Architecture of the yeast cell wall, beta(1-6)glucan interconnects mannoprotein, beta(1-3)-glucan, and chitin

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Architecture of the Yeast Cell Wall

β(1–6)-GLUCAN INTERCONNECTS MANNOPROTEIN, β(1–3)-GLUCAN, AND CHITIN*

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In a previous study (Kollár, R., Petráková, E., Ashwell, G., Robbins, P. W., and Cabib, E. (1995) J. Biol. Chem. 270, 1170–1178), the linkage region between chitin and β(1–3)-glucan was solubilized and isolated in the form of oligosaccharides, after digestion of yeast cell walls with β(1–3)-glucanase, reduction with borotritide, and subsequent incubation with chitinase. In addition to the oligosaccharides, the solubilized fraction contained tritium-labeled high molecular weight material. We have now investigated the nature of this material and found that it represents areas in which all four structural components of the cell wall, β(1–3)-glucan, β(1–6)-glucan, chitin, and mannoprotein are linked together. Mannoprotein, with a protein moiety about 100 kDa in apparent size, is attached to β(1–6)-glucan through a remnant of a glycosylphosphatidylinositol anchor containing five α-linked mannosyl residues. The β(1–6)-glucan has some β(1–3)-linked branches, and it is to these branches that the reducing terminus of chitin chains appears to be attached in a β(1–4) or β(1–2) linkage. Finally, the reducing end of β(1–6)-glucan is connected to the non-reducing terminal glucose of β(1–3)-glucan through a linkage that remains to be established. A fraction of the isolated material has three of the main components but lacks mannoprotein. From these results and previous findings on the linkage between mannoproteins and β(1–6)-glucan, it is concluded that the latter polysaccharide has a central role in the organization of the yeast cell wall. The possible mechanism of synthesis and physiological significance of the cross-links is discussed.

Cell walls are essential for the survival of fungal cells. Digestion of cell walls in the absence of an osmotic protector leads to cell lysis due to the high internal turgor pressure. Thus, substances that interfere with cell wall synthesis may be considered as potential antifungal agents (1). Because of its rigidity, the cell wall determines the shape of fungal cells. For that reason, cell wall formation has been used as a model for morphogenesis (1).

The major components of fungal cell walls are polysaccharides and glycoproteins (2). In the yeast, Saccharomyces cerevisiae, the cell wall contains β(1–3)-β-glucan, β(1–6)-β-glucan, chitin, and mannoprotein(s) (3). The polysaccharides appear to have a structural function, whereas the mannoprotein(s) may act as “filler” and are important for the permeability of the cell wall (4, 5). How can one explain the strength and resilience of the fungal cell wall? Recent results with S. cerevisiae suggest that the answer may be found in the existence of covalent linkages between the different components of the wall that would give rise to a continuous and consequently stronger fabric. Thus, previous studies in our laboratories showed the presence of linkages between chitin and β(1–3)-glucan (6) as well as among glycoproteins, β(1–6)-glucan, and β(1–3)-glucan (7).

The strategy for the investigation of interconnections between chitin and β(1–3)-glucan consisted in the digestion of cell walls with β(1–3)-glucanase, followed by labeling of the exposed reducing ends with borotritide and enzymatic hydrolysis of the chitin. This procedure led to the isolation of a family of trinitiated oligosaccharides that contained the sought after connection, i.e. a β(1–4)-linkage between the reducing end of a chitin chain and the nonreducing end of a β(1–3)-glucan chain (6). The oligosaccharides were separated on a Bio-Gel P-2 sizing column. The void volume fraction of this column contained a fairly large amount of tritium-labeled material of high molecular weight (6). We have now studied the structure of this material. The results described below show that it represents a region or regions of the cell wall where all four major components, β(1–3)-β-glucan, β(1–6)-β-glucan, chitin, and mannoprotein, are linked together. In this complex, chitin is directly attached to a branch of β(1–6)-β-glucan.

EXPERIMENTAL PROCEDURES

Materials—β-N-Acetylhexosaminidase, β-galactosidase, and α-mannosidase (all from jack bean) were from Oxford Glycosystems. β-Glucosidase from sweet almonds and recombinant endoglycosidase-H and protease K were purchased from Boehringer Mannheim; glycopeptidase F was from Life Technologies, Inc.; Zymolase 100 T was from Seikagaku; and protease E (Pronase) was from Sigma. Sodium [3H]borohydride (100 mCi/mmol) was obtained from ICN; [1-34]glucopyranosyl(50–60 mCi/mmol) was from American Radiolabeled Chemicals; and uridine diphospho-[U-14C]galactose (305 mCi/mmol) was from Amersham Corp. Bio-Gel P-2 (fine and extra fine) and Bio-Gel P-4 (extra fine) were from Bio-Rad; concanavalin A-Sepharose and Sephadex G-100 were from Pharmacia Biotech Inc.; Erythrina cristagalli lectin-agarose was from Vector; and Rezex RSO-Oligosaccharides HPLC1 column was from Phenomenex. Polyacrylamide gels were from Novex, and PVDF

The abbreviations used are: HPLC, high performance liquid chromatography; HPAEC, high performance anion exchange chromatography; ConA, concanavalin A; ConA†, cell wall fraction that binds to ConA-Sepharose; ConA±, cell wall fraction that does not bind to ConA-Sepharose; fraction M, high molecular weight material remaining after β(1–6)-β-glucanase digestion of ConA†; endo-H, endo-β-N-acetylglucosaminidase H; GPI, glycosylphosphatidylinositol; ES-MS, electrospray mass spectrometry; CID, collision-induced decomposition; ER, endoplasmic reticulum; PVDF, polyvinylidene difluoride; Mann, mannopryanosyl.

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membranes were from Millipore Corp. Chitinase from S. marcescens was prepared as described (8), and endo-β(1→6)-glucanase was prepared from B. circulans WL-12 as described (9).

Yeast Strains and Yeast Growth—The S. cerevisiae strain used was ECY36–3C (MAT a his1-23 leu2-3,112 trp1-1 ura3-52 leu2-3). Cells were grown in YEPD (1% yeast extract, 2% peptone, 2% glucose) at 30 °C.

Preparation of Cell Walls and Digestion with Chitinase—The procedure was as described previously (6), except that, after cell disintegration with glass beads, cell walls were sedimented by centrifugation, washed twice with 50 mM Tris-chloride, pH 7.5, suspended in the same buffer, and incubated in a boiling water bath for 10 min, followed by five additional washings with 50 mM Tris. The isolated cell walls from 30 g (wet weight) of yeast were suspended in 860 ml of 50 mM Tris-chloride buffer, pH 7.5, and incubated with 163 ml of phenylmethylsulfonyl fluoride-treated Zymolyase (36 mg of Zymolyase 100 T) was dissolved in 154 ml of 66 mM sodium phosphate, pH 7.5, containing 0.8 mM mannitol to which 250 mg of phenylmethylsulfonyl fluoride in 9.4 ml of isopropanol alcohol was added and incubated 1 h at 30 °C for 4 h at 37 °C until the absorbance at 660 nm had decreased to about 15% of the original value. The insoluble residue was recovered by centrifugation. Washing of this material with Tris buffer, 1% SDS, and water, reduction with sodium borotritide, and digestion with chitinase were as described previously (6). Any insoluble residue after chitin digestion was recovered by centrifugation, and supernatant fluid was used directly for Bio-Gel P-2 column chromatography (6). The material eluting in the void volume (fraction V₀) was used for further analysis.

