The role of glucose trimming in the endoplasmic reticulum of *Saccharomyces cerevisiae* was investigated using glucosidase inhibitors and mutant strains devoid of glucosidases I and II. These glucosidases are responsible for removing glucose residues from the N-linked core oligosaccharides attached to newly synthesized polypeptide chains. In mammalian cells they participate together with calnexin, calreticulin and UDP-glucose:glycoprotein glucosyltransferase in the folding and quality control of newly synthesized glycoproteins. In *S. cerevisiae*, glucosidase II is encoded by the GLS2 gene, and glucosidase I, as suggested here, by the CWH41 gene. Using castanospermine (an α-glucosidase inhibitor) and yeast strains defective in glucosidase I, glucosidase II and BiP/Kar2p, it was demonstrated that cell wall synthesis depends on the two glucosidases and BiP/Kar2p. In double mutants with defects in both BiP/Kar2p and either of the glucosidases the phenotype was particularly clear: synthesis of 1,6-β-glucan—a cell wall component—was reduced; the cell wall displayed abnormal morphology; the cells aggregated; and their growth was severely inhibited. No defects in protein folding or secretion could be detected. We concluded that glucose trimming in *S. cerevisiae* is necessary for proper cell wall synthesis, and that the glucosidases function synergistically with BiP/Kar2p in this process.

**Keywords:** BiP/Kar2p/cell wall synthesis/endoplasmic reticulum/glucosidase/1,6-β-glucan

**Introduction**

The secretory pathway is responsible for the synthesis, post-translational modification and sorting of a large variety of proteins, lipids and oligosaccharides targeted to the plasma membrane, the organelles of the vacuolar apparatus, and for secretion. In fungi and plants a large fraction of the secretory activity is devoted to production of enzymes and polysaccharides for the cell wall. Folding and oligomeric assembly of proteins occur in the endoplasmic reticulum (ER) and depend on a complex set of luminal and membrane-bound molecular chaperones and folding enzymes (Gething and Sambrook, 1992; Helenius *et al.*, 1992). Most polysaccharides are synthesized in the Golgi complex or extracellularly with the exception of N-linked oligosaccharides and GPI-anchors which are synthesized in the ER (Kornfeld and Kornfeld, 1985).

When glycoproteins pass through the secretory pathway, the N-linked glycans undergo extensive modifications. In the ER, glucosidases I and II remove the three glucose residues present on the core oligosaccharides (Glc₃Man₉GlcNAc₂). Glucosidase I removes the outermost 1,2-α-linked glucose residue, and glucosidase II the two remaining 1,3-α-linked residues (Kornfeld and Kornfeld, 1985).

In mammalian cells, the trimming of glucoses is connected to a molecular chaperone cycle involving two lectin-like chaperones, calnexin and calreticulin. Calnexin is a type I membrane-bound molecule (Degen and Williams, 1991) and calreticulin a soluble luminal protein (Michalak *et al.*, 1992). They specifically bind to mono-glucosylated N-linked glycans (Glc₁Man₉GlcNAc₂) that are formed after removal of the two outermost glucoses by the ER glucosidases or by the action of the UDP-glucose:glycoprotein glucosyltransferase (UGGTase) (Hammond *et al.*, 1994; Hebert *et al.*, 1995; Peterson *et al.*, 1995; Ware *et al.*, 1995; Spiro *et al.*, 1996). UGGTase is a 170 kDa soluble enzyme that resides in the ER and adds back a single glucose residue to fully deglucosylated N-glycans (Trombetta *et al.*, 1989). Since the glucosyltransferase only reglucosylates incompletely folded proteins, it is thought to serve as a folding sensor in the quality control process (Sousa *et al.*, 1992; Hammond and Helenius, 1993). The addition and removal of glucose residues allows substrates to undergo cycles of binding and release, which promotes correct folding and at the same time prevents incompletely folded proteins from exiting the ER.

BiP/Grp78 is another important molecular chaperone in the ER (Haas and Wabl, 1983). A member of the hsp70 family, it associates with numerous incompletely folded, unassembled or misfolded proteins, and is believed to promote folding through cycles of ATP-dependent binding and release of the substrates (Kassenbrock and Kelly, 1989; Flynn *et al.*, 1991; Gething and Sambrook, 1992; Knittler and Haas, 1992; Gaut and Hendershot, 1993; Simons *et al.*, 1995).

The ER of *Saccharomyces cerevisiae* is similar to that of mammalian cells in many respects. It contains a functional homologue of BiP/Grp78 (N normington *et al.*, 1989; Rose *et al.*, 1989) as well as several proteins that share homology with the components of the calnexin cycle. The latter include glucosidase I (Esmon *et al.*, 1984), glucosidase II (Trombetta *et al.*, 1996), the calnexin...
homologue Cne1p (De Virgilio et al., 1993; Parlati et al., 1995) and Kre5p which is homologous to UGGTase (Fernandez et al., 1996). As in mammalian cells, neither the two glucosidases nor the calnexin homologue are essential for growth.

