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Eukaryotic Cell 2012, 11(4):388. DOI: 10.1128/EC.05328-11.
Published Ahead of Print 24 February 2012.

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Presence of a Large $\beta(1-3)$ 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 $\beta(1-3)$ glucan, may help to suppress cell wall growth at the neck by competing with $\beta(1-6)$ glucan and thereby with mannoproteins for their attachment to the same sites. Here we explored whether the linkage of chitin to $\beta(1-3)$ glucan may also prevent the remodeling of this polysaccharide that would be necessary for cell wall growth. By a novel mild procedure, $\beta(1-3)$ glucan was isolated from cell walls, solubilized by carboxymethylation, 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 $\beta(1-3)$ 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, carboxymethylation, fractionation by affinity chromatography on wheat germ agglutinin-agarose, and fractionation by size chromatography on Sephacryl columns, it was shown that the $\beta(1-3)$ 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 $\beta(1-3)$ 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, $\beta(1-3)$ glucan, the major structural component, $\beta(1-6)$ 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 *claΔ* 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 $\beta(1-6)$ glucan, at a branch point, and $\beta(1-3)$ glucan, at the nonreducing ends (Fig. 1 and references 12 and 13). These nonreducing ends of $\beta(1-3)$ glucan are those to which $\beta(1-6)$ 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 $\beta(1-6)$ glucan and prevent its attachment to those sites. As a consequence, mannoproteins, which attach to $\beta(1-6)$ 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 $\beta(1-3)$ 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 $\beta(1-3)$ glucan, whereas $\beta(1-6)$ 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 $\beta(1-3)$ 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 $\beta(1-3)$ glucan, which is dispersed all over the cell, with that of the chitin-linked $\beta(1-3)$ glucan, which is present only at the neck.

Received 27 December 2011 Accepted 15 February 2012

Published ahead of print 24 February 2012

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Supplemental material for this article may be found at <http://ec.asm.org/>.

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doi:10.1128/EC.05328-11

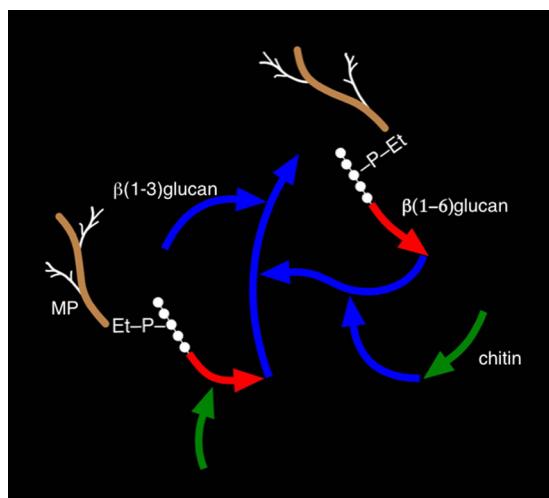


FIG 1 Schematic representation of the cell wall structure of *Saccharomyces cerevisiae* (13). The arrowheads indicate the reducing ends of the different polysaccharides. MP is mannoprotein, where the amino acid chain is brown and the branched mannosyl chains are white. The connection between mannoprotein and β(1-6)glucan consists of the remnant of a glycosylphosphatidylinositol anchor, where Et stands for ethanolamine, P stands for phosphate, and the five white dots represent mannose residues (13). Chitin is attached to β(1-6)glucan through a β(1-3)-linked glucose branch, which is not shown here. Part of the chitin that is free is not shown.

MATERIALS AND METHODS

Strains and growth conditions. The strains used in this study are listed in Table 1. Cells were grown at 30°C in YEPD (2% peptone, 1% yeast extract, 2% glucose), except where indicated otherwise.

For the construction of the *crh1Δ crh2Δ* double mutant (strain NBT014 [Table 1]) in the YPH499 background, the *CRH2* open reading frame (ORF) was deleted from YPH499 using the His3MX6 module as previously described (23). Correct ORF replacement was verified by PCR using primers 5′-GCCAGATGCGAAGTTAAG-3′ and 5′-CGTCGGAGGAGATATTTATTA-3′. In the resulting strain, the *CRH1* ORF was replaced by using a hygromycin B resistance marker (hpHMX4 module). The BsgI insert from plasmid pCG01 was employed as an interruption cassette in this case as previously described (3). Correct ORF replacement was verified by PCR with primers 5′-GCCAGATGCGAAGTTAAG-3′ and 5′-AGCTGAAATGCGAGGATTG-3′.

Preparation of [¹⁴C]CM-β(1-3)glucan and [¹⁴C]CM-chitin. [¹⁴C]CM-β(1-3)glucan (CM stands for carboxymethyl) was prepared as already described (2). For the preparation of [¹⁴C]CM-chitin, strain FY001 was grown and labeled with [¹⁴C]glucosamine as previously reported (4), except that the growth temperature was 38°C rather than 30°C, to increase the incorporation into chitin (3). Cell walls were prepared and treated with sodium hydroxide as described previously (4, 8) and divided into six aliquots, each containing about 800,000 cpm. Each aliquot, in a total volume of 375 μl, was incubated for 3 h at 37°C with 0.05 M sodium acetate, pH 5, and 22 μl (~0.6 mg of protein) of recombinant β(1-6)glu-

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 Centricron 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 ×

TABLE 1 Strains used in this study

Strain	Genotype	Source or reference
YPH499	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1</i>	29
NBT014	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 crh1::HygR crh2::HIS3</i>	This work
ECY46-4-1B	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 chs3::LEU2</i>	7
BY4741	<i>MATa ura3Δ0 his3Δ1 leu2Δ0 met15Δ0</i>	EUROSCARF
YHR307W	<i>MATa ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 gas1::KanMX4</i>	EUROSCARF
FY001	<i>MATα his3Δ200 ura3-52 leu2Δ1 trp1Δ63</i>	3

