein by calnexin/calreticulin. This facilitates correct folding of the protein. The glucose can be removed again by glucosidase II, and the cycle may repeat itself until the protein is folded correctly (Parodi, 1999). However, there is evidence speaking against the idea that Kre5 has a GT-like function: (1) there is no detectable GT activity in S. cerevisiae (Fernández et al., 1994; Jakob et al., 1998), and (2) the essential function of Kre5 is not that of a GT, since KRE5 is essential in a mutant in which the requirement of a GT is bypassed (Shahinian et al., 1998). Although this can hardly be seen as conclusive evidence, an alternative possibility is that the function of Kre5 is related to that of a GT, but that it has diverged and is now involved in 1,6-β-glucan synthesis directly or indirectly. Nonetheless, it has recently been found that the C. albicans Kre5 homolog (CaKre5) can partially restore the kre5 mutant. Although it has not been tested for GT activity, it has a higher similarity to GT than Kre5 (Levinson et al., 2002).

Also other mutations in genes encoding components of the quality control mechanism were found to affect 1,6-β-glucan. These include CWH41/GLS1, ROT2/GLS2, and CNE1, which encode glucosidase I (Romero et al., 1997), glucosidase II (Trombetta et al., 1996), and the yeast homolog of mammalian calnexin/calreticulin (De Virgilio et al., 1993), respectively. The cwh41 mutant has lowered levels of 1,6-β-glucan, but this is not accompanied by a growth defect (Jiang et al., 1996; Shahinian et al., 1998). Also, the rot2 mutant shows a decrease in 1,6-β-glucan levels, and these cells display killer resistance
(Shahinian et al., 1998) just like the cwh41 mutant (Jiang et al., 1996; Shahinian et al., 1998). The CWH41 gene displays strong genetic interaction with KRE6 and KRE1, two genes that are involved in the biogenesis of 1,6-β-glucan (discussed below), as double mutants are inviable or show exacerbated phenotypes, respectively (Jiang et al., 1996). Interestingly, the strong genetic interactions with both genes disappeared upon deletion of ALG5 - encoding the dolichol-P-glucosyltransferase - which results in unglucosylated core N-chains. As this bypasses the requirement for glucosidase I activity, this indicates that indeed this function of Cwh41 is essential in the kre6 mutant. It was found that Kre6 is unstable in the absence of CWH41, suggesting that the effect of Cwh41 on 1,6-β-glucan synthesis is indirect (Abeijon and Chen, 1998).

Cells deleted for CNE1 show a moderate defect in 1,6-β-glucan synthesis (Shahinian et al., 1998), although this conclusion is contradicted by a study of Simons and co-workers, who saw no effect (1998). However, the outer electron dense layer of the cne1Δ mutant was reduced and the cell wall surface had an irregular shape (Simons et al., 1998). Cne1 appears to function in the quality control mechanism, as its deletion results in a marked protein folding defect, yet its function is not essential in S. cerevisiae (Parlati et al., 1995). Deletion of CNE1 enhanced the 1,6-β-glucan defects of kre6Δ, cwh41Δ, and rot2Δ (Shahinian et al., 1998). Another component of the quality control mechanism, BiP/Kar2, also exacerbated the 1,6-β-glucan defect of cwh41Δ and rot2Δ when deleted (Simons et al., 1998). This further demonstrates that defects in the quality control mechanism may result in general cell wall defects, amongst others by indirectly affecting 1,6-β-glucan.

5.4. Golgi

The KRE6 gene encodes a type II transmembrane glycoprotein located in the Golgi (Boone et al., 1990; Roemer and Bussey, 1991; Roemer et al., 1994). Deletion of this gene results in a growth defect, a significant reduction of 1,6-β-glucan content, and cell walls with an aberrant structure (Roemer and Bussey, 1991; Roemer et al., 1994). Furthermore, these cells are defective in the incorporation of α-agglutinin (Lu et al., 1995) and Cwp1 into the cell wall, yet they incorporate more Pir2 protein (Kapteyn et al., 1999). SKN1 was identified as a multi-copy suppressor of the kre6Δ mutant, and encodes a homolog of KRE6. Deletion of SKN1 did not result in a noticeable phenotype, but a kre6Δskn1Δ double mutant is hardly viable and displays a dramatic defect in 1,6-β-glucan synthesis (Roemer et al., 1993).

Hydrophobic cluster analysis showed that Kre6 and Skn1 have similarities to family 16 glycosylhydrolases, indicating that they function as glycosylases or transglycosylases. This seems to exclude a function as nucleotide sugar glucosyltransferase (Montijn et al., 1999, Chapter 3). The cytoplasmic tail of Kre6 can be phosphorylated, which might be a potential mode of regulation. The kre6Δ mutant is synthetic lethal with several components of the cell wall integrity Pkc1 MAP kinase cascade, and overexpression of the KRE6 gene
can suppress the lysis defect of the \(pkc1\Delta\) mutant (Roemer et al., 1994). This implicates a role for \(PKC1\) in whatever function Kre6/Skn1 may have.

