Presence of a Large β(1-3)Glucan Linked to Chitin at the Saccharomyces cerevisiae Mother-Bud Neck Suggests Involvement in Localized Growth Control

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Previous results suggested that the chitin ring present at the yeast mother-bud neck, which is linked specifically to the nonreducing ends of β(1-3)glucan, may help to suppress cell wall growth at the neck by competing with β(1-6)glucan and thereby with mannoproteins for their attachment to the same sites. Here we explored whether the linkage of chitin to β(1-3)glucan may also prevent the remodeling of this polysaccharide that would be necessary for cell wall growth. By a novel mild procedure, β(1-3)glucan was isolated from cell walls, solubilized by carboymethylation, and fractionated by size exclusion chromatography, giving rise to a very high-molecular-weight peak and to highly polydisperse material. The latter material, soluble in alkali, may correspond to glucan being remodeled, whereas the large-size fraction would be the final cross-linked structural product. In fact, the β(1-3)glucan of buds, where growth occurs, is solubilized by alkali. A gas1 mutant with an expected defect in glucan elongation showed a large increase in the polydisperse fraction. By a procedure involving sodium hydroxide treatment, carboymethylation, fractionation by affinity chromatography on wheat germ agglutinin-agarose, and fractionation by size chromatography on Sephacryl columns, it was shown that the β(1-3)glucan attached to chitin consists mostly of high-molecular-weight material. Therefore, it appears that linkage to chitin results in a polysaccharide that cannot be further remodeled and does not contribute to growth at the neck. In the course of these experiments, the new finding was made that part of the chitin forms a noncovalent complex with β(1-3)glucan.

The cell wall imparts shape to the fungal cell. For many years, we have used the cell wall of Saccharomyces cerevisiae and its specialized component, the septum, as models of morphogenesis (5). The yeast cell wall consists of three polysaccharides, β(1-3)glucan, the major structural component, β(1-6)glucan, and chitin, a minor component that is, however, essential for cell survival. In addition, mannoproteins are present as an external layer of the cell wall. All of these constituents are linked together to form a tight network capable of preventing osmotic or mechanical injuries to the cell (Fig. 1; for reviews, see references 11 and 15). During the cell cycle, the cell wall must undergo a constant process of synthesis and remodeling to accompany cell growth. However, after early budding, one area of the cell wall that does not change is the neck at the mother-bud interface. In yeast, this is a crucial region, because it is the site where cytokinesis and septation take place (16, 26). We previously showed that control of growth at the neck is exerted, in a redundant fashion, by the septin and the chitin ring present at that location (27). A defect in either one of the rings, such as in a chs3Δ mutant, which lacks the chitin ring (28), or in a clbΔ mutant, where the septin ring is poorly organized, leads to only minor morphological abnormalities. However, when both rings are faulty, control of growth is lost, the neck widens, and cytokinesis does not take place (27). It is probable that the septin ring acts through its barrier function (6), impeding access to the neck of membrane proteins necessary for cell wall synthesis. As for the chitin ring, we suggested that it may work by interfering with wall assembly (27). By the action of the Crh1p and Crh2p transferases (2, 3), cell wall chitin is attached to both β(1-6)glucan, at a branch point, and β(1-3)glucan, at the nonreducing ends (Fig. 1 and references 12 and 13). These nonreducing ends of β(1-3)glucan are those to which β(1-6)glucan is linked (Fig. 1). It seemed possible that at the neck, where most of the chitin and of Crh2p (23, 24) is localized, chitin could compete with β(1-6)glucan and prevent its attachment to those sites. As a consequence, mannoproteins, which attach to β(1-6)glucan, would also be unable to join the cell wall structure (27). One prediction of this hypothesis is that most of the chitin at the neck would be specifically linked to β(1-3)glucan. By applying new techniques to the analysis of chitin linkages formed during different segments of the cell cycle, we found that, indeed, almost all of the bound chitin at the neck is attached to β(1-3)glucan, whereas β(1-6)glucan is the chitin acceptor in lateral walls (4). However, to restrict formation of the cell wall at the neck, it would also be necessary to prevent remodeling and growth of β(1-3)glucan itself. Could the attachment of chitin to the glucan also accomplish this function? This is the question that we tried to answer in the present work, by comparing the size distribution of the chitin-free β(1-3)glucan, which is dispersed all over the cell, with that of the chitin-linked β(1-3)glucan, which is present only at the neck.
canase (1). After incubation, 18 μl of 1 M phosphate at pH 6.3, 15 μl of 0.2 M sodium hydroxide, and 17 μl of Zymolyase 100T* (Associates of Cape Cod) at 10 mg/ml were added and incubation was continued for 3 h at 37°C to hydrolyze the β(1-3)glucan. Tubes were centrifuged for 10 min at 18,000 × g in a refrigerated centrifuge, and pellets were carboxymethylated as previously reported (4). For each aliquot, the final product contained 600,000 to 700,000 cpm. The carboxymethylated mixtures were pooled and concentrated by centrifugation in an Amicon Centricon with a molecular weight cutoff of 3,000 to a final volume of 1 ml.

**Preparation of carboxymethylated cell walls**. From YPH499 cells (1.2 g, wet weight), cell walls were prepared, treated with sodium hydroxide, and carboxymethylated as previously outlined (4), using volumes of reagents 9 times larger than those described (4). Because of the scaled-up procedure, the mixture was in a 14-ml polypropylene tube and mixing had to be done by either shaking the tube by hand or using a glass rod, rather than in the BeadBeater. The final drying was done by adding ether, centrifuging the mixture, and allowing the ether to evaporate under a hood. The dried pellet was suspended in 3 ml of water. After centrifugation, another 2 ml of water was added to dissolve the remaining pellet. Both fractions were dialyzed against water overnight and then mixed in the original proportions. The resulting solution was clear.

