1,6-beta-glucan synthesis in Saccharomyces cerevisiae

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Chapter 5

General Discussion
Chapter 5

1. Introduction

The cell wall of baker's yeast has a bilayered structure and is composed of four components: 1,3-β-glucan and chitin, which form the inner layer of the cell wall and represent the skeletal network, and mannoproteins which are mainly linked through 1,6-β-glucan to the skeletal network, and which form the outer layer of the cell wall (reviewed by Klis, 1994, Orlean, 1997, and Klis et al., 2002). The mannoproteins and glucans are present in about equal amounts, whereas chitin represents only a minor percentage of the cell wall (1 – 2%). About 80% of the glucan is represented by 1,3-β-glucan, and the remaining 20% is 1,6-β-glucan. In the cell wall of Saccharomyces cerevisiae, 1,6-β-glucan occurs as a highly branched, water-soluble polymer consisting of about 130 residues (Manners et al., 1973).

Roughly six major research questions can be posed with regard to the biogenesis of 1,6-β-glucan: (i) is 1,6-β-glucan synthesis a single- or a multistep process? (ii) at which location are these steps performed? (iii) which enzymes are involved? (iv) does the primary synthetic step involve a single protein or multiple proteins? (v) how is 1,6-β-glucan assembled into the cell wall? (vi) how is 1,6-β-glucan synthesis regulated? It is remarkable how little is known about the biosynthesis of 1,6-β-glucan and its regulation in comparison with the other cell wall components. When this work was started, our knowledge on this subject was mainly based on a genetic screen developed by Bussey and co-workers that exploits the K1 killer toxin (reviewed by Shahinian and Bussey, 2000). As a rule, the sensitivity of cells for this toxin is correlated with their cell wall 1,6-β-glucan levels: cells with lowered levels of 1,6-β-glucan are more resistant to this toxin, and vice versa. Based on the identification of a relatively small number of genes by this screen, it was hypothesized that 1,6-β-glucan synthesis is a stepwise process that starts in the ER, continues along the secretory pathway, and is completed at the cell surface (reviewed by Klis, 1994, and Orlean, 1997). As depicted in figure 1, some of the KRE genes identified in the killer toxin screen allegedly encoded glucan synthases. Kre5 was believed to initiate the synthesis of 1,6-β-glucan in the endoplasmic reticulum (ER), either as a free molecule or attached to precursor forms of cell wall proteins. Extension of 1,6-β-glucan was believed to occur by a related pair of Golgi-located enzymes (Kre6 and Skn1). Finally, maturation steps were believed to take place at the cell surface, and involved Kre1 and Kre9. As the original screen appeared to be exhaustive, it was assumed that most of the important genes in 1,6-β-glucan biogenesis had been identified. Recently, a genome-wide screen was carried out based on K1 killer toxin sensitivity (Pagé et al., 2003). In this study 268 genes were identified with a toxin phenotype, of which 186 displayed increased resistance and 82 hypersensitivity. The genes that were identified in this screen coded for proteins from many different functional classes, such as signaling, transcription, secretion, protein modification, and ribosomal subunits. Unfortunately, no obvious candidate genes were found and/or proposed to encode subunits of the 1,6-β-glucan synthase, although it might be difficult to identify such a gene without
precisely knowing the diagnostic features of the predicted protein. Moreover, although heterozygous mutant diploids for these genes were used for analysis, essential genes are easily missed in this screen, because not all essential genes display a so-called haploinsufficiency phenotype when one copy is disrupted in a diploid.

In the following paragraphs, the contributions of this thesis to solving some of the questions on 1,6-β-glucan synthesis are discussed.

