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Increase in Chitin as an Essential Response to Defects in Assembly of Cell Wall Polymers in the *ggp1*Δ Mutant of *Saccharomyces cerevisiae*

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The *GGP1*/*GAS1* gene codes for a glycosylphosphatidylinositol-anchored plasma membrane glycoprotein of *Saccharomyces cerevisiae*. The *ggp1*Δ mutant shows morphogenetic defects which suggest changes in the cell wall matrix. In this work, we have investigated cell wall glucan levels and the increase of chitin in *ggp1*Δ mutant cells. In these cells, the level of alkali-insoluble 1,6-β-D-glucan was found to be 50% of that of wild-type cells and was responsible for the observed decrease in the total alkali-insoluble glucan. Moreover, the ratio of alkali-soluble to alkali-insoluble glucan almost doubled, suggesting a change in glucan solubility. The increase of chitin in *ggp1*Δ cells was found to be essential since the *chs3*Δ *ggp1*Δ mutations determined a severe reduction in the growth rate and in cell viability. Electron microscopy analysis showed the loss of the typical structure of yeast cell walls. Furthermore, in the *chs3*Δ *ggp1*Δ cells, the level of alkali-insoluble glucan was 57% of that of wild-type cells and the alkali-soluble/alkali-insoluble glucan ratio was doubled. We tested the effect of inhibition of chitin synthesis also by a different approach. The *ggp1*Δ cells were treated with nikkomycin Z, a well-known inhibitor of chitin synthesis, and showed a hypersensitivity to this drug. In addition, studies of genetic interactions with genes related to the construction of the cell wall indicate a synthetic lethal effect of the *ggp1*Δ *kre6*Δ and the *ggp1*Δ *pkc1*Δ combined mutations. Our data point to an involvement of the *GGP1* gene product in the cross-links between cell wall glucans (1,3-β-D-glucans with 1,6-β-D-glucans and with chitin). Chitin is essential to compensate for the defects due to the lack of Ggp1p. Moreover, the activities of Ggp1p and Chs3p are essential to the formation of the organized structure of the cell wall in vegetative cells.

Yeasts and fungi are surrounded by a cell wall that is essential for maintenance of the cell shape, for prevention of lysis, and for regulation of the uptake of substances from the environment (for a review, see reference 18). The cell wall structure is dynamic and can adapt to different physiological states (e.g., conjugation, sporulation, and stationary phase) or to morphological changes, as in *Candida albicans* during the transition from yeast to hyphal growth (5). In recent years, *Saccharomyces cerevisiae* has been subjected to genetic analyses which led to the identification of several genes involved in the biosynthesis of cell wall polymers. In particular, glucans, mannoproteins, and chitin constitute about 60, 40, and 1%, respectively, of the total cell wall (18). The most abundant glucan is alkali insoluble with a degree of polymerization of 1,500 glucose residues. These are composed principally of linear 1,3-β linkages, 3% of which are branched through 1,6-β linkages. This fraction also contains the 1,6-β-D-glucan of about 140 1,6-β-linked residues, with a small portion of 1,3-β-linked residues. This glucan is released by extensive digestion with 1,3-β-D-glucanase and constitutes about 20% of the total carbohydrates of the alkali-insoluble fraction. Also, chitin is always found in the alkali-insoluble fraction. The alkali-soluble fraction consists of 1,6-β-D-glucan and 1,3-β-D-glucan (18). On the basis of several different studies, the alkali-soluble glucan appears to be the precursor of the alkali-insoluble one, and the linkage to chitin appears to be a relevant element for the change in alkali solubility (18, 19). In the cell wall, the mannoproteins are responsible for permeability properties (6). A new class of cell wall mannoproteins, the glucomannoproteins, has recently been discovered. They are connected through a 1,6-β-D-glucan bridge to 1,3-β-D-glucan (16, 21). This finding provides an explanation for the previous observation of mannan-containing 1,6-β-D-glucan and 1,3-β-D-glucan in the alkali-soluble and -insoluble fractions (9). The characterization of the biosynthetic pathway of 1,6-β-D-glucan exploited K1 killer toxin, which allowed the isolation of several *kre* mutants defective in the synthesis of the glucosyl or mannosyl side chains of glucomannoproteins (18). The biosynthesis of 1,6-β-D-glucan occurs along the secretory pathway and requires the sequential function of several gene products.

The knowledge of biosynthesis of 1,3-β-D-glucan is less advanced. Only recently, the gene *FKS1*/*ETG1*/*CWH53*/*PBR1*/*CND1*/*GNS1*, which encodes the putative catalytic subunit of the 1,3-β-D-glucan synthase, and its homolog *FKS2* have been isolated (5, 15). The synthesis of this polymer takes place at the plasma membrane level by a multisubunit enzyme regulated by a soluble G protein (8).

