Molecular Organization of the Alkali-insoluble Fraction of *Aspergillus fumigatus* Cell Wall*

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Physical and biological properties of the fungal cell wall are determined by the composition and arrangement of the structural polysaccharides. Cell wall polymers of fungi are classically divided into two groups depending on their solubility in hot alkali. We have analyzed the alkali-insoluble fraction of the *Aspergillus fumigatus* cell wall, which is the fraction believed to be responsible for fungal cell wall rigidity. Using enzymatic digestions with recombinant endo-β-1,3-glucanase and chitinase, fractionation by gel filtration, affinity chromatography with immobilized lectins, and high performance liquid chromatography, several fractions that contained specific interpolysaccharide covalent linkages were isolated. Unique features of the *A. fumigatus* cell wall are (i) the absence of β-1,6-glucan and (ii) the presence of a linear β-1,3/1,4-glucan, never previously described in fungi. Galactomannan, chitin, and β-1,3-glucan were also found in the alkali-insoluble fraction. The β-1,3-glucan is a branched polymer with 4% of β-1,6 branch points. Chitin, galactomannan, and the linear β-1,3/1,4-glucan were covalently linked to the nonreducing end of β-1,3-glucan side chains. As in *Saccharomyces cerevisiae*, chitin was linked via a β-1,4 linkage to β-1,3-glucan. The data obtained suggested that the branching of β-1,3-glucan is an early event in the construction of the cell wall, resulting in an increase of potential acceptor sites for chitin, galactomannan, and the linear β-1,3/1,4-glucan.

The fungal cell wall is a physically rigid layer that protects the fungal cell from its environment, mediates cell-cell interaction, and is responsible for the shape of the cell. Despite its central role in growth and survival, the fungal cell wall remains poorly studied and its biosynthesis is insufficiently understood (1, 2). Cell wall polysaccharides are separated in two groups according to their solubility in hot alkali solution. The structural skeleton of the cell wall is alkali-insoluble. It has been known for a long time that β-1,3-glucan and chitin (linear polymer of β-1,4-N-acetylglucosamine) are the main components of the alkali-insoluble fraction. The alkali insolubility of glucan is due to its covalent linkage with chitin (3–5). The covalent bond between the two polysaccharides has been characterized in *Saccharomyces cerevisiae* by Kollars et al. (6), who showed that chitin is linked to the nonreducing end of a β-1,3-glucan chain. More recently, the same research group reported that the core of the yeast cell wall is a complex structure with a β-1,6- and β-1,3-glucan to which chitin and some mannoproteins are attached (7). In yeast, cell wall-bound glycoproteins have been described to be covalently linked to β-1,6-glucan (8–10). These proteins are originally GPI-anchored to the membrane (11, 12) and then cleaved to be transferred onto β-1,6-glucan using the sugar moieties of GPI as a bridge (7, 13). Ethanolamine and mannose residues, but not glucosamine and inositol, are in the GPI remnant involved in the protein-glucan linkage (14). Another family of cell wall proteins are directly bound to β-1,3-glucan and released by mild alkali treatments (15). In contrast to yeast, the polymer organization of the cell wall of filamentous fungi has been poorly studied. Basically, it is only known that the alkali insolubility of their cell wall results, like in yeast, from the covalent association between glucan and chitin, with a concentration of chitin (around 10%) that is considerably higher than in yeast (2%) (16).

To better understand the organization of the cell wall components of a filamentous fungus and to gain further insight into the biosynthetic pathways involved in cell wall construction, we have focused our studies toward the chemical characterization of the interpolymer linkages occurring in the structural part of the cell wall, i.e. alkali-insoluble fraction of cell wall. The fungal model used is *Aspergillus fumigatus*. Using specific enzymatic digestion and various carbohydrate chemistry methods, we have shown that four polysaccharide components constituted this fraction: β-1,3-glucan was highly branched and was linked to chitin, galactomannan, and a linear β-1,3/1,4-glucan never described before.

**EXPERIMENTAL PROCEDURES**

**Preparation of Cell Walls**

*A. fumigatus* CBS 144–89 was grown in a 15-liter fermenter in a liquid medium containing a 2% glucose and 1% mycoteptone (Biokar Diagnostics) as described previously (17). After 24 h of culture (linear growth phase), the mycelia were collected by filtration, washed extensively with water and disrupted in a 50 mM Tris-HCl, pH 7.5 buffer containing 50 mM EDTA and 1 mM phenylmethylsulfonyl fluoride in a Dyno-mill (W. A. Bachofen AG, Basel, Switzerland) cell homogenizer in the presence of 1-mm-diameter glass beads at 4 °C. The disrupted mycelial suspension was centrifuged (8000 × g for 10 min), and the cell wall pellet was washed three times with the same buffer and stored at −20 °C.

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Received for publication, December 12, 1999, and in revised form, June 22, 2000 Published, JBC Papers in Press, June 26, 2000, DOI 10.1074/jbc.M909975199

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Fractionation of Cell Walls by Alkali and Enzymatic Treatments

Fractionation and digestion steps of the cell wall are summarized in Fig. 1. The disrupted cell wall pellet (50 g of wet weight, equivalent to 7.5 g of dried material) was incubated twice in 200 ml of 1 M NaOH at 65 °C for 30 min. A third NaOH treatment did not release any extra material from the pellet. The alkali-insoluble pellet was washed five times with water and once with 50 mM Tris-HCl, pH 7.4 buffer. The pellet was resuspended in the same buffer and incubated with 5 mM sodium azide (60 ml) and incubated with 180 μl of Quantazyme (50 units/μl, recombinant endo-β-1,3-glucanase; Quantum Industry, Quebec, Canada) at 37 °C for 5 days. Endoglucanase digestion was repeated once. Pooled supernatants (QsSN) were kept frozen. After Quantazyme digestion, the insoluble pellet was treated twice with 1 M NaOH at 65 °C for 30 min both to inactivate Quantazyme and to extract material that had become alkali-soluble after the glucanase treatment (accounting for 8% of the total alkali-insoluble starting material). After washing with water, the insoluble pellet residue was resuspended in 80 ml of 50 mM Tris-HCl, pH 8.0 containing 5 mM sodium azide and incubated at 37 °C for 5 days with 4 ml of recombinant chitinase A (0.5 mg of protein/ml) from Serratia marcescens produced in Escherichia coli (17) and purified as described previously (18). After centrifugation, the residual pellet was treated again with 1.2 ml of chitinase A in 40 ml of 50 mM Tris-HCl, pH 8.0, for 3 days. After centrifugation, supernatants (ChSN) were pooled. The Quantazyme-chitinase resistant pellet was excluded fractions, eluting at the void volume of the TSK HW40S column. The column was stabilized 20 min before injection. The products were detected by refractometry. The peracetylated product was reduced for 2 h in 100 mM ammonium hydroxide (2 ml) containing 20 mg of NaBH4. Excess of reagent was destroyed by addition of Dowex 50 × 8 (H+ form) resin beads until a pH of 5–6 was reached. After co-distillations with methanol, Smith degradation was performed with 10% acetic acid at 100 °C for 1 h. Degraded products were then separated by gel filtration chromatography on a TSK HW40S column as described above.