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

1,6-β-Glucan synthesis has long been presumed to start in the ER, and to be continued along the rest of the secretory pathway (reviewed in Klis, 1994, and Orlean, 1997). However, as extensively discussed in chapter 3 thus far there is no significant evidence for the presence of intracellular 1,6-β-glucan. Rather, the 1,6-β-glucan seems to be associated with the plasma membrane and the cell wall, suggesting that the major synthase activity is located in the plasma membrane (Figure 2). This was shown by using a temperature-sensitive secretory mutant, which was blocked in exocytosis when grown at the restrictive temperature. Secretory vesicles were allowed to accumulate for the duration of roughly one cell cycle. After fractionation of the cell organelles, the bulk of 1,6-β-glucan appeared to co-localize with the plasma membrane-derived fractions. If its synthesis was to take place intracellularly, one would expect that the intracellular 1,6-β-glucan content would be about equivalent to the amount of 1,6-β-glucan produced by the cells during a generation time. The same secretory mutant grown at the restrictive temperature was also used for immunogold labeling, showing that 1,6-β-glucan was only present at the cell surface and in the cell wall. In addition, using other secretory mutants blocked at various stages of the secretory pathway to allow accumulation of precursor forms of the cell wall protein α-agglutinin, the various precursor forms were easily identifiable, but they never became attached to 1,6-β-glucan, although the cell wall form of α-agglutinin is known to be 1,6-β-glucan-bound (Lu et al., 1995). Together these observations strongly suggest that the bulk of 1,6-β-glucan is synthesized at the plasma membrane (Figure 2). These data contradict the earlier ideas about the biogenesis of 1,6-β-glucan as presented in figure 1. In particular, the idea that Kre5 and Kre6/Skn1 are involved in the initiation and elongation of 1,6-β-glucan chains has become untenable. In the next section, we will discuss alternative functions for these proteins in the biogenesis of 1,6-β-glucan.

3. Role of ER and Golgi proteins in 1,6-β-glucan synthesis

As stated before, a number of genes have been proposed mainly based on genetic evidence to function in 1,6-β-glucan synthesis. As intracellular synthesis of 1,6-β-glucan seems unlikely, the roles of Kre5 and Kre6/Skn1 have to be reinterpreted. The KRE5 gene encodes an ER-localized soluble glycoprotein with a signal sequence and the C-terminal ER-
retention signal HDEL (Meaden et al., 1990; Levinson et al., 2002). Its deletion severely compromises growth in *S. cerevisiae* (Meaden et al., 1990; Shahinian et al., 1998; Levinson et al., 2002). The Kre5 protein has significant homology with UDP-glucose: glycoprotein glucosyltransferase (GT), an enzyme that is involved in quality control of proteins in the ER. However, this enzyme activity has not been detected in *S. cerevisiae* (Fernández et al., 1994; Jakob et al., 1998). The Kre5 function might thus be related to the GT function, yet might have diverged from the quality control of protein folding to a function in 1,6-β-glucan synthesis. For example, Kre5 may be involved in the production of an acceptor structure or a primer structure that is extended by the 1,6-β-glucan synthase (Figure 2). This primer might be a glucosylated protein, as is the case for glycogen synthesis where the self-glucosylating protein glycogenin functions as a primer (Alonso et al., 1995). Another option is that this primer is a glucosylated sterol. It was recently found that cellulose synthesis in plants requires sitosterol-β-glucoside as a primer (Peng et al., 2002). Sterol glucosides have also been identified in *S. cerevisiae*, which are produced by the UDP-glucose: sterol glucosyltransferase encoded by *UGT51*. However, cells deleted for the sterol glucosyltransferase lack sterol glucosides yet exhibit normal growth under several conditions (Warnecke et al., 1999), and therefore it is unlikely that a glucosylated sterol functions as a primer for 1,6-β-glucan synthesis. It has also been proposed that Kre5 may glucosylate the GPI-anchor of precursor forms of GPI-CWPs, a process that might be key in the later attachment of 1,6-β-glucan (Shahinian and Bussey, 2000). However, it is unlikely that these modifications occur on precursor forms of GPI-CWPs, as the assembly of the 1,3-β-glucan-1,6-β-glucan complex in the cell wall does not seem to depend on GPI-CWP incorporation (Roh et al., 2002). Interestingly, there is import of UDP-glucose in the ER of *S. cerevisiae* (Castro et al., 1999). However, up to this point no UDP-glucose dependent reaction in the ER lumen of *S. cerevisiae* has been identified. This nucleotide sugar might thus be a candidate sugar donor for Kre5. Cells deleted for *BIG1*, a gene that encodes an ER transmembrane protein, also show a dramatic reduction in 1,6-β-glucan levels. Although its function is unclear, it is supposed to play a role together with Kre5 in a common pathway, yet with partially distinct functions. This could be the modification of a precursor, or the assembly of a functional synthase complex (Azuma et al., 2002). If indeed Kre5 is involved in synthesizing an acceptor structure, it seems possible that cells deleted for *KRE5* would still be able to produce 1,6-β-glucan, yet the incorporation of 1,6-β-glucan in the cell wall would then be disturbed. It would thus be interesting to test the culture medium of these cells for the presence of 1,6-β-glucan.
General Discussion