Chitin, a 1,4-β-linked homopolymer of *N*-acetylglucosamine, is important for morphogenetic events in yeast. Three genes, *CHS1*, *CHS2*, and *CHS3*, which encode the putative catalytic subunits of chitin synthases 1, 2, and 3, respectively, have been isolated (3, 5). The product of *CHS1* performs repair functions during cell separation, that of *CHS2* is responsible for chitin synthesis in the primary septum, and that of *CHS3* is responsible for chitin synthesis in the ring at bud emergence and along the lateral cell wall. In vegetatively growing yeast cells, chitin is present mainly in the septum and in the ring at bud

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emergence; after cell separation, it remains in the bud scar of the mother cell. The *CTS1* gene encodes a chitinase that is important in the hydrolysis of chitin during cell separation and is active only at acidic pH (20).

The synthesis and assembly of the cell wall polymers are strictly regulated so that an organized structure is formed. Upon electron microscopy analysis, this structure presents a typical outer electron-dense layer composed of mannoproteins and an amorphous inner electron-transparent layer composed of glucans and chitin. Other activities necessary for the remodelling, for example, the activities of endo- or exoglucanases, are being characterized in several laboratories (5). Other genes, *KNR4* and *HKR1*, encode proteins which appear to be involved in the regulation of 1,3-β-D-glucan synthesis (14, 17).

In this work, we investigated cell wall defects and the increase of chitin in the *ggp1*Δ mutant. The *GGP1/GAS1/CWH52* gene (23, 25, 35) encodes a glycoprotein which contains a glycosylphosphatidylinositol that functions as an anchor for plasma membrane attachment, in contrast to cell wall mannoproteins that transiently contain a glycosylphosphatidylinositol to be released when the mannoproteins link to the glucan (7, 21). The phenotype of *ggp1*Δ mutant cells suggests an important role for the Ggp1 protein in morphogenesis (24, 25). A homolog of *GGP1*, *PHR1*, has been isolated in *C. albicans*, and also in this microorganism the related protein was found to be involved in morphogenesis (32).

MATERIALS AND METHODS

Strains and growth conditions. *Escherichia coli* JM101 [Δ(*lac proAB*) *thi strA supE endA sbcB hsdR* F′ *traD36 proAB lacI*q *lacZ*Δ*M15*] was the host strain for recombinant DNA manipulations. The *S. cerevisiae* haploid strain WB2d (*ggp1*::*LEU2*) was generated from the wild-type strain W303-1B (*MAT*α *ade2-1 his3-11,15 trp1-1 ura3-1 leu2-3,112 can1-100*) and from the same strain but of opposite mating type (W303-1A) by one-step gene disruption (30). Strain SEY6210 (*MAT*α *leu2-3,112 ura3-52 his3*Δ*200 trp*Δ*901 lys2-801 suc2*Δ*9*) and its *kre2*Δ (*kre2*::*TRP1*) and *kre6*Δ (*kre6*::*HIS3*) derivatives were kindly provided by Howard Bussey (McGill University, Montreal, Quebec, Canada). Strains 6D (*MAT***a** *ura3 leu2*) and 6B (*MAT***a** *cal1*::*URA3 ura3 leu2*) and the W303-1B strain carrying the *cal1*::*LEU2* disruption were kindly provided by A. Duran (Universidad de Salamanca, Salamanca, Spain). Strain GPY1115 (*MAT***a** *pkc1*::*HIS3 leu2-3,112 ura3-52 his3*Δ*200 trp*Δ*901 suc2*Δ*9 ade2-101*) was kindly provided by G. Paravicini (Glaxo Institute for Molecular Biology, Geneva, Switzerland). Yeast cells were grown in batches at 30°C in Difco yeast nitrogen base medium without amino acids (YNB-aa; 6.7 g/liter) containing 2% glucose and the required supplements or in YEPD (1% yeast extract, 2% Bacto Peptone, 2% glucose). The regeneration of spores was carried out also in medium supplemented with 0.5 M KCl. Cell number, volume distribution, and percentage of budded cells were determined as previously described (38).

DNA manipulations and yeast genetic techniques. Standard procedures were used for recombinant DNA manipulations (31). Transformation of yeast cells was carried out by the lithium acetate procedure (13). Standard techniques were used for diploid construction, sporulation, and tetrad dissection. Null mutants were constructed by the one-step gene replacement procedure using a plasmid containing the *GGP1* gene inactivated by the insertion of a *LEU2* gene (35).

Glucan analysis. Alkali-insoluble 1,3- and 1,6-β-D-glucans were isolated and quantified as previously described by Boone et al. (2). Alkali-soluble glucans were estimated from NaOH extraction supernatants by precipitating carbohydrates with 2 volumes of ethanol at −20°C. The total carbohydrate content of each fraction was measured as hexoses by the borosulfuric acid method (1). The data reported are the means of three experiments.