Chemical Degradation of Polysaccharides and Oligosaccharides

Release of galactofuranoside residues from galactomannan was obtained by mild acid hydrolysis with 15 mM HCl at 100 °C for 20 h (17). Periodate oxidation was performed after incubation of 10 mg of material in 2 ml of sodium meta-periodate, 100 mM, during 7 days at 4 °C in the dark (27). Excess of reagent was destroyed following addition of 200 μl of sodium bisulfite. After dialysis against water (membrane cut-off, 100 Da) or gel filtration chromatography on Sephadex G15 as described above, the oxidized product was reduced for 2 h in 100 mM sodium azide (2 ml) containing 20 mg of NaBH4. Excess of reagent was destroyed by addition of Dowex 50 × 8 (H+ form) resin beads until a pH of 5–6 was reached. After co-distillations with methanol, Smith degradation was performed with 10% acetic acid at 100 °C for 1 h. Degraded products were then separated by gel filtration chromatography on a TSK HW40S column as described above.

Acetylation of soluble polymers was performed according to Ferguson (28). Peracetylated products (40 mg) were treated with 10 ml of an acetic acid/acetic anhydride/sulfuric acid solution (10:1:10 v/v/v) at 25 °C for 37 °C for 3, 5, 7, and 24 h. The reaction was stopped by addition of 40 ml of pyridine and water (1:3 v/v). The peracetylated products were extracted with chloroform and washed with water. Deacetylation was performed in 300 mM NaOH and NaBH4 (10 mg/ml) overnight at room temperature.

Enzymatic Treatments

Hydrolysis with a 74-kDa endo-β-1,3-glucanase (ENG1) purified from A. fumigatus cell wall autolyate was performed as described previously (29). Briefly, 1 mg of sample was digested in 500 μl of a 100 mM imidazole-acetic acid, pH 7.0 buffer with 10 μl of the 74-kDa endo-β-1,3-glucanase solution (specific activity, 1.5 μmol glucose equivalents/min/ml) at 37 °C for 24 h. To remove GlCNase from the terminal nonreducing end of an oligosaccharide, 5 mg of sample were incubated with 5 μl of β-N-acetyl-glucosaminidase from jack bean (10 units/160 μl, Sigma) in 250 μl of 250 mM sodium acetate, pH 5.0 buffer at 25 °C for 24 h. After addition of 50 μl of 1 M NaOH, oligosaccharides were treated with NaBD4 as described above.

Lectin Affinity Chromatography

Fractions containing 10 mg of carbohydrate were applied to a column of concanavalin A-Sepharose (4 ml; Amersham Pharmacia Biotech) in 100 mM NH4OH overnight and desalted over a Sephadex G15 column.
Alkali-insoluble Fraction of A. fumigatus Cell Wall

**Fractionation Scheme**

![Fractionation scheme of A. fumigatus cell wall using alkali and enzymatic treatments.](image)

**Results**

- **Cell wall**
  - Extraction with NaOH 1N, twice
  - Alkali-insoluble fraction
  - Alkali-soluble fraction
  - Digestion with quantzyme, twice
- **Alkaline fraction**
  - Water-soluble fraction QzSN
  - Insoluble fraction
- **Chitinase treatment**
  - Alkali-soluble fraction QzAS
  - Digestion with chitinase A, twice
  - Water-soluble fraction
  - Insoluble fraction
  - Extraction with NaOH 1N, twice
- **Chitinase**
  - Alkali-soluble fraction ChSN
  - Insoluble fraction
- **Final insoluble pellet**
  - FP

**Alkali-insoluble Fraction of A. fumigatus Cell Wall**

The alkali-insoluble fraction of A. fumigatus cell wall was prepared by NaOH treatment of 7.5 g of cell wall dried material (equivalent to 50 g of wet weight) in 200 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, and 1 mM MnCl₂. The amounts of material of each fraction, expressed as percent of original dry weight, and their sugar compositions are shown in Table I. Three out of the four fractions released by NaOH treatment of 7.5 g of cell wall dried material (equivalent to 50 g of wet weight) resulted in the production of 3 g of alkali-insoluble fraction (40% of the wall dry weight). Treatment of this fraction by sequential incubation with recombinant β-1,3-glucanase (Quantzyme) and chitinase, alternated with alkali treatment, made soluble 90% of the alkali-insoluble starting material. (Fig. 1 and Table I). Further purification of the soluble fractions by gel filtration chromatography on TSK-HW40S and Sephadex G100 columns resulted in the separation of nine fractions of different molecular mass (Figs. 2 and 3). The amounts of material of each fraction, expressed as percentages of original dry weight, and their sugar compositions are shown in Table I. Three out of the four fractions released by Quantzyme contained only glucose, whereas the high molecular weight fraction (QzSN I₃) contained glucose associated with galactose and mannose. In a similar way, low molecular weight fractions ChSN III and ChSN IV released by chitinase were exclusively composed of GlcNAc, whereas fractions ChSN I₃, ChSN I₅, and ChSN II contained GlcNAc associated with various amounts of glucose, galactose, and mannose. The chemical analysis of the different fractions resulted in the identifi-
Chemical Characterization of the Water-soluble Products Released by Quantazyme

**Fraction QzSN III: Degradation Products Obtained with Endo-β-1,3-glucanase**—HPAEC, methylation, and MALDI-TOF mass spectrometry analysis showed that QzSN III corresponded to laminaripentaose, which is the product of hydrolysis of β-1,3-glucan by Quantazyme (data not shown).

**Fraction QzSN II: Mixture of Branched Laminarioligosaccharides**—QzSN II (1.5–2.5 kDa) contained a mixture of laminarioligosaccharides that had been reduced with NaBH₄ before separation by HPAEC (Fig. 4). MALDI-TOF mass spectrometry analysis indicated that the degree of polymerization (dp) of oligosaccharide varied from 9 to 15 (Fig. 4). Products with the same Mr gave two peaks on HPAEC analysis, suggesting they were chemically organized differently. Analysis of these oligosaccharides was performed by 1H NMR spectroscopy on two couples of oligosaccharides with 10 and 11 glucose residues (QzSN IIc, QzSN IId, QzSNIIe, and QzSN IIf). The one-dimensional 1H NMR spectra of QzSN IId and QzSN IIf oligosaccharides were similar to a linear β-1,3-glucan with a glucitol residue at the reducing end as described previously (Ref. 38 and data not shown). The one-dimensional spectrum of the QzSN IIe oligosaccharide contained five doublets and two close-lying doublets in the anomeric region between 4.5 and 4.8 ppm (Table II). These chemical shifts and the coupling constants $^3J_{1,2}$ of these doublets were in good agreement with those published for a linear β-1,3-glucan containing a β-1,6 linkage (39, 40). Each signal corresponded to one anomeric proton, except for the signals at 4.76 and 4.80 ppm, which accounted for two and four protons, respectively. The two close-lying doublets corresponding to one proton were due to different populations of conformers. Because glucitol did not give any signal in this part of the spectrum, the NMR results indicated the presence of 11 glucose units/QzSNIIe molecule, in agreement with the MALDI-TOF data. Because of severe overlap of