**Preparation of CM-curdlan**. Carboxymethylation of curdlan was carried out with 0.5 g of the commercial product (Carbomer, San Diego, CA) as described above for yeast cell walls. The final product was dialyzed overnight against water. The solution, 37.5 ml, was extremely viscous and formed a gel after storage at 4°C. It could be liquefied by heating in a boiling water bath for 10 min, and 5-fold dilution with water gave rise to a low-viscosity solution. In the undiluted preparation, the final concentration was 45 mM glucose equivalents, as measured with anthrone (30), using glucose as the standard. The yield of the operation was about 55%.

**Chitinase purification**. Chitinase from *Serratia marcescens* was purified by adsorption-digestion on chitin as previously described (22) and dialyzed against 0.05 M phosphate, pH 6.3. To eliminate traces of β(1-3)glucanase, affinity adsorption on curdlan, an insoluble β(1-3)glucan, was carried out. CurdLAN gel was prepared from a 2% suspension of the polysaccharide as described previously (2), except that the buffer was 0.05 M 2-(N-morpholino)ethanesulfonic acid, pH 6. To gel from 0.3 ml of a curdLAN suspension, 250 μl of dialyzed chitinase was added; this was followed by shaking of the tube in a Mini-BeadBeater (Biospec Products, Bartlesville, OK) for 20 s, rotation at room temperature for 30 min, and centrifugation. The supernatants from two batches were pooled and treated in the same way with curdLAN gel from 0.3 ml of a 2% suspension. The final supernatant was the purified chitinase. In this operation, the chitinase was diluted about 40%.

**Isolation of bulk β(1-3)glucan**. A schematic diagram of the β(1-3)glucan isolation procedure used is shown in Fig. 2A. All centrifugations were for 5 min at 16,000 × g. Yeast cells were labeled during logarithmic growth with [14C]glucose as previously described for the preparation of 14C-labeled β(1-3)glucan (2). To cells from a 20-ml culture (2) in 50 mM Tris chloride, pH 7.5, in a total volume of 1 ml in a 2-ml Mini-BeadBeater tube, 2 g of glass beads (0.5-mm diameter) was added. To break the cells, the tube was shaken in a Mini-BeadBeater for 8 periods of 30 s, with 1-min periods of cooling in ice in between. Washing of the cell walls was done as already described (8). To 200 μl of a cell wall suspension containing 1.3 ×

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**TABLE 1 Strains used in this study**

| Strain   | Genotype                        | Source or reference |
|----------|---------------------------------|---------------------|
| YPH499   | MATα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 | 29                  |
| NBTO14   | MATα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 crh1::HygR crh2::His3 | This work           |
| ECY46-4-1B | MATα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 chs3::LEU2 | 7                   |
| BY4741   | MATα ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 | EUROSCARF           |
| YHR307W  | MATα ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 gas1::KanMX4 | EUROSCARF           |
| FY001    | MATα ura3Δ200 ura3-52 leu2Δ1 trp1Δ63 | 3                   |
106 to 1.6 × 106 cpm, 11.6 μl of 1 M sodium acetate, pH 5, and 20 μl of recombinant β(1-6)glucanase (1) were added. The mixture, in a screw-cap microcentrifuge tube, was incubated overnight at 37°C on a rotator and then centrifuged. The supernatant was saved, and the pellet was washed with 200 μl of water, which was added to the first supernatant. The pellet was suspended in 200 μl of water, 50 μl of 5 M sodium hydroxide was added, and the tube was vortexed for 1 min before centrifugation. The supernatant was saved. The pellet (P1) was washed with 200 μl of water, which was added to the supernatant. To this combined supernatant, 2 volumes of ethanol were added. The tube was stored at 4°C overnight and then centrifuged in a refrigerated microcentrifuge. The resulting pellet (P2) was saved.

In a parallel experiment, cell walls were incubated with β(1-6)glucanase as described above and centrifuged but the pellet (P3) was not treated further. Radioactivity was monitored in all supernatants and pellets. The three pellets, P1, P2, and P3, were transferred to 2-ml BeadBeater tubes with three 0.1-ml portions of 60% sodium hydroxide–0.2% sodium dodecyl sulfate, and carboxymethylation was carried out as previously described (4). As already mentioned (4), extraction of the carboxymethylated material with water gave rise to two fractions, the first of which had a high salt concentration. This fraction was usually desalted by centrifugation in an Amicon Ultra device with a molecular weight cutoff of 10,000 before being mixing with the second fraction.

**Chromatography on Sephacryl S-500.** Chromatography of the pooled fractions on a Sephacryl S-500 column (1 by 76 cm) was carried out with the same buffer (50 mM Tris chloride, pH 7.5, containing 150 mM sodium chloride and 0.02% sodium azide) and conditions previously described for Sephacryl S-300 (4). The void volume of the column is at fractions 24 to 26. For glucose, the position of the peak maximum was at fraction 57, as determined with [14C]glucose as the standard. (D) Chromatography of total β(1-3)glucan on Sephacryl S-500 before and after filtration through WGA-agarose.

