Molecular Microbiology (2000) **35**(3), 477–489

MicroReview

β-1,6-Glucan synthesis in *Saccharomyces cerevisiae*

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Summary

β-1,6-Glucan is an essential fungal-specific component of the *Saccharomyces cerevisiae* cell wall that interconnects all other wall components into a lattice. Considerable biochemical and genetic effort has been directed at the identification and characterization of the steps involved in its biosynthesis. Structural studies show that the polymer plays a central role in wall structure, attaching mannoproteins via their glycosylphosphatidylinositol (GPI) glycan remnant to β-1,3-glucan and chitin. Genetic approaches have identified genes that upon disruption result in β-1,6-glucan defects of varying severity, often with reduced growth or lethality. These gene products have been localized throughout the secretory pathway and at the cell surface, suggesting a possible biosynthetic route. Current structural and genetic data have therefore allowed the development of models to predict biosynthetic events. Based on knowledge of β-1,3-glucan and chitin synthesis, it is likely that the bulk of β-1,6-glucan polymer synthesis occurs at the cell surface, but requires key prior intracellular events. However, the activity of most of the identified gene products remain unknown, making it unclear to what extent and how directly they contribute to the synthesis of this polymer. With the recent availability of new tools, reagents and methods (including genomics), the field is poised for a convergence of biochemical and genetic methods to identify and characterize the biochemical steps in the synthesis of this polymer.

Introduction

Carbohydrate polymers are essential components of many organisms, including plants, bacteria and fungi. Such essential function is invariably provided in the form of the basic building block(s) of the cell wall, giving plants their structural strength, and protecting bacterial and yeast cells from lysis as a result of their internal osmotic pressure, as well as providing many essential cell-surface functions. Although such polymers have long been identified and the subject of considerable research, in many cases the details of their biosynthesis remains poorly understood. Consider cellulose, a β-1,4-glucan found in bacteria, plants and fungi (for reviews, see Carpita and Vergara, 1998; Delmer, 1999). Although the relevant genes had been identified in bacteria, accompanied by the subsequent demonstration of *in vitro* activity (for a review, see Ross *et al*., 1991), screens using this information were unable to identify similar components in plants. Putative cellulose synthase genes have since been identified in cotton using random sequence data (Pear *et al*., 1996) and in Arabidopsis via gene mapping (Arioli *et al*., 1998), however the inability to reconstitute plant cellulose synthesis *in vitro* limits elucidation of the biochemical steps of this process.

The yeast *Saccharomyces cerevisiae* has been extensively studied, having a wall containing mannoproteins, β-1,3-glucan, β-1,6-glucan and chitin (Klis, 1994; Lipke and Ovalle, 1998; Kapteyn *et al*., 1999a). Two main approaches have been used: (i) structural and biochemical analyses of cell wall structure and composition and (ii) genetic methods, which are well developed in this organism. Further, the recent availability of its complete genome sequence has led to additional gene identification, and the current era of yeast functional genomics promises to accelerate this discovery process. As a result, many genes have been identified that are required for various aspects of cell wall polymer synthesis, and the mature polymer structures allow the construction of models to predict how these gene products may function. However, despite these developments, the exact biochemical mechanisms remain unknown. This is especially true for β-1,6-glucan, which plays a critical role in wall structure by interconnecting all other components (Kollár *et al*., 1997; Kapteyn *et al*., 1999a). To date, several genes have been identified that affect its synthesis, but the biochemical activity of only two of these gene products, the ER N-chain glucosidases Cwh41p and Rot2p, are known, and their activities are unlikely to directly participate in polymer biosynthesis (Shahinian *et al*., 1998). In

Received 26 August, 1999; revised 25 October, 1999; accepted 27 October, 1999.
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addition, a plasma membrane 'synthase' protein of the types identified in β-1,3-glucan and chitin synthesis has not been found. Here, we examine our present knowledge of β-1,6-glucan synthesis, and re-evaluate current models and predictions.

Structural characterization of cell wall β-1,6 glucan

Analysis of *S. cerevisiae* cell wall β-1,6-glucan has revealed a highly branched polymer comprising about 10% of the total cell wall glucan (Manners *et al*., 1973), with an average size of 350 glucose residues (Kollár *et al*., 1997). The polymer has been subjected to further structural study, primarily by the work of Klis and co-workers (University of Amsterdam), and mostly with respect to its role in the covalent attachment of mannoproteins to the cell wall (see Kapteyn *et al*., 1999a). An early comprehensive biochemical study of cell wall protein carbohydrate side-chains revealed that their *O*-chains contained mannose, and their *N*-chains were found to contain mannose (*N*-acetyl)glucosamine and some glucose (van Rinsum *et al*., 1991). This latter observation is intriguing as it provides evidence for the attachment of cell wall glucan to protein *N*-chains, which will be discussed further below. Most importantly, a third type of protein-attached carbohydrate was found containing mannose (*N*-acetyl)glucosamine and most of the protein-attached glucose, thus representing the primary mode of attachment of these proteins to cell wall glucan. This third fraction is now thought to be derived from a carboxy-terminal glycosylphosphatidylinositol (GPI) moiety.