**Table I**

| Fraction          | % (weight) | Man | Glc | Gal | GlcNAc | GalNAc |
|-------------------|------------|-----|-----|-----|--------|--------|
| QzSN IA           | 16.4       | 1.1 | 1   | 1.4 |        |        |
| QzSN IB           | 18.7       | 1.1 | 1   | 1   |        |        |
| QzSN II           | 4.8        | 1   | 1   |     |        |        |
| QzSN III          | 12.3       | 1   |     |     |        |        |
| ChSN IA           | 5.7        | 0.9 | 1   | 0.9 | 0.01   |        |
| ChSN IB           | 6.2        | 0.03| 1   | 0.04| 0.02   |        |
| ChSN II           | 0.6        | 0.02| 1   | 0.04| 0.06   |        |
| ChSN III          | 0.2        |     | 1   |     |        |        |
| ChSN IV           | 14.2       |     | 1   |     |        |        |
| QzAS              | 8.3        | 0.5 | 1   | 0.6 | 0.15   | 0.05   |
| ChAS              | 1.8        | 0.34| 1   | 0.36| 0.06   | 0.12   |
| FP                | 11         | 0.36| 1   | 0.39| 1.35   | 2.7    |

**Fig. 2.** Gel filtration chromatography on a TSK HW40S column of the products released by Quantazyme (QzSN) and chitinase (ChSN) digestion of the alkali-insoluble cell wall fraction. The column (90 × 1.4 cm) was eluted with 0.25% (v/v) acetic acid at 0.5 ml/min. Products (20 mg) were applied to the column and detected by refractometry.

**Fig. 3.** Gel filtration chromatography on a Sephadex G100 column of the QzSN I and ChSN I fractions, which eluted at the void volume of the TSK HW40S column (Fig. 2). The column (90 × 1.4 cm) was eluted with 50 mM sodium acetate, pH 6.0, at 0.5 ml/h. Products (50 mg) were applied to the column and detected by refractometry.
other proton resonances, even in the DQF-COSY experiment, RELAYH experiments were performed to identify H3 resonances for further sequential assignment and linkage determination. From the chemical shifts reported in Table II, it can be seen that four units (labeled F) are indistinguishable by their proton resonances, as expected for a linear β-1,3-glucan chain. Chemical shifts of H3 protons are comprised between 3.75 and 3.80 ppm for A, B, D, E, and F. For the same reason, the other proton resonances, even in the DQF-COSY experiment, RELAYH experiments were performed to identify H3 resonances for further sequential assignment and linkage determination. From the chemical shifts reported in Table II, it can be seen that four units (labeled F) are indistinguishable by their proton resonances, as expected for a linear β-1,3-glucan chain. Chemical shifts of H3 protons are comprised between 3.75 and 3.80 ppm for A, B, D, E, and F. For the same reason, the other proton resonances, even in the DQF-COSY experiment, RELAYH experiments were performed to identify H3 resonances for further sequential assignment and linkage determination. From the chemical shifts reported in Table II, it can be seen that four units (labeled F) are indistinguishable by their proton resonances, as expected for a linear β-1,3-glucan chain. Chemical shifts of H3 protons are comprised between 3.75 and 3.80 ppm for A, B, D, E, and F. For the same reason, the other proton resonances, even in the DQF-COSY experiment, RELAYH experiments were performed to identify H3 resonances for further sequential assignment and linkage determination. From the chemical shifts reported in Table II, it can be seen that four units (labeled F) are indistinguishable by their proton resonances, as expected for a linear β-1,3-glucan chain. Chemical shifts of H3 protons are comprised between 3.75 and 3.80 ppm for A, B, D, E, and F. For the same reason, the other proton resonances, even in the DQF-COSY experiment, RELAYH experiments were performed to identify H3 resonances for further sequential assignment and linkage determination. From the chemical shifts reported in Table II, it can be seen that four units (labeled F) are indistinguishable by their proton resonances, as expected for a linear β-1,3-glucan chain. Chemical shifts of H3 protons are comprised between 3.75 and 3.80 ppm for A, B, D, E, and F. For the same reason, the other proton resonances, even in the DQF-COSY experiment, RELAYH experiments were performed to identify H3 resonances for further sequential assignment and linkage determination. From the chemical shifts reported in Table II, it can be seen that four units (labeled F) are indistinguishable by their proton resonances, as expected for a linear β-1,3-glucan chain. Chemical shifts of H3 protons are comprised between 3.75 and 3.80 ppm for A, B, D, E, and F. For the same reason, the other proton resonances, even in the DQF-COSY experiment, RELAYH experiments were performed to identify H3 resonances for further sequential assignment and linkage determination. From the chemical shifts reported in Table II, it can be seen that four units (labeled F) are indistinguishable by their proton resonances, as expected for a linear β-1,3-glucan chain. Chemical shifts of H3 protons are comprised between 3.75 and 3.80 ppm for A, B, D, E, and F. For the same reason, the other proton resonances, even in the DQF-COSY experiment, RELAYH experiments were performed to identify H3 resonances for further sequential assignment and linkage determination. From the chemical shifts reported in Table II, it can be seen that four units (labeled F) are indistinguishable by their proton resonances, as expected for a linear β-1,3-glucan chain. Chemical shifts of H3 protons are comprised between 3.75 and 3.80 ppm for A, B, D, E, and F. For the same reason, the other proton resonances, even in the DQF-COSY experiment, RELAYH experiments were performed to identify H3 resonances for further sequential assignment and linkage determination. From the chemical shifts reported in Table II, it can be seen that four units (labeled F) are indistinguishable by their
for two anomeric carbons C1 B at 98.4 ppm and C1 G at 94.7 ppm, which corresponded, respectively, to the anomeric protons of their anomeric moieties of the reducing end, respectively (39). The gHMBC experiment confirmed that all the units are compatible with a β-1,6 linkage. The C3 D resonance at 87.0 ppm was typical of β-1,3-Glc. These 1H and 13C chemical shifts indicated that the D unit was 3-β-D glucosylated and that the E unit was 3-β-D-glucosylated, supported by the 1H and 13C chemical shifts of these units. The 1H and 13C chemical shifts of the E unit were in agreement with those of the E unit of QzSN II with oligosaccharides of dp higher than 15. In a way similar to QzSN IIe, acetolysis of QzSN IB released a branched oligosaccharides was obtained by an acetolysis assay. The nonequivalent geminal proton resonating at lower field is denoted H1'. The glucose residues were labeled A to F in order of increasing agreement with 1H and 13C chemical shifts of the undecasaccharide QzSN IIe.

