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Altered Extent of Cross-Linking of β1,6-Glucosylated Mannoproteins to Chitin in *Saccharomyces cerevisiae* Mutants with Reduced Cell Wall β1,3-Glucan Content

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Center for Fungal Cell Wall Research, Institute of Molecular Cell Biology, University of Amsterdam, Amsterdam, The Netherlands[1]; Laboratory of Biochemistry and Metabolism, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland 20892[2]; and Instituto de Bioquimica Vegetal y Fotosintesis, Consejo Superior de Investigaciones Cientificas, Universidad de Sevilla, 41080-Seville, Spain[3]

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The yeast cell wall contains β1,3-glucanase-extractable and β1,3-glucanase-resistant mannoproteins. The β1,3-glucanase-extractable proteins are retained in the cell wall by attachment to a β1,6-glucan moiety, which in its turn is linked to β1,3-glucan (J. C. Kapteyn, R. C. Montijn, E. Vink, J. De La Cruz, A. Llobell, J. E. Douwes, H. Shimoi, P. N. Lipke, and F. M. Klis, Glycobiology 6:337–345, 1996). The β1,3-glucanase-resistant protein fraction could be largely released by exochitinase treatment and contained the same set of β1,6-glucosylated proteins, including Cwp1p, as the β1,3-glucanase-extractable fraction. Chitin was linked to the proteins in the β1,3-glucanase-resistant fraction through a β1,6-glucan moiety. In wild-type cell walls, the β1,3-glucanase-resistant protein fraction represented only 1 to 2% of the covalently linked cell wall proteins, whereas in cell walls of *fks1* and *gas1* deletion strains, which contain much less β1,3-glucan but more chitin, β1,3-glucanase-resistant proteins represented about 40% of the total. We propose that the increased cross-linking of cell wall proteins via β1,6-glucan to chitin represents a cell wall repair mechanism in yeast, which is activated in response to cell wall weakening.

The cell wall is crucial for the integrity of *Saccharomyces cerevisiae*. Its rigid structure maintains the shape of the cell and offers protection against harmful environmental conditions (6, 19). The wall is mainly composed of β-glucans and mannoproteins, in addition to smaller amounts of chitin and lipids (6). The glucans, which are interwoven with the chitin fibrils, form the inner skeletal layer of the cell wall, whereas the outer layer consists of mannoproteins. The majority of the cell wall mannoproteins are anchored into the wall through covalent linkages to heteropolymers of β1,6- and β1,3-glucan (18, 26, 41, 43) (Fig. 1). The β1,6-glucosyl moiety of these polymers is phosphodiester-linked to protein as shown by its sensitivity to treatment with ice-cold aqueous hydrofluoric acid (HF) and phosphodiesterases (18). This observation, together with data from other studies, pointed to a glycosylphosphatidylinositol (GPI)-derived structure as the attachment site for β1,6-glucan (3, 23, 40, 42, 45). The β1,3/β1,6-glucan heteropolymer was proposed to be identical to the alkali-soluble β1,3/β1,6-glucan studied by Fleet and Manners (7, 8). In *S. cerevisiae*, this alkali-soluble glucan becomes alkali insoluble through a linkage with chitin (11, 24) via a β1,4 bond from the terminal reducing residue of chitin to the nonreducing end of β1,3-glucan (20). Furthermore, by digesting cell walls with β1,3-glucanase, followed by incubation with exochitinase, a heterogeneous high-molecular-weight complex was isolated (21). Structural studies indicated that in this complex the terminal reducing residue of chitin is linked to β1,6-glucan (21). The nonreducing end of the β1,6-glucan polymer is bound to the GPI-derived glycan part of a cell wall protein, whereas its reducing terminus is linked to β1,3-glucan (21) (Fig. 1).