Attachment to the glycosylphosphatidylinositol (GPI) moiety

GPI modification in *S. cerevisiae* entails the post-translational attachment of an ethanolamine-phosphate-Man_5GlcNH_2-inositol phospholipid to the C-termini of appropriate acceptor proteins, simultaneously displacing the last ≈ 20–30 amino acids, which contain the consensus for GPI attachment (for a review, see Orlean, 1997). The resulting GPI-protein remains membrane anchored by virtue of this lipid modification, and proceeds through the secretory pathway to the cell surface (for a review, see Orlean, 1997). Early results directly implicating GPI modification with wall attachment were obtained via the study of the cell-surface protein α-agglutinin, when it was determined that this protein is indeed GPI modified and that this modification is essential for cell-surface attachment (Wojciechowicz *et al*., 1993), which has been shown by electron microscopy to be specifically localized to the outer fibrillar layer of the wall (Cappellaro *et al*., 1994). This was supported by the study of fusion proteins containing a reporter enzyme fused to either the C-terminal half (Schreuder *et al*., 1993; van Berkel *et al*., 1994) or even the last 30 amino acids (van Berkel *et al*., 1994) of α-agglutinin (containing the GPI consensus), demonstrating that these regions were essential and sufficient to covalently attach a heterologous protein to the wall. A key technological development in this field was the preparation of anti-β-1,6-glucan polyclonal antibodies, which were shown to immunostain discrete bands in a laminarinase-released cell-wall fraction in a highly specific manner (Montijn *et al*., 1994). Such staining was shown not to be derived from *O*- or *N*-chains (Montijn *et al*., 1994) and was removed by treatment with either aqueous HF or phosphodiesterase, which cleave the GPI from the C-termini of proteins (Kapteyn *et al*., 1996). Similar anti-β-1,6-glucan immunodetection was demonstrated for the α-agglutinin fusions described above, thus implicating both the GPI moiety and β-1,6-glucan in the attachment of proteins to the cell wall (van Berkel *et al*., 1994).

Studies of the model wall protein α-agglutinin have shown that it is GPI-, *N*- and *O*-modified as it passes through the secretory pathway, resulting in a large (> 300 kDa) cell-surface form (Lu *et al*., 1994). Mature cell-surface α-agglutinin may be further subdivided into a GPI-anchored plasma membrane-associated form, a soluble (presumably) periplasmic form and a cell wall-attached form, with the interesting observation that the periplasmic and cell wall forms have lost the inositol phospholipid (Lu *et al*., 1994) and only the wall form is attached to β-1,6-glucan (Lu *et al*., 1995). These results suggested that plasma membrane α-agglutinin becomes attached to cell wall β-1,6-glucan with the concomitant loss of the inositol phospholipid anchor. Subsequent studies have identified and characterized other similar GPI-dependent/β-1,6-glucan-attached wall proteins, including Cwp1p and Cwp2p (van der Vaart *et al*., 1995), as well as fusions containing the C-termini of these proteins (van der Vaart *et al*., 1996a; Ram *et al*., 1998). Similarly, introduction of a protease site just N-terminal to the GPI attachment site was shown to enable proteolytic removal of the GPI moiety and with it β-1,6-glucan (van der Vaart *et al*., 1996b). It therefore appeared that β-1,6-glucan serves to attach at least some mannoproteins to the cell wall via their GPI moiety. Similar experiments have identified β-1,6-glucan-anchored cell wall proteins in other fungi such as *Candida albicans* (Kapteyn *et al*., 1994, 1995a,b), *Exophilia (Wangiella) dermatidis* (Montijn *et al*., 1997) as well as filamentous fungi involved in food spoilage (Brul *et al*., 1997).

A recent biochemical and analytical study has greatly advanced our understanding of cell wall ultrastructure and the role of β-1,6-glucan by demonstrating that β-1,6-glucan acts as the central core of the wall protein–carbohydrate network, interconnecting chitin and β-1,3-glucan, and attaching wall mannoproteins to these constituents via a remnant of the C-terminal GPI moiety (Fig. 1), literally