Affinity Chromatography of Fraction V₀ on Sepharose-bound Concanavalin A Column—Tritiated V₀ fraction (5.6 × 10⁶ cpm) was evaporated to dryness, dissolved in 500 ml of 50 mM Tris-chloride, pH 7.4, containing 0.15 mM NaCl (binding buffer), and applied to a concanavalin A (ConA)-Sepharose column (0.9 × 85 cm). The elution was with binding buffer, and 0.45-ml fractions were collected. At fraction 34, the eluting solution was changed to 0.5 mM a-methylmannoside in 50 mM Tris-chloride containing 0.25 mM NaCl to displace the bound material. A portion of each fraction (10 ml) was counted. The fractions containing radioactivity and corresponding either to the unretained (ConA−) or to the bound (ConA+) material were pooled and concentrated by evaporation.

Chromatography—High performance anionic exchange chromatography (HPAEC) and paper chromatography were performed as described previously (6).

SDS-Polyacrylamide Gel Electrophoresis—Cell wall proteins were separated by either 18% or linear gradient (2.2–20%) polyacrylamide gels according to Laemmli (10). For Western analysis, gels (2.2–20%) were blotted onto PVDF membrane, and antibody staining was carried out as described by Montijn et al. (11).

Carbohydrate Determinations—Total carbohydrate was measured with the anthrone reagent (12) or by a modified phenol sulfuric method (13). Samples were diluted to 40 μl with water, mixed with 30 μl of 5% phenol and 250 μl of concentrated sulfuric acid in 96-well microtitration plates, and incubated for 10 min at room temperature. Absorbance at 490 nm was measured in a Bio-Tek EL309 autoreader. Free GlcNAc was quantified by the method of Reissig et al. (22). The incubated mixture was reduced to dryness, dissolved in 50 μl of NaOH, and incubation continued overnight under the same conditions. The sample was neutralized with 0.1 M NaOH and reduced by the direct addition of solid NaBH₄ (1 mg) at room temperature for 16 h. Excess reducing agent was destroyed by the addition of 5 μl of acetic acid, and the solution was dried in a vacuum centrifuge. Borate was removed by the repeated addition and drying with methanol. Periodate–oxidized and reduced oligosaccharides were then methylated as above.

Electrospray Ionization Mass Spectrometry—The instrument used was a TSQ 700 (Finnigan-MAT, San Jose, CA) equipped with an Analytica (Analytica of Branford, CT) electrospray ion source. Methylated oligosaccharides were analyzed by direct infusion at flow rates of 0.85 μl/min of a 6:4 methanol:water electrospray buffer with 0.25 mM NaOH.

RESULTS

Preparation and General Properties of Cell Wall Complex (Fraction V₀)—Yeast cell walls were digested with β(1→3)-glucanase, labeled by reduction with borotritide, and further treated with chitinase as described previously (6). After chromatography of the material solubilized by chitinase on a Bio-Gel P-2 column (6), the fraction emerging at the void volume (fraction V₀) was collected. This early fraction, as well as later peaks, contained radioactivity (6). Initial experiments were directed to ascertain the general composition of the material and its relationships with known cell wall components.
Yeast Cell Wall Cross-links

Fig. 1. Labeling of fraction V₀ with 14C-galactose. The chitinase-solubilized fraction V₀, obtained by digestion of cell walls and gel chromatography (see "Experimental Procedures") was incubated with UDP-[U-14C]galactose and bovine galactosyltransferase, with (a) or without (b) preincubation with jack bean β-N-acetylhexosaminidase. The incubated fraction was applied to an extra fine Bio-Gel P-2 column (2 × 90 cm) and eluted with 0.1 M acetic acid. Fractions of 1.8 ml were collected, and a 10-μl portion of each sample was counted using a program that allowed determination of tritium (●) and 14C (▲) isotope in separate channels.

Labeling with borotritide required prior treatment with β(1→3)-glucanase. When borotritide was used on the intact walls, only 24% of the radioactivity was incorporated into fraction V₀. This result suggested that most substances in the void volume peak had been attached to β(1→3)-glucan in the intact cell wall. Similarly, the solubilization of the material upon chitinase treatment clearly indicated that it had been linked to chitin. In our previous study of the chitin-β(1→3)-glucan connection, it had been easy to identify remaining GlcNAc in the chitin. In our previous study of the chitin-β(1→3)-glucan connection, it had been easy to identify remaining GlcNAc in the chitin.

Further fractionation was required to determine whether the galactose (hence the linkage to chitin, we availed ourselves of a sensitive and specific technique that consists in labeling nonreducing GlcNAc residues with [14C]galactose by incubation with UDP-[14C]Gal in the presence of bovine galactosyltransferase (16). Treatment of fraction V₀ in this fashion resulted in the incorporation of radioactivity (Fig. 1b). A control in which fraction V₀ was pretreated with β-N-acetylglucosaminidase showed no incorporation (Fig. 1a). This result confirms the covalent linkages of substances in fraction V₀ to chitin. It should be kept in mind that, because Serratia chitinase cleaves two GlcNAc residues at a time, even-numbered chains may be excised completely without leaving any amino sugar, as was found for the chitin-β(1→3)-glucan linkage (6). Therefore, assuming a random chain length, the residues labeled in the experiment of Fig. 1 may correspond to only half of the connecting chitin chains, those with an odd number of GlcNAc residues.

The evidence outlined above points to a linkage of the material in fraction V₀ to both β(1→3)-glucan and chitin but does not address the question of how many different substances are present in fraction V₀. Further fractionation was required to deal with this problem. Because of the presence of mannose (see below) in fraction V₀, we used chromatography on ConA-Sepharose columns (see "Experimental Procedures"). Some what more than half of the tritium label went through the column without binding, whereas the remainder was attached and required α-methylmannoside for elution. The two fractions were designated ConA⁻ and ConA⁺, respectively. Both fractions could be labeled with [14C]galactose in the manner outlined above. To ascertain whether the galactose (hence the
GlcNAc) was attached to the same substance(s) that carried the tritium label, the derivatized fractions were applied to a column of agarose-bound E. crista-galli lectin, which binds specifically to galactosyl and Gal-GlcNAc groups (Fig. 2). Both ConA and ConA$_1$ were separated into three fractions, I, II, and III. The first one was not bound to the column and contained only tritium; the second one was retarded and contained tritium and a small proportion of $^{13}$C; the last fraction required lactose plus GlcNAc for elution and contained both isotopes, but with a much higher percentage of $^{13}$C. We interpret these results as follows: (a) the first fraction represents material previously joined to even-numbered chitin chains, which were completely eliminated by chitinase; (b) the second fraction contains substance(s) that were bound to a single odd-numbered chitin chain; and (c) the third fraction consists of material that had multiple linkages to chitin; therefore, it contains several galactosyl residues per molecule and binds more strongly to the lectin column.