We have studied the same β1,3-glucanase-resistant, exochitinase-solubilized, high-molecular-weight complex with different methodologies involving the use of antibodies and lectins and have focused on the protein components. We found that in wild-type strains, the β1,3-glucanase-resistant proteins represented only 1 to 2% of the total amount of covalently bound cell wall proteins. In contrast, in *fks1* and *gas1* deletion mutants, which grow as large spherical cells and which are disturbed in β1,3-glucan synthesis (4, 13) and assembly (30, 33), respectively, this percentage increased to about 40%. These data indicate that, when β1,3-glucan is lacking, cell wall proteins are rather cross-linked to chitin via a β1,6-glucosyl moiety. We therefore propose that the increased cross-linking of proteins to a β1,6-glucan-chitin complex represents a rescue mechanism in yeast to compensate for a reduction in cell wall β1,3-glucan content.

MATERIALS AND METHODS

Strains and growth media. *S. cerevisiae* SEY6210 (*MATα leu2-3,112 ura3-52 his3-Δ200 lys2-801 trp1-Δ901 suc2-Δ9*), FY833 (*MAT***a** *leu2Δ1 ura3-52 his3-Δ200 lys2Δ202 trp1Δ63*), FY834 (*MATα leu2Δ1 ura3-52 his3-Δ200 lys2Δ202 trp1Δ63), AR100 (MATα fks1*::*HIS3* in FY834), AR104 (*MAT***a** *gas1*::*LEU2* in FY833), and AR113.1B (*MAT***a** *gas1*::*LEU2 fks1*::*HIS3* in FY833) were used. Cells were grown at 28°C either in YPD medium (1% [wt/vol] yeast extract, 1% [wt/vol] Bacto Peptone, and 3% [wt/vol] glucose) or in synthetic medium containing 0.17% (wt/vol) yeast nitrogen base without amino acids (Difco), 2% (wt/vol) glucose, 0.5% (wt/vol) casein hydrolysate, 0.5% (wt/vol) ammonium sulfate, 0.25% (wt/vol) succinate, 0.01% (wt/vol) uracil, 0.01 (wt/vol) tryptophan, and 0.01% (wt/vol) adenine sulfate at pH 5.5.

Isolation of cell wall proteins. Cells were harvested, washed, and homogenized at 0°C with glass beads in a Bead-Beater (26, 44). Cell walls were collected by centrifugation and rinsed extensively with 1 M NaCl and 1 mM phenylmethylsulfonyl fluoride. Isolated cell walls were boiled in the presence of sodium dodecyl sulfate (SDS), EDTA, and β-mercaptoethanol to extract noncovalently linked cell wall proteins and to remove any cytosolic contaminants (25, 26, 44).

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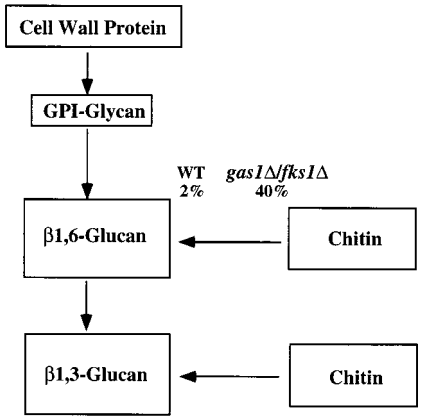

FIG. 1. Known cross-linkages in the yeast cell wall and compensatory changes in yeast cell walls in response to a reduction in β1,3-glucan. This model is based on data from Kapteyn et al. (19), Kollar et al. (22), and this paper. The arrows denote the reducing ends of the polysaccharides.

SDS-extracted walls were subsequently digested with Quantazyme (Quantum Biotechnologies Inc., Montreal, Canada) (1,500 U g^{-1} [wet weight of cell walls]), a recombinant β1,3-glucanase (17). Quantazyme-treated cell walls were digested at 37°C for 18 h with exochitinase (0.3 U g^{-1} [wet weight of cell walls]) by the method of Kollár et al. (20) in 50 mM sodium phosphate buffer, pH 6.3. In other experiments, cell walls were digested with an endo-β1,6-glucanase (0.8 U g^{-1} [wet weight of cell walls]) isolated from *Trichoderma harzianum* (2) or treated with ice-cold aqueous (50% [vol/vol]) hydrofluoric acid (HF) as described previously (18).