Measurement of chitin level. The chitin level was measured as previously described (34). The residue of digestion with Zymolyase 100T (4 mg/ml of Tris HCl [10 mM; pH 7.4] for 20 h at 37°C) of the alkali-insoluble pellet (about 100 mg [dry weight] of cells) was hydrolyzed in 6 N HCl at 105°C overnight. After desiccation, the samples were resuspended in H_2O and glucosamine was measured as follows. A 0.5-ml volume of solution A (1.5 N Na_2CO_3 in 4% acetylacetone) was added to 1 ml of sample, the mixture was incubated at 100°C for 20 min, and 3.5 ml of 96% ethanol was added. Forty-five minutes after the addition of 0.5 ml of solution B (1.6 g of *p*-dimethylaminobenzaldehyde in 30 ml of concentrated HCl and 30 ml of ethanol), the absorbance at 520 nm was measured and compared with a standard curve of 0 to 200 μg of glucosamine.

Electron microscopy. Samples were prepared and then stained with uranyl acetate and lead citrate or with periodic acid-thiocarbohydrazide-silver proteinate (PATAg test) as previously described (24). To label chitin with wheat germ agglutinin (WGA), cells were treated as described previously (24).

TABLE 1. β-Glucan and chitin levels in *ggp1*Δ cells

Strain	Carbohydrate content[a]				Alk$_s$/Alk$_i$[c]	Chitin[d]
	Alkali-insoluble			Alkali-soluble 1,3- + 1,6-β-D-glucan		
	1,3- + 1,6-β-D-glucan	1,6-β-D-glucan	ZP[b]			
W303-1B (*GGP1*)	164	52	16	93	0.6	2.7
WB2d (*ggp1*Δ)	122	27	32	137	1.1	36

[a] Carbohydrate content (measured by the borosulfuric acid method) expressed as micrograms per milligram (dry weight) of cells.
[b] ZP, zymolyase-undigestible pellet.
[c] Alk$_s$, alkali soluble; Alk$_i$, alkali insoluble.
[d] Glucosamine content in ZP fractions (micrograms per milligram [dry weight] of cells).

For 1,3-β-D-glucan immunolabeling, thin sections were incubated for 15 min on 5% normal goat serum (IGS-NGS) (Jansenn, Wantage, Oxons, United Kingdom) and then incubated for 1 h at 18°C with anti-β1,3 rabbit antibodies (22) (Euromedex, Schiltigheim, France) diluted 1:5,000 in 20 mM Tris–20 mM NaN_3–225 mM NaCl–0.5% bovine serum albumin–0.1% Tween 20 or Triton X-100–1% normal goat serum. After incubation for 1 h at 18°C in goat anti-rabbit secondary antibody labeled with gold particles (15-nm diameter; Auroprobe EM [Jansen]), the sections were treated as described previously (24). Observations were made with a Philips CM10 electron microscope at 80 kV.

Liquid broth microdilution assay. To quantify drug resistance, 75 μl of a suspension containing 2×10^5 cells/ml was added to an equal volume of minimal medium containing 1:2 serial dilutions of nikkomycin Z (Calbiochem, La Jolla, Calif.) in a 96-well microtiter plate. All determinations were performed in duplicate. As a control, four wells contained minimal medium without the drug. The plates were then incubated at 30°C for 48 h, and the growth in the presence and absence of the drug was measured by reading the turbidity at 600 nm.

RESULTS

Analysis of the glucan levels of the *ggp1*Δ mutant. We have previously shown that the *ggp1*Δ mutant is characterized by several phenotypic defects. Mutant cells are round and larger, arrest at stationary phase with a percentage of budded cells around 60 to 70%, are resistant to enzymatic digestion by a 1,3-β-D-glucanase (zymolyase), and display in electron microscopy analysis an increased content of chitin in lateral cell walls (24). The growth rate is also particularly reduced at pHs near neutrality. Moreover, *ggp1*Δ cells are hypersensitive to growth in the presence of an osmotic destabilizing agent, sodium dodecyl sulfate (36). This suggests that the cell wall is weakened, allowing the normally nonpermeable molecules of the detergent to penetrate more easily. The *ggp1*Δ cells are also hypersensitive to growth in the presence of Calcofluor White (26).