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![Diagram of isolation and fractionation of β(1-3)glucan](image_url)

**FIG 2** Isolation and fractionation of β(1-3)glucan. (A) Scheme for isolation of β(1-3)glucan from yeast cell walls. All carboxymethylated (CM) fractions are soluble in water. (B) Chemical linkage of acetic acid to hexose in a carboxymethylated sugar. The open structure of the sugar is shown for simplicity. Monochloroacetic acid is the reactant, and an ether linkage is formed as shown. The acetic acid residue may be attached to any of the free hydroxyl groups in a polysaccharide and will be negatively charged at neutral pH. (C) Chromatography of total (no NaOH), alkali-soluble, and alkali-insoluble β(1-3)glucan on Sephacryl S-500. Here as in subsequent columns, fractions of 1 ml were collected. The void volume of the column is at fractions 24 to 26. For glucose, the position of the peak maximum was at fraction 57, as determined with [14C]glucose as the standard. (D) Chromatography of total β(1-3)glucan on Sephacryl S-500 before and after filtration through WGA-agarose.
sured in the Sephacryl S-500 column with dextran blue because dextran blue was in the included volume of the column. By comparison with a Sephacryl S-400 column of the same size, it was judged to be 25 to 26 ml (fractions 25 and 26). We were also able to measure the void volume directly by using colloidal gold particles 60 nm in diameter (nanoCom- posix, San Diego, CA). The colloidal gold can be detected by its absorbance at 534 nm. The void volume measured in this way was at fraction 24, similar to the calculated value.

Preparation of cell walls for observation by microscope. Cell walls were prepared as described in the section on the isolation of bulk β-(1-3)glucan, except that the cell mixture with glass beads was in a 12 ml polycarbonate tube, rather than in a Mini-BeadBeater tube. The tube was vortexed for 6 periods of 1 min, with 1-min periods of cooling in ice in between. Washing of cell walls, incubation with β-(1-6)glucanase, and treatment with sodium hydroxide at room temperature were carried out as in the above-mentioned section.

Isolation of chitin-linked β-(1-3)glucan. Cells were labeled with [14C]glucose, and cell walls were prepared and treated with β-(1-6)glucanase as described above. The β-(1-6)glucanase-resistant pellet was suspended in 200 μl of water, and 50 μl of 3 M sodium hydroxide was added. The mixture was placed in a dry bath at 80°C for 1 h; this was followed by centrifugation and suspension of the pellet in 250 μl of 1 M sodium hydroxide. The tube was heated again at 80°C for 1 h and centrifuged. The pellet was washed with 200 μl of water, subjected to carboxymethylolation, and desalted as outlined above. To the desalted material, 5 M NaCl and 1 M Tris-HCl, pH 7.5, were added to make the final concentration of NaCl 1 M and that of Tris 0.1 M in a total volume of 1 ml.

Wheat germ agglutinin (WGA)-agarose (Vector Laboratories, Burlingame, CA) columns of 0.88 ml were set up in tubes 0.7 cm in diameter. The columns were washed with 2 ml of 1 M NaCl–0.1 M Tris-HCl, pH 7.5. The above-described carboxymethylolated mixture (100,000 cpm) was applied to a column, allowed to percolate, and reapplied. This operation was repeated once more to ensure maximum adsorption of the chitin-bound material. A 1-ml washing with 1 M NaCl–0.1 M Tris-HCl, pH 7.5, was pooled with the column filtrate to yield fraction 1 (total percolate). The column was successively washed with 1 ml of the NaCl-Tris solution (fraction 2) and 1 ml of water (fraction 3). The chitin-containing material was eluted with three 1-ml portions (fractions 4 to 6) of 0.1 M sodium hydroxide. Addition to the sodium hydroxide of CM-curdlan, to a final concentration of 0.05% CM-curdlan, to a final concentration of 9 mM glucose equivalents, somewhat increased the yield of radioactivity in these fractions. Most of the radioactivity applied to the column was found in fractions 1, 4, and 5, and very little was found in the other fractions, which were discarded.

Fraction 1 was desalted and concentrated to 0.4 ml by centrifugation in an Amicon Ultra device with a molecular weight cutoff of 10,000. To the desalted fraction, 40 μl of Tris-HCl, pH 7.5, and 13 μl of 5 M NaCl were added. The mixture was fractionated on Sephacryl S-500 as outlined above.

Fractions 4 and 5 were pooled and neutralized with hydrochloric acid. The following additions were made: 100 μl of 1 M Tris-HCl, pH 7.5; 62 μl of 5 M NaCl; 100 μl of carboxymethylated cell walls; and 200 μl of 0.5% CM-chitin. The last two items were added as carriers because of the very small amount of radioactive material in these fractions. The mixture was subjected to chromatography on Sephacryl S-500.

For treatment of the WGA-agarose fractions with β-(1-3)glucanase, the pH of either desalted fraction 1 or pooled fractions 4 and 5 was adjusted to 7.5 and 40 μl of chitinase-free Zymolyase (2) was added. Mixtures were incubated for 3 h at 37°C and then fractionated on Sephacryl S-500 after the additions mentioned above.

For chitinase digestion, the pH of fraction 1 or fractions 4 and 5 was adjusted to 6.3 and 20 μl of curdland-purified chitinase was added. Incubation was overnight at 37°C. To the digested mixtures, additions were made as described above, followed by chromatography on Sephacryl S-500.
column at the void volume. As mentioned above, the \(\beta\(1\-6\)\)-glucanase-resistant fraction contains, in addition to \(\beta\(1\-3\)\)-glucan, free and \(\beta\(1\-3\)\)-glucan-bound chitin, whose presence could conceivably affect the elution pattern. We assumed that this effect would be negligible, in view of the small amount of chitin present in the cell wall, and this turned out to be the case, as shown below.

The results of these experiments are consistent with the presence in yeast cell walls of two \(\beta\(1\-3\)\)-glucan populations, one of extremely high molecular weight and the other one showing a broad range of smaller sizes.