Figure 1. Previous model for 1,6-β-glucan synthesis in *S. cerevisiae* (adapted from Orlean, 1997). The order of events was based on localization of the gene products, and on epistatic analysis of the genes. In the ER, 1,6-β-glucan synthesis was presumed to be initiated by Kre5. In the Golgi, Kre6/Skn1 were believed to be responsible...
for the extension of 1,6-β-glucan. Finally, at the cell surface the maturation of 1,6-β-glucan was presumably established by Kre1 and Kre9, although during their passage through the secretory pathway these proteins might already have carried out (part of) their function.

Using hydrophobic cluster analysis (HCA), we found that the Golgi-localized Kre6 protein and its close homolog Skn1 share significant homology to family 16 glycoside hydrolases, which indicates that they function as glycoside hydrolases or transglycosidases (Chapter 3). This excludes that, in contrast to what was postulated before (Figure 1), Kre6 and Skn1 function as glucosyltransferases, which, unlike transglycosidases, utilize activated sugars. The action of Kre6 might be to modify a glucose-containing acceptor structure associated with GPI-anchors in the ER or to other such structures (Figure 2).

4. Development of an assay for 1,6-β-glucan synthase activity

Although the genetic screens using the K1 killer toxin have been of great value to the study of cell wall biogenesis, this genetic approach by itself seems to be exhausted. Up to now, no potential candidate genes that encode (components of) the 1,6-β-glucan synthase complex have been identified. In particular, the lack of a biochemical assay to measure 1,6-β-glucan synthase activity has hampered the use of reverse genetics to identify components of this enzyme activity. For this reason, we decided to develop a biochemical assay for in vitro 1,6-β-glucan synthase activity. In principle, purification of the activity in combination with currently available techniques such as mass spectrometry could provide a simple means to identify the components of the synthase. A similar approach has helped to identify the catalytic subunit of the 1,3-β-glucan synthase complex (Inoue et al., 1995). Chapter 4 describes the development of a method for measuring 1,6-β-glucan synthase activity in vitro. The reaction products of a 1,6-β-glucan synthase assay were analyzed in two ways: by a qualitative dot blot assay, and by a quantitative enzyme immunoassay, both using affinity-purified antibodies raised against 1,6-β-glucan. The optimal pH was 6.5 for the in vitro 1,6-β-glucan synthase activity. The structure of the reaction products is unknown, so the question if the 1,6-β-glucan synthase produces linear polymers of 1,6-β-glucan or perhaps a more branched product remains unanswered. If sufficient amounts of reaction product could be produced in vitro, the structure could be resolved using NMR. For example, the 1,3-β-glucan synthase produces linear 1,3-β-glucan polymers of 60 - 80 residues long (Shematek et al., 1980). It would also be interesting to know what the chain lengths are of the 1,6-β-glucan synthase products. These reaction products appear to be water-soluble, in contrast to e.g. the 1,3-β-glucan synthase products and the commercially available 1,6-β-glucan pustulan (with an average chain length of about 110 residues). This suggests that the 1,6-β-glucan synthase produces either relatively short oligomers which are subsequently remodeled, or highly branched polymers. Both hypotheses are in agreement with the fact that 1,6-β-glucan in the
cell wall of *S. cerevisiae* occurs as a highly branched, water-soluble polymer consisting of about 130 residues (Manners et al., 1973).