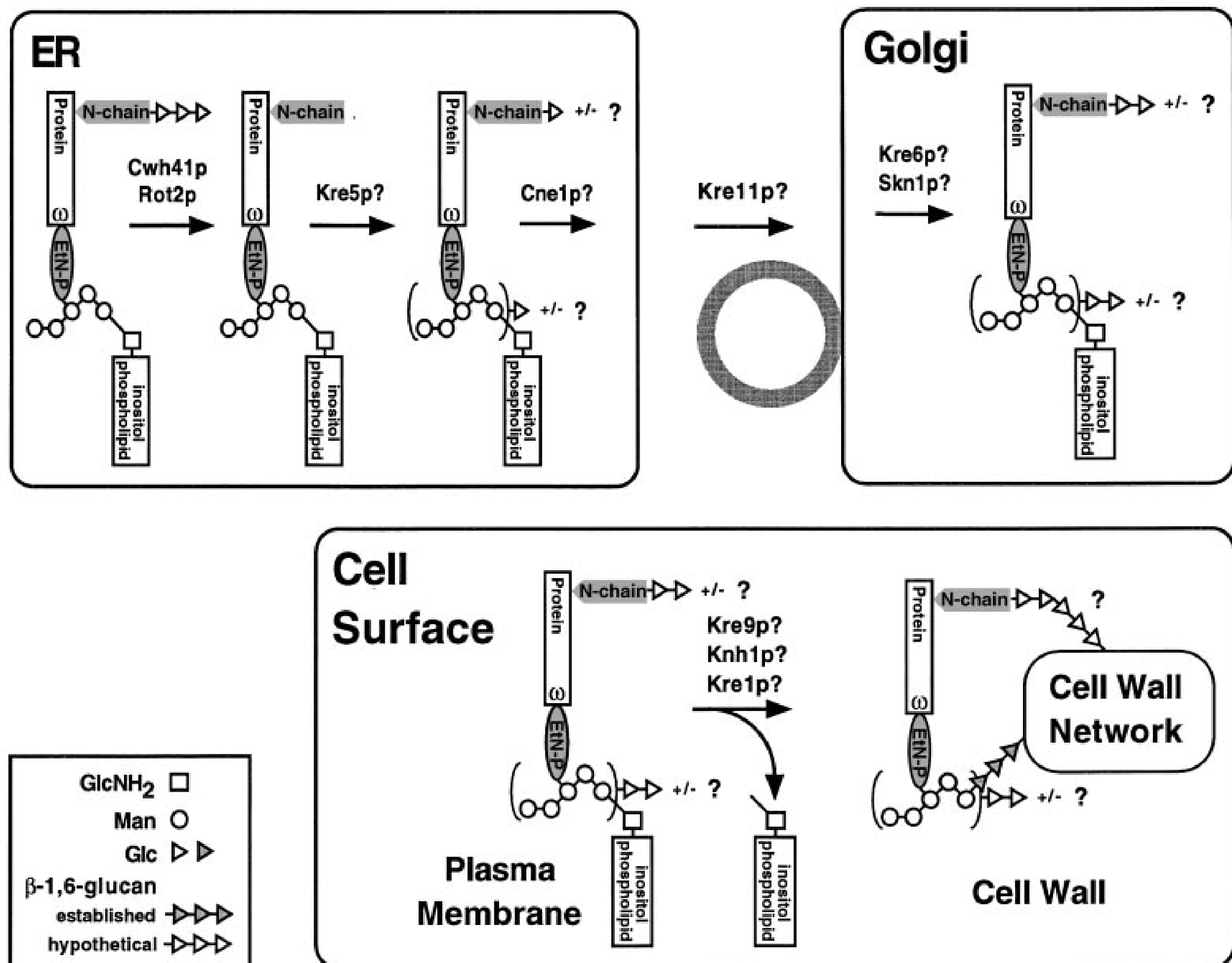

Fig. 1. Schematic model of the putative biosynthetic steps involved in the β-1,6-glucan attachment of a GPI-cell wall protein in *Saccharomyces cerevisiae.* $GlcNH_2$, glucosamine; Man, mannose; Glc, glucose, EtN-P, phosphoethanolamine.

acting as the 'glue' holding this network together (Kollár *et al.*, 1997). It was further shown that GPI-glucan attachment involves the loss of the GPI glucosamine, in addition to the inositol phospholipid noted above (Fig. 1; Kollár *et al.*, 1997). Similar results were obtained via the study of the attachment point of the cell wall protein Tip1p, which was found to be consistent with a GPI structure lacking glucosamine and the inositol phospholipid (Fujii *et al.*, 1999). In addition, it was determined that no mannose is present at the reducing end, suggesting that this is the site of the β-1,6-glucan attachment (Fig. 1; Fujii *et al.*, 1999).

A few studies have addressed the important question of what directs some GPI-proteins to β-1,6-glucan/cell wall attachment while others remain lipid anchored in the plasma membrane. Via the alignment of the C-terminal regions of all predicted yeast GPI-proteins combined with predictions based on known cell wall (for example α-agglutinin; Lu *et al.*, 1994) and plasma membrane (for example Gas1p; see Popolo and Vai, 1999) GPI proteins, it has been determined that cell wall GPI-proteins lack a dibasic motif just N-terminal to the GPI attachment (ω) site (Caro *et al.*, 1997) and possess certain amino acid requirements at the ω-5 and ω-2 positions (Hamada *et al.*, 1998a). Thus, it has been demonstrated that the dibasic motif acts as a negative signal while the ω-5 and ω-2 requirements act as a positive signal for wall attachment (Hamada *et al.*, 1999). These sequence requirements were confirmed experimentally by the conversion of the plasma membrane GPI-protein Yap3p to a cell wall-attached version (Hamada *et al.*, 1998b).

The point of cell wall protein attachment therefore appears to be to via a remnant of the GPI moiety to cell wall β-1,6-glucan, which in turn interconnects other wall components. Such a model is in agreement with the severe phenotypes associated with mutants defective in either β-1,6-glucan synthesis (see *Genetics* section) or GPI modification (reviewed in Orlean, 1997). However, the exact type of linkage involved remains unknown.

Table 1. Genes involved in β-1,6-glucan biosynthesis.

Gene	Basis of identification	Properties of protein	% β-1,6-glucan of null mutant (relative to wild type)	Functional remarks	References
KRE5	K1 toxin res.	ER*, soluble* protein	0	Homologous to UGGT; glucosyltransferase?	Boone *et al.* (1990) Meaden *et al.* (1990) Shahinian *et al.* (1998)
CWH41/*GLS1*	Calcofluor white hypersensitive	ER, N-glycosylated, membrane protein	50	Glucosidase I enzyme	Ram *et al.* (1994) Jiang *et al.* (1996) Romero *et al.* (1997) Shahinian *et al.* (1998)
ROT2/*GLS2*	Mammalian glucosidase II homologue; reversal of *tor2*	ER*, soluble* protein	55	Glucosidase II enzyme	Trombetta *et al.* (1996) Bickle *et al.* (1998) Shahinian *et al.* (1998)
CNE1	Mammalian calnexin/calreticulin homologue	ER, N-glycosylated, membrane protein	70	Protein folding/quality control?	Parlati *et al.* (1995a) Shahinian *et al.* (1998)
KRE6	K1 toxin res.	Golgi, *N*-glycosylated, phosphorylated, membrane protein	50	Homologous to glucan binding proteins and glucanases; expression is cell cycle controlled; glucanase?	Roemer *et al.* (1993) Roemer and Bussey (1991) Roemer *et al.* (1994) Igual *et al.* (1996) Spellman *et al.* (1998)
SKN1	MCS of *kre6Δ*	As *KRE6*	100 (SL in *kre6Δ*)	As *KRE6*	Roemer *et al.* (1993) Roemer *et al.* (1994) Spellman *et al.* (1998)
KRE11	K1 toxin res.	Cytoplasmic*, soluble* protein	50	Component of TRAPP complex; ER-Golgi transport?	Brown *et al.* (1993a) Sacher and Ferro-Novick, personal communication
KRE9	K1 toxin res.	Cell surface (periplasmic/wall), *O*-glycosylated, soluble protein	20	Carbon source-dependent growth, suppressed by two-component regulator *SKN7*	Brown and Bussey (1993) Brown *et al.* (1993b)
KNH1	Homologue of *KRE9*	As *KRE9*	100 (SL in *kre9*Δ)	Carbon source-dependent expression, transcription increased in *kre9*Δ	Dijkgraaf *et al.* (1996)
KRE1	K1 toxin res.	Plasma membrane, *O*-glycosylated, GPI-protein*	60	Cross-linker?	Boone et al. (1990) Roemer and Bussey (1995)

*Predicted. MCS, multicopy suppressor; SL, synthetically lethal.