From the results with the ConA-Sepharose and the E. crista-galli lectin-agarose columns, we conclude that the tritium-labeled chain ends, the GlcNAc to which $[^{13}$C]galactose had previously been attached, remained after borohydride reduction. Chitin would be linked to one or more $\beta(1\rightarrow6)$ branches of the $\beta(1\rightarrow3)$-glucan, a stub of which, with the terminal glucose converted into sorbitol, remained after $\beta(1\rightarrow3)$-glucanase digestion and borohydride reduction. Chitin would be linked to one or more $\beta(1\rightarrow3)$ branches of the $\beta(1\rightarrow6)$-glucan through its reducing terminal GlcNAc, in the same fashion as it is attached directly to $\beta(1\rightarrow3)$-glucan (6). Finally, the nonreducing end of the $\beta(1\rightarrow6)$-glucan would be attached to a mannanprotein through a bridge region consisting of part of a GPI anchor (23). The remainder of the GPI anchor was presumably the leaving group in the transglycosylation process that gave rise to the glucan-protein linkage.

For the ConA$^-$ fraction we propose essentially the same structure, but without the mannosylprotein or the GPI anchor bridge. The reasons for this attribution will be discussed below.

A Tentative Structure for ConA$^+$—Because of the complex nature of the ConA$^+$ and ConA$^-$ fractions, it is convenient at this point to introduce a tentative structure for them, to facilitate understanding of the experiments to be described. Such a structure is shown for ConA$^+$ in Fig. 3.

The structure inside the broken line corresponds to the isolated ConA$^+$, whereas the connections to the remainder of the cell wall are shown outside the line. According to this hypothesis, the central portion of ConA$^+$ would consist of a $\beta(1\rightarrow6)$-glucan chain with some $\beta(1\rightarrow3)$ branches. At the reducing terminus, the $\beta(1\rightarrow6)$-glucan would be attached to $\beta(1\rightarrow3)$-glucan, a stub of which, with the terminal glucose converted into sorbitol, remained after $\beta(1\rightarrow3)$-glucanase digestion and borohydride reduction. Chitin would be linked to one or more $\beta(1\rightarrow3)$ branches of the $\beta(1\rightarrow6)$-glucan through its reducing terminal GlcNAc, in the same fashion as it is attached directly to $\beta(1\rightarrow3)$-glucan (6). Finally, the nonreducing end of the $\beta(1\rightarrow6)$-glucan would be attached to a mannanprotein through a bridge region consisting of part of a GPI anchor (23). The remainder of the GPI anchor was presumably the leaving group in the transglycosylation process that gave rise to the glucan-protein linkage.

For the ConA$^-$ fraction we propose essentially the same structure, but without the mannosylprotein or the GPI anchor bridge. The reasons for this attribution will be discussed below.

ConA$^-$ Contains $\beta(1\rightarrow6)$-Glucan—Acid hydrolysis of ConA$^-$ followed by HPAEC showed the presence of glucose and mannan (Fig. 4d) in approximately equal amounts (Glc:Man ratio of 1:1 in the experiment of Fig. 4, closer to 1:1 in other batches), together with a very small proportion of GlcNAc. A $^{13}$C NMR spectrum of ConA$^-$ clearly showed the presence of signals corresponding to $\beta(1\rightarrow6)$-glucose linkages (Fig. 4c). However, the spectrum of ConA$^-$ (Fig. 4e) was chosen for detailed interpretation, because it is free from mannose sig-
nals. There are two sets of signals in this spectrum (Table I). One set of high intensity signals corresponds well with signals for pustulan (β1→6)-linked glucopyranosyl residues. The signals of the second set, of lower intensity, are in very good agreement with the $^{13}$C chemical shifts of laminarin; therefore, they correspond to β1→3-linked glucopyranosyl units. There are two more signals in the spectrum of ConA, at δ 75.46 and 73.65 ppm, which may be assigned to β1→6-linked glucopyranosyl units of the main chain that are carrying branches composed by β1→3-linked glucose residues. We arrived at this assignment by exclusion of all other possibilities, as follows: a branch linked at C-4 of the polysaccharide backbone would have resulted in signals either at δ 79.5 ppm (β-linked) or 78.4 ppm (α-linked); a branch at C-2 would have yielded signals at δ 82.1 ppm (β-linked) or 79.5 ppm (α-linked); finally, the signal of an α-linked branch at C-3 would have been at δ 99.8 ppm (26). The only remaining possibility is a side chain composed of β(1→3)-linked glucopyranosyl units linked to a C-3 position of the main β1→6 chain. The signals at δ 75.46 and 73.65 ppm can be assigned to C-5 and C-2 of the branched unit, in very good agreement with the 75.4 ppmsignal in spectra of the 3,6-di-O-substituted β1→6-glucopyranosyl residue of a variety of branched β1→3-glucans (27). The difference between the two chemical shifts (δ 75.46 and 73.65 ppm) and those belonging to unbranched glucose units (δ 75.78 and 73.93 ppm) is probably caused by greater shielding due to the presence of the side chain.

The results of the $^1$H NMR spectrum of ConA (Fig. 5) were in agreement with those of the $^{13}$C spectrum. In the anomeric region of this spectrum there are two resolved doublets. The first one, at δ 4.73 ppm (J$_{1,2}$ 7.6 Hz) was assigned to the H-1 proton of the side chain (1→3)-glucopyranosyl unit, the second one, at δ 4.52 ppm (J$_{1,2}$ 7.9 Hz), belongs to the H-1 proton of the main chain, (1→6)-linked. Their coupling constants are in good agreement with those published for β-linked n-glucopyranoses (28, 29). There is a third doublet in the anomeric region, partially overlapped by the doublet at δ 4.52 ppm. We assume that this doublet belongs to the branched glucopyranosyl residues (3,6-linked). The sum of the integrals (22.6 at δ 4.52 ppm) was compared with the integral (22.2) of the doublet at δ 4.22 ppm, which represents H-6$_a$ of the main chain (30). Since they are almost identical, one may conclude that the doublet at δ 4.52 ppm represents all H-1 protons from the backbone, either from branched or unbranched units.

All these data indicate the presence in ConA of a β1→6-linked glucopyranose polysaccharide with β1→3 branches. All of the signals in the NMR spectra of ConA could be found in those of ConA$^+$ and of fraction V$^o$, therefore, these fractions contain the same or a similar polysaccharide.

