The EMBO Journal Vol.17 No.2 pp.396–405, 1998

Cell wall 1,6-β-glucan synthesis in *Saccharomyces cerevisiae* depends on ER glucosidases I and II, and the molecular chaperone BiP/Kar2p

Jan Fredrik Simons[1], Melanie Ebersold and Ari Helenius[2,3]

Department of Cell Biology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520-8002, USA

[1]Present address: CuraGen Corporation, 555 Long Wharf Drive, New Haven, CT 06511, USA
[2]Present address: Laboratory for Biochemistry, Swiss Federal Institute of Technology Zürich, Universitaetstrasse 16, CH-8092 Zürich, Switzerland

[3]Corresponding author
e-mail: ari.helenius@bc.biol.ethz.ch

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 lumenal 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 ($Glc_3Man_{5-9}GlcNAc_2$). 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 lumenal protein (Michalak *et al.*, 1992). They specifically bind to monoglucosylated N-linked glycans ($Glc_1Man_{5-9}GlcNAc_2$) 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 (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.

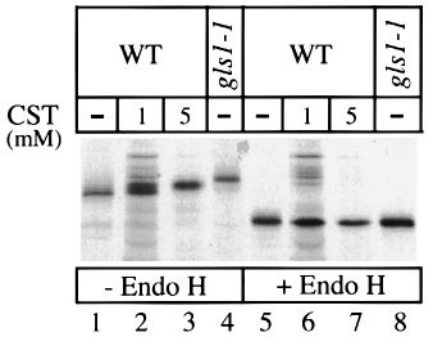

Fig. 1. CST inhibits ER associated glucose trimming in yeast. CPY was analyzed in mock-treated wild-type cells (lane 1) or cells preincubated in the presence of 1 mM (lane 2) or 5 mM CST (lane 3) for 1 h 20 min before radiolabeling for 30 min. To prevent CPY from exiting the ER, DTT was added at 10 mM for 10 min before the radioactive pulse (Simons *et al.*, 1995). A mutant strain, *gls1-1*, lacking glucosidase I activity was similarly radiolabeled in the presence of DTT (lane 4). CPY was recovered by immunoprecipitation from the cell lysates and analyzed by SDS–PAGE. Part of the immunoisolated CPY from each sample was subjected to Endo H digestion before gel analysis (lanes 5–8).

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 ^{35}S-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

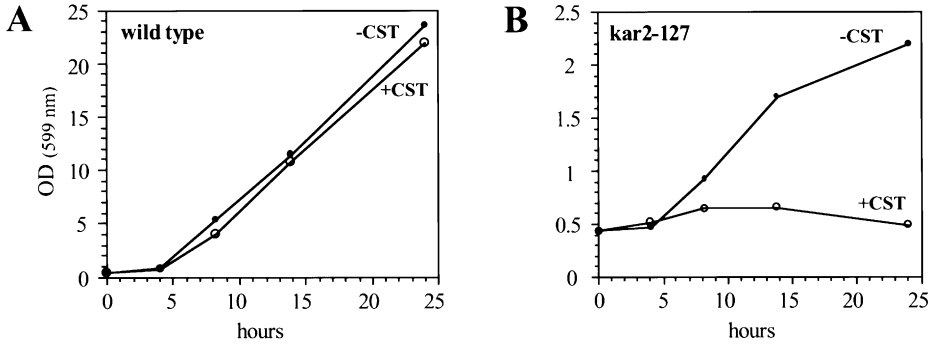

Fig. 2. Growth of the *kar2-127* strain is inhibited by CST. Wild-type (**A**) or *kar2-127* (**B**) cells were grown at 37°C in the absence (●) or presence (○) of 1 mM CST. Cell growth was monitored by measuring optical density (OD) at 599 nm.