Determination of protein content in isolated cell walls. Isolated cell walls (50 mg wet weight) were extracted twice in SDS extraction buffer for 5 min at 100°C, rinsed five times with water, and heated in 1 N NaOH (100 μl) at 100°C for 10 min. Subsequently, the alkaline extract was neutralized by adding 1 N HCl (100 μl), and the protein concentration in the neutralized extract was determined with the bicinchoninic acid protein assay reagents (Pierce) with bovine serum albumin (BSA) as the reference protein.

Determination of protein incorporation by labeling with ^{14}C-protein hydrolysate. Labeling of cells was performed essentially as described by Valentin et al. (39). Cells were precultured at 28°C in synthetic medium to an optical density at 530 nm (OD_{530}) of 1.0 (equivalent to 10^7 cells ml^{-1}). Cells were collected, resuspended in 90 ml of fresh synthetic medium (OD_{530} = 0.5; equivalent to 5×10^6 cells ml^{-1}), and incubated for 15 min at 28°C. Subsequently, 0.46 MBq of ^{14}C-protein hydrolysate (Amersham) was added, and the cultures were grown for 3 h at 28°C. Cells were harvested by centrifugation at 3,000 × *g* for 5 min and washed twice in 20 mM Tris-HCl, pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride. Labeled cell walls were isolated and extracted with SDS essentially as described by Van Rinsum et al. (44). The SDS extracts were separated from the cell walls by centrifugation for 5 min at 14,000 × *g*. The cell wall pellets were washed five times with water and digested with the glucanases and exochitinase according to the procedures described above. Protein contents of the different fractions were calculated as percentages of total label incorporated into SDS-extracted walls.

Determination of chitin content in isolated cell walls. Chitin levels of alkali-extracted cell walls were measured as described by Tracey (38). In brief, the alkali-insoluble wall fraction was hydrolyzed in 6 N HCl at 100°C overnight. After lyophilization, the samples were resuspended in water. 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. One hour 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 absorbance values on a standard curve of 0 to 200 μg of glucosamine (30).

Analysis of cell wall proteins. Cell wall proteins were separated by electrophoresis with linear-gradient 2.2 to 20% polyacrylamide gels and electrophoretically transferred onto an Immobilon polyvinylidene difluoride (PVDF) membrane (26). Cell wall mannoproteins were visualized by probing the membranes with peroxidase-labeled concanavalin A (ConA) (1 μg ml^{-1}) in phosphate-buffered saline (PBS) containing 3% (wt/vol) bovine serum albumin (BSA), 2.5 mM $CaCl_2$, and 2.5 mM $MnCl_2$. Western immunoblot analyses were performed with affinity-purified polyclonal antisera directed against β1,6-glucan and β1,3-glucan, as described before (17, 26). Western analysis was also carried out with anti-Cwp1p antiserum as described by Kapteyn et al. (18). In some cases, the PVDF filters were also probed with peroxidase-labeled wheat germ agglutinin (WGA) (50 ng ml^{-1}) in PBS, containing 3% (wt/vol) BSA. WGA is a lectin that specifically recognizes (chains of) β1,4-linked *N*-acetylglucosamine residues (10). The blots were visualized with enhanced chemiluminescence Western blotting detection reagents (Amersham) according to the manufacturer's instructions.

RESULTS

Analysis of β1,3-glucanase-resistant cell wall proteins. To isolate cell wall proteins that are linked to chitin through β1,6-glucan, cell walls were first digested extensively with Quantazyme, a pure recombinant endo-β1,3-glucanase, to remove all β1,3-glucanase-extractable proteins (18, 21). When the protein content of the cell wall was measured by ^{14}C labeling, it was found that after Quantazyme treatment the protein content of the cell walls—expressed as a percentage of the total label incorporated into SDS-extracted cell walls—diminished by 98% (Table 1). Reincubation with Quantazyme did not release more radiolabel (data not shown), indicating that all β1,3-glucanase-extractable proteins were removed. The high percentage of proteins released by Quantazyme supports our idea that the β1,3/β1,6-glucan heteropolymer is responsible for the anchoring of the majority of the cell wall proteins (18) (Fig. 1). The remaining β1,3-glucanase-resistant ^{14}C-labeled proteins could largely be released by digestion with a pure exochitinase (Table 1) and also with a purified endo-β1,6-glucanase (data not shown). This is consistent with recent observations that the relatively small population of proteins is linked to chitin, with β1,6-glucan as the intermediate moiety (21) (Fig. 1). This was further explored by analyzing the proteins released by exochitinase.