In order to characterize the changes occurring in the cell wall of *ggp1*Δ cells, we determined the levels of the glucans and quantified the increase of chitin in exponentially growing cells. As shown in Table 1, the mutant has a distribution of glucans different from that of the parental strain (W303-1B). The total alkali-insoluble glucan decreases in the mutant. The level of the alkali-insoluble 1,6-β-D-glucan undergoes a 50% decrease. In contrast, by subtracting these values from the alkali-insoluble glucan levels it can be observed that the level of 1,3-β-D-glucan in the mutant is roughly the same as that in the control. The alkali-soluble fraction increases, so that the ratio of alkali-soluble to alkali-insoluble glucan roughly doubles in the mutant. The carbohydrate content of the insoluble material obtained after the digestion of the alkali-insoluble fraction with zymolyase was also determined (ZP in Table 1). This fraction represents the glucan still linked to chitin and eventually the glycogen. The increase of this fraction is consistent with the increase of chitin previously observed (24) since glycogen, being accumulated in stationary-phase cells, should be absent in

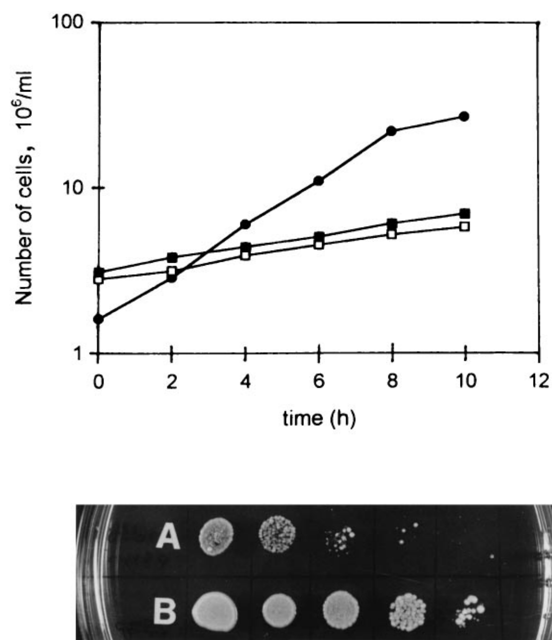

FIG. 1. Growth curve and viability of *chs3*Δ *ggp1*Δ cells. (Top) Growth curve of 6B cells (*chs3*Δ) (●) and 6Bd cells (*chs3*Δ *ggp1*Δ) (■) in minimal medium at 30°C. The growth of 6Bd cells in the presence of 0.5 M KCl is also shown (□). (Bottom) Cell viability assay. Dilutions (1:10) of a concentrated suspension of exponentially growing cells were spotted on YEPD medium and incubated at 30°C for 48 h. A, 6Bd cells (*chs3*Δ *ggp1*Δ); B, 6B cells (*chs3*Δ).

these cells. We also directly measured the chitin content of the ZP fraction, which is known to contain the cell wall chitin, by determining the glucosamine content. An increase of more than 10-fold was found in the mutant cells, in agreement with the previous observation (Table 1, last column).

Thus, for the *ggp1* mutant, the analysis of the cell wall polymers indicates that the glucans have changed their solubility in alkali, being more abundant in the alkali-soluble fraction, while the decrease of the alkali-insoluble fraction is due mainly to the marked decrease of 1,6-β-D-glucan. The increase of chitin could be a response to a stress condition of the cell wall, and the validity of this hypothesis has been tested.

Effects of *chs3* gene disruption in the *ggp1*Δ mutant. The *GGP1* gene was inactivated in a *chs3*Δ haploid strain (6B) which carries an inactivated copy of the *CHS3* gene encoding the putative catalytic subunit of chitin synthase 3. This enzyme is responsible for the deposition of chitin in the lateral cell walls and in the ring present in the septum between the mother cell and the bud (33). Cells of strain 6B (*chs3*::*URA3*) containing double null mutations (*chs3*::*URA3 ggp1*::*LEU2*) were isolated, and the derived strain was named 6Bd. The *GGP1* gene disruption was confirmed by Southern analysis, and the complete lack of the Ggp1 protein in the mutant cells was confirmed by immunoblotting (data not shown). In Fig. 1 (upper panel), the growth curves of strains 6B and 6Bd are shown. The 6Bd cells grow very slowly, with a doubling time of about 8 h, whereas the doubling time of 6B cells is 2 h. No difference between the rates of growth of 6B and the control strain 6D was found (data not shown). The slow-growth defect of 6Bd was not osmotically remediable, since the presence of 0.5 M KCl in the medium did not modify the growth rate. Before starting to grow, the cells exhibited a very long lag period. The viability of these cells decreased dramatically in stationary phase (Fig. 1, lower panel). During stationary phase, the 6Bd cells remain in a budded state, with 70% of the cells having one or more buds and abnormal morphologies, and lysis appears to occur. Finally, exponentially growing cells are resistant to Zymolyase, although less so than the single *ggp1*Δ mutant (24).