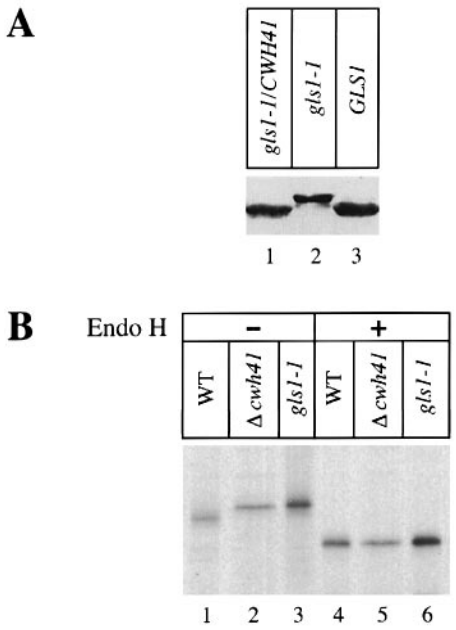

Fig. 3. *CWH41* encodes yeast ER glucosidase I. (**A**) The mobility of PDI from *gls1-1* or wild-type (WT) cells (lanes 2 and 3), or *gls1-1* cells transformed with *CWH41/GLS1* (lane 1) was analyzed by Western blot. (**B**) Wild-type, *CWH41* disrupted (Δ*cwh4I*) or *gls1-1* cells were incubated in the presence of 10 mM DTT to retain CPY in the ER (Simons *et al.*, 1995) and radiolabeled. CPY was immunoprecipitated from the cell lysates and analyzed by SDS–PAGE (lanes 1–3). Part of the samples were subjected to Endo H treatment before gel analysis (lanes 4–6).

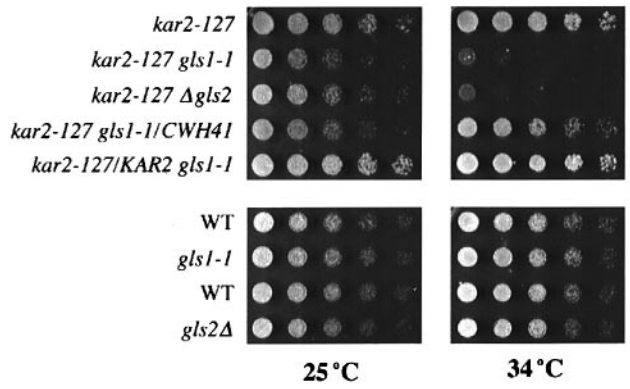

Fig. 4. Glucosidases I and II are essential for the viability of *kar2-127* cells at 34°C. The indicated strains were serially diluted (4-fold) starting at 0.2 OD_{599} units/ml, seeded onto rich medium plates, and grown at either 25 or 34°C.

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 preincubation 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