On the other hand, yeast cells lacking KRE5 grow poorly and have altered morphology (Meaden et al., 1990), suggesting that Kre5p has functions unrelated to a putative calnexin cycle. In S.cerevisiae, BiP/Kar2p is needed for proper protein folding (Simons et al., 1995). In addition, it is essential for translocation of proteins into the ER (Brodsky and Schekman, 1993).

Here, the role of glucose trimming in S.cerevisiae was analyzed using mutant strains and inhibitors. Our data suggest that the previously described CWH41 gene encodes yeast glucosidase I. Disruption of this gene, or of the glucosidase II gene GLS2, caused a synthetic growth defect in cells also containing a kar2 mutation. This suggested a functional relationship between BiP/Kar2p and glucose trimming in the ER. The growth phenotype was not due to extensive inability of the double mutants to support protein folding in the ER, but rather to a decrease in the synthesis of cell wall 1,6-β-glucan.

**Results**

**Effects of a glucosidase inhibitor on S.cerevisiae**

In mammalian cells the activity of ER glucosidases I and II can be inhibited by glucose analogues such as castanospermine (CST) (Elbein, 1991). To test whether glucose trimming is similarly inhibited in S.cerevisiae, wild-type cells were treated with CST for 1.5 h. The SDS–PAGE mobility of 35S-labeled carboxypeptidase Y (CPY) was found to be slower than in untreated cells (Figure 1, lanes 1–3). Treatment of the cell lysates with endoglycosidase H (Endo H) confirmed that the reduced mobility was due to a change in oligosaccharide processing (lanes 5–7). While we could conclude that CST inhibited trimming in S.cerevisiae, the inhibition did not seem to be complete: CPY synthesized in a glucosidase I-deficient strain, gls1-1 (Esmon et al., 1984), migrated somewhat more slowly than the CST-treated species (Figure 1, lane 4). The partial block in glucose trimming by CST had no effect on cell growth at either 37°C (Figure 2A) or at 25°C.

To examine whether CST would have an effect on strains with a compromised ER protein-folding machinery, the growth of five kar2 mutant strains was analyzed at different temperatures in the presence of the inhibitor. Two of the strains, kar2-159 and kar2-203, which are not viable at 37°C (Vogel et al., 1990; Sanders et al., 1992), were analyzed at 25 and 30°C. The three other strains, kar2-1, kar2-127 and kar2-133, were grown and analyzed at 37°C.

The most pronounced defect was seen with kar2-127. The cells were almost completely growth-arrested at 37°C (Figure 2B). At this temperature, mutants kar2-1 and kar2-133 were also affected by CST, judging by increased doubling times. Similar growth inhibition was observed using another α-glucosidase inhibitor, 1-deoxynojirimycin. Mutants kar2-159 and kar2-203, which could not be tested at 37°C, proved insensitive to CST at 30°C. None of the mutants suffered growth defects at 25°C. Taken together, the results indicated that sensitivity to the glucosidase inhibitors correlated with the temperature-induced inactivation of BiP/Kar2p.

**Glucosidase I-deficient kar2 strains have growth defects**

The apparent functional relationship between the glucosidases and BiP/Kar2p was confirmed using double mutants defective in Kar2p and glucosidase I. Several kar2 strains
were crossed with a strain carrying the non-functional glucosidase I allele gls1-1 (Esmon et al., 1984). Growth of haploid daughter cells was assessed after sporulation and tetrad dissection.

With two strains, kar2-127 and kar2-159, mating was successful and the spores showed a growth pattern indicative of a synthetic interaction between glucosidase I and BiP/Kar2p. The defect was most severe for kar2-127 gls1-1 spores whose growth, even at 25°C, was barely sufficient for retrieval of colonies for further analysis. At 37°C, these spores were unable to grow.

That the slow-growing spores carried the gls1-1 allele was confirmed by slower SDS–PAGE mobility of the ER-resident glycoprotein protein disulfide isomerase (PDI) compared with cells with intact glucosidase I (see Materials and methods). The presence of kar2-159 in the slow-growing spores was verified by taking advantage of this allele’s lethal phenotype at 37°C (see Materials and methods). We also found that the viability of representative slow-growing spores from the kar2-127 gls1-1 cross was restored by transforming the cells with a single copy plasmid carrying the KAR2 gene (see Figure 4).

We concluded that the poor growth of the spores obtained in the crosses was caused by the combination of defective glucosidase I and kar2 genes. The more severe growth defect in these experiments compared with those in CST was most likely due to incomplete inhibition of glucose trimming by the inhibitor.