Another important question is if the 1,6-β-glucan synthase can produce 1,6-β-glucan polymers *de novo*, or if it needs a primer structure to which the synthase can transfer 1,6-β-linked glucose residues. This could be investigated by analyzing the terminal reducing glucose, which, if UDP-[¹⁴C]-glucose would be used as substrate, would contain this ¹⁴C-label. This can be done by reduction by sodium borohydride followed by acid hydrolysis. The liberated sorbitol (derived from the terminal reducing glucose) can subsequently be analyzed for the presence of the ¹⁴C-label.

The *in vitro* assay for 1,6-β-glucan synthase activity, as described in Chapter 4, displays a relatively low activity compared to for example the *in vitro* 1,3-β-glucan synthase activity. This might very well be due to non-optimal assay conditions. An interesting possibility is that the putative primer structure is present at a limiting concentration and does not allow the synthase to function at maximal speed. Currently, our *in vitro* 1,6-β-glucan synthase assay is being used and further developed by Drs Manon Gérard-Vincent and Howard Bussey from the McGill University in Montreal, Canada.

Since the 1,6-β-glucan synthase itself has not yet been identified, it is also unknown if the synthase is made up of one or more subunits. The 1,3-β-glucan synthase consists of at least two components: the putative catalytic subunit encoded by *FKS1*, which is a multipass transmembrane protein, and the cytoplasmic regulatory subunit, a small G-protein encoded by *RHO1* (reviewed in Cabib et al., 1998). Although Fks1 lacks the conserved UDP-glucose binding site (reviewed in Klis et al., 2002), it was recently shown that the UDP-glucose analog 5-azido-[beta-³²P]-UDP-glucose binds to the *Neurospora crassa* Fks1 homolog (Schimoler-O’Rourke et al., 2003). This strongly suggests that Fks1 is indeed the substrate-binding subunit of the 1,3-β-glucan synthase. One may presume that the 1,6-β-glucan synthase also requires a multipass transmembrane domain, since such a domain probably facilitates transmembrane transport of the product as for example postulated in the case of the putative 1,3-α-glucan synthase Ags1 in *S. pombe* (Hochstenbach et al., 1998). On the other hand, the 1,6-β-glucan synthase might well consist of multiple proteins, which means that the various functions are not necessarily restricted to one protein. If the 1,6-β-glucan synthase has a function analogous to that of 1,3-β-glucan synthase, it would probably also use UDP-glucose as a substrate. The UDP-glucose analog 5-azido-[beta-³²P]-UDP-glucose may then be exploited for the identification of components of the 1,6-β-glucan synthase as well. When the *in vitro* assay for 1,6-β-glucan synthesis is further refined so that it can be routinely used, a purification strategy might be used after which UDP-glucose binding proteins can be labeled with this substrate analog. Subsequently, mass spectrometry could be used to identify these proteins. Interestingly, Frost and co-workers labeled yeast microsomal fractions with 5-azido-
UDP-glucose and identified several potential UDP-glucose binding proteins (1992). These have not been further investigated, but this observation merits re-investigation.

5. Assembly of 1,6-\(\beta\)-glucan in the cell wall

The speculative assembly of 1,6-\(\beta\)-glucan in the cell wall presumably consists of three enzymatic steps: (1) elongation and remodeling of linear 1,6-\(\beta\)-glucan chains to form mature cell wall 1,6-\(\beta\)-glucan polymers, (2) the attachment of the 1,6-\(\beta\)-glucan polymers to 1,3-\(\beta\)-glucan, and (3) the attachment of GPI-CWPs to the 1,6-\(\beta\)-glucan. It was proposed by Roh and co-workers (2002) that the order of assembly of the cell wall components is as follows: 1,3-\(\beta\)-glucan is produced first, followed by the covalent addition of 1,6-\(\beta\)-glucan to 1,3-\(\beta\)-glucan, and finally the mannoproteins are linked to 1,6-\(\beta\)-glucan. Chitin then is the last component to be added to the complex. Interestingly, mutations in Fks1, putatively encoding the catalytic subunit of 1,3-\(\beta\)-glucan synthase, also affect 1,6-\(\beta\)-glucan levels. This might be caused by a decrease in 1,3-\(\beta\)-glucan levels, and thus decreased acceptor levels for attachment of 1,6-\(\beta\)-glucan at the cell surface (Dijkgraaf et al., 2002).