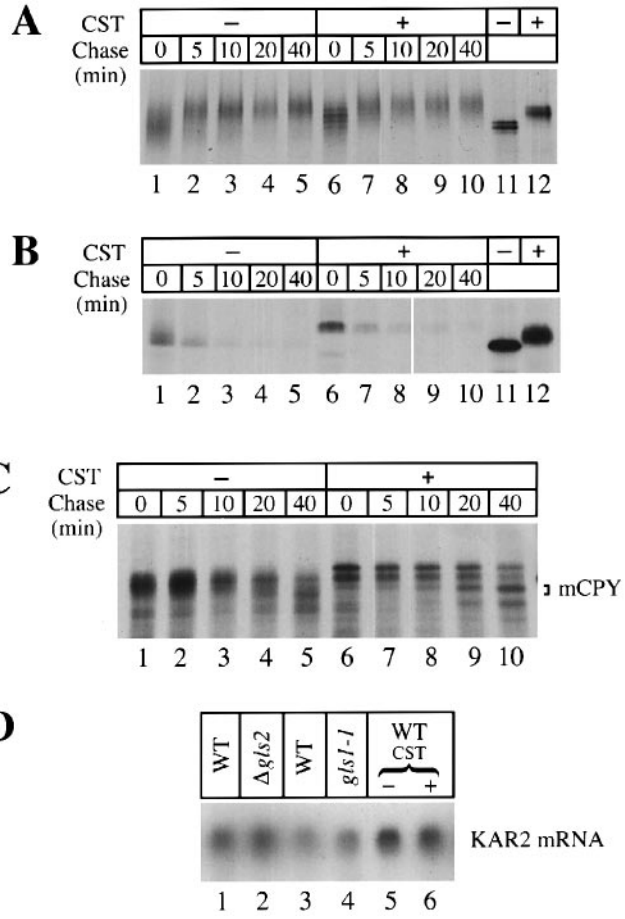

Fig. 5. Protein transport and maturation proceeds normally in CST-treated *kar2-127* cells. *kar2-127* cells were grown at 37°C for 12 h, incubated for 60 min in the absence or presence of 2 mM CST, radiolabeled for 5 min and chased for the indicated times in the presence of cycloheximide and unlabeled methionine and cysteine. Invertase (**A**), pro-α-factor (**B**) and CPY (**C**) were retrieved by immunoprecipitation from the cell lysates and analyzed by SDS–PAGE. A temperature-sensitive strain blocked in transport between ER and Golgi, *sec18-1*, provided markers for the ER-retained forms of invertase (A) and pro-α-factor (B) (lanes 11 and 12). (**D**) Induction of the unfolded protein response pathway in glucosidase-deficient cells (lanes 2 and 4), or cells treated with CST for 30 min (lane 6) was compared with untreated wild-type cells (lanes 1, 3 and 5) by monitoring *KAR2* mRNA levels by Northern-blot analysis. mCPY, mature vacuolar CPY.

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

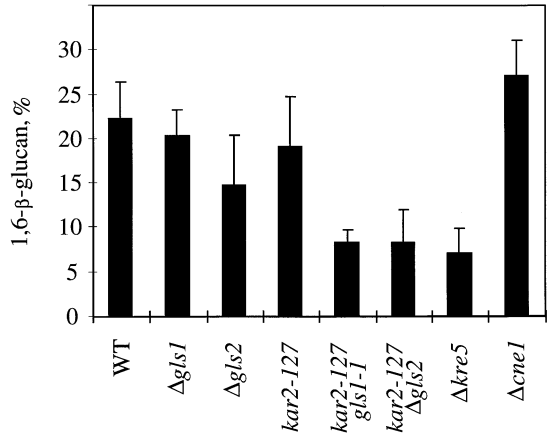

Fig. 6. Pronounced cell wall 1,6-β-glucan deficiency in glucosidase-lacking *kar2-127* strains. Cells were grown until stationary phase and the percentage alkali-insoluble 1,6-β-glucan of total cell wall alkali-insoluble glucans was determined. Each column represents the mean value of three or more experiments. The error bars indicate standard deviation of the mean.

reflected differences in strain background. The *gls1-1* strain (Esmon *et al.*, 1984), on the other hand, did show a 35% decrease of 1,6-β-glucan compared with the isogenic wild-type strain (data not shown). For the *GLS2*-deficient strain a 30% reduction was observed.

In double mutants, the amount of 1,6-β-glucan was greatly diminished. The average 1,6-β-glucan content in the *kar2-127 gls1-1* strain was 6% of total alkali-insoluble glucan—about one-third of the level in wild-type cells. In the *kar2-127* Δ*gls2* strain the 1,6-β-glucan content was 5%. The levels were essentially as low as those we measured for a *KRE5* disruptant (Meaden *et al.*, 1990). The reduction in cell wall glucan was specific for the 1,6-β-form.

We also determined the 1,6-β-glucan level in a *CNE1*-disrupted strain, as this gene encodes the only protein with homology to mammalian calnexin (De Virgilio *et al.*, 1993; Parlati *et al.*, 1995). In these cells there was no reduction of 1,6-β-glucan, instead a slight increase was observed.

Taken together, the results indicated a direct or indirect involvement of not only ER glucosidases I and II, but also of Kar2p in 1,6-β-glucan synthesis. As no defect in 1,6-β-glucan was found for *kar2-127* and only moderate changes in the single glucosidase mutants, the results indicated that Kar2p and the glucosidases contributed to the synthesis of 1,6-β-glucan in a synergistic manner.

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 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 Δ*gls2* 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.