The CWH41 gene encodes glucosidase I

By searching the yeast genome library, we found that a previously described yeast protein called Cwh41p (Jiang et al., 1996) had 33% amino acid sequence identity with human glucosidase I (Kalz-Füller et al., 1995). Like mammalian glucosidase I, it appeared to be a type II transmembrane protein (Kalz-Füller et al., 1995; Jiang et al., 1996).

To determine whether it encoded yeast glucosidase I, a PCR-generated copy of CWH41 was expressed in the kar2-127 gls1-1 double mutant. We found that glucose trimming of PDI was restored (Figure 3A), and the slow electrophoretic mobility of CPY was changed to that of wild-type cells. Furthermore, when CWH41 was disrupted in a haploid strain, the ER form of CPY co-migrated with the ER form obtained from the gls1-1 strain (Figure 3B, lanes 2 and 3). The mobility shift was due to differences in glycan processing, since CPY from wild-type and mutant strains co-migrated after Endo H digestion (lanes 4–6). Importantly, unlike kar2-127 gls1-1, the CWH41-transformed kar2-127 gls1-1 cells were able to grow at elevated temperature (Figure 4).

These results strongly suggested, without formally proving it, that CWH41 is the gene for glucosidase I and that it represents the same gene as GLS1. They furthermore confirmed that lack of glucosidase I activity due to the gls1-1 mutation causes a synthetic growth defect in cells compromised in BiP/Kar2p function.

**Growth defects by disrupting glucosidase II**

To test whether the synthetic growth defect observed with gls1-1 and kar2-127 could be reproduced with glucosidase II, the GLS2 gene was disrupted in the kar2-127 strain by deleting the entire ORF by homologous recombination (Trombetta et al., 1996). Glucosidase II-deficient transformants were identified based on the slower SDS–PAGE mobility of PDI.

The gls2-disrupted kar2-127 strain was essentially non-viable at 34°C. Only after extended incubation periods could some growth be observed. By comparing diluted samples, it was concluded that the severity of the phenotype was similar to that observed in the kar2-127 gls1-1 double mutant strain (Figure 4). Together with the growth defect observed for the kar2-127 strain in the presence of CST, this showed that the temperature-sensitive growth phenotype was not simply a consequence of the accumula-
tion of triglucosylated glycans as in the kar2-127 gls1-1 strain. Assuming that the only activity of the glucosidases is to trim N-glycans, the growth defect was rather due to the failure to generate monoglucosylated or glucose-free N-glycans.

**Protein maturation is normal in kar2-127 lacking glucosidase I and II activity**

Given the role of BiP/GRP78 and glucose trimming in protein folding in mammalian cells, it was of interest to test whether the synthetic growth defect in the double mutants was due to complications in glycoprotein folding and maturation. A variety of experiments were performed to analyze the conformational maturation, intracellular transport and secretion of endogenous glycosylated yeast proteins. The proteins tested were CPY, the mating pheromone α-factor and invertase. The experiments were performed in the kar2-127 strain after 12 h incubation at 37°C and a 60 min precubination in 2 mM CST.

It was found that both pro-α-factor and invertase efficiently exited the ER both in the presence and absence of CST. The high-mannose post-ER form of invertase appeared simultaneously in control and CST-treated cells after a 5 min radiolabeling (Figure 5A). The kinetics by which the intracellular form of α-factor disappeared from the cells was also unaffected by the presence of the inhibitor (Figure 5B). In pulse–chase experiments, the ability of the CST-treated mutant to secrete proteins into the medium was also unchanged.

The transport of CPY to the vacuole was less efficient in the kar2-127 mutant compared with wild-type cells (J.F.S. and A.H., unpublished results); however, the presence of CST did not further affect its transport rate (Figure 5C). Neither was any difference observed in the rate of disulfide bond formation in newly synthesized CPY. The transport kinetics of CPY in the early secretory pathway could not be determined due to the fuzzy appearance of the ER and Golgi forms in the kar2-127 strain. This heterogeneity was most likely caused by incomplete glycan processing in the ER of the kar2-127 strain, as discrete bands were seen in the presence of CST.

We also monitored the unfolded protein response (Shamu et al., 1994), by determining the level of KAR2 mRNA by Northern blot analysis in CST-treated kar2-127 cells and in the double mutants grown at 25°C. While the amount of KAR2 mRNA was several fold higher in all three mutant strains than in the wild-type, no difference was observed between kar2-127 alone and the double mutants. The unfolded protein response was also tested in wild-type cells disrupted for either glucosidase I or II, or cells treated with CST. As shown in Figure 5D, no increase in KAR2 mRNA levels was observed compared with control cells.

It was apparent that, although the combination of defects in Kar2p and ER glucosidases resulted in a dramatic growth defect, the folding and maturation of glycoproteins appeared normal. The major cause for inhibition of growth was therefore likely to be elsewhere.