In conclusion, data obtained by 1H and 13C NMR, methylation analysis, and HPAEC separation of QzSN IB, submitted to gel filtration chromatography, and the action of Quantazyme. The ENG1-digested product was boro-hydride-reduced and submitted to gel filtration chromatography, and the action of Quantazyme. The ENG1-digested product was boro-hydride-reduced and submitted to gel filtration chromatography, and the action of Quantazyme. The ENG1-digested product was boro-hydride-reduced and submitted to gel filtration chromatography.

| Residues   | H1   | H1'  | H2   | H3   | H4   | H5   | H6   | H6'  |
|------------|------|------|------|------|------|------|------|------|
| 3Glcitol   | 3.68 | 3.79 | 4.03 | 4.06 | 3.66 | 3.91 | 3.65 | 3.86 |
| A          | 4.56 | 4.56 | 3.52 | 3.75 | 3.52 | 3.52 | 3.75 | 3.93 |
| 3Glcβ1–6   | 4.68 | 3.59 | 3.79 | 3.53 | 3.52 | 3.75 | 3.52 | 3.75 |
| B          | 4.76 | 3.37 | 3.53 | 3.415| 3.49 | 3.72 | 3.92 |
| 3Glcβ1–3glucitol |      |      |      |      |      |      |      |      |
| C          | 4.77 | 3.58 | 3.80 | 3.61 | 3.70 | 3.90 | 3.70 | 4.22 |
| 3Glcβ1–3   | 4.77 | 3.56 | 3.79 | 3.53 | 3.52 | 3.75 | 3.52 | 3.75 |
| 3Glcβ1–3A  | 4.80 | 3.56 | 3.79 | 3.53 | 3.52 | 3.75 | 3.52 | 3.75 |
| 3Glcβ1–3   |      |      |      |      |      |      |      |      |

All the methylenic carbon signals, easily identified in the geHSQC experiment, had typical chemical shift values of β-1,3-glucan (between 63.3 and 63.5 ppm) except for 4-O-substituted glucose containing a β-1,6 glucosylated and that the E unit was 3-β-D glucosylated, supported by the 1H and 13C chemical shifts of these units. The 1H and 13C chemical shifts of the E unit were in agreement with those of the E unit of QzSN II with oligosaccharides of dp higher than 15. Fraction QzSN IB: Identification of a New β-1,3/1,4-Glucan—Methylation analysis of QzSN IB showed the presence of 2,3,6-Glc and 2,4,6-Glc in the molar ratio 1:16. The glucan containing 4-O-substituted glucose was also recovered from mycelium grown in a chemically defined medium in flasks containing 4-O- and 6-O-disubstituted. Resonance values lying between 105.0 and 105.5 ppm were in agreement with the β-1,3-glucan containing a β-1,6 linkage, except for two anomeric carbons C1 G at 94.7 ppm, which corresponded, respectively, to the β and a Glc moieties of the reducing end, respectively (39). The gHMBC experiment confirmed that all the units are β-1,3 linked except for the A residue, which was β-1,6 linked with the D residue. Further confirmation that QzSN IB contained a mixture of branched oligosaccharides was obtained by an acetolysis assay. In a way similar to QzSN IIe, acetolysis of QzSN IB released a large number of linear β-1,3 linked oligosaccharides of variable size (dp 2–20 and higher; Fig. 5).

In conclusion, data obtained by 1H and 13C NMR, methylation analysis, and HPAEC separation of QzSN IB, submitted to gel filtration chromatography, and the action of Quantazyme. The ENG1-digested product was boro-hydride-reduced and submitted to gel filtration chromatography, and the action of Quantazyme.
phy on the TSK HW40S column (Fig. 6). Gel permeation profile showed laminari-oligosaccharides of low dp (mainly 2 and 3), branched β-1,3/1,6 laminari-oligosaccharides (dp 6–9, fraction b), and a fraction a of high Mr that was further analyzed. Methylation analysis showed that a polymer with an equimolar ratio of glucose residues substituted in position 3 and substituted in position 4 was recovered in the fraction a. 1H and 13C NMR data of this fraction are presented in Table IV and Fig. 7. Two main doublets were observed in the anomeric region at 4.53 and 4.77 with a 3J1,2 coupling constant value of 7.9 Hz typical of β-linked units. Integration of these two signals shows that A and B residues were in the ratio 1:1. H3 and H4 resonances determined with RELAYH and TOCSY experiments showed that residue A was substituted in position 3 and residue B in position 4. The gGHMBC experiment showed interglycosidic couplings between H1A and C4B and between H1B and C3A, indicating that A was linked to B in position 4 and B was linked to A in position 3 (Fig. 7). Degradation of this fraction by periodic oxidation and Smith degradation yielded a monosaccharide glycoside isolated from the TSK HW40S column as a dp 2 (Fig. 6). GLC analysis indicated that it was composed of glucose and erythritol. Erythritol residue was produced by cleavage of glucose residue substituted in position 4 by sodium periodate. GLC-MS analysis using the chemical ionization mode showed that the permethylated compound had a Mr of 382, corresponding to an hexose plus erythritol. Analysis with electronic impact mode after methanolysis indicated that the glucose residue was bound to carbon 2 of erythritol. NMR and methylation data indicated that the linear β-1,3/1,4-glucan has the following repeating unit: \( \text{3Glc}_β 1–4\text{Glc}_β \).

**TABLE III**

| Residues | \( ^1H \) chemical shifts in ppm | \( ^13C \) chemical shifts in ppm |
|----------|---------------------------------|---------------------------------|
|          | \( \text{H1} \) | \( \text{H2} \) | \( \text{H3} \) | \( \text{H4} \) | \( \text{H5} \) | \( \text{H6} \) | \( \text{H6} ' \) |
| A 3Glcβ1–6 | 4.55 | 3.52 | 3.75 | 3.52 | 3.52 | 3.75 | 3.92 |
| B 3Glcβ1–6 | 4.67 | 3.44 | 3.74 | 3.52 | 3.50 | 3.52 | 3.74 |
| C 3Glcβ1–3 | 4.74 | 3.36 | 3.53 | 3.41 | 3.49 | 3.72 | 3.92 |
| D 3Glcβ1–3 | 4.75 | 3.57 | 3.79 | 3.59 | 3.70 | 3.89 | 4.22 |
| E 4Glcβ1–3 | 4.76 | 3.40 | 3.67 | 3.67 | 3.62 | 3.82 | 3.98 |
| F 3Glcβ1–3 | 4.79 | 3.56 | 3.78 | 3.53 | 3.52 | 3.74 | 3.92 |
| G 3Glcα | 5.24 | 3.72 | 3.92 | 3.52 | 3.87 | 3.79 | 3.84 |
| reducing end | 94.7 | 73.8 | 85.2 | 70.8 | 73.9 | 63.3 |