Cell wall ultrastructure of the *chs3*Δ *ggp1*Δ mutant. We analyzed the ultrastructure of the cell wall of strains 6B and 6Bd. The 6B cells show a normal structure, whereas the walls of the double-mutant cells have lost the typical layered structure, which has been replaced with a fibrillar electron-dense matrix (Fig. 2). The cell wall appears less compact, especially in the septum region, where an increase in thickness was often observed. In this analysis, as a control of the absence of chitin in the lateral cell walls of the *chs3*Δ mutant, the cells were labeled with WGA-gold. Labeling all over the lateral cell walls and in the ring was not detected as expected for cells with a deletion in the *CHS3* gene (Fig. 2, inset). Moreover, by Calcofluor White staining, 6B cells showed almost no fluorescence, whereas the control cells (6D) showed definite fluorescence in the bud scars and at the mother-daughter junction (ring), in agreement with the expected phenotype (data not shown).

Analysis of the glucan levels in the *chs3*Δ *ggp1*Δ mutant. To better characterize the changes affecting the cell wall of the *chs3*Δ-derived mutant cells, we determined the glucan levels. As shown in Table 2, the lack of Chs3 and Ggp1 proteins determines changes in glucan level and solubility. The total alkali-insoluble fraction shows a 32% decrease in the double mutant (6Bd) compared to that of the single mutant (6B) and 55% in comparison with the control cells (6D). The alkali-soluble fraction progressively increases, raising the ratio of alkali-soluble to alkali-insoluble fractions from 0.5 to 1.1. As a control, the chitin level was also measured, and it decreases from 1.7 in the control cells to 0.3 μg/mg of cells (dry weight) in the *chs3*Δ cells, in agreement with previous data which indicate a decrease of chitin in *chs3*Δ cells (37). Chitin molecules synthesized by chitin synthase 3 can bind through a 1,4-β linkage to the nonreducing end of 1,3-β-D-glucan chains (19), and, as mentioned in the introduction, this linkage appears to be relevant to the formation of the alkali-insoluble glucan (11, 29). In agreement with this, we found a 20% decrease in the alkali-insoluble glucan in 6B cells in comparison with the level in control cells (6D). In order to determine if other *chs3*Δ strains gave similar results, we measured the glucan levels of a different disruptant obtained by replacement of the Chs3p gene with a *chs3*::*LEU2* construct in a W303 genetic background. As reported in Table 2, a similar decrease in the alkali-insoluble fraction was found. Thus, our data confirm that Chs3p is involved in the formation of alkali-insoluble glucan, although the contribution we detected is less relevant than that reported by other authors using different types of experimental protocols which employ measurements of radioactive glucose (11, 29).

We performed an immunoelectron microscopy localization of the 1,3-β-D-glucan with anti-1,3-β-D-glucan antiserum. Figure 3 shows that in strain 6Bd the 1,3-β-D-glucan is uniformly distributed along the cell wall with a labeling less intense than that in 6B cells, in agreement with the results obtained with the chemical assay. In the micrographs, it can also be observed that the labeling is restricted to the cell wall. No labeling is present inside the cell, as expected from the fact that the plasma membrane is the site of synthesis of 1,3-β-D-glucan.

In conclusion, these data indicate that the synthesis of chitin in the lateral cell walls is crucial for the growth and viability of *ggp1*Δ cells and that Chs3p and Ggp1p cooperate in the construction of an organized cell wall.