**Defective cell wall 1,6-β-glucan synthesis in double mutants**

When considering alternative causes for the growth defect, we noted that the CWH41 gene had been previously implicated in the synthesis of cell wall 1,6-β-glucan (Jiang et al., 1996). Kre5p, the UGGTase homologue of yeast, is also involved in the same process (Meaden et al., 1990). In addition, it had been reported that disruption of KRE5 resulted in cell aggregation (Meaden et al., 1990), a defect also observed for the kar2-glucosidase double mutants.

To determine whether the kar2-127 glucosidase double mutant had a defect in cell wall synthesis, we measured the cell wall content of 1,6-β-glucan using an alkali extraction-1,3-β-glucanase protocol (Brown et al., 1994). In agreement with previously reported measurements for wild-type cells (Brown et al., 1994), the 1,6-β-glucan in wild-type and kar2-127 cells constituted ~20% of the total alkali-insoluble glucan (Figure 6). In contrast to the reported decrease in 1,6-β-glucan in a CWH41-deficient strain (Jiang et al., 1996), we did not observe a clear-cut reduction in our CWH41 disrupted strain. This may have
et al. (1995). They form an amorphous layer in which the 1,6-β-glucan is thought to serve as an anchor for the mannoproteins (Meaden et al., 1990). The electron-dense mannoprotein rim staining was absent. The outer boundary of the cell wall was rough and ill-defined. The thickness of the wall was variable. In some regions the cell wall was almost absent. The cells also displayed changes in the cytoplasm. Numerous vacuolar structures that were only occasionally seen in wild-type cells were present. There was also a clear increase in ER-like membranes throughout the cell.

Interestingly, the CNE1-disrupted strain had a cell wall morphology that resembled that of the double mutants, although less perturbed. Cells with an uneven outer cell wall surface and a reduction in the electron density of the mannoprotein layer were frequently observed (Figure 7, panel g).

We also examined the morphology of the KRE5 disruptant (Meaden et al., 1990) (Figure 7, panel f). Similar changes as in the double mutants were observed, although both the cell wall defects and the accumulation of membranous and vacuolar structures were more severe. Cell wall alterations resembling those observed have also been reported for mutants lacking the KRE1, KRE6 or KRE9 genes, all of which have reduced cell wall 1,6-β-glucan (Boone et al., 1990; Brown and Bussey, 1993; Roemer et al., 1994).

**Discussion**

Glucosidases I and II are present in the ER of almost all eukaryotes from yeast to man. Their primary role is to trim core N-linked glycans added to newly synthesized glycoproteins. In mammalian cells this process is linked to glycoprotein folding and quality control (see Helenius et al., 1997). Our results suggested that in *S.cerevisiae* their function may have a different emphasis. Instead of playing a general role in glycoprotein maturation, it was found that the glucosidases participated directly or indirectly in cell wall synthesis. More specifically, the two glucosidases and BiP/Kar2p were found to be synergistically involved in the production of 1,6-β-glucan, a cell wall component.

The cell wall of *S.cerevisiae* is a large and complex structure with a distinctive composition and morphology. During the cell cycle it undergoes dynamic changes and its properties are strictly regulated (Cid et al., 1995). The various mutants, wild-type and mutant strains were grown at 25°C, harvested in early logarithmic phase, fixed, sectioned and analyzed by electron microscopy. In the wild-type strain the cell wall was even and had a defined thickness all around the cell (Figure 7, panel a). On higher magnification (see insert), the typical layered structure was apparent, with the dark-staining outer layer of mannoproteins clearly distinguishable. The cell wall of the kar2-127 mutant was morphologically indistinguishable from that of the wild-type strain (Figure 7, panel b). Also the glucosidase I- and II-deficient single mutants were largely normal (panel c, only Δgls2 strain shown). A slight roughness in the outer part of the cell wall was, however, apparent in some of these cells. This was also observed in some wild-type cells, but the frequency of appearance was higher in the glucosidase mutants.

Analysis of the kar2-127 glucosidase double mutants revealed marked changes both in cell wall architecture and in the appearance of the cell body (Figure 7, panels d and e). The electron-dense mannoprotein rim staining was absent. The outer boundary of the cell wall was rough and ill-defined. The thickness of the wall was variable. In some regions the cell wall was almost absent. The cells also displayed changes in the cytoplasm. Numerous vacuolar structures that were only occasionally seen in wild-type cells were present. There was also a clear increase in ER-like membranes throughout the cell.

We also determined the 1,6-β-glucan level in a CNE1-disrupted strain (Boone et al., 1990; Brown and Bussey, 1993; Roemer et al., 1994).

**Aberrant cell wall morphology in the double mutants**

The outermost layer of the *S.cerevisiae* cell wall is composed of mannoprotein fibrils that make up ~25% of its dry weight. It has an electron-dense appearance in ultra-thin sections. Underlying the mannoproteins are the 1,6-β- and 1,3-β-glucans that comprise 5% and 60% of cell wall mass, respectively (for a recent review see Cid et al., 1995). They form an amorphous layer in which the 1,6-β-glucan is thought to serve as an anchor for the mannoproteins (Meaden et al., 1990).