**FIG. 6.** Gel filtration chromatography on a TSK HW40S column of QzSN IA fraction after periodic oxidation or enzymatic digestion with the 74-kDa endo-β-1,3-glucanase ENG1 of \( A. \) fumigatus. A, QzSN IA without treatment. B, QzSN IA after enzymatic digestion with ENG1. C, QzSN IA fraction after peridote oxidation and Smith degradation. A TSK HW40S column (90 x 1.4 cm) was eluted with 0.25% (v/v) acetic acid solution at 0.5 ml/min. Products were applied to the column and monitored by refractometry. dp was established with malto-oligosaccharides as standard.
ments preferentially released a tetrasaccharide consistent with the presence of a repeating mannose unit [6Manα1–2Manα1–2Manα1–2Manα1] (data not shown). As indicated by methylation analysis, two of the α-1,2 linked mannose residues were substituted in position 3 or 6 and were branching point for the galactofuran side chain (Table V). Because galactomannan binds to Concanavalin A, the galactomannan containing molecules were purified by affinity chromatography on a ConA-Sepharose column. The fraction bound to the ConA-Sepharose and released with 0.2 M α-methylmannoside accounted for 80% of QzSN 1A. It was composed of mannose, galactose, and glucose residues in a molar ratio of 2.5:2.7:1. This result indicated that the galactomannan polymer was covalently bound to the glucan moiety. The polysaccharide bound to ConA-Sepharose was reduced with NaBH₄, hydrolyzed, and derivatized. GLC analysis showed the absence of mannitol and the presence of glucitol, indicating that a glucose residue was located at the reducing end (data not shown). This fraction was sequentially submitted to mild acid hydrolysis to remove galactofuran side chains and to periodate oxidation to degrade the mannan moiety. The products were separated by gel filtration chromatography on TSK HW40S column (Fig. 8). Fraction b contained typical products of degradation by periodate oxidation. Fraction a was composed of a mixture of oligosaccharides with sizes varying between 2 and 11 residues. Only glucose and arabinol were detected by GLC analysis in the fraction a with a glucose/arabinol ratio of 2.9. Arabinol was produced by periodate oxidation, which cleaves glucose residues at the reducing end between carbon 1 and 2, indicating that the β-1,3-glucan chain was at the reducing end. This result was in agreement with the composition analysis performed in the reduced undegraded QzSN 1A. MALDI-TOF mass spectrometry analysis of the fraction a showed the presence of a series of oligosaccharides containing 1 arabinol residue plus an increasing number of glucose residues (Fig. 9). The major oligosaccharides contained 2–5 glucose units. Methylation of the fraction a showed the presence of several methyl ethers: 1,3,4,5-arabinol, 2,3,4,6-Glc, 2,4,6-Glc, and 2,4-Glc in the ratio of 0.4:1:3:2:0.2, indicating that this fraction was composed of short β-1,3 linked glucan chains with some β-1,6 branch points. The predominance of glucan chains with 2–5 glucose units constituting the glucan moiety of QzSN 1A resistant to hydrolysis by Quantzyme suggested that QzSN 1A fraction was composed of a galactomannan chain linked to the nonreducing end of a short β-1,3-glucan chain as follows.

| Residues | H₁ | C₁ | H₂ | C₂ | H₃ | C₃ | H₄ | C₄ | H₅ | C₅ | H₆ | C₆ | H₆’ |
|----------|----|----|----|----|----|----|----|----|----|----|----|----|----|
| A        | 4.53 | 3.52 | 3.76 | 3.52 | 3.51 | 3.75 | 3.92 |    |    |    |    |    |    |
| 3Glcβ1–4 | 105.0 | 75.6 | 86.6 | 70.6 | 78.2 | 63.2 |    |    |    |    |    |    |    |
| B        | 4.77 | 3.40 | 3.67 | 3.67 | 3.61 | 3.80 | 3.98 |    |    |    |    |    |    |
| 4Glcβ1–3 | 105.2 | 75.8 | 76.8 | 81.3 | 77.5 | 62.6 |    |    |    |    |    |    |    |

Fig. 7. Two-dimensional gHMBC NMR spectrum of fraction a purified by gel filtration on TSK HW40S column after ENG1 digestion of the QzSN 1B fraction. Only chemical shifts of H1 and C1 were presented on the spectrum.
**Table V**

Ratios of methyl ethers obtained after methanalysis of permethylated fractions from the most complex polysaccharide structure of the AIS fraction of A. fumigatus

| Methyl ethers | QzSN IA | ChSN IA | FP |
|---------------|---------|---------|----|
| 2,3,5,6-Gal   | 1.8     | 1.5     | 2.1|
| 2,3,6-Gal     | 4.3     | 3.2     | 13.8|
| 2,3,4,6-Man   | 0.1     | 0.1     | 0.6|
| 3,4,6-Man     | 2       | 1.6     | 2.9|
| 2,3,4-Man     | 0.8     | 0.7     | 4 |
| 3,4-Man       | 1       | 1       | 1 |
| 4,6-Man       | 0.4     | 0.3     | 1.4|
| 2,3,4,6-Glc   | 0.2     | 0.1     | 2.1|
| 2,4,6-Glc     | 7.1     | 3.8     | 50.6|
| 2,3,6-Glc     | 0.9     | 2.3     | 21 |
| 2,4-Glc       | 0.5     | 0.2     | 4.1|
| 2,6-Glc       |         |         | 6.7|
| 3,4,6-GlcNAc  |         | traces  |    |
| 3,6-GlcNAc    |         | 57      |    |
| 3,6-GalNAc    |         | 81      |    |

**Figure 8.** Analysis by gel filtration chromatography on a TSK HW40S column of oligosaccharides resistant after periodate oxidation of the fraction of QzSN IA binding to ConA-Sepharose column. Continuous line, product after mild acid hydrolysis (15 mM HCl, 100 °C 24 h); broken line, product after mild acid hydrolysis, periodate, and Smith degradation. TSK HW40S column (90 x 1.4 cm) was equilibrated with 0.25% (v/v) acetic acid solution at 0.5 ml/min. Products were applied to the column and detected by refractometry. dp was established with maltose-oligosaccharides as standard.

Fraction QzSN IA 2: Linkage between Linear β-1,3/1,4-Glucan and β-1,3-Glucan—The unbound ConA-Sepharose fraction contained only glucose residues. Methylation and GLC-MS analysis revealed the presence of five methyl ethers: 1,2,4,5,6-Glc, 2,3,4,6-Glc, 2,4,6-Glc, 2,3,6-Glc, and 2,4-Glc in the molar ratio of 0.1:0.8:16:4:6:5:1. The unbound ConA-Sepharose fraction was treated with ENG1. The TSK HW40S gel filtration pattern was similar to the one obtained after ENG1 treatment of the fraction QzSN Ia and shown in Fig. 6 (data not shown). The fraction obtained at the void volume was submitted to periodic oxidation. A single disaccharide peak was obtained. GLC-MS analysis showed that this disaccharide was composed of glucose linked to erythritol. These results indicated that the unbound ConA-Sepharose fraction contained both branched β-1,3/1,6-glucan and a linear β-1,3/1,4-glucan. According to the methylation data, the linear β-1,3/1,4-glucan represents 52% of the unbound ConA-Sepharose fraction.

When periodate oxidation and Smith degradation, degrading the β-1,3,1,4-glucan, was performed on the unbound ConA fraction, without previous ENG1 enzymatic digestion, small laminarioligosaccharides of dp 2–5 were released. This short size of the laminarioligosaccharides linked to the β-1,3,1,4-glucan, resulted from the Quantzyme digestion of the alkali-insoluble fraction of the cell wall, and indicated that the linear β-1,3,1,4-glucan was linked to β-1,3-glucan chains.

**Characterization of Water-soluble Products Released by Chitinase**

Fractions ChSN III and ChSN IV: Products of Degradation of Chitinase—Fractions ChSN III and ChSN IV contained only N-acetylglucosamine residues (GlcNAc). MALDI-TOF mass spectrometry showed that ChSN IV corresponded to N-acetylchito-bose and ChSN III to a mixture of N-acetylchito-rose and N-acetylchitotetraose (data not shown).