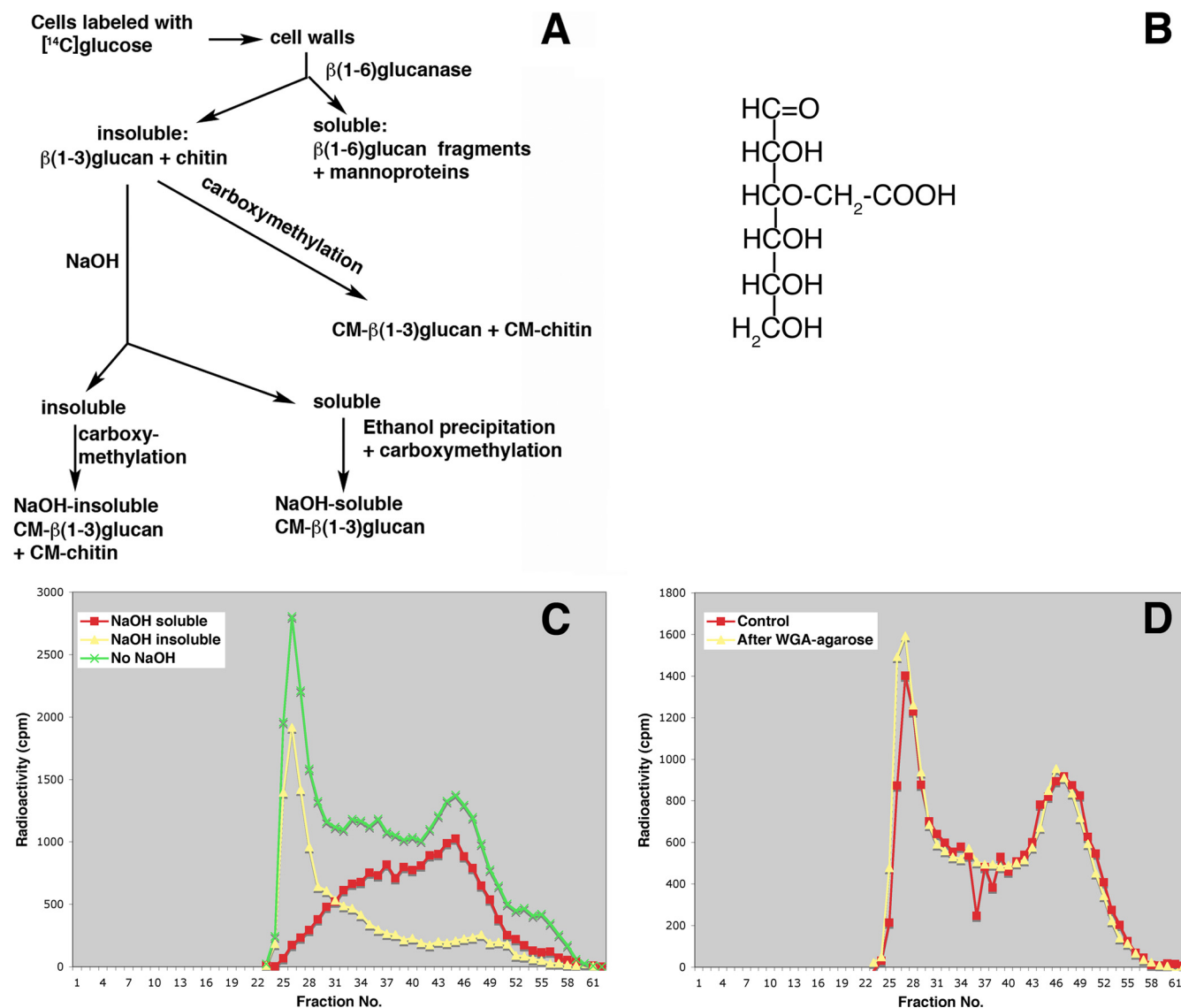


FIG 2 Isolation and fractionation of $\beta(1-3)$ glucan. (A) Scheme for isolation of $\beta(1-3)$ glucan from yeast cell walls. All carboxymethylated (CM) fractions are soluble in water. (B) Chemical linkage of acetic acid to hexose in a carboxymethylated sugar. The open structure of the sugar is shown 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 $\beta(1-3)$ 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 $[^{14}\text{C}]$ glucose as the standard. (D) Chromatography of total $\beta(1-3)$ glucan on Sephacryl S-500 before and after filtration through WGA-agarose.

10^6 to 1.6×10^6 cpm, $11.6 \mu\text{l}$ of 1 M sodium acetate, pH 5, and $20 \mu\text{l}$ of recombinant $\beta(1-6)$ 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 $200 \mu\text{l}$ of water, which was added to the first supernatant. The pellet was suspended in $200 \mu\text{l}$ of water, $50 \mu\text{l}$ 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 $200 \mu\text{l}$ 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 $\beta(1-6)$ 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 could not be mea-

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 (nanoComposix, 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 [14 C]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 μ l of water, and 50 μ l of 5 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 μ l of 1 M sodium hydroxide. The tube was heated again at 80°C for 1 h and centrifuged. The pellet was washed with 200 μ l of water, subjected to carboxymethylation, 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 carboxymethylated 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-curdan, 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 μ l of Tris-HCl, pH 7.5, and 13 μ l 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 μ l of 1 M Tris-HCl, pH 7.5; 62 μ l of 5 M NaCl; 100 μ l of carboxymethylated cell walls; and 200 μ l 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 μ l 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 μ l of curdlan-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.

TABLE 2 Isolation of β (1-3)glucan by β (1-6)glucanase and sodium hydroxide treatment of cell walls^a

Strain	β (1-6)glucanase supernatant ^b	NaOH-soluble fraction ^c
YPH499 (wild type)	53.2 \pm 3.3 (33)	55.6 \pm 3.8 (10)
ECY46-4-1B (<i>chs3</i> Δ)	47.1 \pm 4.6 (9)	79.8 \pm 2.6 (5)
NBT014 (<i>crh1</i> Δ <i>crh2</i> Δ)	46.8 \pm 3 (18)	67.9 \pm 5 (4)
BY4741 (wild type)	59.3 (1)	58 (1)
YHR307W (<i>gas1</i> Δ)	48.4 \pm 5 (3)	52.8 \pm 1.7 (3)

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

^b Mean percentage of cell wall radioactivity in β (1-6)glucanase supernatant \pm standard deviation. Values in parentheses indicate how many independent determinations were carried out in each case.

^c Mean percentage of the radioactivity of the β (1-6)glucanase-insoluble fraction in the 1 M NaOH supernatant \pm standard deviation. Values in parentheses indicate how many independent determinations were carried out.