To examine the morphology of the cell wall in the
Cell wall synthesis in yeast

Fig. 7. Changed cell wall morphology and accumulation of intracellular membranes in glucosidase-deficient kar2-127 strains. Cells were grown to early logarithmic phase and prepared for electron microscopy as described in Materials and methods. Panels: (a) wild-type cells; (b) kar2-127 single mutant; (c) Δgls2 cells; (d) kar2-127 gls1-1 double mutant; (e) kar2-127 Δgls2 cells; (f) Δkre5 cells; (g) Δcne1 cells.
outermost layer consists of highly glycosylated manno-
proteins that make up ~25% of the total weight of the cell wall. They form fibrillar structures which give an electron- dense appearance in electron micrographs (Horrisberg and Vonlanthen, 1977). The other major constituents are the 1,6-β- and 1,3-β-glucans that comprise 5% and 60% of the cell wall, respectively. Together with small amounts of chitin, they form an amorphous, less intensively stained layer below the mannoproteins (Cid et al., 1995). The 1,6-β-glucan serves as an anchor for the mannoproteins and is necessary for the structural integrity of the mannoprotein layer (Boone et al., 1990; Brown and Bussey, 1993; Roemer et al., 1994).

The 1,6-β-glucans differ from the other cell wall poly-
saccharides in being synthesized intracellularly. Bussey and co-workers have identified several genes that affect their synthesis (Boone et al., 1990). Unlike the synthesis of chitin and 1,3-β-glucan, which occurs at the plasma membrane (Cid et al., 1995), the proteins and enzymes involved in 1,6-β-glucan synthesis are localized to sites along the secretory pathway. Kre5p and Cwh41p are ER proteins, Kre6p appears to reside in the Golgi, and Kre1p is thought to occur at the plasma membrane (Boone et al., 1990; Meaden et al., 1990; Roemer et al., 1994; Jiang et al., 1996). The cellular location of Kre9p is unknown, but it also seems to be somewhere in the secretory pathway (Brown and Bussey, 1993). That at least part of the synthetic steps leading to the formation of 1,6-β-glucan occur within the organelles of the secretory pathway is supported by the observed intracellular presence of 1,6- β-glucan by immunogold labeling (Horrisberg and Clerk, 1987).

Of previously identified proteins involved in 1,6-β- glucan synthesis, Cwh41p and Kre5p are both related to the glucose-processing machinery in the ER of mammalian cells. CWH41 is, as shown here, most likely the gene for glucosidase I, while KRE5 encodes a homologue of UDP-glucose:glycoprotein glucosyltransferase (UGGTase), an ER enzyme found in most eukaryotic cells including other yeasts such as Schizosaccharomyces pombe (Parodi et al., 1984; Fernandez et al., 1994; Parker et al., 1995). Together with glucosidase II and the two ER resident chaperones calxin and calreticulin, these enzymes constitute a chaperone cycle important for the maturation of N-glycosylated proteins in mammalian cells (Ou et al., 1993; Hammond et al., 1994; Hebert et al., 1995; Peterson et al., 1995).

Although we could not observe any effects of defective glucose trimming on the maturation of several major glycoproteins, it is possible that a calnexin-like cycle does exist in yeast. It might facilitate proper folding and maturation of a selected group of proteins including one or more of the enzymes involved in 1,6-β-glucan synthesis. Folding of such proteins could occur without the calxin cycle if the molecular chaperone BiP/Kar2p was fully functional. Redundancy in the folding machinery would allow the BiP/Kar2p system to take over if the calxin cycle were not operational. We have seen examples of such redundancy between calxin/calreticulin and BiP/Grp78 in the retention of proteins in the ER of mammalian cells (Hammond and Helenius, 1994; Zhang et al., 1997). The fact that the double mutants had highly abnormal cell walls and a strong growth defect, whereas the kar2 or glucosidase single mutants had wild-type or only slightly abnormal phenotypes, suggested that the function of the glucosidases is closely related to the chaperone function of BiP/Kar2p.

It is noteworthy that if S.cerevisiae has a calnexin/calreticulin pathway, it must represent a stripped-down version. Despite the homology of Kre5p with the UGGTase no transferase activity has been detected in yeast lysates (Fernandez et al., 1994). In addition, there is only one protein that is homologous to calnexin and calreticulin. It is Cne1p, which like calnexins carries a C-terminal transmembrane region, but lacks the typical negatively charged cytoplasmic domain and the ER retrieval signal (De Virgilio et al., 1993; Parlati et al., 1995). While Cne1p is not essential for cell growth, it has been suggested to have a role in ER retention and quality control because cell surface expression of α-factor receptor and secretion of α1-antitrypsin occurs somewhat faster in cells devoid of CNE1 than in wild-type cells (Parlati et al., 1995). Whether Cne1p associates with these or other newly synthesized proteins is not known.