Exochitinase liberated a high-molecular-mass, polydisperse smear (>180 kDa) that was reactive with both peroxidase-labeled ConA (Fig. 2A, lane 1) and WGA (Fig. 2A, lane 3), indicating that these proteins were mannosylated and that they were associated with chitin. Binding to WGA was not affected by periodate treatment of the proteins (data not shown), which is in agreement with chitin being insensitive to periodate oxidation. The exochitinase-liberated smear was also highly reactive with β1,6-glucan antiserum (Fig. 2A, lane 5) but reacted very weakly with β1,3-glucan antiserum (data not shown), demonstrating that these proteins were β1,6-glucosylated but hardly contained any β1,3-glucan epitope. The weak immunoreactivity with the β1,3-glucan antiserum was expected, as these proteins were pretreated with Quantazyme. When the exochitinase-released material was probed with the anti-Cwp1p antiserum, a smear was seen (Fig. 2A, lane 7), suggesting that Cwp1p released by the exochitinase had a high and heterogeneous molecular mass, presumably because it was associated with β1,6-glucan and chitin.

As a next step, the exochitinase-solubilized proteins were treated with purified endo-β1,6-glucanase to investigate

TABLE 1. Chitin and protein levels in *S. cerevisiae* cell walls and solubilization of SDS-resistant ^{14}C-labeled cell wall proteins

Strain	Chitin content[a] (μg mg^{-1})	Protein content[b] (μg mg^{-1})	% Solubilized[c]	
			Quantazyme	Exochitinase after Quantazyme
FY833	16	25.5	98.5	99.7
*gas1*Δ	85	37.5	58.4	91.2
*fks1*Δ	128	47.9	62.7	83.2
*gas1*Δ *fks1*Δ	106	46.7	59.7	92.0

[a] Determined by the Elson-Morgan method and expressed as micrograms per milligram (dry weight) of SDS- and alkali-extracted cell walls.
[b] Determined by the bicinchoninic acid-protein assay and expressed as micrograms per milligram (dry weight) of SDS-extracted cell walls.
[c] Determined by ^{14}C labeling of cell wall proteins.

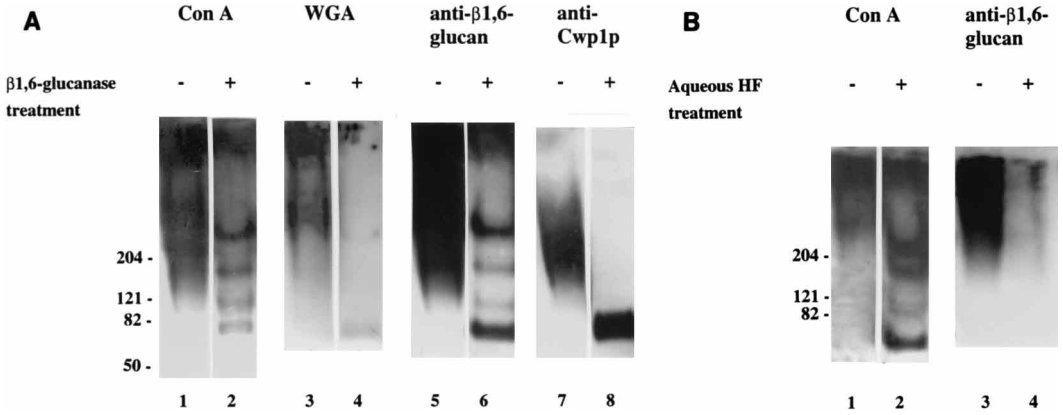

FIG. 2. Analysis of β1,3-glucanase-resistant cell wall proteins of *S. cerevisiae* SEY6210 released by exochitinase. The liberated proteins were detected with peroxidase-labeled ConA, peroxidase-labeled WGA, anti-β1,6-glucan antibodies, or anti-Cwp1p antibodies. (A) Analysis of exochitinase-released proteins before and after treatment with endo-β1,6-glucanase; (B) analysis of exochitinase-released proteins before and after treatment with aqueous HF. The sizes of standard molecular mass markers are indicated. The enzymes used did not react with the lectins and antisera used (data not shown).