Attachment to N*-glycosyl chains*

Although not extensively studied, some evidence exists supporting the attachment of β-1,6-glucan to protein *N*-glycosyl chains. An early study utilizing chromatographic analyses of *in vivo* synthesized and radiolabelled cell wall material identified a fraction of glucan originally attached to protein *N*-chains (Tkacz, 1984). As noted above, a similar analytical study of wall material identified a minor proportion of (cell wall glucan derived) glucose copurifying with PNGaseF-released *N*-chains (van Rinsum *et al*., 1991). Similarly, in the study of Kollár *et al*. (1997), a small amount of reducing end label, perhaps derived from cell wall glucan, co-purified with the PNGaseF- or EndoH-released *N*-chain fraction. Finally, alteration of *N*-chain structure via genetic means has been shown to affect cell wall β-1,6-glucan levels (Shahinian *et al*., 1998), an observation discussed in the *Genetics* section. Taken together, these results suggest the attachment of cell wall β-1,6-glucan to protein *N*-chains either directly or via an unknown linker structure. However, as not all cell wall GPI-proteins are necessarily *N*-glycosylated, and mutations affecting this mode of attachment do not result in significant growth phenotypes or defects in cell wall protein anchorage (Shahinian *et al*., 1998), it appears to be a secondary non-essential attachment point of GPI-proteins to cell wall β-1,6-glucan. This linkage may only be demonstrated via detailed structural analyses, as those utilized in the elucidation of GPI-β-1,6-glucan attachment.

Genetic studies: gene products involved in β-1,6-glucan synthesis

Although structural studies have made a significant contribution to the elucidation of the cell wall organization of β-1,6-glucan, genetic methods have identified most of the genes known to be involved in its biosynthesis. Two approaches have been especially useful, one based on resistance to the K1 killer toxin (Al-Aidroos and Bussey, 1978; Boone *et al*., 1990; Brown *et al*., 1993a) and the other on hypersensitivity to the drug calcofluor white (Ram *et al*., 1994; Lussier *et al*., 1997). The former screen is more directed at identifying mutants with defects in β-1,6-glucan synthesis because the K1 killer toxin is a pore-forming protein that binds to a β-1,6-glucan-associated component in the yeast cell wall and subsequently kills yeast, thus cells with lower amounts of this polymer exhibit a degree of resistance to the toxin (for a review, see Bussey, 1991). Calcofluor white hypersensitivity enables the identification of mutants containing a broader range of cell wall defects because virtually any mutant with a weaker/compromised cell wall will, when given the drug (which binds nascent chitin microfibrils and halts their assembly), fail to grow at a drug concentration lower than that tolerated by the wild type (Elorza *et al*., 1983; Murgui *et al*., 1985; Ram *et al*., 1994; Lussier *et al*., 1997). Such screens have led to the identification of a number of *KRE* (killer toxin resistant), *CWH* (calcofluor white hypersensitive) and *ECM* (extracellular mutant) genes, whose gene products have been localized along the secretory pathway and at the cell surface, suggesting that multiple intracellular and cell-surface events are required for the proper biosynthesis of this polymer.

Endoplasmic reticulum (ER)

The *KRE5*, *CWH41/GLS1*, *ROT2/GLS2* and *CNE1* genes encode ER proteins that have been shown to be involved in β-1,6-glucan synthesis to varying extents (see Table 1). Loss of Kre5p results in no detectable β-1,6-glucan and extremely slow growth (Meaden *et al*., 1990), or inviability in some strains such as SEY6210 (Shahinian *et al*., 1998). Disruptants appear as large multiple-budded cells (Meaden *et al*., 1990) with an aberrant cell wall which completely lacks the electron-dense outer mannoprotein layer, and have a significant accumulation of ER membranous and vacuolar structures within the cell (Simons *et al*., 1998). Disruptants are severely defective in the anchorage of the cell wall protein α-agglutinin (Lu *et al*., 1995) and possess higher than wild-type amounts of cell wall β-1,3-glucan (S. Shahinian, unpublished) as well as increased levels of proteins anchored to the wall via β-1,3-glucan rather than β-1,6-glucan, such as Pir2p (Kapteyn *et al*., 1999b). The biochemical activity of Kre5p is unknown, but the protein has limited, yet significant, similarity with UDP-glucose:glycoprotein glucosyltransferase (UGGT) enzymes identified in Drosophila (Parker *et al*., 1995) and *S. pombe*. (Fernández *et al*., 1996). UGGT catalyses the attachment of a glucose residue to the *N*-glycosyl chains of misfolded proteins in the ER, enabling the binding of the chaperone calnexin that facilitates another round of protein folding (for reviews, see Helenius *et al*., 1997; Parodi, 1999). However, no detectable UGGT activity exists in *S. cerevisiae* (Fernández *et al*., 1994; Jakob *et al*., 1998), and the essential activity of Kre5p is not that of a UGGT because disruption of *KRE5* remains lethal in a genetic background with constitutively monoglucosylated *N*-chains in which UGGT activity would not be required (Shahinian *et al*., 1998). Given the similarity with UGGT and the linkage of cell wall β-1,6-glucan to the GPI remnant of cell wall proteins, it has been suggested that Kre5p may be a glucosyltransferase which may glucosylate the GPI moiety of cell wall proteins in the ER (Shahinian *et al*., 1998). A precedent for such modification exists because a glucose-containing GPI structure has been observed in the GPI anchor of acetylcholinesterase from *Torpedo californica* (Mehlert *et al*., 1993). Also consistent with this hypothesis is the

observation that *S. cerevisiae* has the ability to transport into the ER UDP-glucose (Castro *et al*., 1999), the likely substrate of Kre5p. UDP-glucose is thought to be the donor not only because of its use by UGGT enzymes (reviewed in Parodi, 1999) but also from the observation that a loss of ER dolichyl-phosphate-glucose (synthesized by Alg5p; te Heesen *et al*., 1994) is not required for β-1,6-glucan synthesis as an *alg5Δ* mutation has no effect on this process (Shahinian *et al*., 1998).