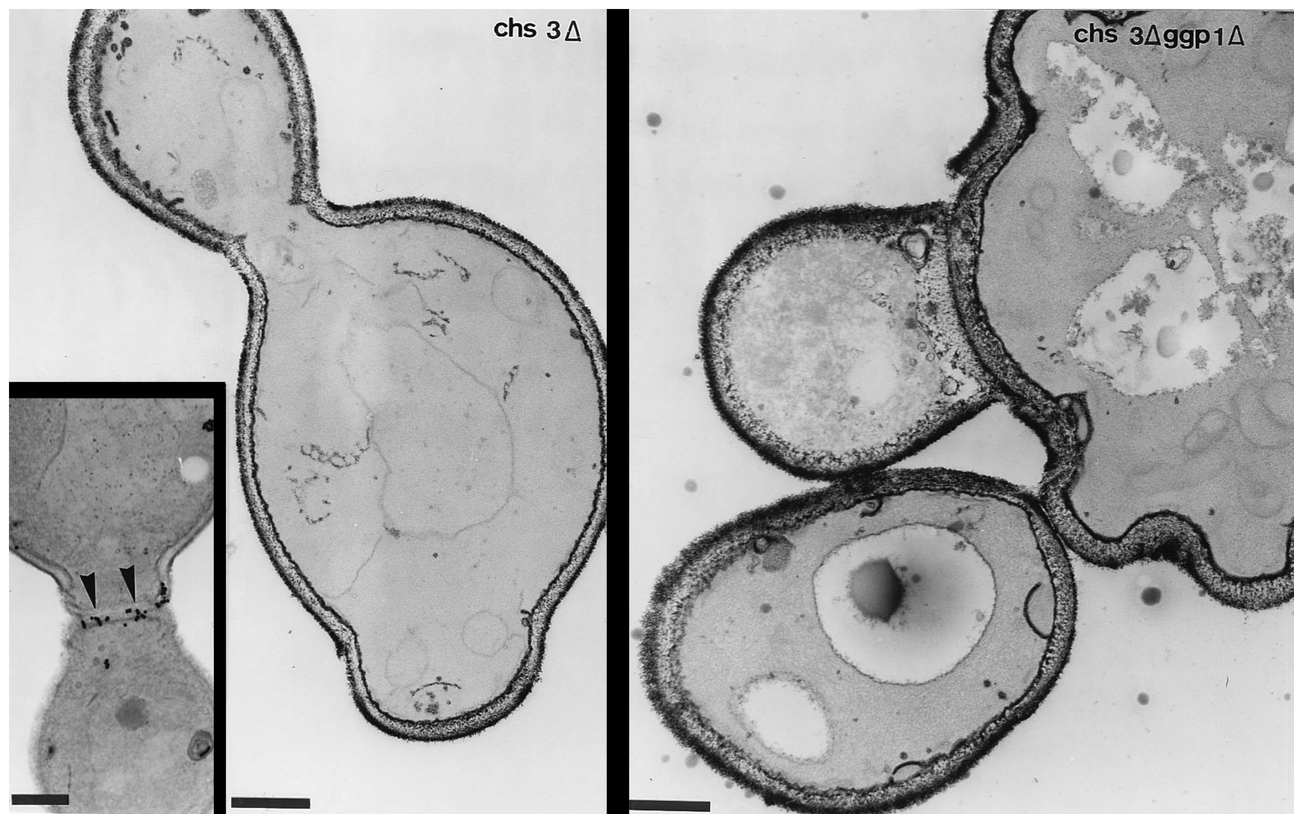

FIG. 2. Cell wall ultrastructure of *chs3Δ ggp1Δ* cells. 6B cells (*chs3Δ*) show a thin cell wall reactive with PATAg staining for polysaccharide. The labeling with the complex WGA-gold allows location of chitin exclusively over the septum (inset, arrowheads), as expected in a *chs3* mutant (33). 6Bd cells (*chs3Δ ggp1Δ*) show abnormal bud scars and a wall with a loose texture and an abundant electron-dense outer layer. An increase of thickness of the cell wall is particularly evident in the septum area. Bars, 0.5 μm.

***ggp1Δ* mutant cells are hypersensitive to nikkomycin Z.** If the increase of chitin in the *ggp1Δ* mutant is a cellular response necessary to counteract the defects of a weakened cell wall, *ggp1Δ* cells should be more sensitive than wild-type cells to the action of an inhibitor of chitin synthesis. We tested the sensitivity of cells of strain W303-1B and its derivative WB2d, containing the *ggp1* null mutation, to growth in the presence of nikkomycin Z, a competitive inhibitor of chitin synthases 2 and 3 in vitro and a selective inhibitor of chitin synthase 3 in vivo (4, 10). The growth of the parental strain is not affected even at high concentrations of inhibitor, whereas the growth of WB2d cells begins to be inhibited at a low concentration and is completely blocked at a concentration of 100 μM (Fig. 4).

Thus, by two different approaches to inhibiting the chitin synthesis directed by chitin synthase 3, one based on a mutation and the other on an inhibitor, a common effect of reduced growth of *ggp1Δ* cells is observed.

TABLE 2. β-Glucan levels of *chs3Δ ggp1Δ* cells

Strain	Carbohydrate content[a]		Alk_s/Alk_i[b]
	Alkali-insoluble 1,3- + 1,6-β-D-glucan	Alkali-soluble 1,3- + 1,6-β-D-glucan	
6D (*CHS3 GGP1*)	260	129	0.5
6B (*chs3Δ GGP1*)	212	120	0.6
6Bd (*chs3Δ ggp1Δ*)	144	156	1.1
W303-1B (*CHS3*)	265	99	0.4
W303-1B (*chs3Δ*)	219	124	0.6

[a] Expressed as micrograms per milligram (dry weight) of cells.
[b] Alk_s, alkali soluble; Alk_i, alkali insoluble.

Analysis of genetic interaction with *KRE* genes and the *PKC1* gene. Since the results obtained suggest that Ggp1p is involved in the assembly of cell wall polymers, the effect of the combination of a *ggp1* null mutation with null mutations in genes related to the construction of the cell wall and its regulation was investigated. We studied the effects of the combination of a *ggp1* null mutation and disruption of *KRE1*, involved in the remodeling of the 1,6-β-D-glucan chains (2); *KRE2*, which is involved in the elongation of O-linked oligosaccharide chains (12); and *KRE6*, involved in the elongation of the 1,6-β-D-glucan chains (27). The *GGP1* gene was disrupted in the haploid *kre1Δ* and *kre2Δ* mutants and in the corresponding parental strain. The *kre1Δ ggp1Δ* and the *kre2Δ ggp1Δ* cells showed phenotypes very similar to that of the single *ggp1Δ* mutant, indicating no additional effects. Since no *kre6Δ ggp1Δ* clone was isolated, we crossed a strain carrying a *ggp1Δ* disruption with the strain containing the *kre6Δ* mutation. A total of 42 tetrads were analyzed, and no spore with the phenotype (Leu^+ His^+) expected for the double mutation was isolated. Dissection and regeneration of the spores were also performed in the presence of 0.5 M KCl to prevent possible lytic effects due to the combination of the two mutations. This strongly suggests that *ggp1Δ* is synthetically lethal in combina-