In confirmation of these results, digestion of [14C]Gal-labeled ConA$^+$ or ConA with bacterial β1→6-glucanase followed by chromatography on Sephadex G-100 showed extensive degradation of the material, with all of the $^{14}$C and part of the tritium in a low molecular weight wide peak, whereas the remainder of the $^3$H was still in the void volume fraction (Fig. 6b). When the three fractions isolated by chromatography of ConA$^+$ on the E. crustagalli lectin-agarose column (Fig. 2a) were individually incubated with the glucanase, all of them showed a similar pattern of degradation, with part of the tritium eluted in the void volume fraction (results not shown). Since the $^{14}$C represents galactose attached to GlcNAc, the generation of $^{14}$C-labeled low molecular substances by β1→6-glucanase treatment indicates that chitin is linked to β1→6-glucan.

Chitin Is Directly Attached to β1→6-Glucan, Probably through a β1→3 Branch—Fractionation of the low molecular weight material released by β1→6-glucanase on a E. crustagalli lectin-agarose column completely separated the $^{14}$C-containing material (B), which was retarded, from the tritium-labeled substances (A), which were not absorbed by the column (Fig. 7a). Each one of the E. crustagalli lectin-agarose fractions was rechromatographed on a Bio-Rad P-2 column (Fig. 7, b and c). The tritium-containing fraction gave rise to several labeled

![Fig. 4. $^{13}$C NMR spectra of different fractions and corresponding HPAEC chromatograms of their acid hydrolysates. $^{13}$C NMR spectra of fraction V$^o$.](image-url)

![Fig. 5. Proton NMR spectrum of ConA fraction.](image-url)

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<tr>
<th>Table I</th>
<th>$^{13}$C NMR chemical shifts for ConA$^+$</th>
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<tr>
<td>Pustulan$^a$</td>
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<tr>
<td>Laminarin$^b$</td>
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$^a$ Our measurements.

$^b$ Our measurements, which coincide with published results (24, 25).
peaks, at positions corresponding to oligosaccharides in the 2–6 hexose residue range (Fig. 7b). Carbohydrate determinations revealed the presence of unlabeled oligosaccharides in a similar range of sizes but eluting at slightly different positions (Fig. 7b). The unlabeled oligosaccharides presumably originate in inner portions of the \( \beta \)-d-glucan chain (see Fig. 3) and consist exclusively of glucose, whereas the tritiated compounds are derived from the reducing terminus previously attached to \( \beta \)-linked glucan and contain sorbitol. This difference in composition explains the slight variation in elution volume (6).

The \( ^{13} \text{C} \)-labeled oligosaccharides fractionated in the P-2 column into three main peaks, I, II, and III (Fig. 7c). These oligosaccharides represent linkage points of \( \beta (1\rightarrow6) \)-glucan to chitin, because they contain GlcNAc. Since the linkage points are very few relative to the total length of the glucan chain, this fraction contains very little carbohydrate, which was undetectable under our conditions (Fig. 7c). Peak III was subjected to further analysis. After labeling the reducing end by reduction with borotritide, the \( ^{13} \text{C} \)-galactose at the nonreducing end was cleaved off with \( \beta \)-galactosidase (see “Experimental Procedures”). The liberated tritiated oligosaccharide emerged from the P-2 column at a position consistent with the composition GlcNacGlc3Glcol. The same composition was indicated by sep-
paration of the sugars on HPAEC after acid hydrolysis. Incubation of the oligosaccharide with \( \beta \)-N-acetylglucosaminidase, followed by \( \beta \)-glucosidase, resulted in the liberation of tritiated sorbitol (results not shown).

Further studies of degalactosylated peak III were carried out by mass spectrometry before and after derivatization, a technique that provides structural information with very small amounts of material. Electrospray mass spectrometry (ES-MS) of methylated degalactosylated peak III produced two major peaks at \( m/z \) 584.7 and 1146.6 (Fig. 8a) corresponding to doubly and singly charged ions (charge arising by the addition of sodium), with a mass that is consistent with a methylated oligosaccharide alditol containing one N-acetyl hexosamine and four hexose residues. Since oligosaccharide permethylation eliminates acyl modifications, the underivatized oligosaccharide was also examined by ES-MS in the negative ionization mode, and this was also consistent with a composition of a methylated oligosaccharide alditol containing one N-acetyl hexosamine and four hexose residues (results not shown).

The CID spectrum of the derivatized oligosaccharide was examined to obtain information about the sequencing and branching structure. The collision spectrum of the doubly charged molecular ion at \( m/z \) 584 (Fig. 8c) produced a prominent \( Y_1/Y_2 \) ion pair (31) at \( m/z \) 282 and 887 (Scheme 1a, Fig. 8c). The loss of an additional hexose from the \( m/z \) 887 \( Y_1 \) ion is observed (\( m/z \) 683, \( Y_3 \)), as is another hexose loss (\( m/z \) 479, \( Y_2 \)), to form a partial set of sequence ions containing the reducing end. The final \( Y_1 \) ion is not observed, since the reducing end glucose had been reduced, and the pyranose ring structure that would stabilize the addition of a sodium cation on this fragment was destroyed. The set of \( Y \) ions at \( m/z \) 887, 683, and 479 indicates a linear topology; however, there is another sequence of \( Y \) ions that indicates a branched structure. These are the tri- and disaccharide \( Y/Z \) fragments at \( m/z \) 669/651 and \( m/z \) 465/447, showing multiple glycosidic losses (Scheme 1b, Fig. 8c). Although the fragmentation pattern does indicate both a linear and branched structure, it provides a poor quantification of the relative amounts of these structures.

**Fig. 8. Mass spectrometry of degalactosylated peak III.**

**a**, ES-MS spectrum of permethylated sample. The \( m/z \) 584.7 peak is doubly charged, adducting two sodium ions, and the \( m/z \) 1146.6 peak is singly charged, adducting a single sodium. The mass is consistent with a permethylated oligosaccharide alditol having one N-acetyl hexosamine and 4 hexose residues. **b**, ES-MS spectrum of the periodate-oxidized, NaB\( \text{H}_4 \)-reduced and permethylated degalactosylated peak III. A triplet of ion peaks is observed in the single and double charge states. **c**, ES-MS-CID-MS spectrum of the doubly natriated, permethylated oligosaccharide alditol at \( m/z \) 584.7. The spectrum is plotted as a relative entropy surface (34), which suppresses noise spikes and allows the graphical presentation of high and low amplitude peaks in a single scale, similar to a log scale. **d**, ES-MS-CID-MS of the singly natriated peak at \( m/z \) 939 from the periodate-oxidized, NaB\( \text{H}_4 \)-reduced and permethylated oligosaccharide. The spectrum is again an entropy surface.
The glycosidic linkages in the oligosaccharide were also investigated by CID. The glycosidic bond on the reducing side of N-acetyl hexosamine residues is rapidly cleaved under the collision conditions of the triple quadrupole, and this results in the prompt loss of a nonreducing terminal GlcNAc. This, unfortunately, largely prevented the formation of ring-opening fragments that can be used for linkage assignment with tandem mass spectrometry of permethylated oligosaccharides (32, 33). The only ring-opening fragments observed were a minor m/z 301 (542) and 329 (523) pair (Fig. 8c), and these suggest a terminal 6-linked hexose.