One of the genes identified in the screen for K1 killer toxin resistant mutants, \(KRE2/MNT1\), appeared to encode a Golgi mannosyltransferase involved in \(O\)- and \(N\)-linked glycosylation (Hill et al., 1992; Hausler et al., 1992). It would be interesting to see what the underlying mechanism is of the \(KRE\) phenotype of the \(kre2\Delta\) mutant. One possibility might be that in this mutant Kre1 (discussed below) is not properly \(O\)-glycosylated, but there may be more proteins suffering from this defect.

5.5. Cytoplasm

Also \(KRE1\) was identified in the screen for K1 toxin resistant genes. It encodes a putative cytoplasmic protein. Deletion of this gene results in lowered 1,6-\(\beta\)-glucan levels (Brown et al., 1993). Kre11 was identified as a component of TRAPP (Sacher et al., 2000), a complex on the \(cis\)-Golgi that functions in docking and/or fusion of ER-to-Golgi transport vesicles (Sacher et al., 1998). The synthetic lethality of \(krel\Delta\) with \(kre6\Delta\) (Brown et al., 1993) might thus be explained by the incorrect delivery of Kre6 and Skn1 to the Golgi in a \(krelkrel\) background.

5.6. Plasma membrane

The \(KRE1\) gene is predicted to encode a heavily \(O\)-glycosylated GPI-anchored plasma membrane protein (Boone et al., 1991; Roemer and Bussey, 1995), and it was confirmed by immunofluorescence that Kre1 indeed localizes to the cell surface (Roemer and Bussey, 1995). Deletants for \(krel\Delta\) show an aberrant morphology of the cell wall, demonstrated by a disperse electron dense outer layer. These cells display a decrease in 1,6-\(\beta\)-glucan levels, mostly due to much shorter chains of 1,6-\(\beta\)-glucan (Boone et al., 1991). Recently, it was found that Kre1 is the receptor for the K1 killer toxin. The K1 toxin directly binds to the Kre1 protein and probably allows the subsequent and lethal formation of the ion channel. It was proposed that the other receptor for the K1 toxin, 1,6-\(\beta\)-glucan, functions to concentrate the toxin (Breining et al., 2002).

5.7. Extracellular space

\(KRE9\) encodes a 276-amino acid secretory protein, with a predicted molecular weight of 30 kDa. It has many potential \(O\)-glycosylation sites, many of which presumably are occupied since the protein has an apparent molecular weight of about 55 - 60 kDa on SDS-PAGE (Brown and Bussey, 1993). This might be another important target of the Kre2 mannosyltransferase. The \(kre9\Delta\) deletants display severely reduced 1,6-\(\beta\)-glucan levels, and the electron dense layer of their cell wall appears to be absent (Brown et al., 1991; Brown and Bussey, 1993). In addition, these mutants fail to form a mating projection upon \(\alpha\)-factor treatment and they are defective in mating (Brown and Bussey, 1993). The \(KNH1\) gene is a functional homolog of \(KRE9\), which when overexpressed can suppress the \(kre9\Delta\) mutation. Deletion of \(KNH1\) does not result in a clear phenotype, but in a \(kre9\Delta\) background this
deletion is lethal. Overexpression of KNHI is able to suppress the kre9Δ mutant. The expression of KNHI is upregulated when the cells are grown on galactose, explaining the partial suppression of the kre9Δ phenotypes by growth on galactose.

5.8. Localization of 1,6-β-glucan synthesis

Based on the above described genes - identified on the basis of their resistance to K1 killer toxin - it was thought for a long time that the synthesis of 1,6-β-glucan was a process that started intracellularly, and that the resulting polymer was extended while traveling through the secretory pathway. However, this was all based on genetic evidence, and no biochemical data is available to support this idea. Using immunogold labeling, Montijn and co-workers (1999, Chapter 3) showed that there was no detectable intracellular 1,6-β-glucan in a sec1-1 mutant grown at the restrictive temperature. This mutant accumulates post-Golgi secretory vesicles, and after 2 hours of growth at the restrictive temperature one would expect at least some 1,6-β-glucan to accumulate intracellularly if the hypothesis above was correct. Microsomes isolated from these cells were separated by gel filtration and analyzed for the presence of 1,6-β-glucan in different fractions. The 1,6-β-glucan co-eluted with the plasma membrane fraction, supporting the idea that the bulk of 1,6-β-glucan synthesis occurs at the plasma membrane (Montijn et al., 1999, Chapter 3).

5.9. Regulation of 1,6-β-glucan

Little is known about the regulation of 1,6-β-glucan synthesis. The original screens for K1 killer toxin resistant mutants did not result in the isolation of genes with potential regulatory functions. In contrast, a more general screen for genes involved in cell wall biogenesis - using calcofluor white - did yield potential regulatory genes with defects in 1,6-β-glucan synthesis (Ram et al., 1994; Jiang et al., 1995; Vink et al., 2002, Chapter 2). The PTC1/CWH47 gene encodes a protein phosphatase type 2C, and mutations in this gene resulted in resistance to the K1 killer toxin. Ptc1 appeared to have a negative regulatory function on the Pbs2-Hog1 pathway (Jiang et al., 1995), which later proved to be a direct dephosphorylation of Hog1 (Warmka et al., 2001). Accordingly, overexpression of the kinase of Hog1, PBS2, also results in K1 toxin resistance (Jiang et al., 1995), whereas the pbs2Δ mutant is hypersensitive (Jiang et al., 1995; Vink et al., 2002, Chapter 2). This clearly indicates that 1,6-β-glucan synthesis is inhibited by the Pbs2-Hog1 MAP kinase pathway which is otherwise known to control the response to high osmolarity (reviewed in Hohmann, 2002). The mechanism of how the Pbs2-Hog1 pathway affects 1,6-β-glucan synthesis is not clear.