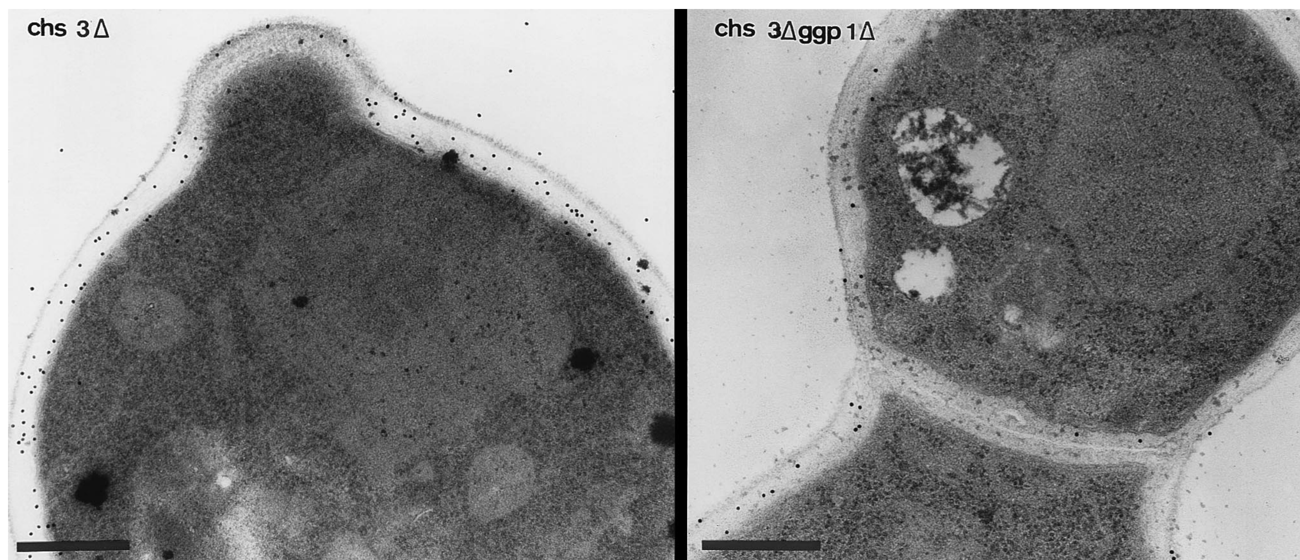

FIG. 3. Ultrastructure immunolocalization of 1,3-β-D-glucan in *chs3*Δ and *chs3*Δ *ggp1*Δ cells. In the *chs3*Δ cells, the gold particles are widespread along the whole wall thickness. In the *chs3*Δ *ggp1*Δ cells, the number of the particles is lower. Bars, 0.4 μm.

tion with the *kre6*Δ mutation and points toward a genetic interaction essential for spore germination and possibly also for vegetative growth of yeast cells.

A synthetically lethal combination was also found between *ggp1*Δ and *pkc1*Δ; the *ggp1*Δ *pkc1*Δ double heterozygote was sporulated and dissected onto osmotically supplemented YEPD medium. Analysis of spores of 41 tetrads indicated that the *ggp1*Δ *pkc1*Δ cells were inviable.

DISCUSSION

In this work, we focused our attention on the cell wall defects of a *ggp1*Δ mutant. The phenotype of the mutant and the cell surface localization of Ggp1p led us to hypothesize that the function of Ggp1p is expressed at the level of the cell wall matrix. The abnormal resistance to Zymolyase digestion exhibited by cells lacking Ggp1p could be ascribed to (i) a decreased accessibility of the enzyme to the substrate (1,3-β-D-glucan),

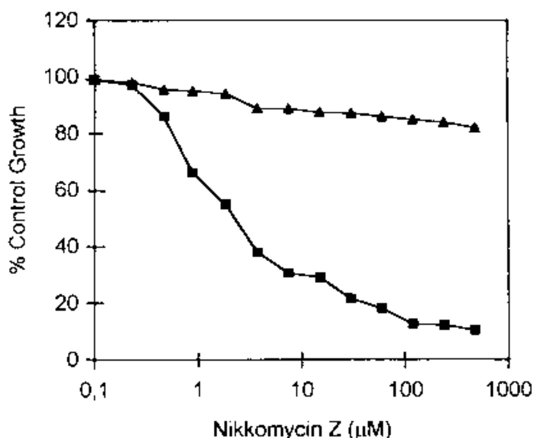

FIG. 4. Sensitivity of *ggp1*Δ cells to nikkomycin Z. Wild-type cells (strain W303-1B) and *ggp1*Δ mutant cells (strain WB2d) were tested for sensitivity to nikkomycin Z by microdilution assay as described in Materials and Methods. ▲, W303-1B; ■, WB2d.