None of the enzymatic activities for covalently connecting 1,6-\(\beta\)-glucan to other macromolecules in the wall has been identified, yet some known genes might play a role in these steps. The Kre1 O-glycoprotein is probably associated with the plasma membrane through a GPI-anchor, and could play a role in the elongation and remodeling of 1,6-\(\beta\)-glucan chains since the average length of the 1,6-\(\beta\)-glucan polymers in the mutant strain was reduced to about 50% of the wild type. The total amount of 1,6-\(\beta\)-glucan was reduced by 40% (Boone et al., 1990; Roemer and Bussey, 1995). Kre1 might thus be involved in the remodeling of freshly synthesized 1,6-\(\beta\)-glucan polymers resulting in more branched chains (Figure 2).

The Kre9 O-glycoprotein and its homolog Knh1 are soluble secretory proteins that also influence 1,6-\(\beta\)-glucan levels (Brown and Bussey, 1993; Dijkgraaf et al., 1996). The 1,6-\(\beta\)-glucan in the cell wall of kre9\(\Delta\) cells had an altered structure, and the levels were reduced to 10 - 20% of the wild type level (Brown and Bussey, 1993). While knh1\(\Delta\) mutants did not show defects in 1,6-\(\beta\)-glucan synthesis, overexpression of KNH1 in kre9\(\Delta\) mutants almost completely restored the 1,6-\(\beta\)-glucan levels. Moreover, deletion of both KRE9 and KNH1 was lethal (Dijkgraaf et al., 1996). This suggests a role for Kre9 and Knh1 in remodeling and incorporating the 1,6-\(\beta\)-glucan (Figure 2).

118
Figure 2. Current model for 1,6-β-glucan synthesis. Kre5 initiates the synthesis of a primer structure in the ER. Kre6/Skn1 are responsible for the maturation of this primer, indicated by an asterisk. At the cell surface, the actual 1,6-β-glucan is synthesized using the primer. Subsequently, 1,6-β-glucan is remodeled by Kre1, and Kre9/Knh1 are involved in the coupling of 1,6-β-glucan to other cell wall macromolecules. Rho3 may directly regulate the activity of the synthase. Kic1 and Pbs2 – Hog1 play opposite roles in controlling 1,6-β-glucan levels, probably in an indirect way.

6. Regulation of 1,6-β-glucan synthesis

The original screen for mutants that are resistant to the K1 killer toxin, did not identify potential regulatory proteins involved in controlling the levels of 1,6-β-glucan. However, another genetic screen which was devised to identify genes involved in cell wall biogenesis in general and which exploits the observation that cell wall mutants are often
hypersensitive to calcofluor white, did result in the identification of genes encoding proteins involved in regulating 1,6-β-glucan levels. The corresponding mutants were not only hypersensitive to calcofluor white, but also displayed resistance to the K1 killer toxin (Ram et al., 1994). Among these genes were PTC1, a protein phosphatase type 2C that is directly involved in the regulation of the Pbs2-Hog1 pathway (see below) (Jiang et al., 1995; Warmka et al., 2001), and KIC1, encoding a serine/threonine protein kinase that is involved in cell wall integrity (Sullivan et al., 1998; Chapter 2). Studies on both these genes have shown that the Pbs2-Hog1 pathway, a MAP kinase pathway that regulates adaptation to high osmolarity (reviewed in Hohmann, 2002), also appears to play a role in the regulation of 1,6-β-glucan synthesis (Jiang et al., 1995; Chapter 2). The precise mechanism of how the Pbs2-Hog1 pathway influences 1,6-β-glucan levels is unclear. The Pbs2-Hog1 pathway possibly plays an indirect role in 1,6-β-glucan synthesis by influencing the expression of genes that modulate 1,6-β-glucan levels in the wall. Yet, it is also conceivable that the Pbs2-Hog1 pathway is more directly involved in 1,6-β-glucan synthesis, for example by regulating the expression of genes encoding subunits of the synthase.