whether the heterogeneity of exochitinase-released Cwp1p and other proteins was due to their association with a complex of chitin remnants and β1,6-glucan and to confirm that β1,6-glucan was responsible for linking these proteins to chitin. In that case, it was expected that the exochitinase-liberated proteins would decrease in size and would lose their ability to bind WGA but would keep part of their antigenicity towards the β1,6-glucan antiserum. The antigenicity of the β1,6-glucanase-treated proteins towards the β1,6-glucan antiserum was probably not lost entirely, as an endoglucanase leaves small chains of glucose residues which are too short to be digested further. Indeed, the endo-β1,6-glucanase treatment resulted in the disappearance of the ConA- and WGA-reactive, β1,6-glucosylated smear containing Cwp1p and also resulted in the appearance of four major ConA-reactive, β1,6-glucosylated bands having apparent molecular masses of 230, 145, 93, and 68 kDa (Fig. 2A, lanes 2 and 6). Most likely, the observed weak reactivity of the ConA-reactive protein bands with WGA (Fig. 2A, lane 4) can be attributed to the binding of this lectin to the Manβ-1,4-GlcNAcβ-1,4-GlcNAcβ-*N*-Asn units in the *N*-glycosidically linked side chains of the proteins, as has been reported before (10). As expected, the four ConA-stained bands were not recognized by the β1,3-glucan antiserum (data not shown). The 68-kDa band was the only one which reacted positively with the anti-Cwp1p antiserum (Fig. 2A, lane 8). The set of β1,3-glucanase-resistant, β1,6-glucosylated mannoproteins could also be released directly by digestion of the Quantazyme-resistant cell wall fraction by endo-β1,6-glucanase (Fig. 3, lanes 1, 3, and 5). Taken together, the data demonstrate that the heterogeneity of the exochitinase-released proteins, including Cwp1p, is explained by their association with a complex containing β1,6-glucan and remnants of chitin. Moreover, the observation that the exochitinase-released, endo-β1,6-glucanase-treated proteins still contained β1,6-glucan, but no chitin epitope, demonstrates that β1,6-glucan functions as a bridge connecting protein and chitin (Fig. 1).

β1,6-glucan is phosphodiester-linked to β1,3-glucanase-resistant proteins. We have reported earlier (16–18) that the Quantazyme-extractable proteins have a phosphodiester-bound β1,6-glucosyl moiety, as these moieties were sensitive to cleavage by phosphodiesterases and ice-cold aqueous HF. This also held true for the Zymolyase-resistant exochitinase-released protein fraction (21). It was therefore investigated whether aqueous HF could release the β1,6-glucan epitope from the Quantazyme-resistant proteins. This turned out to be the case: after treatment with aqueous HF the smear of β1,6-glucosylated mannoproteins (Fig. 2B, lanes 1 and 3) disappeared and, subsequently, none of the four major cell wall mannoproteins (Fig. 2B, lane 2) were recognized by the β1,6-glucan antiserum (Fig. 2B, lane 4). Accordingly, the molecular sizes of the HF-treated proteins were on average about 14 kDa smaller than those of the β1,6-glucanase-treated ones. Again, the lowest band strongly reacted with the anti-Cwp1p antiserum (data not shown). Consistent with these observations, aqueous HF extraction of β1,3-glucanase-pretreated cell walls also resulted in the release of the yeast cell wall proteins, including Cwp1p, without β1,6-glucan (Fig. 3, lanes 2, 4, and 6) and chitin epitopes (data not shown). Consequently, the data strongly support the findings by Kollár et al. (21) that a β1,6-glucan moiety is phosphodiester-linked to the β1,3-glucanase-resistant, exochitinase-extractable cell wall proteins.