Fraction ChSN II: Linkage between Chitin and Glucan—Fraction ChSN II (molecular mass, 1–2 kDa) was composed of glucose and GlcNAc residues in a molar ratio of 16:1 (Table I). Because of the low amount of material recovered, this fraction was analyzed in toto without further purification. MALDI-TOF mass spectrometry showed that ChSN II contained a mixture of oligosaccharides of dp 7–13. After 9 h of chitinase digestion, half of the oligosaccharides contained one GlcNAc residue (Fig. 10). If chitinase incubation was prolonged for 24–72 h, the amount of oligosaccharides containing the GlcNAc residue gradually decreased over time (data not shown), indicating that GlcNAc residues bound to glucan chain were hydrolyzed by the chitinase treatment. Methylation was performed after reduction with NaBD₄ of ChSN II treated with chitinase for 24 h. GLC analysis of methyl ethers showed the presence of 2,3,4,6-Glc, 2,4,6-Glc, 2,3,6-Glc, 2,4-Glc, and 3,4,6-O-methyl 2-N-methyl 1,5-O-acetyl 2-N-acetyl glucosaminitol in a molar ratio of 0.7:10:1.6:9.4:0.6. A 24 h incubation of ChSN II with β-glucosaminidase removed 70% of the GlcNAc residues and 4-O-stabilized glucosamine residues, indicating that GlcNAc was located at the nonreducing end of the oligosaccharide (data not shown). These methylation data suggested that the GlcNAc residue was linked to the nonreducing end of the β-1,3-glucan oligosaccharide via a β-1,4 glycosidic linkage.

The one-dimensional ¹H NMR spectrum of the ChSN II (after 24 h of chitinase digestion) fraction contained five doublets in the anomeric region between 4.5 and 4.8 ppm (Table VI). Chemical shifts of 4.52, 4.75, and 4.80 for residues A, C, and E and the associated coupling constants values J₁2 for these doublets were similar to those obtained with QzSN II and QzSN III and indicated the presence of a linear β-1,3-glucan containing a β-1,6 branching point. H3 resonances, determined

Man-Man-Man-Man-Man-Man-Man-Man-Man-Man-Glc-Glc-Glc-Glc-Glc-

where Man is α-mannose, Gal is β-galactofuranose, and Glc is β-1,3-glucose.

Acetolysis degradation of the fraction bound to ConA-Sepharose resulted in the release of laminarioligosaccharides free from mannose residues. As acetolysis cleaves preferentially 1,6 glycosidic linkage, these data suggested, although indirectly, that the mannan was linked through a 1,6 linkage to the nonreducing end of the glucan chain.
with RELAYH experiments and $^{13}$C resonances, assigned from the gHSQC and gHSQC-TOCSY experiments confirmed the presence of a $\beta$-1,3-glucan chain with $\beta$-1,6 linkage (Glc A, C, and E; Table VI) (34). The B residue was unambiguously identified to be a GlcNAc residue from the gHMBC experiment through the observed long range couplings between the carbon of the carbonyl group at 177.1 ppm and the proton chemical shift of the CH$_3$ group at 2.06 ppm on the one hand and the ring H2 proton at 3.74 ppm on the other hand. Proton chemical shift values were typical of a nonreducing end GlcNAc residue (41). Sequence analysis, obtained from the gHMBC experiment, showed that all the units are $\beta$-1,3 linked except for the non-reducing terminal GlcNAc residue, which is $\beta$-1,4-linked with the D unit and for the A residue, which is $\beta$-1,6 linked with the reducing-end residue. Specific anomeric $^1$H and $^{13}$C signals were not identified at the reducing end. Moreover, methylation and GLC-MS analysis showed the presence of an unknown methyl ether. Methylation and GLC-MS analysis of reducing ends in QzSN I$_B$ treated with hot NaOH showed that the modification of the reducing end was due to a peeling reaction, which was sequential and stopped at a 1,6 branching point. From these data, it can be deduced that GlcNAc was linked to $\beta$-1,3-glucan side chains through a 1,4 linkage according to the following structure.

$$\text{GlcNAc} \rightarrow \beta_1\text{-}4\text{Glc} \rightarrow \beta_1\text{-}3\text{Glc} \rightarrow \beta_1\text{-}3\text{Glc} \rightarrow \beta_1\text{-}3\text{Glc} \rightarrow \beta_1\text{-}3\text{Glc}$$

**Structure 4**

 Fraction ChSN I$_B$: Complex Glucan Structures Are Also Linked to Chitin—ChSN I$_B$ had a molecular mass of 5–10 kDa (Fig. 3) and was mainly composed of glucose with a ratio Glc:GlcNAc of 50. (Table I). Methyl ethers of glucose residues obtained after methylation were 1,2,4,5,6-Glc, 2,3,4,6-Glc, 2,4,6-Glc, 2,3,6-Glc, and 2,4-Glc in a molar ratio of 0.3:0.9:14:10:1. This fraction was characterized by the presence of $\beta$-1,3-glucan with a high amount of 4-$\text{O}$-substituted glucose residues and was analyzed as described previously for QzSN I$_B$, using hydrolysis by endo-$\beta$-1,3-glucanase (ENG1), gel permeation fractionation, NMR, periodate oxidation, and GLC-MS (data not shown). Results were very reminiscent of the ones obtained with the QzSN I$_B$ fraction and indicated that the glucan structures of ChSN I$_B$ were very similar to the glucan structures of QzSN I$_B$: (i) The $\beta$-1,3-glucan molecule was branched through $\beta$-1,6 linkages; (ii) the molecule containing the 4-$\text{O}$-substituted glucose had the following repeating unit [Glc $\rightarrow \beta_1\text{-}4\text{Glc} \rightarrow \beta_1\text{-}3\text{Glc} \rightarrow \beta_1\text{-}3\text{Glc} \rightarrow \beta_1\text{-}3\text{Glc}$] and was linked to the nonreducing end of the $\beta$-1,3-glucan side chain.

The only chemical difference between QzSN I$_B$ and ChSN I$_B$ was the presence of GlcNAc residues in ChSN I$_B$. To identify the linkages and the position of GlcNAc residues to the sugar core, transgalactosylation of GlcNAc residues was performed. Acetylation of ChSN I$_B$ released a mixture of oligosaccharides of variable size with a maximal dp of 10 (data not shown). The mixture of oligosaccharides was incubated with galactosyltransferase and UDP-galactose and then applied to an E. cris-tagalli lectin-agarose column. The fraction retarded on the
affinity column. The Glc/GlcNAc ratio was 6, and all oligosaccharides recovered contained the sequence Gal[b1–4GlcNAc, indicating that all GlcNAc residues were bound in a β anomeric configuration to the branched β-1,3/1,6-glucan. The size of the side chains containing one GlcNAc residue varied from 2 to 8 residues (Fig. 11). These chemical data showed that all GlcNAc residues were bound to the nonreducing end of the branched β-1,3/1,6-glucan as in ChSN II, in agreement with the following structure.

\[
\text{Glcβ1–3Glcβ1–3Glcβ1–3Glcβ1–3Glcβ1–3Glcβ1–3Glcβ1–3Glcβ1–3Glcβ1–3Glcβ1–3Glcβ1} \\
\text{GlcNAcβ1–4Glcβ1–3Glcβ1–3Glcβ1} \\
\]

where \( n \) is between 0 and 7.