KIC1 encodes a protein kinase that was identified as a two-hybrid interactor with Cdc31, revealing a novel function for this yeast centrin homolog in the control of cell wall integrity (Sullivan et al., 1998). A mutation in this gene was identified in a screen for calcofluor white hypersensitivity (Ram et al., 1994; Vink et al., 2002, Chapter 2). Recently, Vink and co-workers (2002, Chapter 2) found that kic1 mutant cells displayed K1 killer toxin
resistance, which was accompanied by a defect in 1,6-β-glucan. Reciprocally, overexpression of \textit{KICl} resulted in hypersensitivity to the Kl toxin and a slight increase in 1,6-β-glucan levels. The Kic1 gene was therefore proposed to participate in the regulation of 1,6-β-glucan synthesis. Interestingly, a multicopy suppressor of the \textit{kic1} calcofluor white hypersensitivity was isolated that shared some of the \textit{kic1} phenotypes. This was the \textit{RHO3} gene, that, when deleted, also resulted in a higher degree of resistance to the K1 toxin, concomitant with reduced 1,6-β-glucan levels. Overexpression of \textit{RHO3} resulted in K1 toxin hypersensitivity (Vink et al., 2002, Chapter 2). The \textit{RHO3} gene encodes a Rho-type small G-protein that also is involved in bud growth, in the actin cytoskeleton, and in exocytosis (Matsui and Toh-E, 1992b; Imai et al., 1996; Robinson et al., 1999; Adamo et al., 1999). In the absence of \textit{RHO3}, overexpression of \textit{RHO4} can suppress the mutant, and the \textit{rho3Δ rho4Δ} double mutant is inviable. This suggests that \textit{RHO4} is functionally related to \textit{RHO3} (Matsui and Toh-E, 1992a). However, \textit{RHO4} does not share the regulating function of \textit{RHO3} in cell wall synthesis (Vink et al., 2002, Chapter 2). It is tempting to speculate that the function of Rho3 in 1,6-β-glucan synthesis is analogous to that of Rho1 in 1,3-β-glucan synthesis, \textit{i.e.} in the direct regulation of the 1,6-β-glucan synthase itself. However, evidence for this is lacking since 1,6-β-glucan synthase has not yet been identified.

\textbf{5.10. Biochemical assay for 1,6-β-glucan}

As yet, no genes have been identified as potential candidates encoding for proteins that are (part of) the putative plasma membrane 1,6-β-glucan synthase complex. How can this important enzyme have been overlooked, whereas (at least some of the) components of both chitin- and 1,3-β-glucan synthase have been isolated? One major help in the identification of chitin- and 1,3-β-glucan synthase components has been the availability of biochemical assays to measure these activities (Shematek et al., 1980; Kang et al., 1984). In this thesis a method is described for measuring the activity of 1,6-β-glucan synthesis, making use of antibodies for the detection of 1,6-β-glucan rather than expecting it to behave as 1,3-β-glucan (Chapter 4). This might be key in the development of an assay for 1,6-β-glucan synthesis activity, since the reaction product appeared to be water-soluble in contrast to the products of chitin- and 1,3-β-glucan assays. As this method uses a membrane extract as starting material, this implicates that there is at least some activity associated with membranes. Further work is needed to optimize this approach, but it is expected to become an important tool to identify components of the 1,6-β-glucan synthase complex.

\textbf{6. References}

\textbf{Adamo J.E., Rossi G., and Brennwald P. (1999).} The Rho GTPase Rho3 has a direct role in exocytosis that is distinct from its role in actin polarity. \textit{Mol. Biol. Cell} 10: 4121-4133


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Shematek E.M., and Cabib E. (1980). Biosynthesis of the yeast cell wall. II. Regulation of beta-(1 leads to 3)glucan synthetase by ATP and GTP. *J. Biol. Chem.* 255: 895-902


Toh-e A., Yasunaga S., Nisogi H., Tanaka K., Oguchi T., and Matsui Y. (1993). Three yeast genes, PIR1, PIR2 and PIR3, containing internal tandem repeats, are related to each other, and PIR1 and PIR2 are required for tolerance to heat shock. Yeast 9: 481-494


Trombetta E.S., Simons J.F., and Helenius A. (1996). Endoplasmic reticulum glucosidase II is composed of a catalytic subunit, conserved from yeast to mammals, and a tightly bound noncatalytic HDEL-containing subunit. J. Biol. Chem. 271: 27509-27516