**Cell wall mutants show an abnormal size distribution of \(\beta\(1\-3\)\)-glucan.** To validate our procedure, we applied it to mutants known or presumed to be affected in cell wall structure. One of them was a \(gas1\) mutant. Gas1p, a protein identified about 27 years ago, has been found to exhibit a transglucosidase activity *in vitro*, transferring glucose chains from a \(\beta\(1\-3\)\)-linked glucose oligosaccharide to another (20). On that basis, it has been speculated that the enzyme functions in the elongation of \(\beta\(1\-3\)\)-glucan, but no *in vivo* evidence that this is the case has been published. We performed the experiment shown in Fig. 2C with a \(gas1\) deletion mutant and with the corresponding wild-type strain, BY4741. The results obtained with the wild type (Fig. 3A) were very similar to those obtained with strain YPH499, but those obtained with the mutant were quite different, showing a sharp shift toward the lower molecular weights in both the NaOH-soluble and insoluble fractions (Fig. 3B). Because the shift occurred in both fractions, the percentage of NaOH-soluble material changed little from that of the wild type (Table 2). These results indicate that Gas1p participates somehow in the polymerization of \(\beta\(1\-3\)\)-glucan *in vivo*.

Another mutant we tested was a \(chs3\) mutant strain, in the same genetic background as YPH499. This strain lacks the chitin attached to both \(\beta\(1\-3\)\) and \(\beta\(1\-6\)\)-glucan (28). Rather surprisingly, here too there was some shift toward the smaller sizes of \(\beta\(1\-3\)\)-glucan, although it occurred only with the alkali-soluble fraction (Fig. 3C). A very similar pattern (Fig. 3D) was found with a \(crh1\Delta\ crh2\Delta\) mutant strain, which has a normal level of chitin (23) but in which the chitin is not bound to either \(\beta\(1\-3\)\) or \(\beta\(1\-6\)\)-glucan (2, 3). In contrast to the \(gas1\) mutant, in both the \(chs3\) and \(crh1\ crh2\) mutant strains, all of the increase in smaller-size \(\beta\(1\-3\)\)-glucan occurred in the NaOH-soluble fraction (Fig. 3C and D; Table 2), which points to a different mechanism. We will return to these results in the Discussion.

**Morphological assessment of the effect of sodium hydroxide.** A question raised was whether the solubilization of part of the

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**FIG 3 Chromatography of total, NaOH-soluble, and NaOH-insoluble fractions of different strains on Sephacryl S-500.** (A) Strain BY4741 (wild type of the \(gas1\Delta\) mutant). (B) Strain YHR307W (\(gas1\Delta\)). (C) Strain ECH46-4-1R (\(chs3\)). (D) Strain NBT014 (\(crh1\Delta\ crh2\Delta\)). The two latter mutations are in the YPH499 background. In all cases, the procedure was as that used for Fig. 2A.
β(1-3)glucan by sodium hydroxide could be somehow visualized by observation of untreated and treated cell walls. The cell walls prepared with the BeadBeater were unsuitable for this purpose because their shredding had gone too far. However, walls prepared by vortexing with glass beads (see Materials and Methods) better maintained the original cell shape and in many cases (26%, n = 414) showed attached walls of the buds (Fig. 4A). After treatment with β(1-6)glucanase, the walls were less dark but the general morphology did not change and the percentage of joined mother-bud cell walls was 23.4% (n = 393; Fig. 4B). However, when those cell walls were briefly treated with 1 M sodium hydroxide at room temperature, practically all of the bud cell walls disappeared, with only 0.6% (n = 314) remaining (Fig. 4C). Thus, these results strongly suggest that it is mainly the β(1-3)glucan of daughter cells, the ones that are growing, that is solubilized by alkali.

Both the wild type and a crh1Δ crh2Δ mutant contain β(1-3)glucan apparently bound to chitin. Having established the size distribution pattern of the bulk β(1-3)glucan, we turned to that portion of the polysaccharide that is bound to chitin. Previous results indicated that the chitin-glucan complex is resistant to alkali at relatively high temperatures (19). Therefore, in an attempt to solubilize as much of the free β(1-3)glucan as possible, the β(1-6)glucanase-resistant fraction was incubated with 1 M NaOH at 80°C for 1 h and the treatment was repeated. As shown in Table 3, about 30% of the radioactivity remained insoluble. This fraction was incubated overnight with chitinase, which solubilized some radioactivity, and the insoluble residue was treated with 1 M NaOH at room temperature, in the same way as was done for bulk glucan. This treatment solubilized about two-thirds of the radioactivity (Table 3), which should represent part or all of the glucan previously bound to chitin. Surprisingly, a crh1Δ crh2Δ mutant strain, which should have no glucan bound to chitin, also yielded soluble material, although only about half of the amount of the wild type (Table 3). Since the lack of β(1-3)- or β(1-6)glucan covalently linked to chitin in such a mutant was previously shown by three different methods (2), we reasoned that perhaps part of the chitin was forming a noncovalent complex with β(1-3)glucan in both the wild-type and mutant strains. Support for this notion came from previous experiments in which we found that finely divided chitin, obtained by adding water to a chitin solution in N,N′-dimethylacetamide—6% LiCl, efficiently adsorbed CM–β(1-3)glucan, showing an affinity between the two molecules (results not shown). The challenge was to distinguish between a covalent and a noncovalent complex of the polysaccharides.