When we analyzed a CNE1-disrupted strain, we detected normal or somewhat increased amounts of 1,6-β-glucan compared with wild-type cells. However, in electron micrographs, the cell wall was clearly abnormal. It had the uneven appearance of the kar2-127 glucosidase double mutants and the KRE5-disrupted strain, albeit less pronounced. Attempts at creating a CNE1-disrupted kar2-127 strain failed, suggesting that such a mutant might be non viable. Attempts to cross the kar2-127 strain with a Δcne1 strain also failed. This was probably due to unsuccessful mating caused by the karyogamy defect in the kar2-127 strain. While inconclusive at this point, these results are consistent with a synthetic interaction between the KAR2 and CNE1 genes. The role of Cne1p in cell wall synthesis as well as in protein secretion thus remains unclear and needs to be further explored.

Another possible explanation for the effects on the cell wall is that glucosidases I and II, Kre5p and Cne1p are directly involved in 1,6-β-glucan synthesis or processing. While little is known about the biosynthesis of 1,6-β-glucan and its integration into the cell wall, it is likely to involve glycoproteins and lipid precursors synthesized in the ER. Based on its homology to the UGGTase, one may hypothesize that Kre5p is a transferase needed for the synthesis of polyglucose itself or other oligosaccharide structures (such as GPI anchors) needed in the generation of 1,6-β-glucans or for their attachment to other cell wall and plasma membrane components. In the case of α-agglutinin and possibly other cell wall proteins, anchorage to the cell wall occurs through covalent linkage of a 1,6-β-glucan chain to the protein’s C-terminal glycosyl phosphatidylinositol (GPI) module (de Nobel and Lipke, 1994; Lu et al., 1995).

In summary, we have observed a functional connection between ER glucosidases and Bip/Kar2p in cell wall 1,6-β-glucan synthesis in S.cerevisiae. Given the importance of the cell wall for yeast and for many other species, including plants, and considering the complexity of polysaccharides, it is not surprising that its synthesis involves the coordinated action of many ER enzymes and factors.
Materials and methods

**Yeast strains**

Yeast strains used in this study are listed in Table 1.

**Reagents and media**

All reagents were purchased from Sigma Chemical Co. (St Louis, MO) unless otherwise stated. Restriction enzymes were from New England Biolabs (Beverly, MA). For PCR reactions, two DNA polymerase from Boehringer-Mannheim (Indianapolis, IN) was used. Cells were grown in YPD medium [1% yeast extract, 2% bacto-agar (Difco Laboratories) as sole carbon source], Yeast Prototrophic strains were cultured at 30°C, and auxotrophic strains were grown at 30°C in YPD medium [1% yeast extract, 2% bactopeptone (Difco Laboratories, Boehringer-Mannheim (Indianapolis, IN) was used. Cells were grown in YPD medium [1% yeast extract, 2% bacto-agar (Difco Laboratories) as sole carbon source], Yeast Prototrophic strains were cultured at 30°C, and auxotrophic strains were grown at 30°C in YPD medium [1% yeast extract, 2% bactopeptone (Difco Laboratories), 0.05% dextrose, 2% bacto-agar (Difco Laboratories). For transformation, the yeast cells were grown in 2% bacto-agar (Difco Laboratories) as sole carbon source], Yeast Prototrophic strains were cultured at 30°C, and auxotrophic strains were grown at 30°C in YPD medium [1% yeast extract, 2% bactopeptone (Difco Laboratories), 0.05% dextrose, 2% bacto-agar (Difco Laboratories). Yeast cells were cultured in YPD medium to stationary phase, washed once in H2O, and spread onto selective minimal medium agar plates.

**Plasmid constructs and PCR**

The KAR2 gene was excised as a PstI fragment from plasmid pMR48 (Rose et al., 1989) and inserted into Smal-digested pRS316 (Sikorski and Hieter, 1989) to obtain pRS316-KAR2. To construct plasmid pH1-CWH41/GLS1, CWH41 was amplified from yeast genomic DNA using specific primers and the presence of acid-washed glass beads. Proteolysis was inhibited by the addition of 1 mM PMSF (Boehringer-Mannheim) and 10 μM LiSORB. The proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH) using a semi-dry blotting apparatus (Hoefer Scientific Instruments, San Francisco, CA). The membrane was incubated for 16 h in 3% (w/v) powdered milk in PBS at 4°C, followed by anti-PDI antibody for 1 h at room temperature. The membrane was washed with PBS and incubated for 1 h with a goat anti-rabbit antibody conjugated to horseradish peroxidase (Pierce, Rockford, IL). After additional washings in PBS the membrane was incubated with Luminol (Pierce) and exposed to X-ray film.