Periodate oxidation, reduction, and permethylation of the oligosaccharide can be used to obtain linkage information at high sensitivity (22). The ES-MS spectrum of the periodate-treated and methylated oligosaccharide (Fig. 8b) shows a triplet of ions, adding one or two sodium cations. The increments of 4 atomic mass units exhibited by the oxidation products (e.g. the singly adducted triplet at m/z 931, 935, and 939 (Fig. 8b, inset)) correspond to the oxidation of additional diols by the periodate anion (4 atomic mass units, since NaB\(_4\)H\(_4\) was used as the reducing agent). The m/z 939 peak is consistent with the presence of both a branched and a linear structure, and in this case periodate oxidation cannot directly assign the molar fraction of the two structures by a specific mass shift. The oxidation product total mass, together with the knowledge that the reducing end is a hexose alditol, does indicate specific combinations of oxidized and unoxidized residues; however, their sequence in the oligosaccharide requires tandem mass spectrometry (Scheme 2: a, linear structure, m/z 939; b, branched structure, m/z 939). The m/z 939 ion was dissociated by collisional activation, and the mass spectrum of the product ions was taken (Fig. 8d). The ion fragments at m/z 818, 676, and 468 are common to both structures and indicate an oxidized nonreducing terminal N-acetyl hexosamine, 2- or 4-linked to a hexose (Scheme 2). The m/z 761 fragment corresponds to the loss of an oxidized hexose and could only arise from the branched structure. The hexose linked to the sorbitol was not oxidized, and this is consistent with it being linked at the 3-position (or branched). The tandem mass spectrometry spectra of the m/z 935 and 931 peaks show that the 4-atomic mass unit decrement arises from a failure to oxidize either the terminal N-acetyl hexosamine or the adjacent hexose (m/z 935) or both (m/z 931). Failure to oxidize the terminal N-acetyl hexosamine suggests that incomplete oxidation chemistry and not linkage arrangement is responsible for these peaks.

Both the mass spectrometry results and the chemical and enzymatic determinations indicate the presence in degalactosylated peak III of a pentasaccharide, containing one GlcNAc and four hexose residues, one of which had been reduced to the corresponding alditol. The fragmentation pattern shows the GlcNAc at the nonreducing terminus, as confirmed by its cleavage by β-N-acetylgalactosaminidase. All hexoses are β-linked glucose residues, as shown by the action of β-glucosidase and by HPAEC (see above). The acetylated hexosamine is attached to the next glucose by a β(1→2) or a β(1→4) linkage. CID data, both of the permethylated material and of the periodate-oxidized, reduced, and permethylated material indicate the presence of two isomers, one linear and the other branched (Schemes 1 and 2). In both structures, one of the hexoses appears to be β(1→3)-linked. In the branched structure, there is a β(1→6)-linked glucose. All of these results are compatible with a linkage of the GlcNAc at the reducing end of a chitin chain to a glucose linked β(1→3) to the main chain of β(1→6)-glucan. Cutting at different locations by the β(1→6)-glucanase used in the preparation of peak III would give rise either to the branched or to the linear isomer (Scheme 3; arrows show two possible cutting patterns). Notice that the mass spectrometry results would also be compatible with the (1→3)-linked glucose in the linear isomer being directly attached to sorbitol. However, this would imply that β(1→3) linkages would be occasionally present in the main β(1→6) chain. There is no evidence for this; in fact, all four internal oligosaccharides from β(1→6)-glucan that we analyzed after reduction were completely hydrolyzed to glucose and sorbitol by β-glucosidase (see example below in Fig. 9b), an indication that there were no β(1→3) linkages between glucose and sorbitol (6). Furthermore, \(^{13}\)C NMR spectra indicate the presence of branches attached to the main β(1→6) chain by a β(1→3) linkage, as discussed above. Another piece of evidence for attachment of chitin to a β(1→3) branch comes from a study of oligosaccharide I (Fig. 7c), a tetrasaccharide. After reduction with borohydride and incubation with β-galactosidase and β-N-acetylgalactosaminidase, β-glucosidase was unable to hydrolyze the residual reduced disaccharide (data not shown), a result suggestive of a β(1→3) linkage between glucose and sorbitol (6). We also considered the possibility that the GlcNAc was directly attached to a glucose residue of the main β(1→6) chain, but we found that all structures that would have resulted in that case were incompatible with the mass spectrometry results.

The Reducing End of the β(1→6) Chain Is Attached to β(1→3)-Glucan—In a previous study on the chitin-β(1→3)-glu-
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Fig. 9. β-Glucosidase digestion of oligosaccharides released from ConA\(^+\) by β(1→6)-glucanase. a, a sample of tritiated pentasaccharide (2 \times 10^6 \text{ cpm}) isolated by Bio-Gel P-2 (Fig. 7b) was evaporated to dryness, dissolved in 280 μl of 50 mM sodium acetate, pH 5.0, and digested with 0.3 mg of sweet almond β-glucosidase for 16 h at 37 °C in the presence of 0.02% NaN\(_3\). After incubation, the sample was applied to a Bio-Gel P-2 column, and the digestion product, eluting at the position of a reduced hexose disaccharide, was recovered. Pooled fractions were evaporated to dryness, dissolved in 30 μl of water, and subjected to paper chromatography. Segments (1 cm) of the paper were counted. Standards were glucose ([14C]glucose, internal standard) (1), glucitol (2), lamanariibitol (3), sophoritol (4), cellobibitol (5), and gentio-bibitol (6). b, a sample of nonreduced tetrasaccharide, isolated from the same chromatography (Fig. 7b), was labeled by reduction with sodium borotritide. Part of the recovered reduced tetrasaccharide (1 \times 10^6 \text{ cpm}) was evaporated to dryness, dissolved in 280 μl of 50 mM sodium acetate, pH 5.0, and digested with 1.2 mg of sweet almond β-glucosidase as in a. A portion of incubation mixture (20,000 cpm) was subjected to paper chromatography as in a. Standards were the same for both panels.

Based on all of these results, it is clear that fraction M contains a high molecular weight mannose polysaccharide of the type normally found in yeast cell walls.

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The High Molecular Weight Material Remaining after β(1→6)-Glucanase Digestion Contains Mannan.—The material eluting in the void volume of the Sephadex G-100 column after β(1→6)-glucanase digestion (fraction M, Fig. 6b) contains all of the mannose and very little of the glucose associated with ConA\(^+\) (Fig. 4). This fraction also contains part of the original tritium label (Fig. 6b). The nature of the radioactive component will be discussed below.