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

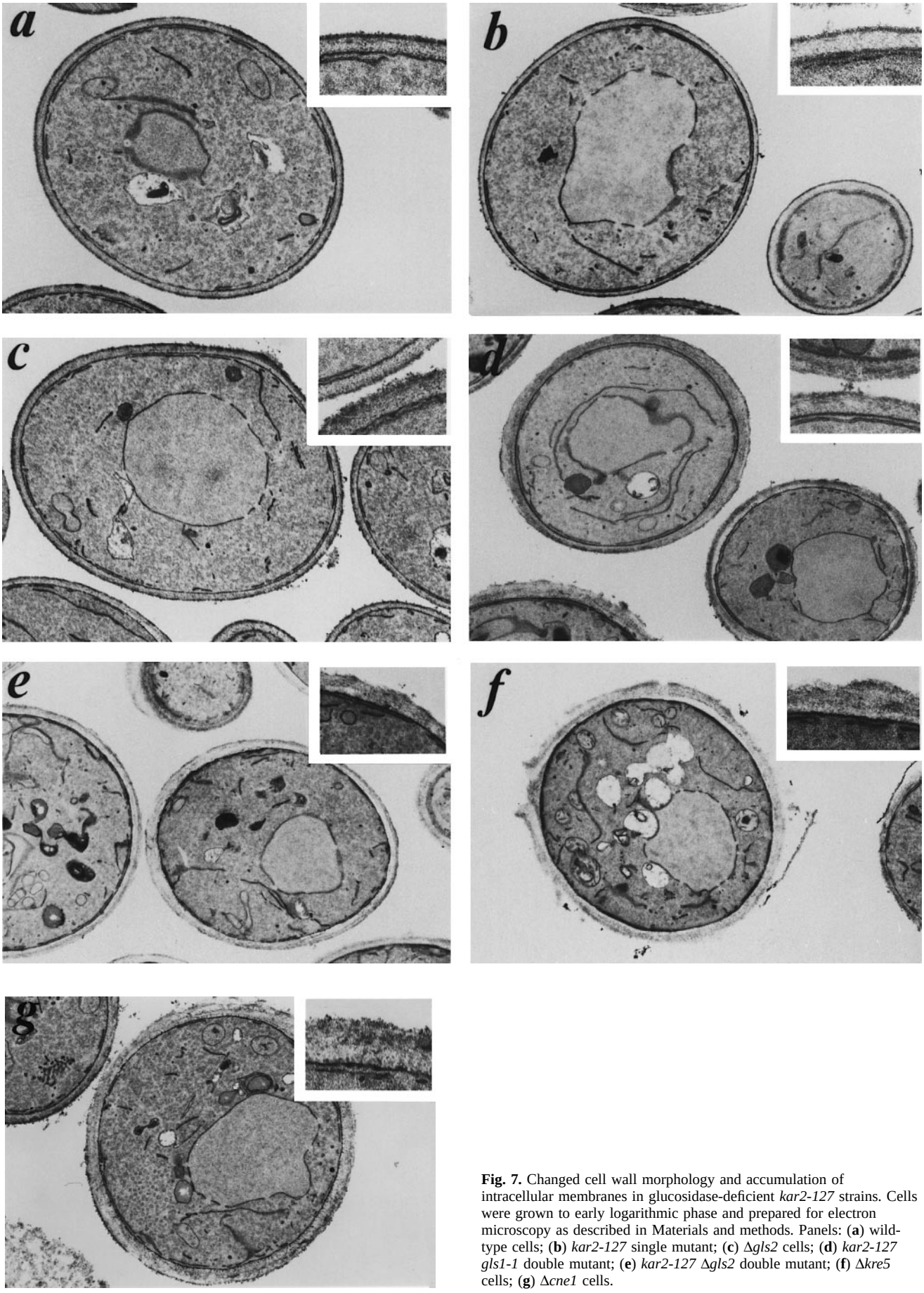

Fig. 7. Changed cell wall morphology and accumulation of intracellular membranes in glucosidase-deficient *kar2-127* strains. Cells were grown to early logarithmic phase and prepared for electron microscopy as described in Materials and methods. Panels: (**a**) wild-type cells; (**b**) *kar2-127* single mutant; (**c**) Δ*gls2* cells; (**d**) *kar2-127 gls1-1* double mutant; (**e**) *kar2-127* Δ*gls2* double mutant; (**f**) Δ*kre5* cells; (**g**) Δ*cne1* cells.

outermost layer consists of highly glycosylated mannoproteins 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 polysaccharides 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 calnexin 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 calnexin 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 calnexin cycle were not operational. We have seen examples of such redundancy between calnexin/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 α_1-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 Δ*cne1* 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.