RESULTS

Two populations in bulk β (1-3)glucan. In the past, β (1-3)glucan was prepared by extraction of intact yeast cells with sodium hydroxide and acetic acid at high temperatures (18). Treatment of cell walls with hot alkali results in the extraction of part of β (1-3)- and β (1-6)glucan and of proteins, which are degraded in the procedure. The β (1-3)glucan may also be partly degraded, but the portion bound to chitin remains insoluble (19). We wished to use mild conditions conducive to the formation of a product as similar as possible to the native polysaccharide. To this end, we first labeled wild-type cells (strain YPH499) *in vivo* with [14 C]glucose to tag all of the cell wall components. Cell walls were then isolated and digested with recombinant β (1-6)glucanase (Fig. 2A and Materials and Methods). Hydrolysis of β (1-6)glucan by the endoglucanase also liberated the mannoproteins, which are water soluble (see Fig. 1). About half of the radioactivity was found in the supernatant after centrifugation (Table 2), in line with previously published results (17). The insoluble fraction consists mostly of β (1-3)glucan, with a small amount of chitin, some of it previously linked to β (1-6)glucan, some free, and some bound to β (1-3)glucan. Accordingly, sequential treatment of this material with chitinase and β (1-3)glucanase (Zymolyase) solubilized all of the radioactivity. To make the insoluble residue accessible to fractionation, it was carboxymethylated (Fig. 2B), a procedure that results in the attachment of acetic acid residues to the polysaccharide. The negative charges thus introduced counteract the attraction between carbohydrate chains due to hydrogen bonds, and both β (1-3)glucan and chitin become soluble in water (4). The solubilized material was fractionated on a Sephacryl S-500 size exclusion column. The elution profile showed a sharp peak at the void volume position, followed by radioactivity extending through most of the column included volume (Fig. 2C, green line). When a portion of the fraction that remains insoluble after β (1-6)glucanase digestion was briefly treated with 1 M NaOH at room temperature, more than half of the radioactivity was solubilized (Table 2). The solubilized polysaccharide was precipitated with ethanol. Both this material and the fraction that remained insoluble in alkali were carboxymethylated (Fig. 2A) and fractionated on Sephacryl S-500 (Fig. 2C). Here it can be seen that the alkali-insoluble fraction (yellow curve) contained mostly high-molecular-weight material, whereas the soluble polysaccharide (red curve) was highly polydisperse. The range of molecular weights in the alkali-insoluble material cannot be assessed because most of it emerges from the

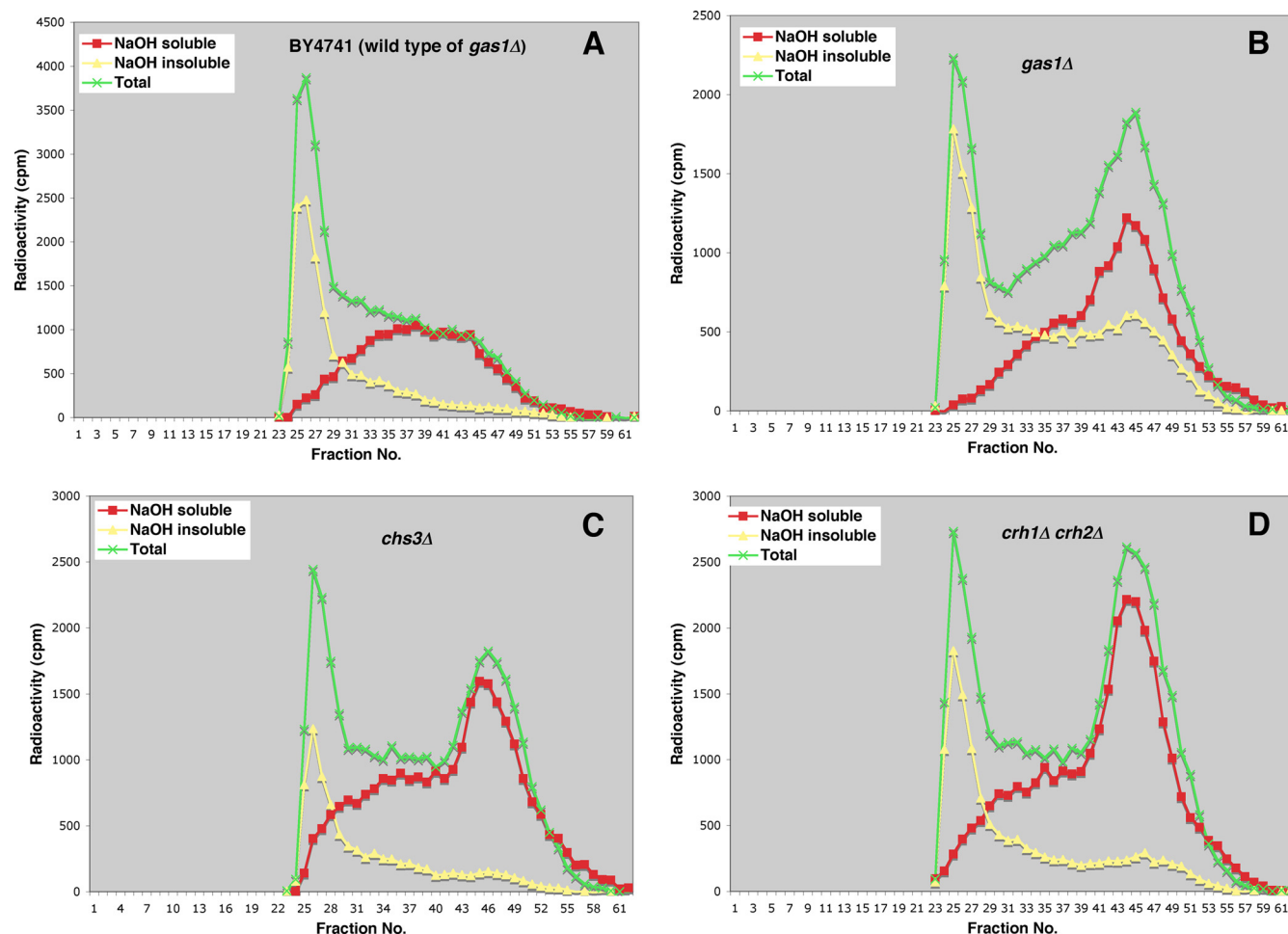


FIG 3 Chromatography of total, NaOH-soluble, and NaOH-insoluble fractions of different strains on Sephacryl S-500. (A) Strain BY4741 (wild type of the *gas1Δ* mutant). (B) Strain YHR307W (*gas1Δ*). (C) Strain EY46-4-1B (*chs3Δ*). (D) Strain NBT014 (*crh1Δ crh2Δ*). The two latter mutations are in the YPH499 background. In all cases, the procedure was as that used for Fig. 2A.