**Genotype analysis of spores**

Sporing of strain NY914, carrying a ura3-52 and an ade2-101 mutation, was carried out as described before by Trombetta et al. (1996). The ura3-52 mutation alone is non-viable, and the ade2-101 mutation alone is slow-growing. The ura3-52 mutation alone causes cell death. For every tetrad analyzed, 2 of every 4 spores were viable and 2 non-viable. All glucosidase deficient colonies that grew poorly at 25°C were found to carry the kar2-127 allele.

**Yeast transformation**

Cells were transformed by a modified lithium acetate protocol. Typically, 20 ml of cells were grown to 0.5–1 ODU/ml harvested by centrifugation and resuspended in 2 ml LiSORB (100 mM Li-acetate, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 M sorbitol). After incubation at 30°C for 30 min, cells were pelleted and resuspended in 100 μl LiSORB. 25 μl of cell suspension was added to 10 μl sonicated and boiled salmon sperm DNA (5 mg/ml) and 10 μl LiSORB and 0.3–1 μl of the transforming DNA, and the mixture was incubated for 30 min at 30°C. 450 μl 40% (w/v) PEG 3350 (Sigma) in LI (100 mM Li-acetate, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA) was added and the incubation was continued for 30 min at 30°C. Cells were then heat-treated for 5 min at 42°C, washed once in H2O, and spread onto selective minimal medium agar plates. Transformants were subjected to a second round of growth on selective plates before further characterized.

**Western blot analysis**

Cell lysates were made by vortexing the cells in 1% SDS in PBS in the presence of acid-washed glass beads. Proteolysis was inhibited by the addition of 1 mM PMSF (Boehringer-Mannheim) and 10 μg/ml each of chymostatin, leupeptin, antipain and pepstatin (CLAP) (Sigma). Cell debris was removed by centrifugation and cell lysate corresponding to 1–2 ODU of cells was separated by SDS-PAGE. The proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH) using a semi-dry blotting apparatus (Hoefer Scientific Instruments, San Francisco, CA). The membrane was incubated for 16 h in 3% (w/v) powdered milk in PBS at 4°C, followed by anti-PDI antibody for 1 h at room temperature. The membrane was washed with PBS and incubated for 1 h with a goat anti-rabbit antibody conjugated to horseradish peroxidase (Pierce, Rockford, IL). After additional washings in PBS the membrane was incubated with Luminol (Pierce) and exposed to X-ray film.

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Sporing of strain NY914, carrying a ura3-52 and an ade2-101 mutation, was carried out as described before by Trombetta et al. (1996). The ura3-52 mutation alone is non-viable, and the ade2-101 mutation alone is slow-growing. The ura3-52 mutation alone causes cell death. For every tetrad analyzed, 2 of every 4 spores were viable and 2 non-viable. All glucosidase deficient colonies that grew poorly at 25°C were found to carry the kar2-127 allele.

**Yeast transformation**

Cells were transformed by a modified lithium acetate protocol. Typically, 20 ml of cells were grown to 0.5–1 ODU/ml harvested by centrifugation and resuspended in 2 ml LiSORB (100 mM Li-acetate, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 M sorbitol). After incubation at 30°C for 30 min, cells were pelleted and resuspended in 100 μl LiSORB. 25 μl of cell suspension was added to 10 μl sonicated and boiled salmon sperm DNA (5 mg/ml), 10 μl LiSORB and 0.3–1 μl of the transforming DNA, and the mixture was incubated for 30 min at 30°C. 450 μl 40% (w/v) PEG 3350 (Sigma) in LI (100 mM Li-acetate, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA) was added and the incubation was continued for 30 min at 30°C. Cells were then heat-treated for 5 min at 42°C, washed once in H2O, and spread onto selective minimal medium agar plates. Transformants were subjected to a second round of growth on selective plates before further characterized.