The protein kinase Kic1 also appears to play a role in regulating 1,6-β-glucan synthesis. Deletion of the KIC1 gene results in K1 killer toxin resistance and a decrease in 1,6-β-glucan levels in the wall. Reciprocally, overexpression of the KIC1 gene results in hypersensitivity to the killer toxin and an increase in 1,6-β-glucan levels (Chapter 2). Kic1 interacts with and is activated by Cdc31, the yeast centrin, demonstrating a novel function of Cdc31 in cell wall integrity (Sullivan et al., 1998; Ivanovska and Rose, 2001). As there are no known targets of the Kic1 kinase, it is unclear by which mechanism Kic1 is able regulate 1,6-β-glucan levels. One way to identify downstream targets of a kinase is by using a multicopy suppressor screen, where cells mutated in the gene of interest are transformed with a genomic library and screened for suppression of an associated phenotype of the mutation. Using this technique, the small G-protein encoding RHO3 was identified. Small G-proteins are regulated by a set of specific proteins, such as GTPase activating proteins (GAPs), guanine nucleotide exchange factors (GEFs), and GDP dissociating inhibitors (GDIs) (reviewed in Matozaki, 2000). As Kic1 does not belong to any of these classes of proteins, it is unlikely that Kic1 directly interacts with Rho3. It might be possible that Kic1 modulates the activity of the above-mentioned G-protein regulatory proteins, but there is no evidence for this as a yeast two-hybrid screen using Kic1 as bait did not result in the identification of such G-protein regulators (Ito et al., 2001). This two-hybrid screen did not yield interactors that clarify the role of Kic1 in cell wall biogenesis, but recently it was established in a large scale analysis of protein complexes by mass spectrometry that Kic1 complexes with Slt2 (Ho et al., 2002). The Slt2 protein is the MAP kinase of the PKC1 cell wall integrity pathway (reviewed in Heinisch et al., 1999), and therefore represents a link to cell wall biogenesis. However, it must be noted that mutants of slt2 do not share the kic1 defects in 1,6-β-glucan synthesis.
(Pagé et al., 2003; Chapter 2). As pkcl mutants display K1 killer toxin hypersensitivity, which is not shared by components of the downstream MAPK pathway (Pagé et al., 2003), this suggests that the interaction of Kic1 with Slt2 is either unrelated to 1,6-β-glucan synthesis or causes feedback to Pkcl. Perhaps Kic1 regulates 1,6-β-glucan synthesis through one (or more) of its two-hybrid interactors with yet unknown functions (Ito et al., 2001). In this model, Kic1 would probably function in parallel of Rho3. Interestingly, Kic1 and the Pbs2-Hog1 pathway seem to have counteracting effects on 1,6-β-glucan levels.

The exact role of the small G-protein Rho3 in 1,6-β-glucan biogenesis is also unknown, but one may predict that Rho3 has a role in 1,6-β-glucan synthesis, which is similar to the activating role of Rho1 in 1,3-β-glucan synthesis (Figure 2). Although this is an appealing scheme, another possibility is that the stimulatory role of Rho3 in exocytosis (Adamo et al., 1999; Robinson et al., 1999) contributes to 1,6-β-glucan synthesis, perhaps through the correct delivery of 1,6-β-glucan synthetic components. The most obvious location for the 1,6-β-glucan synthase is at the sites of growth, as is the case for the 1,3-β-glucan synthase (Yamochi et al., 1994; Qadota et al., 1996). Interestingly, Rho3 localizes to the bud tip of cells with a small bud (Robinson et al., 1999), a place where active cell growth occurs. However, this does not distinguish between a direct and an indirect role in 1,6-β-glucan synthesis. Finally, one could speculate that the 1,6-β-glucan synthase is co-localized with the 1,3-β-glucan synthase, which probably would facilitate the coordination of the synthesis of both cell wall components. Also, this would simplify the delivery of the different biosynthetic components to the site(s) of growth.