High-molecular-weight β(1-3)glucan and chitin bind to each other covalently and noncovalently. If our hypothesis were correct, the components of the alkali-insoluble residue should include free chitin, a covalent and a noncovalent chitin–β(1-3)glucan complex, and possibly some high-molecular-weight β(1-3)glucan (Fig. 5A, left). The crh1Δ crh2Δ mutant should have the same components, except for the covalent complex. Obviously, it would not be possible to analyze this mixture as long as all of the components are insoluble. Therefore, the first step should be solubilization, which can be attained by carboxymethylation of the material. This would also partially simplify the mixture, since the noncovalent complex should give rise to free CM-chitin and CM–β(1-3)glucan. These should not bind to each other because the negative charges introduced by the carboxymethylation would induce repulsion between the polysaccharide chains. Thus, the components of the solubilized mixture should be CM-chitin, CM-glucan, and the covalent CM–chitin–β(1-3)glucan in the wild type (Fig. 5A, right) and only the first two in the crh1Δ crh2Δ mutant. It seemed that a first step in the separation of these components could be adsorption-elution on WGA-agarose because WGA specifically binds compounds with β(1-4)N-acetylglucosamine linkages, such as those of chitin. Thus, CM-chitin, free or in a complex, should be adsorbed, but β(1-3)glucan should not. However, we found that a large percentage of [14C]CM–β(1-3)glucan was adsorbed, suggesting that it might be the covalent complex of the alkali insoluble. To determine the nature of the alkali-insoluble residue, we fractionated it with the BeadBeater by three different methods (2), which should give material that is free chitin, a chitin–β(1-3)glucan complex, and possibly some high-molecular-weight β(1-3)glucan (Table 3).

**Table 3** Fractionation of β(1-3)glucan-insoluble material by treatment with sodium hydroxide at 80°C, followed by incubation with chitinase and new treatment with sodium hydroxide at room temperature

| Fraction | Wild type | crh1Δ crh2Δ mutant |
|----------|-----------|---------------------|
| NaOH (80°C) insoluble | 22.2 ± 2.2 | 38.1 ± 2.3 |
| NaOH (room temp) soluble | 20.7 ± 0.8 | 10.6 ± 3 |

a Cells were labeled with [14C]glucose as described in Materials and Methods.
b Values represent the radioactivity in each fraction as a percentage of the β(1-3)glucanase-insoluble radioactivity. The data in the first row are averages of five experiments, and those in the second row are averages of two experiments.
FIG 5 Isolation of the covalent chitin–β(1-3)glucan complex. (A) Left, components of the sodium hydroxide-insoluble fractions. The covalent complex of chitin and β(1-3)glucan is symbolized by a hyphen between the two, whereas the noncovalent complex has a dot. Right, components of the fraction solubilized by carboxymethylation. Note that the number of components decreased from 4 to 3 after carboxymethylation. The carboxymethylated mixtures from the wild type and the crh1Δ crh2Δ mutant were applied to WGA–agarose columns (B) Sephacryl S-500 chromatography of material not retained by WGA–agarose for YPH499 (wild type) and NBT014 (crh1Δ crh2Δ). This represents free CM–β(1-3)glucan (C) fractionation of 0.1 NaOH eluates of WGA–agarose columns on Sephacryl S-500. For the wild type, the first peak contains the CM–chitin–β(1-3)glucan covalent complex, while the second peak corresponds to free chitin. Only the free chitin peak is present in the mutant fraction. See text and Materials and Methods for details.
Further characterization of WGA-agarose fractions by enzymatic treatments. To verify the chemical nature of the WGA-agarose fractions, both percolates and NaOH eluates were treated with either β(1-3)glucanase or chitinase before Sephacryl chromatography. Incubation of a CM–β(1-3)glucan sample with chitinase-free Zymolyase (2), followed by fractionation on Sephacryl S-500, resulted in a large displacement of the peak toward the lower-molecular-weight area (see Fig. S1 in the supplemental material). Total hydrolysis of β(1-3)glucan by this enzyme should result in an oligosaccharide of 5 glucose units (10). In the Sephacryl S-500 column, it would probably run about the same as glucose, whose maximum is found in fractions 56 and 57. The digested peak emerges somewhat earlier (see Fig. S1), indicating that the breakdown of the polysaccharide is not complete, most probably because of the presence of acetate groups that hinder the action of the enzyme. Incubation of the WGA-agarose percolates with Zymolyase gave a similar result (Fig. 6A and B), confirming that these fractions consist of CM–β(1-3)glucan. In the alkali-eluted fraction of the wild type, the first peak was shifted in the same way (Fig. 6C), as expected if it contained CM–β(1-3)glucan. Because of the superimposition of the digested material with the position of CM-chitin, no information can be furnished by this experiment about the position of the glucan-bound chitin or of the second peak. Finally, the alkali-eluted material from the crh1Δ crh2Δ mutant strain was somewhat displaced, although not as much as for the percolates, by glucanase digestion (Fig. 6D). This result was unexpected and may indicate the presence of some β(1-3)glucan in this fraction.

When chitinase was used on the WGA-agarose percolates, a surprising result was obtained because the peaks, which should not contain chitin, were significantly shifted to the right (see Fig. S2 in supplemental material). Although when the chitinase was first prepared we found no β(1-3)glucanase activity in it with laminarin as the substrate (22), this result indicated the possibility of a small amount of contamination with such an activity. To eliminate the putative glucanase, we treated the chitinase preparation with curdlan gel. Curdlan is a β(1-3)glucan from bacteria that is very insoluble in water and forms a gel at 56°C. It seemed probable that the gel could adsorb the glucanase, for which it is a substrate. After curdlan treatment, CM-chitin was still degraded by the chitinase to the same extent as before (Fig. 7A), with limitations similar to those previously observed for β(1-3)glucanase acting on β(1-3)glucan. However, the purified chitinase only slightly modified the profiles of the percolates (Fig. 7B and C). The small effect may be due to remaining traces of β(1-3)glucanase activity. No effect of the purified chitinase on the first peak of the wild-type alkaline eluate was observed, whereas the second peak was shifted, confirming that it consists of CM-chitin (Fig. 7D). Although the shift is small, it is comparable to that obtained with CM-chitin (Fig. 7A). The lack of displacement of the first peak may be explained by the relatively small amount and low molecular weight of the chitin bound to β(1-3)glucan. In the case of the crh1Δ crh2Δ mutant, chitinase caused a pronounced shift, supporting the notion that this peak corresponds to CM-chitin (Fig. 7E).