Table I. Yeast strains used in this study

Strain	Genotype	Source
NY914	*his3Δ200, leu2-3,112, ura3-52,* MAT**a**	P.Novick
NY432	*ura3-52, sec18-1,* MATα	P.Novick
MS539	*ade2-101, ura3-52, kar2-127,* MATα	M.Rose
MS177	*ade2-101, ura3-52, kar2-159,* MATα	Vogel *et al*., 1990
MS533	*trp1Δ1, ura3-52, kar2-133,* MATα	M.Rose
MS1000	*trp1Δ1, ura3-52, kar2-1,* MATα	M.Rose
MS1351	*ade2-101, leu2-3,112, trp1Δ1, ura3-52, kar2-127,* MATα	M.Rose
MLY1601	*leu2-3,112; pep4::URA3, gls1-1,* MAT**a**	Esmon *et al*., 1984
TA405/Δkre5	*leu2-3,112, can1-100, kre5::HIS3,* MATa	Meaden *et al*., 1990
JFSY5-1	*ade2-101, ura3-52, kar2-127, gls1-1* (from cross between MS539 and MLY1601)	This study
JFSY11-1	*ade2-101, gls2::URA3, kar2-127,* MATα; derived from MS539	This study
JFSY13-1	JFSY5-1 transformed with pRS316–KAR2	This study
JFSY14-1	JFSY5-1 transformed with pBPH1–CWH41/GLS1	This study
JFSY17-1	*his3D200, leu2-3,112; gls1::URA3,* MAT**a**; derived from NY914	This study
JFSY18-1	*his3D200, leu2-3,112; cne1::URA3,* MAT**a**; derived from NY914	This study
JFSY19-1	*his3D200, leu2-3,112; gls2::URA3,* MAT**a**; derived from NY914	Trombetta *et al*., 1996

Materials and methods

Yeast strains

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

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, Pwo DNA polymerase from Boehringer-Mannheim (Indianapolis, IN) was used. Cells were grown in YPD medium [1% yeast extract, 2% bactopeptone (Difco Laboratories, Detroit, MI) and 2% glucose]. Tetrad formation was induced by growing cells on sporulation plates containing 1% potassium acetate, 0.1% bacto-yeast extract, 0.05% dextrose, 2% bacto-agar (Difco Laboratories).

Plasmid constructs and PCR

The *KAR2* gene was excised as a *Pvu*II fragment from plasmid pMR48 (Rose *et al*., 1989) and inserted into *Sma*I-digested pRS316 (Sikorski and Hieter, 1989) to obtain pRS316–KAR2. To construct pBPH1–CWH41/GLS1, *CWH41* was PCR-amplified from yeast genomic DNA isolated according to standard procedures (in Ausubel, 1994). The 5′ end primer was JFS00396 (5′-CCCAAGCTTACGCG TCGACGTGG-ATAATAACGGTTCAGG-3′) and the 3′ end primer JFS00496 (5′-CCCAAGCTTGCTTGTAAACAATAACATTCCC-3′). The PCR product was digested with *Sal*I and *Hin*dIII and inserted under the control of the alcohol dehydrogenase promoter into plasmid pBPH1 cut with the same enzymes. pBPH1 contains a *CEN4* origin of replication and a *URA3*-selective marker.

For disruption of *GLS1*, the *URA3* gene was amplified from plasmid pSFNB44 using two sets of overlapping primers: for the 5′ end JFS00596 (5′-GTAATTTAGAAGTTCAATTATTGTATGCGTGACAC-ACTATCTTTTTTTTGATTTCGG-3′) and JFS00197 (5′-GTATCGTG-ACCAATTTTAGATCATTTCGGTCAGAACAGCCACATTTTCGCG-TAATTTAGAAGTTC-3′), and for the 3′ end JFS00696 (5′-AAA-TGCTGGCTCTAACTAAGAGATGCGAGGACCCTTTTCGCCAATT-TTTTTTTTTTCGTC-3′) and JFS00297 (5′-TCTCACAGCCG-CATCGAAATGTAAATAAACGACGAGAAAAAAAAAGCTGAAA-ATGCTGGCTCTAAC-3′). This gave a PCR product in which the *URA3* gene was flanked on both sides by 90 nucleotides corresponding to *GLS1*. The construct for disruption of *GLS2* was generated as described before by Trombetta *et al*. (1996).