Retention of cell wall proteins in *gas1*Δ, *fks1*Δ, and *gas1*Δ *fks1*Δ strains. As shown in Table 1, in wild-type cells only a very small minority of the covalently linked cell wall proteins were resistant to β1,3-glucanase digestion, because they were retained in the cell wall through phosphodiester linkages to

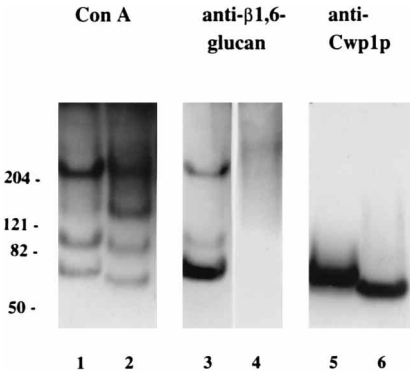

FIG. 3. Analysis of β1,3-glucanase-resistant cell wall proteins of *S. cerevisiae* SEY6210 released by endo-β1,6-glucanase or aqueous HF. The liberated proteins were detected with peroxidase-labeled ConA, anti-β1,6-glucan antibodies, or anti-Cwp1p antibodies. Lanes 1, 3, and 5, β1,6-glucanase-released proteins; lanes 2, 4, and 6, aqueous HF-released proteins. The sizes of standard molecular mass markers are indicated.

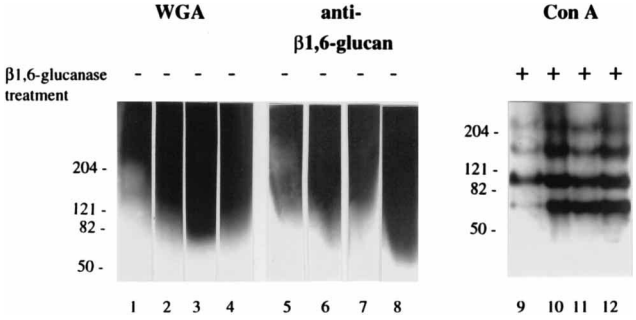

FIG. 4. Analysis of β1,3-glucanase-resistant, exochitinase-released cell wall proteins obtained from various cell wall mutants of *S. cerevisiae*. Liberated proteins were detected with peroxidase-labeled WGA (lanes 1–4), anti-β1,6-glucan antibodies (lanes 5–8), or peroxidase-labeled ConA (lanes 9–12). Lanes 1, 5, and 9, strain FY833; lanes 2, 6, and 10, *gas1*Δ strain; lanes 3, 7, and 11, *fks1*Δ strain; lanes 4, 8, and 12, *gas1*Δ *fks1*Δ strain. Before analysis (lanes 9–12), the liberated proteins were treated with endo-β1,6-glucanase. Note that for the mutants, four times less cell equivalents were applied than for the wild type. The sizes of standard molecular mass markers are indicated.

β1,6-glucan–chitin heteropolymers (Fig. 2 and 3). This is probably a reflection of chitin being a minor cell wall component in wild-type cells. For this reason, we decided to explore this mechanism of cell wall protein anchorage in the *fks1*Δ and *gas1*Δ mutants of *S. cerevisiae*, which are known to have elevated amounts of chitin, presumably as compensation for the diminished β1,3-glucan content resulting from the lost Fks1p and Gas1p activities, respectively (30, 31, 33). This was confirmed here, as the chitin levels were increased in *gas1*Δ, *fks1*Δ, and *fks1*Δ *gas1*Δ cell walls by approximately five-, eight-, and sevenfold, respectively. Interestingly, as measured by ^{14}C labeling, about 40% of the covalently bound cell wall proteins in *fks1*Δ, *gas1*Δ, and *fks1*Δ *gas1*Δ cells appeared to be Quantazyme resistant (Table 1). Subsequent digestion with exochitinase released most Quantazyme-resistant proteins from the *gas1*Δ, *fks1*Δ, and *fks1*Δ *gas1*Δ cell walls, leaving approximately 10, 17, and 8%, respectively, of the ^{14}C-labeled material behind in the cell wall of the tested mutants (Table 1). It is also notable that approximately 47% more cell wall proteins were incorporated into *gas1*Δ cell walls than into walls of the corresponding wild type, whereas the protein contents of *fks1*Δ and *fks1*Δ *gas1*Δ cell walls were raised by about 85% (Table 1). One should, however, take into account that as a consequence of normalizing the data on the basis of cell wall dry weight, the observed increases in protein content might to a large extent reflect the reductions in β1,3-glucan levels. Western analyses confirmed that the deletion mutants contained considerably larger amounts of exochitinase-extractable, WGA-stainable, β1,6-glucan-containing cell wall proteins than did wild-type cells (Fig. 4; compare lane 1 with lanes 2, 3, and 4). As revealed after β1,6-glucanase treatment of the exochitinase-released wall material, Cwp1p in particular (Fig. 4, lanes 9–12; first band from the bottom) was very abundant in the exochitinase-solubilized protein fraction of the mutant cells. Similar to wild-type cell wall proteins, upon β1,6-glucanase treatment the exochitinase-solubilized *gas1*Δ, *fks1*Δ, and *fks1*Δ *gas1*Δ cell wall proteins still possessed a small β1,6-glucan side chain but did not contain β1,3-glucan and chitin epitopes (data not shown). Consequently, the data indicate that as a consequence of a reduction in β1,3-glucan synthesis and/or assembly the cross-linking of cell wall proteins through β1,6-glucan to chitin is enhanced (Fig. 1).