It is not trivial, however, to reconcile genetic predictions with the structural data described above, which have shown that the GPI moiety in the cell wall contains five mannose residues (probably GPI derived) and up to several glucose residues (cell wall β-1,6-glucan derived), with the mannose reducing end blocked (see Fig. 1). As this reducing end was originally the attachment point for the glucosamine-inositol phospholipid which is only lost at the cell surface, such single-point attachment dictates that coupling to β-1,6-glucan may only occur once this reducing end is available, i.e. at the cell surface. Therefore, prior glucosylation of the GPI-glycan at the mannosyl reducing end cannot occur, and thus cannot be performed by Kre5p in the ER (or by other intracellular components such as Kre6p; see below). In addition, structural analysis of bulk purified lipid-anchored intracellular GPI structures in *S. cerevisiae* failed to detect any attached glucose residues (Fankhauser *et al*., 1993). However, in the case of the last observation, it may be argued that such bulk purification may not detect wall-destined GPI because it is in transit and may only represent a small proportion of total GPI. Such issues have raised the question as to the exact cellular location of β-1,6-glucan polymer synthesis. The existence of plasma membrane synthase enzymes for β-1,3-glucan and chitin synthesis (reviewed in Orlean, 1997), coupled with the fact that β-1,6-glucan has yet to be immunodetected intracellularly (F. Klis, personal communication), suggests that this polymer is also synthesized at the cell surface. However, a similar cell-surface β-1,6-glucan synthase has not been found, and the inability to detect the polymer does not rule out that it may be present intracellularly at a smaller size and/or very low amounts which hamper immunodetection. Indeed, the localization of various *KRE* and *CWH* gene products along the secretory pathway combined with the severity of some of the phenotypes observed upon their loss provides evidence for intracellular biosynthetic steps. The true mechanism probably encompasses both of the above, perhaps involving the preparation of a proper acceptor-type structure in the ER and Golgi, which is attached to the larger polymer at the cell surface. Assuming Kre5p is a GPI-glucosyltransferase required to initiate a unique 'wall-targeting' process, it may glucosylate the GPI mannosyl core at another residue, perhaps at the fourth or fifth mannoses which are unique to yeast and absent from the mammalian structure (Englund, 1993; Fankhauser *et al*., 1993). Subsequently, this glucose may either be removed or elaborated by a later β-1,6-glucan biosynthetic component such as the Golgi protein Kre6p (discussed further below). To date, however, no direct structural evidence exists in support of a Kre5p glucosyltransferase activity.

A final possibility to account for Kre5p activity is that its involvement in β-1,6-glucan biosynthesis is indirect. Interestingly, *kre5Δ* cells share certain phenotypes with the *mcd4-174* mutant, which also secretes cell wall mannoproteins into the medium and exhibits an intracellular accumulation of ER/vesicular membranes (Gaynor *et al*., 1999). Mcd4p is required for ER-to-Golgi transport of GPI-proteins and is thought to be a GPI-biosynthetic component, possibly for phosphoethanolamine addition (Gaynor *et al*., 1999). It is possible therefore that Kre5p may play a similar role which indirectly contributes to β-1,6-glucan synthesis, given the importance of GPI modification in this process. Similarly, Kre5p could also exert its effects indirectly via such processes as protein folding and secretion. Although much study remains to distinguish between the various models proposed above, it is clear that Kre5p performs an early essential step in this process, which results in the attachment of proteins to cell wall β-1,6-glucan.

The *CWH41/GLS1* and *ROT2/GLS2* genes encode the ER glucosidase I (Romero *et al*., 1997) and glucosidase II (Trombetta *et al*., 1996) enzymes respectively (see Table 1). These enzymes are responsible for the trimming of the three *N*-chain glucose residues following transfer of *N*-chains to protein asparagine residues (reviewed in Orlean, 1997). *cwh41Δ* disruptants have a β-1,6-glucan defect yet no detectable growth or wall protein anchorage phenotype (Jiang *et al*., 1996; Shahinian *et al*., 1998). However, its disruption is lethal in a *kre6Δ* background and exacerbates both the growth and β-1,6-glucan defects of *kre1Δ* cells, accompanied by a defect in the anchorage of the cell wall protein Cwp1p (Jiang *et al*., 1996). Such genetic interactions imply that *CWH41* operates in a pathway of β-1,6-glucan synthesis that differs from that involving *KRE6* or *KRE1*, which may be *N*-chain dependent rather than GPI dependent (see below). The *ROT2/GLS2* gene was first identified based on its homology to the alpha subunit of the mammalian glucosidase II enzyme (Trombetta *et al*., 1996) and later identified via the ability of the *rot2* mutant to suppress the loss of the phosphatidylinositol kinase homologue Tor2p, an effect also demonstrated with other cell wall mutants (Bickle *et al*., 1998). As with *cwh41Δ* disruptants, *rot2Δ* cells have a partial β-1,6-glucan defect and exhibit killer toxin resistance (Shahinian *et al*., 1998), yet have no growth (Trombetta *et al*., 1996) or wall protein anchorage defects (Shahinian *et al*., 1998). As the biochemical activities of Cwh41p and Rot2p are known, their role(s) in β-1,6-glucan synthesis have been investigated and found to be limited to their

glucosidase activity (Shahinian *et al.*, 1998). Specifically, it is simply the failure to remove these three *N*-chain glucose structures which results in a β-1,6-glucan defect. Such results, combined with previous structural evidence, suggest that β-1,6-glucan may be attached to protein *N*-chains and that the persistent presence of the glucose residues may sterically hinder such attachment, resulting in the observed defect. An alternative mechanism has recently been proposed, suggesting that Cwh41p is required for the stability of the Golgi β-1,6-glucan biosynthetic protein Kre6p (see below) and implying that the observed β-1,6-glucan defect of *cwh41Δ* cells is due to a resulting lack of Kre6p (Abeijon and Chen, 1998). However, *cwh41Δ* cells do not possess the slow growth phenotype (Roemer and Bussey, 1991) or defective α-agglutinin cell wall anchorage (Lu *et al.*, 1995; Shahinian *et al.*, 1998) typical of *kre6* mutants. In addition, the lethality of *cwh41Δkre6Δ* cells noted above suggests that Cwh41p has a role in β-1,6-glucan independent of Kre6p, perhaps more *N*-chain dependent for the former and GPI dependent for the latter. Finally, the inability of a *cwh41Δ* disruption to cause a β-1,6-glucan defect in non-glucosylated *N*-chain strains such as *alg5Δ* (Abeijon and Chen, 1998; Shahinian *et al.*, 1998) and the observed inherent instability of Kre6p even under wild-type conditions (H. Bussey, unpublished) make such a mechanism unlikely, and strongly suggest that the observed β-1,6-glucan defect is manifested only by the absence of the glucose trimming activity, supporting the steric hindrance model of β-1,6-glucan-*N*-chain attachment. Direct structural evidence of β-1,6-glucan attachment to *N*-chains will, however, be required to validate such a model.