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 *chs3Δ* 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 *crh1Δ crh2Δ* 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

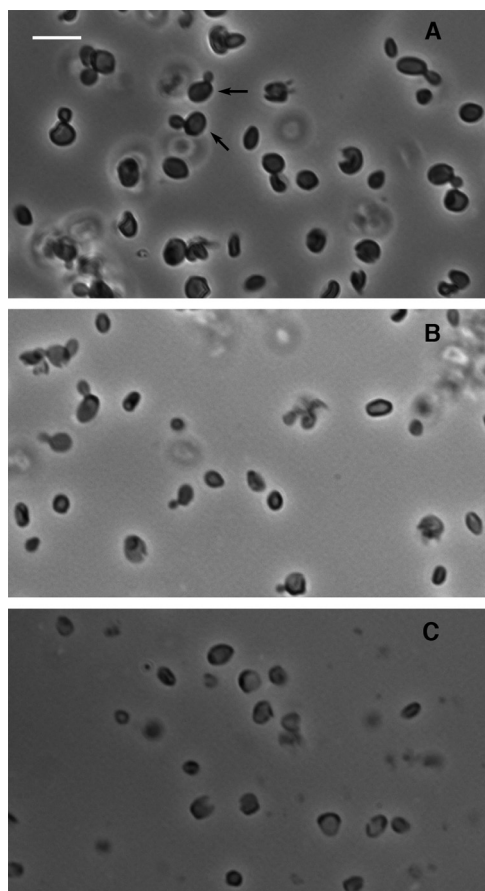


FIG 4 Cell walls before and after different treatments. (A) Untreated cell walls. Note that bud cell walls are in several cases attached to mother walls (arrows). (B) Cell walls after incubation with $\beta(1-6)$ glucanase. (C) Cell walls after further treatment with 1 M sodium hydroxide at room temperature. No bud cell walls are visible here. Scale bar represents 10 μm .

$\beta(1-3)$ 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 $\beta(1-6)$ 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 $\beta(1-3)$ glucan of daughter cells, the ones that are growing, that is solubilized by alkali.

Both the wild type and a $crh1\Delta crh2\Delta$ mutant contain $\beta(1-3)$ glucan apparently bound to chitin. Having established the size distribution pattern of the bulk $\beta(1-3)$ 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 $\beta(1-3)$ glucan as possible, the

$\beta(1-6)$ 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 $crh1\Delta crh2\Delta$ 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 $\beta(1-3)$ - or $\beta(1-6)$ 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 $\beta(1-3)$ 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– $\beta(1-3)$ 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 $\beta(1-3)$ 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– $\beta(1-3)$ glucan complex, and possibly some high-molecular-weight $\beta(1-3)$ glucan (Fig. 5A, left). The $crh1\Delta crh2\Delta$ 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– $\beta(1-3)$ 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– $\beta(1-3)$ glucan in the wild type (Fig. 5A, right) and only the first two in the $crh1\Delta crh2\Delta$ 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 $\beta(1-4)$ N -acetylglucosamine linkages, such as those of chitin. Thus, CM-chitin, free or in a complex, should be adsorbed, but $\beta(1-3)$ glucan should not. However, we found that a large percentage of [^{14}C]CM– $\beta(1-$

TABLE 3 Fractionation of $\beta(1-6)$ glucanase-insoluble material by treatment with sodium hydroxide at 80°C, followed by incubation with chitinase and new treatment with sodium hydroxide at room temperature^a

Fraction	Wild type ^b	$crh1\Delta crh2\Delta$ mutant
NaOH (80°C) insoluble	29.5 \pm 3.2	23.1 \pm 3.1
NaOH (room temp) soluble after chitinase	20.7 \pm 0.8	10.6 \pm 3

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

^b Values represent the radioactivity in each fraction as a percentage of the $\beta(1-6)$ 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.