DISCUSSION

The majority of the wall proteins of *S. cerevisiae* wild-type cells are retained by covalent association with β1,6-glucan which is itself coupled to β1,3-glucan (18) (Fig. 1). This was confirmed here, as 98% of all SDS-resistant proteins in the cell wall could be solubilized by β1,3-glucanase digestion of the cell wall (Table 1). The linkage between these proteins and β1,6-glucan was reported to be of the phosphodiester type (18), which is in line with other studies providing evidence for a GPI-derived structure as an attachment site for β1,6-glucan (3, 23, 40, 42, 45). There are conflicting data concerning the exact structure of the GPI remnant of mature incorporated cell wall proteins. Müller et al. (28) presented some evidence that the complete GPI anchor is removed from the wall-bound form of Gce1p, a cyclic AMP-binding protein, in a two-step process involving phospholipase C and subsequent proteolytic activity. In contrast, Lu et al. (22) reported that the mature wall-bound form of α-agglutinin had a trimmed GPI anchor, without the original inositol residue and the lipid moiety but still containing ethanolamine. Recently, Kollár et al. (21) established that β1,6-glucan was bound to the protein through a GPI-derived moiety containing only five α-linked mannosyl residues. Our observations that the β1,6-glucan side chains of isolated cell wall proteins are insensitive to nitrous acid deamination (15a), a common procedure to cleave the glucosamine-inositol linkage in GPI anchors (5), are consistent with the attachment of β1,6-glucan to a GPI anchor remnant lacking glucosamine and inositol.

In this paper, we further demonstrated that a small subpopulation of proteins, representing only 1 to 2% of all covalently bound cell wall proteins in yeast wild-type strains (Table 1), is retained in the wall by a slightly different mechanism (Fig. 1). These proteins, including Cwp1p, are resistant to β1,3-glucanase extraction of the wall, because their β1,6-glucan side chains are cross-linked not only to β1,3-glucan but also to chitin. This was shown by the extractability of the Quantazyme-resistant proteins with an exochitinase and an endo-β1,6-glucanase and by their reactivity with WGA and the β1,6-glucan antiserum, respectively (Table 1; Fig. 2A). It was further demonstrated that the proteins are attached to the chitin part of the complex through a β1,6-glucosyl moiety. Similar to the β1,3-glucanase-extractable cell wall proteins (18), the β1,6-glucosyl moiety was shown to be linked to proteins through phosphodiester bridges (Fig. 2B and 3), confirming that the attachment of β1,6-glucan to the β1,3-glucanase-resistant proteins was also GPI dependent (21). Consequently, the data of this and previous work (18, 21), together with the observations that severe β1,6-glucan defects in numerous *kre*Δ mutant strains lead to secretion of cell wall proteins (14, 23), point to an essential "cementing" function of β1,6-glucan, interconnecting all other components of the yeast cell wall.