The *CNE1* gene encodes the *S. cerevisiae* homologue of mammalian calnexin/calreticulin proteins (see Table 1; Parlati *et al.*, 1995a). Based on this homology, *cne1Δ* cells were examined for the presence of a strong protein folding defect, but a significant phenotype was not apparent (Parlati *et al.*, 1995a). It has recently been shown that *cne1Δ* cells have a mild β-1,6-glucan defect, yet do not have a growth phenotype nor any defects in the cell wall anchorage of α-agglutinin (Shahinian *et al.*, 1998). However, *CNE1* disruption exacerbated the β-1,6-glucan defect of *kre6Δ*, *cwh41Δ* or *gls2Δ* cells, suggesting a broad, perhaps unique, role in this process. Several observations suggest that Cne1p does not function as a classic calnexin: the absence of a significant folding phenotype in *cne1Δ* cells, its inability to bind calcium as its mammalian counterparts (Parlati *et al.*, 1995a), the fact that it is not essential unlike its *cnx1*$^{+}$ homologue in *Schizosacchromyces pombe* (Parlati *et al.*, 1995b), and the inability to restore the β-1,6-glucan defect of *cne1Δ* cells via the heterologous expression of the dog or *S. pombe* calnexin proteins (Shahinian *et al.*, 1998). However, the similar ability of *kar2* mutants, which lack the major *S. cerevisiae* folding chaperone Kar2p/BiP, to exacerbate the β-1,6-glucan defect of *cwh41Δ* or *rot2Δ* cells (Simons *et al.*, 1998) supports a quality control role for Cne1p. Therefore, the nature of Cne1p activity in β-1,6-glucan synthesis is unclear. Its lack of growth or α-agglutinin anchorage phenotypes and its genetic interactions with the ER *N*-chain-dependent components *CWH41* and *GLS2* coupled with its ability to exacerbate the phenotype of *kre6Δ* cells implies a more *N*-chain-dependent role in β-1,6-glucan synthesis, consistent with the *N*-chain involvement of calnexin proteins elsewhere. However, the mild nature of the observed β-1,6-glucan defect of *cne1Δ* cells does not point to a strong direct involvement in its synthesis.

An interesting observation regarding the ER components implicated in β-1,6-glucan biosynthesis is that their homologues in other systems are involved in the ER quality control of protein folding; specifically, the *N*-chain glucosidases I (Cwh41p) and II (Rot2p), UGGT (Kre5p homologue) and calnexin (Cne1p homologue; Helenius *et al.*, 1997; Parodi, 1999). Although Cwh41p and Rot2p retain the same biochemical activities as their counterparts, Kre5p and Cne1p, the critical components of this system, apparently do not. Although evidence exists for a monoglucosylated *N*-chain-dependent quality control system in *S. cerevisiae* (Jakob *et al.*, 1998), it appears not to utilize a UGGT/calnexin-mediated mechanism, suggesting that this organism directs these activities to β-1,6-glucan synthesis.

Golgi

Located in the Golgi is the membrane glycoprotein Kre6p (Boone *et al.*, 1990; Roemer and Bussey, 1991; Roemer *et al.*, 1994) and its homologue Skn1p (see Table 1; Roemer *et al.*, 1993; 1994). *kre6Δ* disruptants are defective in growth and cell wall β-1,6-glucan, and tend to clump and have a large aberrant morphology (Roemer and Bussey, 1991) with a cell wall having an amorphous glucan layer and lacking a dense mannoprotein layer (Roemer *et al.*, 1994). They are also defective in the anchorage of cell wall α-agglutinin (Lu *et al.*, 1995; Shahinian *et al.*, 1998), but have increased levels of cell wall β-1,3-glucan (Shahinian *et al.*, 1998) and proteins attached exclusively to the wall via β-1,3-glucan, such as Pir2p (Kapteyn *et al.*, 1999b). Kre6p has been shown to be phosphorylated and to interact genetically with components of the Pkc1p-mediated MAP kinase pathway (Roemer *et al.*, 1994), suggesting that it may account for a degree of regulation of β-1,6-glucan synthesis. In addition, both *KRE6* and *SKN1* (see below) gene expression exhibits some periodicity during the cell cycle, with peaks of expression during G_1/S and M phases respectively (Igual *et al.*, 1996; Spellman *et al.*, 1998). Such differential expression