Preliminary experiments on digestion of ConA\(^+\) with Pronase\(^2\) followed by Sephadex G-100 chromatography suggested that the mannose could be a component of a large polysaccharide, such as yeast cell wall mannan. This mannan consists of a core region that includes the N-linkage to protein and of an extended (1→6)-linked mannosyl chain with α(1→2) and α(1→3) branches 1–4 mannoses long (35). Acetylation of mannan results in breakage of the main chain α(1→6) bonds and release of the short side chains (35). High molecular weight carbohydrate-containing material was isolated from a Pronase digest of ConA\(^+\) by Sephadex G-100 chromatography and subjected to acetylation followed by deacetylation (see “Experimental Procedures”). The products were separated by chromatography on a Bio-Gel P-2 column into four sugar peaks, eluting at the position of free hexose and di-, tri-, and tetrasaccharide (M2, M3, and M4, respectively; see Fig. 10). The structure of the oligosaccharides was studied by \(^1\text{H NMR} and \(^13\text{C NMR}. \)

Comparison of spectra with those reported in the literature (36–44) resulted in the assignment of the structure Manp-α(1→2)-Manpα-α(1→3)-Manpα-α(1→3)-Manpα-α(1→3)-Manpα for M4, M3, and M2, respectively (Fig. 4), and Manp-α(1→3)-Manpα-α(1→2)-Manpα for M4 (data not shown). Signals for the same linkages were found in the \(^1\text{H NMR} spectrum (not shown) and \(^13\text{C NMR} spectrum (Fig. 4g) of fraction M.

\(^2\)In the course of this study it was found that the commercial preparation of Pronase used contained endo-β(1→6)-glucanase activity. Therefore, Pronase could not be used in most experiments. Here, however, the contaminating activity was beneficial, because it allowed us to isolate mannan free of β(1→6)-glucan by collecting the void volume fraction in gel columns.
b of the mannoprotein and of through a Portion of a GPI Anchor—presence of 0.02% NaN₃ and subsequently applied to a Sephadex G-100 column (1 × 85 cm) and eluted with 0.1 m acetic acid. Fractions of 0.45 ml were collected, and a 40-μl portion of every second fraction was either counted (1) or used for the assay of total carbohydrates (C) (see “Experimental Procedures”). Positions of molecular mass standards (in kDa) are indicated.

ConA⁺ Contains Mannoprotein—Since yeast mannoprotein is invariably found attached to protein, the presence of mannann in fraction M suggested that protein should also be a component of this fraction. Mannan is N-linked to protein, and it can be cleaved off with either glycopeptidase F or endoglycosidase H. When fraction M was relabeled by borotritide reduction and treated with glycopeptidase F, chromatography of the digest on Sephadex G-100 separated unlabeled carbohydrate that emerged in the void volume from most of the tritiated material that was eluted in later fractions and presumably contained the protein (Fig. 11). Similar results were obtained with endoglycosidase H (data not shown). The latter finding suggested a procedure to label specifically the protein, by transgalactosidation onto the GlcNAc that remains attached to protein after endo-H hydrolysis. Accordingly, fraction M was treated with endo-H (45) and fractionated on Sephadex G-100. The liberated material containing the intrinsic tritium label (similar to the tritiated peak of Fig. 11) was concentrated and incubated with UDP[¹⁴C]Gal in the presence of galactosyltransferase (see “Experimental Procedures”). Upon Sephadex G-100 chromatography, the incorporated ¹⁴C radioactivity eluted in the same position as the tritiated material. Electrophoresis of the ¹⁴C-labeled material on polyacrylamide in the presence of SDS, followed by autoradiography, showed a broad band at about 100 kDa and some material that trailed behind (Fig. 12a). Fraction M or ConA⁺ remained at the origin.

Mannoprotein in ConA⁺ Is Attached to β(1→6)-Glucan through a Portion of a GPI Anchor—The simultaneous binding of the mannoprotein and of β(1→6)-glucan to ConA-Sepharose indicated that these two components are covalently linked, because ConA has no affinity for β-linked glucose. The nature of the linkage remained to be determined. Several cell wall proteins are attached to β(1→6)-glucan through what seems to be a remnant of a former GPI anchor (7), part of which was lost in a putative transglycosylation. To verify whether that was the case here, fraction M was relabeled by reduction with borotritide and treated in the cold with hydrofluoric acid, under conditions that lead to hydrolysis of the linkage between phosphate and mannose in a GPI anchor (46, 47). When the reaction mixture was applied to a Sephadex G-100 column, a large portion of the tritium label was eluted at a position corresponding to low molecular weight material (Fig. 13b). This material contained the tritium introduced in the relabeling of fraction M, because when the original fraction M was treated with hydrofluoric acid, almost all of the tritium remained in the void volume fraction (Fig. 13c). If the anchor hypothesis were correct, the low molecular weight material released by HF would consist of a mannose oligosaccharide attached to a stub of β(1→6)-linked glucoses left over after β(1→6)-glucanase hydrolysis of ConA⁺ (Fig. 3). This proved to be the case. The tritiated material yielded one peak, corresponding to an oligosaccharide of 8 residues upon HPLC on a Rezex RSO-Oligosaccharides column (Fig. 14a). However, when this material was rechromatographed on a Bio-Gel P-4 column, it split into two peaks eluting at positions corresponding to oligosaccharides of 7 and 8 residues (results not shown). These substances were resistant to β-glucosidase (Fig. 14b). They were, however, hydrolyzed by α-mannosidase, and they were resistant to α-glucosidase (Fig. 14b). They were, however, hydrolyzed by α-mannosidase, and they were resistant to α-glucosidase (Fig. 14b). They were, however, hydrolyzed by α-mannosidase, and they were resistant to α-glucosidase (Fig. 14b).
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been separated from the protein and therefore was lost in the 

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Fig. 13. Release of an oligosaccharide from fraction M by hydrofluoric acid. a, a portion of fraction M (3 × 10^6 cpm) was evapo-
rated to dryness, dissolved in 200 μl of precooled 50% hydrofluoric acid, and incubated at 0 °C for 72 h. The digest was pipetted onto frozen 10 mM NaOH (400 μl) in a microcentrifuge tube placed in ice. The resulting suspension was spun down, and the NaF precipitate was washed twice with 100 μl of water. Supernatants were combined and applied to a Sephadex G-100 column (1 × 85 cm), which was eluted with 25 mM Tris-chloride, pH 7.5, containing 0.02% NaN₃. Fractions of 0.45 ml were collected, and a 400-μl portion of every second fraction was counted. Positions of molecular mass standards (in kDa) are indicated. b, a portion (3 × 10^6 cpm) of 1H-relabeled (see legend of Fig. 14) fraction M was treated with hydrofluoric acid and chromatographed as in a.