In comparison to wild-type cells, in which only a small number of cell wall proteins were attached to chitin through β1,6-glucan (Table 1), *gas1*Δ and *fks1*Δ cells, which were defective in β1,3-glucan assembly and concomitantly showed an increased deposition of chitin (30, 31) and an enhanced incorporation of Cwp1p (32, 33), contained about 20- to 30-fold more β1,3-glucanase-resistant cell wall proteins (Table 1; Fig. 4). Especially Cwp1p was very abundant in the β1,3-glucanase-resistant, chitinase-soluble wall fraction of *gas1*Δ and *fks1*Δ cells (Fig. 4, lanes 9–12; first band from the bottom). The relatively high percentage of β1,3-glucanase-resistant cell wall proteins in *fks1*Δ and *gas1*Δ cells is in line with the observed Zymolyase resistance of these mutants (31, 31a). The *gas1*Δ *fks1*Δ strain contained an amount of β1,3-glucanase-resistant cell wall pro-

tein approximately similar to those of both single-deletion strains (Table 1).

As proposed by Popolo et al. (30) and Ram et al. (33), the increased amounts of chitin and chitin-bound β1,6-glucosylated proteins in *fks1*Δ and *gas1*Δ cell walls might be attributed to a rescue mechanism responsive to cell wall weakening. In support of this, in *knr4*Δ cells, which have a weakened cell wall due to a 50% reduction in the amount of β1,3-glucan, the level of chitin was found to be five times higher than in wild-type strains (12). Moreover, we found that wild-type cells treated with sublethal concentrations of papulacandin B, a known β1,3-glucan synthesis inhibitor, became large and spherical and that this was accompanied by increased levels of chitin (10a). The putative compensatory mechanism might be mediated by the protein kinase C-dependent signal transduction pathway, which is supposed to be activated upon plasma membrane stretch, presumably via mechanosensitive channels, in response to cell wall weakening (15). This idea was supported by the finding that *pkc1*Δ *gas1*Δ and *pkc1*Δ *fks1*Δ double disruptants are synthetically lethal (9, 30). Interestingly, synthetic lethality of the *pkc* null mutant with several *kre*Δ mutations has also been reported (35). In addition, all *cwh* cell wall mutants isolated by Ram et al. (34) were found to be hypersensitive to staurosporine (15a), a well-known inhibitor of Pkc1p (37, 46), suggesting that cell wall mutations become lethal in yeast when the Pkc1p-mediated rescue mechanisms are inactivated. Interestingly, Popolo et al. (30) observed that the double-null mutants in *GAS1* and *CHS3* grow very poorly and have a disorganized cell wall, supporting the idea that chitin plays a key role in the proposed rescue mechanism, which is operative in cells with *gas1*Δ and, probably, also *fks1*Δ genetic backgrounds. These authors also reported a synthetically lethal combination between *gas1*Δ and *kre6*Δ cells, which is in agreement with our findings that the formation of β1,6-glucan is absolutely required for the cross-linking of proteins to chitin.

It seems likely that in nature yeast, and fungi in general, are often exposed to attack by foreign β1,3-glucanases from various sources, leading to a loss in β1,3-glucan and a concomitant weakening of the cell wall. We therefore propose that the compensatory mechanisms observed in, e.g., *fks1*Δ and *gas1*Δ cells, in which the β1,3-glucan level was lowered by genetic means, and in papulacandin B-treated wild-type cells, in which β1,3-glucan synthesis was inhibited biochemically, might be generally used by fungal cells.

Finally, β1,3-glucanase-extractable β1,6-glucosylated cell wall proteins have also been identified in the yeasts *Exophiala dermatitidis* (27) and *Hansenula polymorpha* (1a), in the dimorphic pathogenic fungus *Candida albicans* (16, 17), and in several filamentous fungi belonging to the *Ascomycetes* (1, 36). This raises the question of whether these fungal species also contain cell wall proteins that are linked to chitin through β1,6-glucan. Especially in filamentous fungi, which in general have higher levels of chitin in their cell walls than yeasts, this particular cell wall protein retention mechanism might be important.

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