(ii) changes in the linkage among polymers, or (iii) absence of the substrate. The first hypothesis is not valid, since the resistance to Zymolyase is exhibited also in isolated cell walls (data not shown). The substrate is present in the cell walls without substantial changes in its level, as the analysis of the glucans indicates. It appears more probable that the chains of 1,3-β-D-glucan are arranged in such a way that the enzymatic reaction is impaired. Consistent with these observations, the glucan analysis presented here indicates that the network of glucans is modified in the *ggp1*Δ cells. The different distributions of the glucan in the alkali-soluble and -insoluble fractions indicate that the levels of alkali-insoluble 1,3-β-D-glucan are almost the same. This is in agreement with preliminary indications that the *cwh52* mutant has no changes in the activity of the 1,3-β-D-glucan synthase (26). The level of 1,6-β-D-glucan appears to be particularly affected. This glucan is responsible for the anchorage of mannoproteins to the 1,3-β-D-glucan fibrils (16, 21). This decrease could be due to reduced 1,6-β-D-glucan synthesis or to a reduction of the linkage with the 1,3-β-D-glucan fibrils. Experiments performed in another laboratory indicate that *ggp1*Δ cells release 1,3-β-D-glucan into the medium (17a), suggesting that the 1,3-β-D-glucan molecules or also the complex mannoprotein–1,6-β-D-glucan–1,3-β-D-glucan could be less cross-linked in the mutant. Further experiments will be performed to test this hypothesis and to test if Ggp1p can catalyze transglucosylation reactions.

The synthetic lethality we found for the combination of *ggp1*Δ and *kre6*Δ mutations suggests that the 1,6-β-D-glucan level, which is reduced to 50% in a single *kre6*Δ mutant (27), further decreases in the double mutant, thus becoming incompatible with cell viability.

The level of chitin is increased in *ggp1*Δ cells, and the results obtained with the *chs3*Δ *ggp1*Δ mutant and with nikkomycin Z indicate that this increase is essential to *ggp1*Δ cell viability. More chitin synthesized by chitin synthase 3 is laid down in the lateral cell walls of the *ggp1*Δ mutant than in the wild type, and this appears to be important for its integrity. Alternatively, it can be hypothesized that a mechanism of coordination of chitin synthesis and linkage to 1,3-β-D-glucan in which Ggp1p

would be involved is not functional in *ggp1*Δ cells, thus causing the increase in the chitin level and the alkali-soluble fraction.

The increase of chitin in *ggp1* cells does not appear to be mediated by regulation at the transcriptional level of the catalytic subunit of chitin synthase 3. In fact, preliminary experiments suggest that the level of *CHS3* mRNA in the mutant cells does not change compared to that of the wild type (data not shown). This is consistent with other observations concerning chitin synthase 3 regulation which indicate that the enzyme is modulated by other factors (*CAL2*/*CSD4* or *CAL3* gene products) which are present in limiting amounts (4).

We have examined the relationship to the *PKC1* gene. The basis of the synthetic lethality of *ggp1*Δ *pkc1*Δ double mutants is not known but is consistent with a further weakening of the *pkc1* cell wall, presumably by exaggeration of the 1,6-β-D-glucan defect, as previously observed for the *kre6*Δ *pkc1*Δ double mutant (28). Alternatively, it can be proposed that the signal transduction pathway of PKC1 and the downstream mitogen-activated protein kinase cascade, indicated to be involved in maintenance of cell integrity (5), could be important for the signalling of the cell wall defects of the *ggp1*Δ mutant. Experiments to test this hypothesis are under way.

In conclusion, the lack of Ggp1p and Chs3p damages the cells and causes a loss of the organized structure of the cell wall. Thus, the presence of both Chs3p and Ggp1p is important for the organization of the yeast cell wall. Our data indicate that the yeast cells are endowed with the capability to compensate for alterations of the structure and/or composition of the cell wall matrix. The existence of such compensatory mechanisms for cell wall defects, together with the presence of gene redundancy, may provide an explanation for the absence of lethality associated with the disruption of many cell wall-related genes. In this regard, it must be emphasized that for the *GGP1* gene four homologs have been identified in the whole yeast genome sequence and that future studies to determine the function and regulation of these genes will be important.

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