The results of treatments with β(1-3)glucanase and chitinase are in general agreement with the notion that the material not adsorbed by the WGA-agarose columns is CM–β(1-3)glucan, whereas that absorbed and later eluted consists of a covalent CM-chitin–β(1-3)glucan complex plus free chitin for the wild type and

### Table 4 Fractionation of carboxymethylated, NaOH-insoluble fraction on WGA-agarase

| Fraction | Wild type | crh1Δ crh2Δ mutant |
|----------|-----------|---------------------|
| Column percolate | 54.8 ± 3.1 | 74 ± 0.55 |
| 0.1 M NaOH eluate | 26.6 ± 3 | 20.7 ± 1.5 |
| Total recovery | 81.4 | 94.7 |

a Cells were labeled with [14C]glucose as described in Materials and Methods.

b Data represent percentages of the radioactivity applied to the columns and are the averages of three experiments ± the standard deviations.

3)glucan from yeast was adsorbed by WGA-agarose. In view of the specificity of WGA, it seemed probable that this adsorption was unspecific and due to the negative charges introduced by carboxymethylation. A high ionic strength should abolish that kind of binding. Accordingly, when a [14C]CM–β(1-3)glucan solution containing 1 M sodium chloride was applied to WGA-agarose, 90% of the radioactivity was recovered in the filtrate. Under the same conditions, 100% of a sample of [14C]CM-chitin from yeast was adsorbed. There still remained the problem of eluting the chitin-containing material from the column. Several procedures, such as high concentrations of N-acetylglucosamine or of unlabeled CM-chitin, did not work at all. Finally, it was found that 0.1 M sodium hydroxide eluted 85 to 95% of the adsorbed [14C]CM-chitin.

With this knowledge, β(1-6)glucanase-resistant fractions of both the wild-type and crh1Δ crh2Δ mutant strains were treated with alkali at 80°C and solubilized by carboxymethylation. After the sodium chloride concentration was adjusted to 1 M, they were applied to WGA-agarose columns. The percolates were expected to consist of free β(1-3)glucan. The columns were then washed with 0.1 M sodium hydroxide to elute any CM-chitin and CM–(1-3)glucan present. The amount of radioactivity not retained by the column was always higher for the mutant than for the wild type (Table 4), and so was the total recovery. In the case of the wild type, some radioactivity was tenaciously retained by the column. In the alkali-eluted fraction of the wild type, the first peak was shifted in the same way as for CM-chitin from yeast (Fig. 7A). The lack of displacement of the first peak may be explained by the small amount of contamination with such an activity. To eliminate the putative glucanase, we treated the chitinase preparation with curdlan gel. Curdlan is a β(1-3)glucan from bacteria that is very insoluble in water and forms a gel at 56°C. It seemed probable that the gel could adsorb the glucanase, for which it is a substrate. After curdlan treatment, CM-chitin was still degraded by the chitinase to the same extent as before (Fig. 7A), with limitations similar to those previously observed for β(1-3)glucanase acting on β(1-3)glucan. However, the purified chitinase only slightly modified the profiles of the percolates (Fig. 7B and C). The small effect may be due to remaining traces of β(1-3)glucanase activity. No effect of the purified chitinase on the first peak of the wild-type alkaline eluate was observed, whereas the second peak was shifted, confirming that it consists of CM-chitin (Fig. 7D). Although the shift is small, it is comparable to that obtained with CM-chitin (Fig. 7A). The lack of displacement of the first peak may be explained by the relatively small amount and low molecular weight of the chitin bound to β(1-3)glucan. In the case of the crh1Δ crh2Δ mutant, chitinase caused a pronounced shift, supporting the notion that this peak corresponds to CM-chitin (Fig. 7E).

The results of treatments with β(1-3)glucanase and chitinase are in general agreement with the notion that the material not adsorbed by the WGA-agarose columns is CM–β(1-3)glucan, whereas that absorbed and later eluted consists of a covalent CM-chitin–β(1-3)glucan complex plus free chitin for the wild type and
only the latter for the \textit{crh1Δ crh2Δ} mutant. Thus, the results are in agreement with those predicted in Fig. 5A. The WGA-agarose procedure provided an opportunity to verify whether the presence of chitin affected the elution pattern of the bulk \(\beta(1-3)\)glucan (as in Fig. 2C, green curve). To this end, a preparation of carboxymethylated total \(\beta(1-3)\)glucan was split in two. One portion was left untreated, and the other was filtered through a WGA-agarose column to remove both free and bound chitin. Fractionation of both samples on Sephacryl S-500 yielded the same elution pattern (Fig. 2D), showing that the effect of chitin on those chromatographic results is negligible.