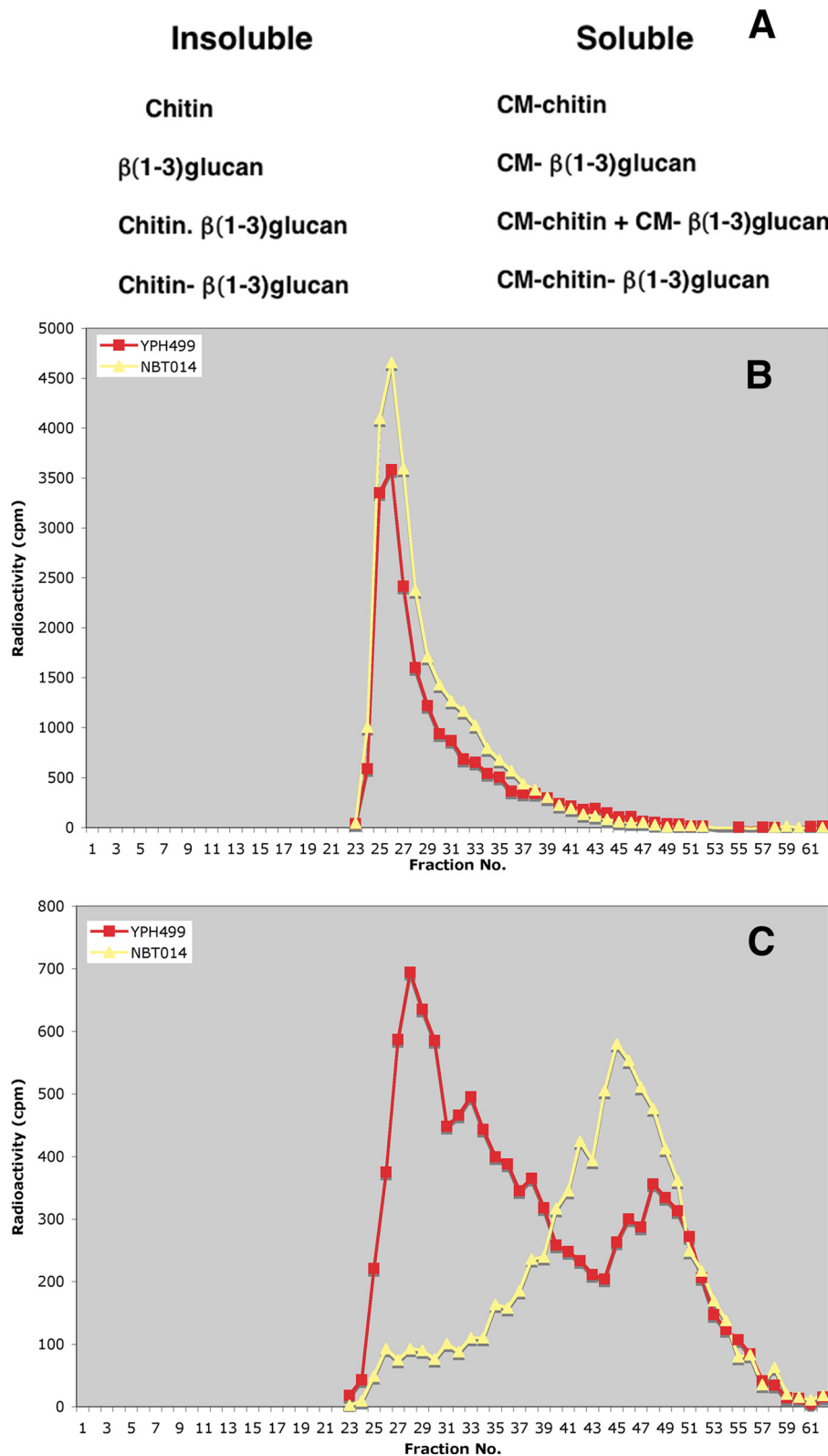


FIG 5 Isolation of the covalent chitin- $\beta(1-3)$ glucan complex. (A) Left, components of the sodium hydroxide-insoluble fractions. The covalent complex of chitin and $\beta(1-3)$ glucan is symbolized by a hyphen between the two, whereas the noncovalent complex has a dot. Right, components of the 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 *crh1* Δ *crh2* Δ 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 (*crh1* Δ *crh2* Δ). This represents free CM- $\beta(1-3)$ 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- $\beta(1-3)$ 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.

TABLE 4 Fractionation of carboxymethylated, NaOH-insoluble fraction on WGA-agarose^a

Fraction	Wild type ^b	<i>crh1Δ crh2Δ</i> 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 then washed with 0.1 M sodium hydroxide to elute any CM-chitin and CM-chitin- β (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 WGA-agarose. When, after the elution, the WGA-agarose was suspended and transferred to a scintillation vial, very little, if any, radioactivity was found in the mutant column. The wild-type column had a much larger amount, which brought the total recovery to a value similar to that for the *crh1Δ crh2Δ* mutant when added to the other fractions. We assume that this tightly bound material is high-molecular-weight CM-chitin- β (1-3)glucan.

Both the column percolates and the material eluted with alkali were fractionated on Sephacryl S-500 columns (Fig. 5B and C). The profiles of the percolates were very similar for both strains and showed single peaks of very high molecular weight (Fig. 5B). Those of the alkali-eluted material, however, were sharply different: the wild type gave rise to two peaks, the first of which was somewhat jagged (Fig. 5 C); the *crh1Δ crh2Δ* mutant yielded a single peak that emerged a little earlier than the second peak of the wild type (Fig. 5 C). The position of the second wild-type peak is the same as for CM-chitin from yeast (see Fig. 7A). The single peak of the mutant is also believed to be CM-chitin, because in this strain the free chitin is significantly larger than that of the wild type, presumably because none of it is transferred to β (1-3)glucan or β (1-6)glucan (2). Therefore, the first peak of the wild type should be the CM-chitin- β (1-3)glucan covalent complex, in agreement with its higher molecular weight. This material is lacking in the *crh1Δ crh2Δ* mutant, as expected.

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. 7 D). 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