is consistent with the potential for regulation via the cytoplasmic domains of these proteins because this is the area of least homology between Kre6p and Skn1p (Roemer *et al*., 1993) and is supported by their phosphorylation and the presence of consensus sequences for various kinases (Roemer *et al*., 1994). Homologues of both *KRE6* and *SKN1* occur in *Candida albicans* and are involved in β-1,6-glucan synthesis and exhibit differential expression, however in this case changes in expression are related to the yeast and hyphal phases of this organism (Mio *et al*., 1997). Data base searches have identified several other proteins that contain regions with some similarity to *KRE6*, namely the *Rhodothermus marinus* β-1,3-glucanase (Spilliaert *et al*., 1994), horseshoe crab β-1,3-glucan-sensitive clotting factor (Seki *et al*., 1994), *Pyrococcus furiosus* endo-β-1,3-glucanase precursor (accession no. AF013169), *Clostridium thermocellum* endo-1,3(4)-β-glucanase (accession no. X89732), and laminarinases from *Thermotoga maritima* (accession no. AE001690) and *Thermotoga neapolitana* (Zverlov *et al*., 1997). The fact that all but one of these homologues are glucanases is intriguing because it suggests that Kre6p may actually perform a processing rather than an elaboration function. This would invoke a model of transient glucosylation with addition in the ER by Kre5p and removal in the Golgi by Kre6p (see Fig. 1), both of these steps being required for proper cell wall targeting. Indeed, transient modification has been discussed with respect to phosphoethanolamine modification of the GPI core glycan (Sütterlin *et al*., 1998; Gaynor *et al*., 1999). Such transient glucosylation would also correlate with the current difficulty in detecting glucosylated forms intracellularly. However, it should be emphasized that such a model is purely speculative, and an elaboration function or an entirely indirect role for Kre6p (as discussed for Kre5p) may be equally possible but must be tested experimentally. Interestingly, *KRE6* also has a dominant negative effect as its overexpression in wild-type cells results in very mild killer toxin resistance (Roemer and Bussey, 1991). Taken together, these results suggest that Kre6p is responsible for an intermediate step in β-1,6-glucan synthesis and provide a possible input for regulatory control into this process.

SKN1 is essentially a *KRE6* homologue, encoding a protein structurally similar to Kre6p (see Table 1; Roemer *et al*., 1993; 1994). Although single *skn1Δ* disruptants have a wild-type appearance and possess neither a growth nor a β-1,6-glucan phenotype, disruption of *SKN1* in a *kre6Δ* background results in extremely slow growth or lethality and a severe defect in β-1,6-glucan (Roemer *et al*., 1993). Because of the sequence and protein structural similarity of *SKN1* with *KRE6*, and its genetic interactions with *kre6Δ* cells, it appears that Skn1p is simply a functional homologue of Kre6p. Its differential cell cycle expression, however, suggests that it may also possess a unique role, which, like *KRE6*, is subject to regulation.

Cytoplasm

KRE11 encodes a predicted cytoplasmic protein (see Table 1; Brown *et al*., 1993a), which when absent results in defects in β-1,6-glucan (Brown *et al*., 1993a) and the cell wall anchorage of α-agglutinin (Lu *et al*., 1995). The epistasis of *kre11Δ* to the cell-surface mutant *kre1Δ* and the synthetic lethality of *kre11Δ* with *kre6Δ* (Brown *et al*., 1993a; Roemer *et al*., 1993) and *pkc1Δ* (Roemer *et al*., 1994) disruptants suggest a broad role for Kre11p in this process. Very recently, Kre11p has been identified as a component of TRAPP (M. Sacher and S. Ferro-Novick, personal communication), a novel *cis*-Golgi complex required for the docking and fusion of ER-derived vesicles (Sacher *et al*., 1998). This observation suggests that Kre11p may play a trafficking role which moves β-1,6-glucan biosynthetic components through the secretory pathway (see Fig. 1). Such a model is similar to the chitin synthesis paradigm, where it has been demonstrated that Chs5p (Santos and Snyder, 1997), Chs6p (Ziman *et al*., 1998) and Chs7p (Trilla *et al*., 1999) are required for various stages of the transport of Chs3p (the chitin synthase III enzyme; see Orlean, 1997) to the cell surface.

Cell surface

The yeast cell surface is a broad term referring to proteins that may be anchored to the plasma membrane either by an integral transmembrane domain or a C-terminal GPI moiety, soluble proteins which may reside in the periplasm and proteins associated with the cell wall. The *S. cerevisiae kre1-1* mutant (Al-Aidroos and Bussey, 1978) was used to clone the *S. cerevisiae KRE1* gene (Boone *et al*., 1990) as well as a functional and structural homologue in *Candida albicans* (Boone *et al*., 1991) by complementation. *kre1Δ* disruptants have a rough cell-surface appearance and defects in β-1,6-glucan (Boone *et al*., 1990) and α-agglutinin wall anchorage (Lu *et al*., 1995). Kre1p is an *O*-glycosylated predicted GPI-protein which was found in both membrane-associated and wall-associated fractions (Roemer and Bussey, 1995). As genomic analyses predict Kre1p to be a plasma membrane-anchored GPI protein as opposed to the wall-attached variety (Caro *et al*., 1997), the wall-associated fraction noted above is probably due to plasma membrane contamination of wall samples because their preparation did not include SDS extraction (Roemer and Bussey, 1995), a phenomenon previously observed in the study of wall proteins (see Klis, 1994). Immunofluorescence microscopy localizes Kre1p to the cell surface as predicted, with an enrichment of the protein in mother cells (Roemer and Bussey, 1995). Although this result was obtained using high heterologously

expressed levels of Kre1p and its significance is unclear, a putative cross-linking/fortification role has been proposed for Kre1p to complete the glucan network in a late step of wall maturation (Roemer and Bussey, 1995).