Fraction ChSN IA: Chitin, β-1,3/1,4-Glucan and Galactomannan Are Linked to Different β-1,3/1,6-Glucan Branches—ChSN IA had a molecular mass of 40 kDa (Fig. 3) and contained galactose, mannose, and glucose in equivalent amounts with the presence of traces of GlcNAc residues in a Glc/GlcNAc ratio of 100 (Table I). Methylation analysis showed that the fraction released by the chitinase treatment was originally composed of galactomannan, β-1,3/1,4-glucan and chitin, as shown by the presence of GlcNAc in this fraction resulting from the action of chitinase (Table V). The main question addressed in the analysis of ChSN IA was the identification of the linkage between chitin, galactomannan, and glucan. To address this question, ChSN IA was incubated with the 74-kDa endo-β-1,3-glucanase, ENG1. Degradation products were separated by gel filtration chromatography on a TSK HW40S column (Fig. 12). Monosaccharide composition showed that GlcNAc residues were only found in the tetraoligosaccharide peak (dp 4) and in fraction b containing oligosaccharides of dp 5–10, whereas galactose and mannose associated to glucose residues were in fraction a excluded at the void volume. MALDI-TOF analysis of fractions b and dp 4 showed that GlcNAc residues were covalently linked to the glucan fragment (Fig. 13). Methylation analysis of fraction b showed the presence of four methyl ethers: 1,2,4,5,6-Glc, 2,4,5,6-Glc, 2,4,6-Glc, and 2,4-Glc in a molar ratio of 0.7:2:2:9:1, respectively, indicating that this fraction consisted of a highly branched β-1,3/1,6-glucan with short chains of linear β-1,3-glucans, which explained its resistance to the 74-kDa endo-β-1,3-glucanase (see above). These data suggested that ChSN IA had a structure similar to that of QzSN IA with the presence of galactomannan and the β-1,3/1,4-glucan associated to β-1,3-glucan and also similar to ChSN IB or ChSN II where GlcNAc residue was linked to branched β-1,3-glucan. This result indicated that GlcNAc, β-1,3/1,4-glucan and galactomannan were covalently associated to three different β-1,3-glucan side chains of the branched β-1,3/1,6 core glucan.

**Characterization of the Quantzyme/Chitinase-resistant Pellet**—The residual insoluble pellet (FP) was very rich in glucosamine and galactosamine, because hexosamine accounted for 80% of this material (Table I). The insolubility of this residual pellet made the analysis of this fraction difficult, and only partial information was obtained on the chemical organization of FP. The most striking features were the following: (i) the glucan moiety was a branched β-1,3/1,6 polymer similar in composition to the one analyzed in QzSN IA and ChSN IB; (ii) treatment of FP with the 74-kDa endoglucanase (ENG1) solubilized 15% of FP. Gel permeation and MALDI-TOF analysis showed that the soluble oligosaccharides had a composition similar to those released by ENG1 from ChSN IA with a dp of 2–10 and the presence of GlcNAc linked to some of the laminarigosaccharides (data not shown); (iii) n-N-acetylglactosamine residues, which is the major monosaccharide of FP, was substituted in position 4 and should correspond to a poly-N-acetylglactosamine polymer.

**FIG. 11.** MALDI-TOF mass spectra of oligosaccharides of the fraction retarded on E. cristagalli lectin-agarose chromatography, after transgalactosylation of ChSN IB. Mass spectra were recorded in the positive ion mode and accelerated to an energy of 5 keV before entering the flight tube. Samples were prepared by mixing directly on the target 1 μl of oligosaccharide solution (about 25 pmol) and 1 μl of 2.5-dihydroxybenzoic acid matrix solution (12 mg/ml dissolved in CH3OH/H2O (80:20 v/v)). Mass (m/z) correspond to the oligosaccharide mass plus sodium.
The eluate was monitored by refractometry. dp was established with malto-oligosaccharides as standard.

**Fig. 12.** Gel filtration chromatography on a TSK HW40S column of products released after 74-kDa endo-β-1,3-glucanase digestion of the ChSN I₃ fraction. TSK HW40S column (90 × 1.4 cm) was eluted with 0.25% (v/v) acetic acid solution at 0.5 ml/min. The elution was monitored by refractometry. dp was established with malto-oligosaccharides as standard.

**Fig. 13.** MALDI-TOF mass spectra of oligosaccharides of the fraction b of Fig. 12 isolated by gel filtration on a TSK HW40S column after 74-kDa endo-β-1,3-glucanase digestion of the ChSN I₃ fraction. Spectra were recorded in the positive ion mode and accelerated to an energy of 5 keV before entering the flight tube. Samples were prepared by mixing directly on the target 1 ml of oligosaccharide solution (about 25 pmol) and 1 ml of 2.5-dihydroxybenzoic acid matrix solution (12 mg/ml dissolved in CH₃OH/H₂O (80:20 v/v)). Masses (m/z) correspond to the oligosaccharide mass plus sodium.

**DISCUSSION**

Cell wall biosynthesis is a key process in the formation, growth, and morphogenesis of fungal cells. Despite its essential function in fungal development, cell wall structure and biosynthesis, especially polymer cross-linking, remain poorly studied. In this study, a fractionation procedure using alkali extraction and enzymatic digestions of the cell wall, followed by a physico-chemical analysis of the soluble polysaccharides, was followed by the synthesis of the general organization of the polysaccharides (β-glucans, chitin, and galactomannan) in the alkali-insoluble fraction of the *A. fumigatus* cell wall. Care has been taken to use exclusively recombinant β-1,3-glucanase and chitinase, which are free of any contaminating enzymes. Most previous reported studies in cell wall chemistry, including the study of Kollar et al. (7) have used commercial enzymes such as laminiarase or zymolyase, which also contain protease and exo-β-glucanase activities, or chitinase, which is contaminated by chitobiase and β-glucanase activities. Such contaminating activities can mislead the identification of sugar linkages in the cell wall.

From the data presented in this paper, a tentative scheme of the organization of the β-1,3-glucan and its covalently associated polymers found in the cell wall of *A. fumigatus* can be presented (Fig. 14). β-1,3-Glucan is the main component of the alkali-insoluble fraction of *A. fumigatus* cell wall, and it is highly branched with β-1,6 linkages (4% of branch points), constituting a three-dimensional network with a large number of nonreducing ends. Chitin, galactomannan, and β-1,3/1,4-glucan are covalently anchored onto these nonreducing ends, producing a large heteropolymer complex. Chitinase digestion (without previous Quantzyme treatment) degraded 80–90% of the total amount of the chitin of the alkali-insoluble fraction. Chitinase treatment only released GlcNAc and did not release other water-soluble polymers such as β-glucan or galactomannan (data not shown), indicating that these components are not directly linked to chitin. Based on Fig. 14, our current hypothesis of the chronological events occurring in the biosynthesis of the *A. fumigatus* polysaccharide network is as follows: (i) biosynthesis of the individual polysaccharides (β-1,3-glucan, β-1,3/1,4-glucan, chitin, and galactomannan); (ii) branching of β-1,3-glucan, increasing the number of acceptor sites; and (iii) covalent addition of chitin, galactomannan, and β-1,3/1,4-glucan to glucan branches.