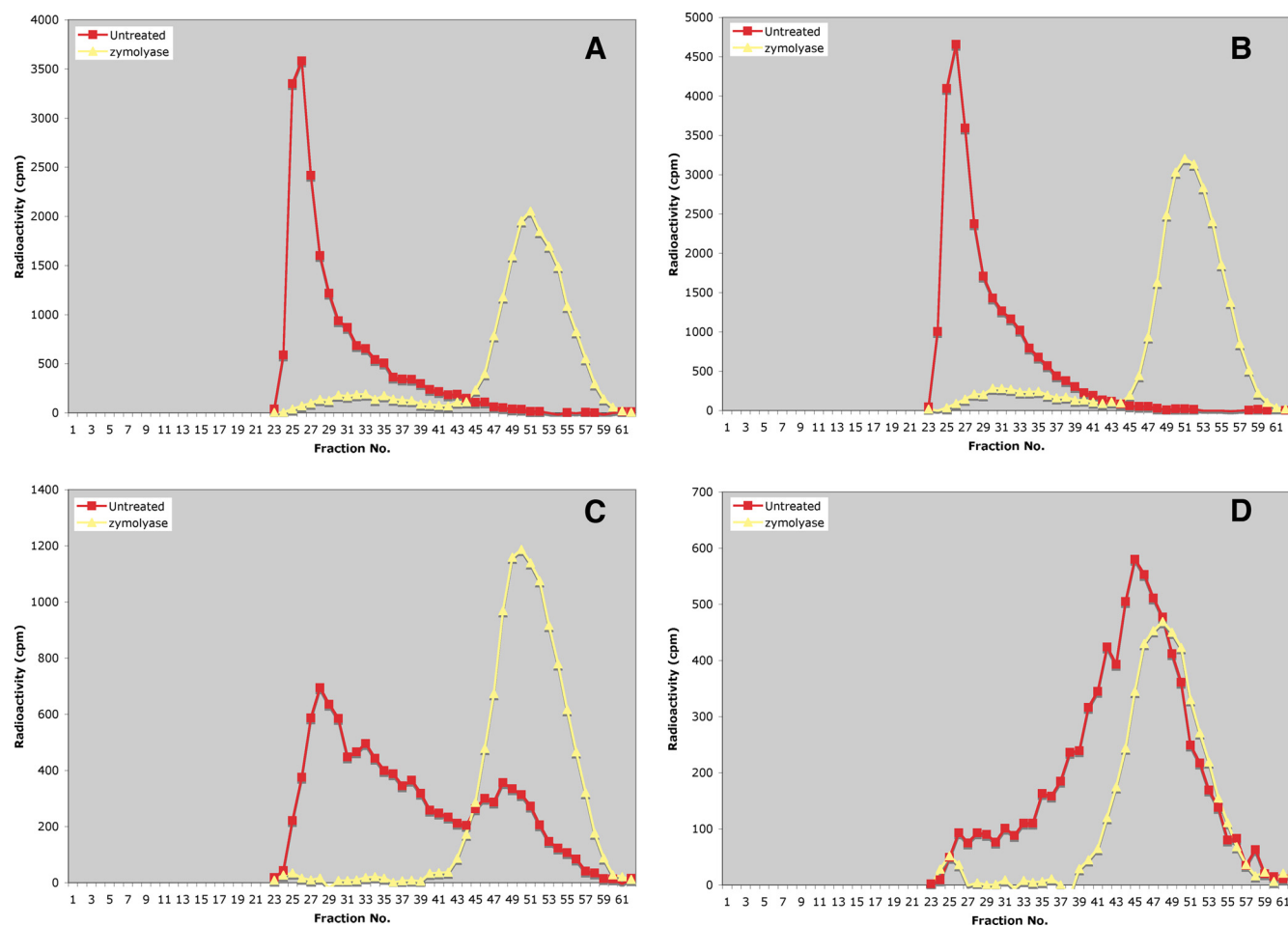


FIG 6 Chromatography of WGA-agarose fractions on Sephacryl S-500 after treatment with Zymolyase [$\beta(1-3)$ glucanase]. (A) Percolates of strain YPH499 (wild type). (B) Percolates of NBT014 (*crh1* Δ *crh2* Δ). (C) NaOH fractions of the wild type. (D) NaOH fractions of the *crh1* Δ *crh2* Δ mutant.

only the latter for the *crh1* Δ *crh2* Δ 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 [14 C]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 *crh1* Δ *crh2* Δ mutant strain after glucanase treatment, we felt the need for independent verification of the experimental evidence. To this end, cells were labeled with [14 C]glucosamine rather than with [14 C]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 *crh1* Δ *crh2* Δ 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 [14 C]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

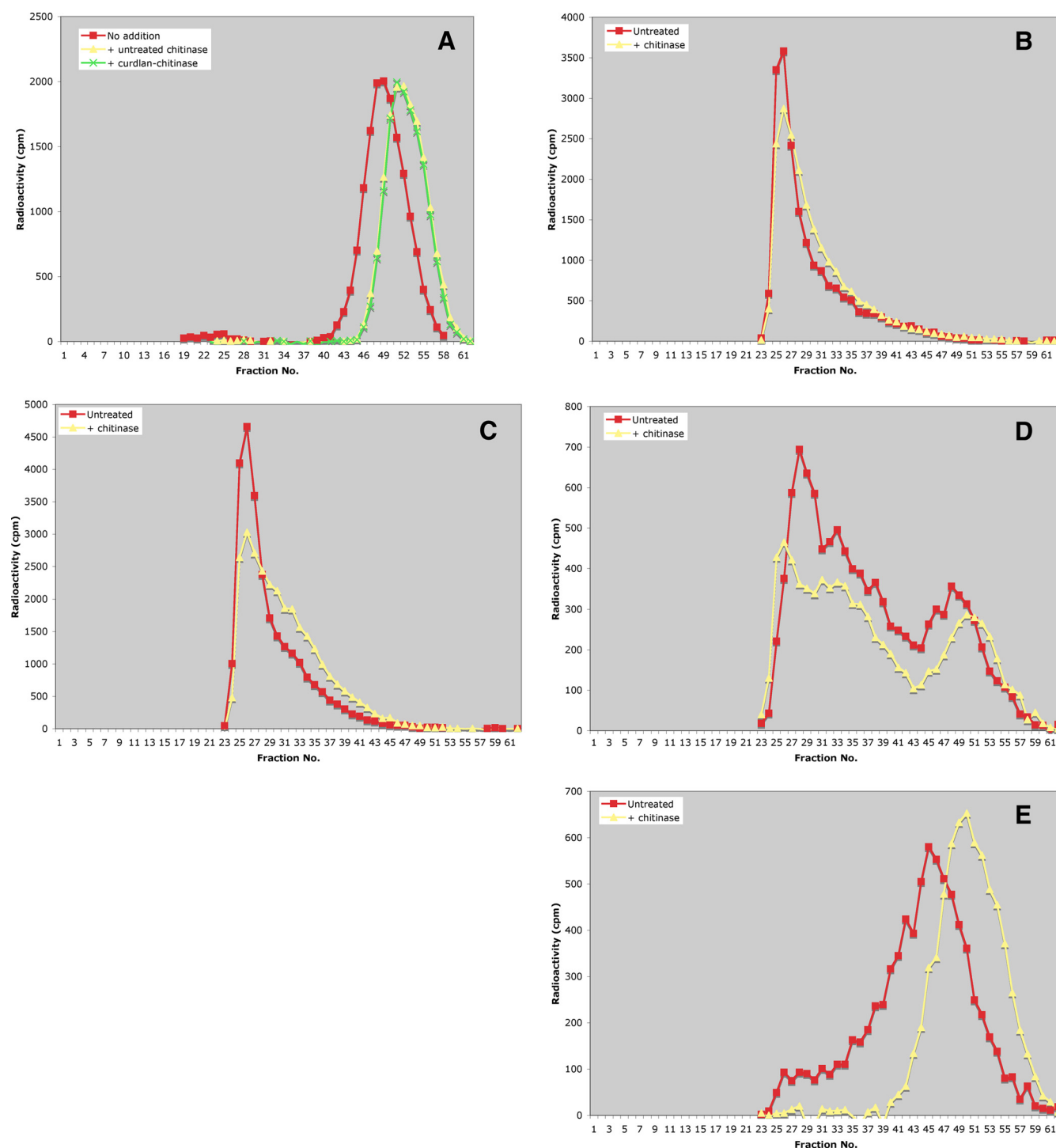


FIG 7 Chromatography of WGA-agarose fractions on Sephacryl S-500 after treatment with chitinase. (A) Chromatography of [^{14}C]CM-chitin before and after treatment with untreated or curdlan-treated chitinase. (B) Percolates of the wild type. (C) Percolates of the *crh1* Δ *crh2* Δ mutant. (D) NaOH fractions of the wild type. (E) NaOH fractions of the *crh1* Δ *crh2* Δ mutant.