**Western blot analysis**

Cell lysates were made by vortexing the cells in 1% SDS in PBS in the presence of acid-washed glass beads. Proteolysis was inhibited by the addition of 1 mM PMSF (Boehringer-Mannheim) and 10 μg/ml each of chymostatin, leupeptin, antipain and pepstatin (CLAP) (Sigma). Cell debris was removed by centrifugation and cell lysate corresponding to 1–2 ODU of cells was separated by SDS-PAGE. The proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH) using a semi-dry blotting apparatus (Hoefer Scientific Instruments, San Francisco, CA). The membrane was incubated for 16 h in 3% (w/v) powdered milk in PBS at 4°C, followed by anti-PDI antibody for 1 h at room temperature. The membrane was washed with PBS and incubated for 1 h with a goat anti-rabbit antibody conjugated to horseradish peroxidase (Pierce, Rockford, IL). After additional washings in PBS the membrane was incubated with Luminol (Pierce) and exposed to X-ray film.
Radiolabeling and immunoprecipitations
Before radiolabeling, cells were grown for 12–16 h in bacto-yeast nitrogen base (Difco) supplemented with 2% glucose and the appropriate amino acids and/or nucleotide bases. When cells were labeled in the presence of CST (1 or 5 mM) a 30- to 90-min preincubation with the drug preceded the radioactive pulse. In experiments that required DTT during the pulse, the reducing agent was added at 10 mM 10 min before the pulse and kept throughout the labeling reaction. Invertase synthesis was induced by incubating the cells in 0.1% glucose for 1 h. Radiolabeling was typically performed in 200–300 μl of minimal medium and 200–300 μCi of [35S]Promix (Amersham Corp., Arlington Heights, IL). Chases were initiated by the addition of 1/10 volumes of non-radioactive methionine and cysteine, 100 mM each, and 10 mM cycloheximide. Radiolabeling or the chase was terminated by the addition of 2 volumes of ice-cold 40 mM Na azide in PBS. Cells were disrupted by vortexing in 50–100 μl of 1–2% SDS in PBS in the presence of glass and followed by 5 min boiling. Proteolysis was inhibited by the addition of 1 mM PMSF and CLAP (see above). Cell debris was pelleted and the lysate was added to 10 volumes of 1% Triton X-100 in PBS. The lysate was incubated for 12–16 h at 4°C with the appropriate antibodies and Protein A-Sepharose beads (Sigma). The immunocomplexes were washed twice at room temperature in 10 mM Tris, pH 7.5, 100 mM NaCl, 0.2% SDS, 0.01% Triton X-100 and boiled in SDS–PAGE sample buffer in the presence of 25 mM DTT. The samples were analyzed by 7.5% SDS–PAGE.

Northern blot analysis
Cells were grown to stationary phase in YPD. Cells from each sample were separated on a 1% denaturing agarose gel, and transferred to a Hybond-N membrane as described by the manufacturer (Amersham International plc, Little Chalfont, UK). Transfer was done using the Turboblotter (Schleicher and Schuell, Keene, NH). Hybridization conditions were as described previously by Ausubel (1994).

The DNA probe was generated by PCR using pRS316–KAR2 as a template together with primers JFS01295 (5'-ATGTTTTTCTAAACAGAC- TAAGCGCTGGC-3') and JFS01395 (5'-CTCAATTCGTCGTTGC-3'), and spanned the entire open reading frame of KAR2. 125 μCi [α-32P]dCTP was added to a 100 μl PCR reaction containing unlabeled nucleotides at 50 μM each. The probe was separated from unincorporated nucleotides on a Sephadex G-25 column (Pharmacia Biotech, Sweden) before being used for hybridization.

Glanuc determination
Cell wall glucans were determined as previously described (Brown et al., 1994). Briefly, cells were grown to stationary phase in YPD. Cells from 10 ml suspension were washed in H2O and divided equally into four tubes. One tube was used for measuring the dry weight. Cells in the remaining tubes were extracted three times, each for 1 h, in 3% NaOH at 75°C. The samples were neutralized in 100 mM Tris–HCl, pH 7.5 followed by 10 mM Tris–HCl, pH 7.5, and digested for 16–20 h with 1 mg/ml Zymolyase 100T (Seikagaku Kogyo, Tokyo, Japan) at 37°C. Half of the digested samples were dialyzed for 16 h using a Spectra/Por tubing with 6000–8000 kDa pore size (Spectrum Medical Industries, Inc., Laguna Hills, CA). The amount of O-linked glucan in the various fractions was determined by the phenol–sulfuric acid assay (McKelvey and Lee, 1969). To ensure that the Zymolyase digestion was complete, the amount of pelletable undigested glucan was measured. The amount remaining was <10% of total alkali insoluble glucan in each sample.

Electron microscopy
50 ml of cells grown to early logarithmic phase (1–2 ODU/ml) were fixed by adding 1 ml of 50% glutaraldehyde in double-distilled H2O. The cells were typically pelleted at 5000 g for 10 min at 4°C and washed in 10 ml H2O. 200 μl of pelleted cells were resuspended in 5 ml of 4% K2MnO4 in H2O and incubated on a nutator in the dark for 2–4 h at 4°C. After five washes in 10 ml H2O, the cells were resuspended in 5 ml of 3% uranyl acetate and incubated for 18 h at 4°C on a nutator in the dark. The cells were washed five times in 10 ml water and dehydrated by incubation in increasing (70–100%) concentrations of ethanol. The samples were infiltrated for 18 h at room temperature in a 1:1 mixture of ethanol with Spurr-low viscosity embedding medium (Electron Microscopy Sciences, Ft Washington, PA). The mixture was replaced with pure embedding medium for 3 h, and after changing for another 16 h. 50 μl of a 1:1 mixture of cells and fresh embedding medium were applied to the tip of a beam capsule, and cured at 60°C for 48 h. Ultrathin sections were cut on a Reichert/Jung microtome, and further contrasted using lead citrate and uranyl acetate.

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