The hydrofluoric acid treatment was also applied to the intact ConA⁺. After HF hydrolysis, the material was subjected to polyacrylamide electrophoresis and Western blotting with an antibody against β(1→6)-glucan (11). The amount of immuno-
reactive material was much diminished after the HF treatment and completely eliminated by incubation with β(1→6)-glucanase (Fig. 12b). Similarly, ConA⁺ that had been labeled with [14C]galactose was incubated with HF, subjected to electrophoresis on polyacrylamide gel, and transferred to a PVDF membrane. The membrane was exposed for autoradiography under conditions in which only 14C but not tritium would be detected. The sample treated with HF yielded a negative result, whereas an intact sample showed a band at about the same position as found before for intact ConA⁺ (Fig. 12c). In both cases, the results indicate that the β(1→6)-glucan had been separated from the protein and therefore was lost in the transfer to the PVDF membrane. The split between the two moieties of the complex could also be shown by ConA-Sepha-
rose chromatography, but it was found that hydrofluoric acid, even at the low temperature used in the experiment, led to rather extensive degradation of β(1→6)-glucanase (results not shown).

Hexitol Residues Are Linked to Protein in ConA⁺—After degradation of ConA⁺ with β(1→6)-glucanase, tritiated material was still found in the Sephadex G-100 void volume peak (frac-
tion M; see Fig. 6b). This result was unexpected, because most of the glucose had been liberated by the glucanase (Fig. 4b); therefore, all of the labeled sorbitol attached to the glucose should be in the low molecular weight fraction. The puzzle was solved when fraction M was hydrolyzed with trifluoroacetic acid and the labeled material was tentatively identified by paper chromatography as mannositol, rather than sorbitol (results not shown). The alditol appears to be directly or indirectly attached to protein, because it remains with the protein after treatment of fraction M with glycopeptidase F (Fig. 11), endo-H, or HF (Fig. 13). Treatment of fraction M with proteinase K, followed by Sephadex G-100 chromatography, yielded a peak containing about 45% of the radioactivity in a position corresponding to low molecular weight material, whereas the remainder was still in the void volume fraction (results not shown). The structure of the alditol-labeled material and the manner in which it is attached to protein remain undeter-
mined. From the practical point of view, it served as a convenient label during the isolation of the protein moiety of ConA".

ConA Is Similar to ConA but Lacks Mannoprotein—Although most of the emphasis of this study was on the ConA fraction, many experiments were also done with ConA. The structure of ConA appears to be similar to that of ConA, except for the absence of mannoprotein in ConA, on the strength of the following evidence. (a) ConA is linked to chitin and to β(1→3)-glucan, based on the incorporation of [14C]galactose and binding to the E. crista argali lectin-agarose column (Fig. 2b) as well as on the production of both 13C- and tritium-labeled oligosaccharides after incubation with β(1→6)-glucanase (Fig. 6, c and d). (b) The 13C NMR spectrum of ConA shows the presence of β(1→6)-glucan, but the signals for mannose are missing (Fig. 4e). Accordingly, ConA contains no mannose (Fig. 4f). The absence of this sugar is also consistent with the inability of ConA to bind to ConA-Sepharose. (c) No "mannitol tritium" was found after β(1→6)-glucanase digestion of ConA (Fig. 6d).

From the Sephadex G-100 elution pattern of intact ConA, it is clear that this material has a lower average molecular weight than ConA and is quite heterogeneous in size. This heterogeneity presumably reflects variability in length of the β(1→6)-glucan chains.

DISCUSSION

A Family of Compounds in the Isolated Complex—The fraction solubilized from the cell wall by successive treatments with β(1→3)-glucanase and chitinase and isolated in the void volume of a P-2 column turned out to be surprisingly complex. This material, expected to represent a linkage region between chitin and β(1→3)-glucan, was separated on a ConA-Sepharose column into the ConA and ConA fractions. After transferring [14C]galactose to terminal GlcNAc residues in ConA and ConA, each one of them could be further fractionated on an E. crista argali lectin-agarose column into portions containing different amounts of galactose, hence of GlcNAc. Added to this complexity is a certain variability in size evidenced during chromatography of ConA on Sephacryl S-400 or of ConA on Sephadex G-100. Despite all these signs of heterogeneity, there are many indications that the substances comprising either the ConA or the ConA fraction share the same basic structure. Thus, the NMR spectra are relatively uncomplicated and of straightforward interpretation, the sugar composition is simple, and all fractions are degraded by β(1→6)-glucanase in a similar fashion. Furthermore, some of the fractions obtained by chromatography of ConA contain mannoprotein, β(1→6)-glucan, tritated sorbitol residues, and GlcNAc (indicated by the presence of [14C]galactose): these compounds could be separated only after treatment of the complex with either enzymes or hydrofluoric acid. The heterogeneity in size can be accounted for by variability in length of the β(1→6)-glucan chains. Still, the complexity of the material, together with its scarcity, reflected often in samples at the nanomolar level, severely limited the range of usable methodologies and prevented the complete determination of some of the sugar to sugar linkages.

Components of the Complex—The main components of the ConA fraction are β(1→6)-glucan and mannoprotein. β(1→6)-glucan was identified by the NMR spectrum and by its susceptibility to hydrolysis by β(1→6)-glucanase. The proton NMR spectrum provided evidence that β(1→3) branches are attached to the main β(1→6) chain. The size of the glucan molecules is somewhat in doubt. In ConA only β(1→6)-glucan makes a significant contribution to the molecular weight, because the mannoprotein is missing and chitin and β(1→3)-glucan are represented by only one or a few sugar residues. Chromatography of ConA on Sephadex G-100 (Fig. 6c) shows a quite heterogenous distribution, with sizes between approximately 10 and 100 kDa, which would correspond to chains of between 60 and 600 glucose residues, with an average of perhaps 300–350 residues. By comparison, β(1→6)-glucan isolated from yeast by alkali and acetic acid extraction seems to consist on average of 140 glucose units (48). For ConA, it is more difficult to estimate the glucan size because of the presence of mannoprotein. Hydrofluoric acid cuts the linkage between glucan and protein but also partially hydrolyzes the glucan (see "Results"). An attempt was made to degrade the mannoprotein by sequential incubation of ConA with glycopeptidase F and proteinase K, followed by chromatography on Sephadex G-100. The treatment caused a decrease in size, with part of the material now entering the included volume (results not shown). However, we lacked criteria to determine whether the action of the enzymes used had been complete; therefore, the size of β(1→6)-glucan in ConA remains in doubt.

The presence of protein in ConA was inferred indirectly from the finding that mannann was a component. The polysaccharide is a typical yeast cell wall mannan, as judged from its NMR spectrum, the oligosaccharides generated by acetylation and their NMR spectra, and the size shown in gel filtration after excision from protein with glycopeptidase F or endo-H. Use of the latter enzyme allowed us to label the protein by transfer of [14C]galactose onto the residual GlcNAc and thus detect it after the subsequent gel electrophoresis. The apparent molecular weight of the deglycosylated protein, in the vicinity of 100,000, is remarkably similar to that we previously found (94,000) for the main component of cell wall proteins after β(1→3)-glucanase and endo-H treatment (4). In addition to the N-linked mannan, it is possible that the protein has O-linked mannose oligosaccharides, but the small amounts of material available did not allow investigation of this point. The radioactivity incorporated into the protein upon reduction of the complex with borohydride was found to be in an alditol residue tentatively identified as mannotol, an indication that mannose with its reducing group exposed was attached, either directly or indirectly, to the untreated protein. This is a very surprising result, because in glycoproteins both N-linked and O-linked sugars are attached through their reducing group, making the latter unavailable to borohydride. The exceptions are GPI anchors (see below), but the alditol could not be part of an anchor because it was not released by hydrofluoric acid (Fig. 13). Preliminary results indicate that a substantial portion of the tritium incorporated by reduction of intact cell walls is in the alditol, confirming that the reducing carbon of the original sugar, presumably mannose, was unlinked (results not shown).