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 $\beta(1-3)$ 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 2×10^7 . A molecule of that size would contain more than 120,000 glucose units. According to a determination of $\beta(1-3)$ glu-

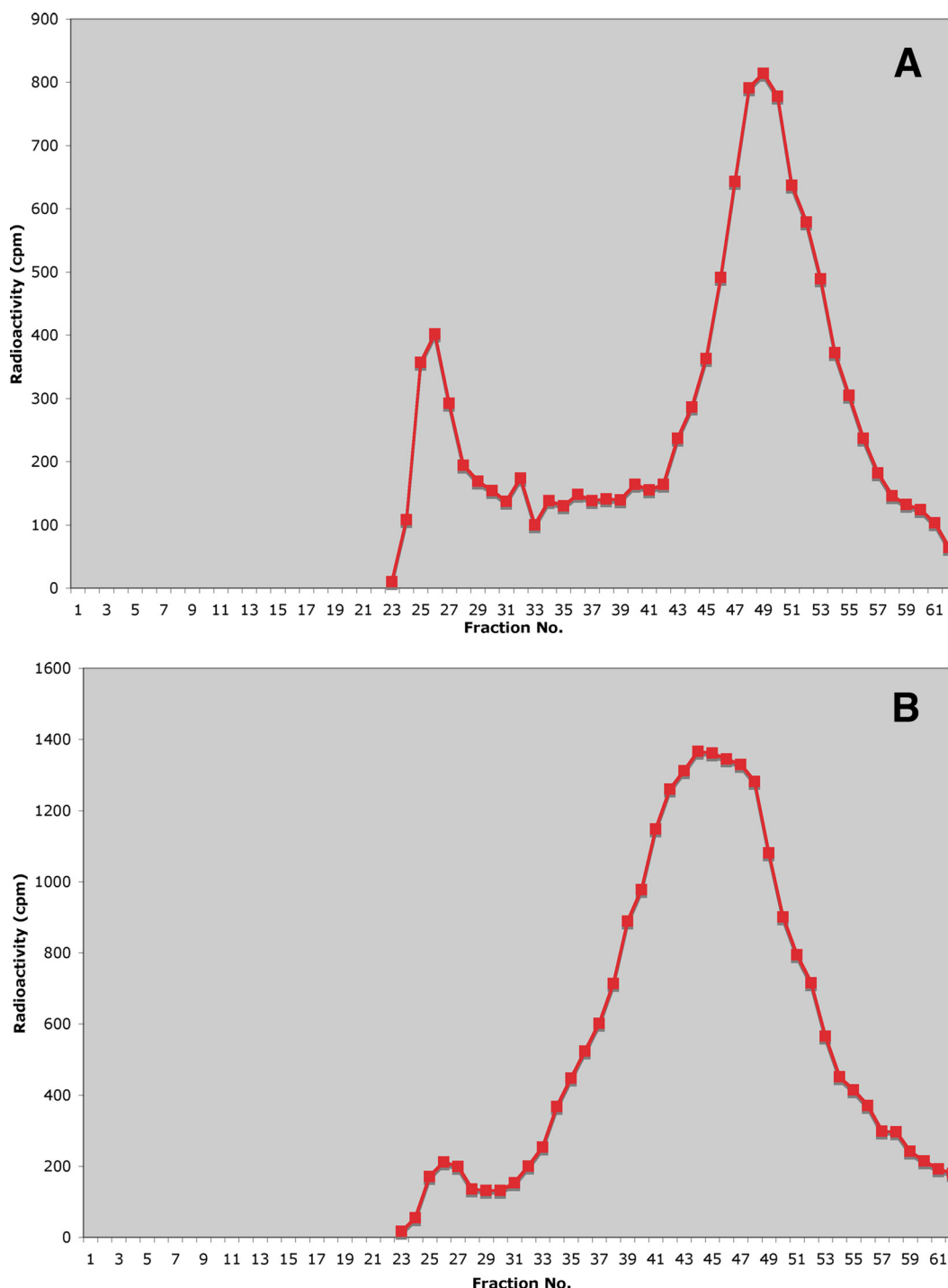


FIG 8 Chromatography on Sephacryl S-500 of WGA-agarose fractions from cells labeled with [^{14}C]glucosamine. (A) NaOH fraction of the wild type. (B) NaOH fraction of the *crh1Δ crh2Δ* mutant. The positions of peaks in these graphs should be compared to those in Fig. 5C.

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

material and would be expected to give rise to polysaccharide chains of many different sizes. We propose that the highly polydisperse alkali-soluble fraction represents the material that is going through remodeling. This notion receives some support from the results obtained with the *gas1* mutant. Because of its *in vitro* transglycosylase activity, Gas1p 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* mutant, 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 Gas1p in the size increase of the polysaccharide, although elucidation 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 Gas1p in the cross-linking process, as previously suggested (20). While this is a rather speculative concept, what is fairly clear is that the reduction in the high-molecular-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 concept 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 disappeared 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 *chs3 Δ* and *crh1 Δ crh2 Δ* 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 causes the difference 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 *chs3 Δ* and *crh1 Δ crh2 Δ* 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 established, 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 *crh1 Δ crh2 Δ* 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 solubilizing 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 Sephacryl S-500. The results are consistent with the presence, in both the wild type and the *crh1 Δ crh2 Δ* 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)glucan 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 *crh1 Δ crh2 Δ* 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? Although 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, because 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 populate 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 [14 C]glucosamine rather than [14 C]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 metabolized, in agreement with our hypothesis. The recent report that localization of Gas1p at the mother-bud neck requires Crh1p and Crh2p (25), together with our previous finding that Crh2p is concentrated at the same location (23), suggests that these enzymes cooperate in the maturation of the cell wall at the neck: Gas1p 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 mannoproteins (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 chitin 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 $\beta(1\rightarrow3)$ 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 $\beta(1\rightarrow3)$ glucan polymerization and a weaker wall. Therefore, we assume that the large-size material is the final structural polysaccharide, whereas the polydisperse fraction represents the portion undergoing remodeling during cell wall growth. The $\beta(1\rightarrow3)$ glucan attached to chitin at the mother-bud neck is mostly of high molecular weight, supporting the idea that the linkage between the two polysaccharides contributes to maintenance of the cell wall in a quiescent state at the neck. Finally, a noncovalent complex of $\beta(1\rightarrow3)$ glucan and chitin has been identified for the first time.

Aside from these findings, the novel techniques developed in this work may well be useful in future studies of the mechanism of cell wall growth and its control.

ACKNOWLEDGMENTS

We thank V. Farkas for a sample of carboxymethylchitin.

This study was supported by a National Institutes of Health grant (Intramural Research Program, NIDDK). J.A. and N.B. were supported by projects BIO2010-22146 (Ministerio de Ciencia e Innovación [MICINN], Spain) and GR58/08 (reference no. 920640; Universidad Complutense de Madrid) and the European Science Foundation Research Networking Programme (06-RNP-132). N.B. is the recipient of an FPI Ph.D. fellowship (BES-2008-003171) from MICINN.