Labeling with \([14C]\)glucosamine confirms the presence of covalently linked \(\beta(1-3)\)glucan and chitin only in the wild type. Because of uncertainty about some results, such as the slight effect of even purified chitinase on WGA-agarose percolates or the shift observed in the NaOH-eluted fraction of the \textit{crh1Δ crh2Δ} mutant strain after glucanase treatment, we felt the need for independent verification of the experimental evidence. To this end, cells were labeled with \([14C]\)glucosamine rather than with \([14C]\)glucose. As we showed previously, under these conditions, chitin is labeled specifically and no radioactivity is found in the glucans (4). Labeled cells were processed as before, and the carboxymethylated, NaOH-insoluble fraction was applied to a WGA-agarose column. Here one would expect no radioactivity in the percolate, if it indeed consists of CM–\(\beta(1-3)\)glucan. This was the case for the \textit{crh1Δ crh2Δ} mutant, but in the wild type, some radioactivity, about 10 to 15% of the total, was not retained by the column. We do not know the nature of this material, which could be glucan with a very small amount of chitin. The alkali eluates, when fractionated on Sephacryl S-500, showed patterns similar to those in which \([14C]\)glucose was the label (compare Fig. 8A and B with Fig. 5C). This result confirms that the first wild-type peak contains chitin in addition to \(\beta(1-3)\)glucan, whereas the second wild-type peak and the only peak of the mutant consist of chitin only.

Although the results are in general agreement with the previous data, in Fig. 8A, the amount of chitin bound to \(\beta(1-3)\)glucan seems a very small percentage of the total chitin. However, when one adds to it the fraction of radioactivity present in the WGA-agarose percolate and that remaining tightly bound to the same column, the final result is 43% of the total (average of two determinations), which is comparable to the \(\sim 40\%\) that was found with a different methodology for the same strain (4).

**DISCUSSION**

The data presented here offer the first glimpse of the size distribution of \(\beta(1-3)\)glucan, the main structural component of the yeast
cell wall. Because of the mild conditions used in the isolation of the polysaccharide and its solubilization, it seems probable that the results of fractionation on a Sephacryl column are a fairly faithful reflection of the *in vivo* distribution of β(1-3)glucan. Sodium hydroxide at room temperature solubilizes roughly half of the glucan. In size chromatography, the soluble fraction shows a greatly polydisperse distribution, whereas the insoluble portion, emerging at the void volume, consists of very high-molecular-weight material. According to the manufacturer, the limit for inclusion of a carbohydrate polymer in Sephacryl S-500 is a molecular weight of $2 \times 10^7$. A molecule of that size would contain more than 120,000 glucose units. According to a determination of β(1-3)glu-
can chain length by methylation, there would be about 1,500 glucose units per chain (18). The explanation for the much greater size that we found most probably is that the chains are cross-linked through the $\beta(1-6)$ linkages that were also found in the polysaccharide (18), as depicted in Fig. 1. The cross-links would allow $\beta(1-3)$ glucan to form an almost continuous network over the surface of the cell. Such a network was detected by electron microscopy (14). Because of its very high molecular weight, the alkali-insoluble fraction probably represents the structural network. Although the cell wall must at all times present a strong and continuous frame to counteract the internal turgor pressure and prevent lysis of the cell, it is also a dynamic structure that needs to be constantly remodeled to accompany cell growth. This remodeling may consist of breakage of some bonds and addition of new

**FIG 8** Chromatography on Sephacryl S-500 of WGA-agarose fractions from cells labeled with $[^{14}\text{C}]$glucosamine. (A) NaOH fraction of the wild type. (B) NaOH fraction of the crh$\Delta$ crh$\Delta$ mutant. The positions of peaks in these graphs should be compared to those in Fig. 5C.
material and would be expected to give rise to polysaccharide
chains of many different sizes. We propose that the highly poly-
disperse alkali-soluble fraction represents the material that is go-
ing through remodeling. This notion receives some support from
the results obtained with the gas1 mutant. Because of its in vitro
transglycosylase activity, Gaslp has been proposed to participate
in the elongation of β(1-3)glucan (20). However, no in vivo data
were available to corroborate this idea. An increase in the ratio
of alkali-soluble to insoluble total glucan was reported in a gas1 mu-
ant, but it was attributed to a decrease in alkali-insoluble β(1-
6)glucan (21). Our results (Fig. 3) clearly show that a gas1 deletion
mutant manifests a large increase in the polydisperse fraction of
β(1-3)glucan, which is observed in both the alkali-soluble and
alkali-insoluble fractions. This result is consistent with a role for
Gaslp in the size increase of the polysaccharide, although elucid-
ation of the mechanism by which this protein acts in vivo will
require further work. The large perturbation in the presumably
cross-linked β(1-3)glucan observed in a gas1 mutant (Fig. 3B) is
consistent with a function of Gaslp in the cross-linking process, as
previously suggested (20). While this is a rather speculative con-
cept, what is fairly clear is that the reduction in the high-molecu-
lar-weight glucan found here in the mutant weakens the cell wall.
The somewhat larger and rounder aspect of gas1 mutant cells is
consistent with a defect in cell wall maturation. Furthermore, gas1
mutants have a greatly increased content of cell wall chitin (9),
which probably helps to stabilize the cell wall, because elimination
of that chitin by a chs3 mutation results in gas1 chs3 mutant cells
that grow very poorly (21). These findings support our argument
that the high-molecular-weight β(1-3)glucan is the structural
component of the polysaccharide. Further backing for this con-
cept came from observation in the microscope of cell walls before
or after different treatments (Fig. 4). About one-fourth of the
mother cell walls were attached to bud cell walls. This morphology
survived β(1-6)glucanase digestion, but the bud cell walls disap-
peared upon mild sodium hydroxide treatment. Since the bud is
where growth and cell wall remodeling occur, this result strongly
supports our proposal that the alkali-soluble fraction represents
material undergoing remodeling.