7. Proposed model for 1,6-β-glucan synthesis

Considering the data presented in this thesis and those described in the literature, we would like to propose the following model for 1,6-β-glucan synthesis (Figure 2): Kre5 is involved in the production of a primer structure in the ER. Although more proteins are probably involved (discussed above), these are omitted for clarity. The proposed primer structure is further processed by Kre6/Skn1 in the Golgi. The mature primer can then function as an acceptor for the 1,6-β-glucan synthase in the plasma membrane. We propose that the small G-protein Rho3 is a regulatory subunit of the 1,6-β-glucan synthase complex. The protein kinase Kic1 controls the levels of 1,6-β-glucan, counteracting the Pbs2-Hog1 pathway. Its influence may be indirect, for example through transcriptional regulation. The 1,6-β-glucan synthase then produces 1,6-β-glucan precursor chains for cell wall assembly, possibly followed by the removal of the primer structure. The assembly of 1,6-β-glucan in the cell wall may involve enzymatic activities for elongation and remodeling, and the attachment to 1,3-β-glucan. At the cell surface, Kre1 and Kre9/Knh1 are probably involved in these processes. The KRE1 gene is not essential, and the average chain length of 1,6-β-glucan is reduced by 50% in krelΔ mutants. This suggests that Kre1 is involved in the elongation and
remodeling of 1,6-β-glucan. On the other hand, the *KRE9/KNH1* gene pair is essential suggesting a role in the incorporation of 1,6-β-glucan in the cell wall.

8. Perspectives

The study of 1,6-β-glucan synthesis was initiated by using a genetic approach, *i.e.* the K1 killer toxin resistance screen (reviewed by Shahinian and Bussey, 2000). This screen was further extended by a genome-wide screen for altered killer toxin sensitivity (Pagé *et al.*, 2003). Although many genes were identified that influence 1,6-β-glucan levels, this approach far from solved the many questions about 1,6-β-glucan synthesis. Probably, this has to do with the limitations of using a single genetic approach. In this thesis, 1,6-β-glucan synthesis was shown to take place mainly at the cell surface, excluding the existence of an intracellular biosynthetic machinery for 1,6-β-glucan. We have further shown that Kic1 and Pbs2-Hog1 have an opposite role in controlling 1,6-β-glucan levels. Also, we propose that the small G-protein Rho3 might play a direct role in activating the 1,6-β-glucan synthase. Finally, an *in vitro* biochemical assay for the detection of 1,6-β-glucan synthase activity has been developed. This assay will contribute to finding an answer to many of the questions posed in the beginning of this chapter, such as (i) if 1,6-β-glucan synthesis is a single- or a multistep process. This can be done by analysis of the terminal reducing glucose to determine if it is derived from UDP-[14C]-glucose. The identification of the catalytic subunit of the 1,6-β-glucan synthase is crucial for the further study of the various aspects of 1,6-β-glucan synthesis, as has been the case for 1,3-β-glucan synthesis. Using the 1,6-β-glucan synthase activity assay, the purification of the activity could lead to the identification of the synthase components (questions iii & iv). When these components are known, it is relatively simple to study their exact subcellular localizations (question ii), for example by the addition of a GFP-tag or by immunofluorescence. The assay itself might also give further insight in how 1,6-β-glucan is synthesized, by detailed analysis of the structures of the reaction products. In addition, knowing the structures of the 1,6-β-glucan synthase reaction products could be of value in the understanding of the remodeling and incorporation reactions, since the substrates for these enzymes would then be identified (question v). Finally, the identification of the catalytic subunit of the 1,6-β-glucan synthase will allow the analysis of its regulation on the transcriptional level, and the discovery of proteins interacting with this subunit. For example, interacting proteins might be involved in the regulation of the synthase or its correct localization (question vi).
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