REFERENCES

- Bom IJ, et al. 1998. A new tool for studying the molecular architecture of the fungal cell wall: one step purification of recombinant *Trichoderma* $\beta(1\rightarrow6)$ -glucanase expressed in *Pichia pastoris*. *Biochim. Biophys. Acta* 1425:419–424.
- Cabib E. 2009. Two novel techniques for determination of polysaccharide cross-links show that Crh1p and Crh2p attach chitin to both $\beta(1\rightarrow6)$ - and $\beta(1\rightarrow3)$ glucan in the *Saccharomyces cerevisiae* cell wall. *Eukaryot. Cell* 8:1626–1636.
- Cabib E, Blanco N, Grau C, Rodríguez-Peña JM, Arroyo J. 2007. Crh1p and Crh2p are required for the cross-linking of chitin to $\beta(1\rightarrow6)$ glucan in the *Saccharomyces cerevisiae* cell wall. *Mol. Microbiol.* 63:921–935.
- Cabib E, Durán A. 2005. Synthase III-dependent chitin is bound to different acceptors depending on location on the cell wall of budding yeast. *J. Biol. Chem.* 280:9170–9179.
- Cabib E, Roh D-H, Schmidt M, Crotti LB, Varma A. 2001. The yeast cell wall and septum as paradigms of cell growth and morphogenesis. *J. Biol. Chem.* 276:19679–19682.
- Caudron F, Barral Y. 2009. Septins and the lateral compartmentation of eukaryotic membranes. *Dev. Cell* 16:493–506.
- Crotti LB, Drgon T, Cabib E. 2001. Yeast cell permeabilization by osmotic shock allows determination of enzymatic activities *in situ*. *Anal. Biochem.* 292:8–16.
- Drgonová J, Drgon T, Roh D-H, Cabib E. 1999. The GTP-binding protein Rho1p is required for cell cycle progression and polarization of the yeast cell. *J. Cell Biol.* 146:373–387.
- Kapteyn JC, et al. 1997. Altered extent of cross-linking of $\beta(1\rightarrow6)$ -glucosylated mannoproteins to chitin in *Saccharomyces cerevisiae* mutants with reduced cell wall $\beta(1\rightarrow3)$ -glucan content. *J. Bacteriol.* 179:6279–6284.
- Kitamura K, Kaneko T, Yamamoto Y. 1974. Lysis of viable yeast cells by enzymes of *Arthrobacter luteus*. II. Purification and properties of an enzyme, Zymolyase, which lyses viable yeast cells. *J. Gen. Appl. Microbiol.* 20:323–344.
- Klis FM, Boorsma A, De Groot PWJ. 2006. Cell wall construction in *Saccharomyces cerevisiae*. *Yeast* 23:185–202.
- Kollár R, Petraková E, Ashwell G, Robbins PW, Cabib E. 1995. Architecture of the yeast cell wall. The linkage between chitin and $\beta(1\rightarrow3)$ -glucan. *J. Biol. Chem.* 270:1170–1178.
- Kollár R, et al. 1997. Architecture of the yeast cell wall. $\beta(1\rightarrow6)$ -Glucan interconnects mannoprotein, $\beta(1\rightarrow3)$ -glucan, and chitin. *J. Biol. Chem.* 272:17762–17775.
- Kopecká M, Phaff HG, Fleet GH. 1974. Demonstration of a fibrillar component in the cell wall of the yeast *Saccharomyces cerevisiae* and its chemical nature. *J. Cell Biol.* 62:66–76.
- Lesage G, Bussey H. 2006. Cell wall assembly in *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* 70:317–343.
- Lippincott J, Li R. 1998. Sequential assembly of myosin II, an IQGAP-like protein, and filamentous actin to a ring structure involved in budding yeast cytokinesis. *J. Cell Biol.* 140:355–366.
- Magnelli P, Cipollo JF, Abejón C. 2002. A refined method for the determination of *Saccharomyces cerevisiae* cell wall composition and β -1,6-glucan fine structure. *Anal. Biochem.* 301:136–150.
- Manners DJ, Masson AJ, Patterson JC. 1973. The structure of a $\beta(1\rightarrow3)$ -D-glucan from yeast cell walls. *Biochem. J.* 135:19–30.
- Mol PC, Wessels JGH. 1987. Linkages between glucosaminoglycan and glucan determine alkali-insolubility of the glucan in walls of *Saccharomyces cerevisiae*. *FEMS Microbiol. Lett.* 41:95–99.
- Mouyna I, et al. 2000. Glycosylphosphatidylinositol-anchored glucanoyltransferases play an active role in the biosynthesis of the fungal cell wall. *J. Biol. Chem.* 275:14882–14889.
- Popolo L, Gilardelli D, Bonfante P, Vai M. 1997. Increase in chitin as an essential response to defects in assembly of cell wall polymers in the *ggp1Δ* mutant of *Saccharomyces cerevisiae*. *J. Bacteriol.* 179:463–469.
- Roberts RL, Cabib E. 1982. *Serratia marcescens* chitinase: one-step purification and use for the determination of chitin. *Anal. Biochem.* 127:402–412.
- Rodríguez-Peña JM, Cid VJ, Arroyo J, Nombela C. 2000. A novel family of cell wall-related proteins regulated differentially during the yeast life cycle. *Mol. Cell. Biol.* 20:3245–3255.
- Rodríguez-Peña JM, Rodríguez C, Alvarez A, Nombela C, Arroyo J. 2002. Mechanisms for targeting of the *Saccharomyces cerevisiae* GPI-anchored cell wall protein Crh2p to polarized growth sites. *J. Cell Sci.* 115:2549–2558.
- Rolli E, et al. 2009. Immobilization of the glycosylphosphatidylinositol-anchored Gas1 protein into the chitin ring and septum is required for proper morphogenesis in yeast. *Mol. Biol. Cell* 20:4856–4870.
- Schmidt M, Bowers B, Varma A, Roh D-H, Cabib E. 2002. In budding yeast, contraction of the actomyosin ring and formation of the primary septum at cytokinesis depend on each other. *J. Cell Sci.* 115:293–302.
- Schmidt M, Varma A, Drgon T, Bowers B, Cabib E. 2003. Septins, under Cla4p regulation, and the chitin ring are required for neck integrity in budding yeast. *Mol. Biol. Cell* 14:2128–2141.
- Shaw JA, et al. 1991. The function of chitin synthases 2 and 3 in the *Saccharomyces cerevisiae* cell cycle. *J. Cell Biol.* 114:111–123.
- Sikorski R, Hieter P. 1989. A system of shuttle vectors and host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 122:19–28.
- Trevelyan WE, Harrison JS. 1952. Studies on yeast metabolism 1. Fractionation and microdetermination of cell carbohydrates. *Biochem. J.* 50:298–303.