Cross-links between the Components—As found in other cases (7), the connection between mannoprotein and β(1→6)-glucan is through a portion of GPI anchor. Previously, the presence of anchor material was inferred somewhat indirectly, from the susceptibility of the protein-glucan linkage to hydrofluoric acid and phosphodiesterase (7) and from the attachment to cell wall of hybrid proteins containing the anchor-bearing domain of α-agglutinin (49). In the present study, ConA was digested with β(1→6)-glucanase, reduced with borohydride, and hydrolyzed with HF. The resulting oligosaccharides were isolated and shown to consist of five o-linked mannose residues, attached to the nonreducing end of β(1→6)-linked reduced disaccharide and trisaccharide, respectively. This result identifies the residual portion of the original GPI anchor as ethanalamine-phosphate-Man3. The glucosaminyl residue was eliminated together with the phosphatidylinositol group in the putative transglycosylation reaction that originated the protein-glucan connection.
Connections of the mannoprotein-β(1→6)-glucan complex to β(1→3)-glucan and chitin were postulated on account of the need of both β(1→3)-glucanase and chitinase for solubilization of the complex and were confirmed by the presence of remnants of both polysaccharides in the complex. Thus, β(1→3)-linked oligosaccharides were connected to the reducing end of β(1→6)-glucan, and GlcNAc that survived chitin degradation by chitinase was found to be attached to the same glucan, probably onto β(1→3) side branches. The GlcNAc at the reducing end of the chitin chain is connected to glucose either by a β(1→2) or a β(1→4) linkage. We favor the latter possibility by analogy to the bond between chitin and β(1→3)-glucan (6).

If our hypothesis that chitin is attached to a β(1→3)-linked glucose residue is correct, one may ask whether the previously found linkage of chitin to glucan might not have been to a β(1→3) side chain of β(1→6)-glucan rather than to the long β(1→3)-linked polysaccharide. This notion seems unlikely for the following reasons: first, the endo-β(1→3)-glucanase activity in Zymolyase requires a minimum of 5 glucose residues to cut the chain (50), whereas β(1→3) branches of β(1→6)-glucan are quite short, probably not more than one or two residues in length (48); second, we found that mutant krc5, despite its low content of β(1→6)-glucan (51), shows a normal complement of oligosaccharides indicative of a chitin-β(1→3)-glucan linkage (results not shown).

The ConA⁺ material is similar to ConA⁺, except for the absence of mannoprotein. Thus, it may be either a precursor or a degradation product of ConA⁺.

The role of β(1→6)-Glucan in the Organization of the Yeast Cell Wall—From the foregoing analysis, it appears that β(1→6)-glucan is the central molecule or “glue” that keeps together the other components of the cell wall, including β(1→3)-glucan, mannoprotein, and part of the chitin (Fig. 3). Thus, it is not surprising that defects in β(1→6)-glucan formation, as found in several mutants (52, 53), can interfere with cell wall assembly and have severe effects on cell growth (52, 53). For this reason, it is possible that compounds that specifically inhibit β(1→6)-glucan synthesis, if such can be found, would behave as antifungal agents.

The chitin participating in linkages to both β(1→3)- and β(1→6)-glucan is synthesized by chitin synthase 3 (6). This chitin is found both in the ring formed at the base of an emerging bud and, in dispersed form, throughout the cell wall (54). It is of interest to compare the results of the present investigation with those of an earlier study on the distribution of chitin in the cell wall (55). In that study, we found that most of the chitin present in lateral walls and part of the bud scar chitin could be solubilized by incubation of alkali-treated cell ghosts with a β(1→6)-glucanase preparation that was contaminated with a small amount of chitinase. The same amount of chitinase without the glucanase had no effect. Those results are in good agreement with the present findings about chitin synthase 3 involvement in chitin synthesis and about the role of β(1→6)-glucan in chitin cross-linking. From both sets of results it may be concluded that the chitin interspersed in the cell wall and possibly part of that in the ring are attached to β(1→6)-glucan, whereas another portion of the ring chitin is directly linked to β(1→3)-glucan (6). It is possible that the β(1→6)-glucan molecules attached to a single chitin chain come from a different region than those bearing several chains.

It is difficult to estimate what proportion of the cell wall is involved in the linkages studied in this work. Fraction Vc contained about 4% of the total cell wall carbohydrate. To this, one should add the chitin and β(1→3)-glucan chains that had been eliminated by treatment with the corresponding hydrolytic enzymes.

**Fig. 15. Proposed flexible building block of the yeast cell wall.** Some of the components may be missing at different locations; e.g. absence of mannoprotein would result in a ConA⁻ like structure, absence of mannoprotein and β(1→6)-glucan would leave a chitin-β(1→3)-glucan polymer, and so on.

From this and previous studies (6, 7), the concept emerges of a “flexible building block” of the yeast cell wall (Fig. 15). The complete structure would comprise mannoprotein, β(1→6)-glucan, β(1→3)-glucan, and chitin. Part of the blocks (ConA⁻) would miss mannoprotein, especially in inner layers of the wall (4), or chitin, mostly in the outer layer (7).

**Biosynthesis of Cross-linkages—How are the components of the yeast wall building blocks joined together in the cell?** From the available evidence it appears that chitin (54) and β(1→3)-glucan (56) are synthesized at the plasma membrane with simultaneous secretion into the periplasmic region. On the other hand, mannoprotein is synthesized at the ER and modified during its transport through the secretory pathway (57). Finally, at least part of β(1→6)-glucan synthesis may occur in the ER or Golgi (53). It is clear that all of the components will only meet in the periplasmic region, which is then the location where the linkage reactions must take place. Because it is unlikely that high energy compounds are present in that location and on account of the nature of the connecting bonds, transglycosidation is the most likely reaction leading to formation of the cross-linkages. Identification of the enzymatic activities involved in these reactions awaits further work.

Although the mechanism of biosynthesis of the cross-linkages remains obscure, we have some information about its timing in the cell cycle. The chitin interspersed in the cell wall that is synthesized by chitin synthase 3 is laid down after septum formation, in the final phase of bud growth (58). As discussed before (6), that must be the phase of the cell cycle in which cross-links involving chitin are formed. Walls of mutants lacking chitin synthase 3 have been found to be less resistant to osmotic shock than those of wild type (59). This invites speculation that the cross-links are not created during most of the period of active growth lest they interfere with the plasticity of the developing wall, but are instead added at the end to increase rigidity of the final product.

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