KRE9 (Brown and Bussey, 1993) and *KNH1* (Dijkgraaf *et al*., 1996) are a pair of homologues which encode soluble cell-surface proteins (see Table 1). *kre9Δ* disruptants are partially defective in α-agglutinin wall anchorage (Lu *et al*., 1995) and β-1,6-glucan, and have an aberrant multiple-budded morphology (Brown and Bussey, 1993), all of which are phenotypes similar to some of the other *kre* mutants described above. However, unlike other *kre* mutants, *kre9Δ* cells are unable to form mating projections in the presence of α-factor pheromone, implying a defect in directing polarized cell wall growth (Brown and Bussey, 1993). *kre9Δ* cells also exhibit carbon source dependence because their considerable growth defect seen on glucose is partially suppressed when they are grown on galactose. The last property is attributed to its functional homologue *KNH1*, which is expressed at higher levels in cells grown on galactose (Dijkgraaf *et al*., 1996). The synthetic lethality of *kre9Δ* disruption in *kre1Δ*, *kre6Δ* or *kre11Δ* backgrounds implies a pleiotropic influence on β-1,6-glucan synthesis, yet *kre5Δ* remains epistatic to *kre9Δ* because double disruptants retain the *kre5Δ* phenotype. With the exception of its differential expression, *KNH1 has* a similar relationship with *KRE9* as does *SKN1* with *KRE6*. Specifically, overexpression of *KNH1* can suppress both the growth and β-1,6-glucan phenotype of *kre9Δ* cells and although single *knh1Δ* disruptants have neither a growth nor a β-1,6-glucan phenotype such disruption is synthetically lethal in a *kre9Δ* background (Dijkgraaf *et al*., 1996). Interestingly, a screen for multicopy suppressors of *kre9Δ* slow growth identified the *SKN7* gene, which restored such growth without any restoration of β-1,6-glucan levels (Brown *et al*., 1993b). Skn7p appears to act as a transcription factor and a eukaryotic two-component response regulator (Brown *et al*., 1993b, 1994; Morgan *et al*., 1997), which has been shown to be involved in the high osmolarity glycerol (HOG) pathway of *S. cerevisiae* (Ketela *et al*., 1998). Based on the diversity of these observations, assigning a role to Kre9p in β-1,6-glucan synthesis is not entirely straightforward. The observed defects in growth, β-1,6-glucan and the anchorage of wall proteins, coupled with its genetic interaction with other *kre* mutants and its cell-surface location, imply a late function in β-1,6-glucan and wall synthesis, perhaps as a type of cross-linker. However, its carbon source dependence and suppression by the signal transduction component Skn7p may support a regulatory role. The identification of functional homologues in both *Candida albicans* (Lussier *et al*., 1998) and *Candida glabrata* (Nagahashi *et al*., 1998) reflect its important conserved function among fungi, and its potential as an antifungal target.

Future directions

Much work remains to elucidate cell wall β-1,6-glucan biosynthesis. Structural analyses have been valuable as the characterization of the mature wall cross-linked material has directed speculation on possible models of its synthesis. Genetic analyses have provided insight into the route of synthesis and the regulation and dynamic nature of this polymer. The current era of study therefore requires a convergence of structural and genetic information. Most important in this respect will be the elucidation of the biochemical activities of the known gene products involved and further structural analyses focused on biosynthetic intermediates obtained in various genetic backgrounds. It is only upon obtaining such results that it may be determined whether these gene products contribute directly or indirectly to this process, a distinction which is currently unclear. A critical requirement to further advances is the development of a useful *in vitro* assay of β-1,6-glucan synthesis.

Several hurdles remain, however, in the elucidation of this process. The first is the essential nature of β-1,6-glucan synthesis, which may suggest that key genes involved have yet to be identified, having been missed in screens using haploid strains. A possible solution to this problem is the analysis of diploid strains which are heterozygous for a given mutation because, in at least some cases, induced haploinsufficiency has been observed in response to specific drugs, with the added advantage of being performed on a genomic scale (Giaever *et al*., 1999). A similar approach may therefore be attempted using drugs that perturb cell wall synthesis. Another strategy to overcome the problem of lethality is the preparation and characterization of conditional alleles, using methods such as error-prone polymerase chain reaction (Muhlrad *et al*., 1992) or transposon insertional mutagenesis (Ross-Macdonald *et al*., 1997). The study of such alleles, however, will require the development of an *in vitro* assay for β-1,6-glucan synthesis because current methods are not conducive to measuring activity under non-permissive conditions. Unfortunately, attempts to adapt assays developed for *in vitro* β-1,3-glucan synthesis (Kang and Cabib, 1986; Douglas *et al*., 1994) have not been successful because β-1,6-glucan is a less abundant component of the cell wall, and therefore the great majority of radiolabelled UDP-glucose is incorporated into β-1,3-glucan *in vitro* (G.J.P. Dijkgraaf, personal communication), giving a very poor signal to noise ratio, a problem also encountered in *in vitro* cellulose synthesis (see Delmer, 1999). In addition, as the cell wall is a highly regulated dynamic structure (reviewed in Smits *et al*., 1999), mutations affecting β-1,6-glucan synthesis are often accompanied by an increase in β-1,3-glucan (for example *kre6*Δ; Shahinian *et al*., 1998) and chitin (for example *kre5*Δ; S. Shahinian, unpublished results), as

well as the upregulation of other modes of wall protein attachment (for example β-1,3-glucan-Pir2p; Kapteyn *et al.*, 1999b), making interpretation of *in vitro* synthesized glucan results especially difficult. Current methods of optimization are directed at the utilization of a combination of factors; using radiolabelled substrate for sensitivity, anti-β-1,6-glucan polyclonal antibodies (Montijn *et al.*, 1994) for the direct isolation of *in vitro* synthesized product to diminish background, and the verification of its identity using a cloned purified β-1,6-glucanase (De La Cruz *et al.*, 1995; Bom *et al.*, 1998). However, the identification of other essential factors may be required to achieve synthesis *in vitro*, as was the case in bacterial cellulose synthesis (Ross *et al.*, 1987).

A growing and powerful area in *S. cerevisiae* research is that of functional genomic analyses. Methods such as transcriptional profiling, which has already been used to show changes in gene expression associated with processes such as sporulation (Chu *et al.*, 1998), metabolic state (DeRisi *et al.*, 1997) and those that exhibit periodicity during the cell cycle (Spellman *et al.*, 1998), may help identify new genes involved in β-1,6-glucan synthesis. In addition, the full set of *S. cerevisiae* disruptants should be available in the near future, allowing gene characterization via genomic-scale cell wall phenotypic analyses. The disruption set will also identify all essential genes, which will allow a systematic approach to the studies described above. The next phase of the study of β-1,6-glucan synthesis will probably use these new approaches, ultimately enabling the identification and characterization of the biochemical events which comprise this system.

Acknowledgements

We thank Peter Dijkgraaf, Robin Green, Troy Ketela, Huijuan Li and Terry Roemer for their thoughts and comments. We also wish to thank F. Klis (University of Amsterdam), M. Sacher and S. Ferro-Novick (Yale University) for providing results before publication. S. Shahinian was supported by a Fonds de la Recherche en Santé du Québec Postdoctoral Fellowship. Work from our laboratory on glucan synthesis has been supported by the Natural Sciences and Engineering Research Council of Canada.

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