For disruption of the *CNE1* gene, a PCR fragment was generated from plasmid pBS–CNE1::URA3 using the *CNE1*-specific primers JFS00895 (5′-TACACGATTCTTGTACGC-3′) and JFS00995 (5′-CAATGGACCTGCCAATG-3′). The PCR product contained the *URA3* gene flanked by ~500 and 300 nucleotides of *CNE1* at the 5′ and 3′ ends, respectively. Transformation with this construct caused an internal deletion of 5/6 of the *CNE1* open reading frame.

All disruptions were confirmed by PCR analysis of chromsomal DNA isolated from the transformed cells.

Growth in the presence of CST

Cells were grown in the presence of 1 mM CST (Sigma) in YPD medium in a total volume of 400 μl. 20 μl of the cell suspension was removed at timed intervals and the OD_{599} was measured. That CST remained active throughout the 23 h incubation was confirmed by subjecting cell lysates from the 23 h time-point to Western blot analysis using an anti-protein disulfide isomerase (PDI) antibody. Only PDI of the slower mobility diagnostic of incomplete glucose trimming was present.

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 μg of the transforming DNA, and the mixture was incubated for 30 min at 30°C. 450 μl 40% (w/v) PEG 3350 (Sigma) in LiTE (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 H_2O 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 preincubated 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

Spores were screened for the presence of glucosidase I activity by assessing the electrophoretic mobility of PDI by Western blot analysis.

To determine which spores from the *gls1-1 kar2-159* cross carried the *kar2* mutation, the spores were grown at 37°C. At this temperature the *kar2-159* mutation alone causes cell death. For every tetrad analyzed, two spores were viable and two non-viable. All glucosidase deficient colonies that grew poorly at 25°C were found to carry the *kar2-159* allele.

To demonstrate that the *kar2-127* allele caused the growth phenotype in the presumptive *gls1-1 kar2-127* double mutants, two representative slow-growing *gls1-1* carrying spores were transformed with the single copy plasmid, pRS316-KAR2, which contained the wild-type *KAR2* gene. The growth of the transformants was compared with that of the untransformed mother cells at 25 and 34°C.

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 [^{35}S]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 beads, 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 at 25°C to a density of 1 ODU/ml, and RNA was prepared from 10 ml of cell suspension. Total RNA was isolated by extraction with hot acidic phenol (Ausubel, 1994). 15 µg RNA of each sample was 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′-ATGTTTTTCAACAGAC-TAAGCGCTGGC-3′) and JFS01395 (5′-CTACAATTCGTCGTGTTCG-3′), and spanned the entire open reading frame of *KAR2*. 125 µCi [α-^{32}P]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.

Glucan 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 H_2O 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 Corp., 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 glucose in the various fractions was determined by the phenol–sulfuric acid assay (McKelvy 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 H_2O. The cells were immediately pelleted at 5000 *g* for 10 min at 4°C and washed in 10 ml H_2O. 200 µl of pelleted cells were resuspended in 5 ml of 4% $KMnO_4$ in H_2O and incubated on a nutator in the dark for 2–4 h at 4°C. After five washes in 10 ml H_2O, 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.

Acknowledgements

We want to thank Dr M.D.Rose for kindly providing the *kar2* mutant strains, and Dr S.Ferro-Novick for many helpful discussions and for generously providing reagents, including anti-invertase and anti-α-factor antibodies, and laboratory space. We are also grateful to Dr R.Schekman for the MLY1601 strain, Dr H.Bussey for the TA405 Δ*kre5* strain, Dr W.J.Lennarz for providing the anti-PDI antibody, and Drs P.Novick and P.Brennwald for the anti-CPY antibody. We also want to thank Dr Antti Seppo for technical advice and Drs J.Peterson, S.Trombetta, B.Sodeik, J.-X.Zhang and M.Aebi for critical review of the manuscript. This work was supported by a postdoctoral fellowship from Human Frontiers Science Program (LT-140/93) to J.F.S. and a grant from the National Institute of Health (CA46128) to A.H. Support was also provided by a grant awarded to S.Ferro-Novick from the National Institute of Health (CA46128).

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Received August 20, 1997; revised and accepted November 13, 1997