A rather surprising result was the increase in polydisperse β(1-
3)glucan, albeit smaller than that in a gas1 mutant strain, found
both in a chs3 mutant, which lacks chitin both in lateral walls and
at the neck, and in a crh1 crh2 mutant, which has the chitin but all
in a free form (2). An increase in total glucan soluble in hot alkali
in the crh1 crh2 mutant was reported earlier (23). The fact that
both the chs3Δ and crh1Δ crh2Δ mutants yield very similar size
distributions of β(1-3)glucan implies that it is the attachment of
chitin to the polysaccharide, rather than its mere presence, that
differs the cause from the wild type. In lateral walls, chitin
appears in the daughter cell after septation and during the residual
growth before new budding (28). The defect found in the chs3Δ
and crh1Δ crh2Δ mutants suggests that this chitin, mostly bound
to β(1-6)glucan (4), may have some role in the arrest of growth at
the end of the cell cycle, although the mechanism of such an action
is still unknown.

Once the size distribution of the bulk β(1-3)glucan was estab-
lished, it was possible to address the main subject of this work, i.e.,
the structure of the polysaccharide linked to chitin in the neck
region, which, as we previously determined, is almost all β(1-
3)glucan (4). Initially, isolation of this fraction of β(1-3)glucan
was attempted by digestion of cell walls with β(1-6)glucanase,
followed by alkali treatment, incubation with chitinase, and a new
brief exposure to sodium hydroxide. This procedure led to the
unexpected result that a crh1Δ crh2Δ mutant, in which all chitin
had been found to be free, apparently had some chitin bound to
β(1-3)glucan. An answer to this conundrum was found after sol-
ubilizing by carboxymethylation the material resistant to β(1-6)-
glucanase and alkali and subjecting it to fractionation on WGA-
agarose columns, followed by chromatography on Sephacyrl S-500.
The results are consistent with the presence, in both the wild type and the crh1Δ crh2Δ mutant, of a tight, alkali-resistant,
noncovalent complex of β(1-3)glucan and chitin. Because of the
possible presence in the analyzed mixture of some free β(1-3)glu-
can of high molecular weight, it is not possible to calculate how
much glucan is bound to chitin in this manner. A minimal
amount is 10% of the total, the fraction solubilized by alkali after
chitinase treatment in the crh1Δ crh2Δ mutant (Table 3). The
localization of this complex is unknown, and it may well be in
the lateral cell wall. The glucan in the noncovalent complex is part of
the percolate of the WGA-agarose columns, which is all of high
molecular weight (Fig. 5B); therefore, it appears to belong to the
final structural product. Is the noncovalent chitin–β(1-3)glucan
complex present in vivo, or could it be an extraction artifact? Al-
though the mild conditions used here suggest that the complex
was already present in the cell, it is conceivable that stripping away
the β(1-6)glucan and mannoproteins somehow brought chitin and
β(1-3)glucan together. This is, however, rather unlikely, be-
cause β(1-6)glucan and attached proteins are in the external layer
of the cell wall, while chitin and β(1-3)glucan are believed to pop-
ulate an inner layer (11).

In contrast to the physical complex of chitin and β(1-3)glucan,
the covalent one is found only in the wild type (Fig. 5C). That this
material contains both glucan and chitin was shown both by the
enzymatic treatments and by labeling of the cells with [14C]gluco-
samine rather than [14C]glucose (Fig. 6 to 8). Our calculations
indicate that about 7% of the total β(1-3)glucan is covalently
linked to chitin, in consonance with its presence only at the
mother-bud neck, a small portion of the cell wall. Most of the
complex is of high molecular weight, supporting the notion that
the β(1-3)glucan linked to chitin at the neck is not being metab-
olized, in agreement with our hypothesis. The recent report that
localization of Gaslp at the mother-bud neck requires Crhlp and
Crh2p (25), together with our previous finding that Crh2p is con-
centrated at the same location (23), suggests that these enzymes
collaborate in the maturation of the cell wall at the neck: Gaslp
could act in glucan cross-linking and the transferases in blocking
further remodeling of the polysaccharide by adding chitin chains
to it. However, we are not implying that the cell wall at the mother-
bud neck consists only of the chitin–β(1-3)glucan complex. In
fact, electron micrographs show at the neck, as in the remainder
of the cell surface, an external darker and hairy-cell-like wall layer
that has been generally associated with the presence of mannopro-
teins (see, for instance, Fig. 2 of reference 28). Most probably, the
cell wall at the neck in the early phase of budding has the same
composition as the remainder, but as the chitin ring and the septin
ring are formed, they redundantly block further synthesis by the
mechanisms discussed in the introduction. One may ask why
there should be two overlapping programs, one based on the chit-
in ring and the other on the septin ring, for the control of growth
at the neck. The answer may be that the structural preservation
at the neck, the future site of cytokinesis, is so important that a dou-
ble protection mechanism conferred an evolutionary advantage under conditions encountered in the wild.

One prediction of our hypothesis, as formulated in the introduction, is that free chitin, not covalently linked to glucan, such as in a crh1Δ crh2Δ mutant, would not be effective in preventing growth at the neck when septins are defective. In another study (N. Blanco, M. Reidy, J. Arroyo, and E. Cabib, unpublished data), we addressed this question and verified that the predicted outcome was correct.

In conclusion, we have shown that the cell wall β(1-3)-glucan consists of a very high-molecular-weight fraction and another smaller fraction that varies in size. The latter material is dramatically increased in a gas1 mutant believed to have problems in β(1-3)glucan polymerization and a weaker wall. Therefore, we conclude that the large-size material is the final structural polysaccharide, whereas the polydisperse fraction represents the portion undergoing remodeling during cell wall growth. The β(1-3)glucan attached to chitin at the mother-bud neck is mostly of high molecular weight, supporting the idea that the linkage between the two attached to chitin at the mother-bud neck is mostly of high molecular weight, supporting the idea that the linkage between the two

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