The first class of biosynthetic events, synthesis of β-1,3-glucan and chitin, is the only one known in *A. fumigatus* (42–44). Synthesis of β-1,3-glucan in *A. fumigatus*, like in yeast, is under the control of a transmembrane glucan-synthase complex, using UDP-glucose as substrate to extrude linear glucan chain in the periplasmic space (45). Two components of this complex, the regulatory GTPase Rho1 and the putative catalytic subunit FKS have been characterized at the molecular level in yeast (46–50) and recently in *A. fumigatus*.² The enzymes responsible for the second and third classes of biosynthetic events (branching and cross-linking) are totally unknown. Two glucanoseyltransferases have been recently characterized in *A. fumigatus*, but none of them could synthesize the β-1,3/1,6 branched glucan of the cell wall. The first glucanoseyltransferase, homologous to Bgl2p in *S. cerevisiae* and *Candida albicans*, is able to construct a β-1,6 linkage inside a β-1,3-glucan chain (39, 51, 52). However, this enzyme (i) requires a free reducing end to act on the β-1,3-glucan chain and (ii) does not make a branched glucan but makes a kinked linear glucan. Moreover, disruption of the gene encoding this protein in *A. fumigatus* and homologous yeast genes did not result in a phenotype distinct from the parental strain, suggesting that this glucanoseyltransferase does not play a role in cell wall construction (40, 53, 54). In contrast, the second glucanoseyltransferase identified in *A. fumigatus* (Gelp) plays a major role in fungal morphogenesis. This transferase is responsible for the elongation of β-1,3-glucan chains (38). Molecular characteriza-

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² A. Beauvais and P. Mol, personal communication.
the polysaccharide organization in the structural alkali-insoluble core of the A. fumigatus cell wall.

![Diagram](image_url)

- glucose
- N-acetylglucosamine
- mannosone
- galactofuranose

Alkali-insoluble Fraction of A. fumigatus Cell Wall

Fig. 14. Tentative representation of the polysaccharide organization in the structural alkali-insoluble core of the A. fumigatus cell wall.

Comparison of polysaccharide structures showed that these two fungal species present striking similarities: (i) the β-1,3-glucan chain of S. cerevisiae is branched via β-1,6 linkages. The β-1,3/β-1,6-glucan fraction represents 85% of the total cell wall glucons and contains 3% of branch points (60) and (ii) the alkali-insoluble fraction is a complex structure associated to other cell wall polymers. Its presence in FP could be only due to its co-precipitation with the alkali-insoluble material. A linear β-1,3/1,4-glucan never described before in fungi was released with Quanzyme and/or chitinase from the alkali-insoluble cell wall fraction in A. fumigatus. This β-1,3/1,4-glucan from A. fumigatus has a structure close to vegetal and lichen glucan. Indeed, lichenin, isolated from Cetraria islandica, barley, and oat β-glucans are β-1,3/1,4-glucans constituted with β-1,4-glucosidoglycosacharides of dp 2 or 3 mainly, joined by a single β-1,3-linkage (63–65). Methylation data from the total soluble fraction released by Quanzyme suggested that it represented 10% of total β-glucan. The identification and characterization of this β-1,3/1–4-glucan could explained the presence of 4-O-substituted β linked glucose residue reported 30 years ago in the cell wall of Ascomycete species (66).

(iii) Galactomannan with galactofuran side chains has been only described in Penicillium and Aspergillus species. Among these filamentous fungi, this polymer has been previously isolated from a culture filtrate or from the alkali-soluble fraction of the mycelial wall (17, 67, 68). It is the first time that galactomannan was described to be covalently associated to β-1,3-glucan in fungi.

In yeast, cell wall bound glycoproteins have been described to be covalently linked to β-1,3-glucan through a β-1,6-glucan moiety (8, 9, 10). These proteins are originally GPI-anchored to the membrane (11, 12) and then cleaved to be transferred onto β-1,6-glucan using the sugar moiety of GPI as a bridge (13, 7). Another family of cell wall proteins are directly bound to β-1,3-glucan through an unknown linkage (15). In our study, the use of hot NaOH to prepare the alkali-insoluble fraction of A. fumigatus cell wall destroyed all proteins putatively associated to the cell wall and did not allow to study the covalent incorporation of proteins in the A. fumigatus cell wall. The presence of such proteins is being presently investigated.

Despite the differences observed in the composition of the structural polysaccharides of S. cerevisiae and A. fumigatus, branching of β-1,3-glucan appears as a central event for the cell wall construction. Recent analysis of C. albicans cell wall also indicated the presence of branched β-1,3/1,6-glucan and the linkage of the β-1,6-glucan to the nonreducing end of the β-1,3-glucan chain. The enzymatic activity responsible for the branching of β-1,3-glucan has not yet been identified and is presently under study in our laboratory since the understanding of this biosynthetic step in the cell wall construction may lead to the discovery of new antifungal drugs for the treatment of life-threatening invasive aspergillosis.

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Molecular Organization of the Alkali-insoluble Fraction of *Aspergillus fumigatus* Cell Wall

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*J. Biol. Chem.* 2000, 275:27594-27607. 
*originally published online September 1, 2000*

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Vol. 275 (2000) 27594–27607

Molecular organization of the alkali-insoluble fraction of Aspergillus fumigatus cell wall.

Thierry Fontaine, Catherine Simenel, Guy Dubreucq, Olivier Adam, Muriel Delepierre, Jérôme Lemoine, Constantin E. Vorgias, Michel Diaquin, and Jean-Paul Latgé

Pages 27603 and 27604: The mass spectra of Figs. 9 and 11 are inverted. Fig. 9 is described by the Fig. 11 legend and Fig. 11 is described by the Fig. 9 legend. These figures with their correct legends are shown below:

FIGURE 9. MALDI-TOF mass spectra of oligosaccharides resistant to periodate oxidation of the fraction of Qsn I₄ binding to ConA-Sepharose column and fractionated on the TSK HW40S column (fraction a of Fig. 8). Mass spectra were recorded in the positive ion mode and accelerated to an energy of 5 keV before entering the flight tube. Samples were prepared by mixing directly on the target 1 μl of oligosaccharide solution (about 25 pmol) and 1 μl of 2,5-dihydroxybenzoic acid matrix solution (12 mg/ml dissolved in CH₃OH/H₂O (80/20 v/v)). Mass (m/z) corresponds to the oligosaccharide mass plus sodium.

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.

41528
Identification and characterization of two members of a novel class of the interleukin-1 receptor (IL-1R) family. Delineation of a new class of IL-1R-related proteins based on signaling.

Teresa L. Born, Dirk E. Smith, Kirsten E. Garka, Blair R. Renshaw, Jeanette S. Bertles, and John E. Sims

Page 29954: We inadvertently failed to reference a previous publication of the sequence that we called TIGIRR-1. That sequence had been published previously with the name IL1RAPL2 (GenBank™ accession number AF181285) in an article by Jin et al., "Two novel members of the interleukin-1 receptor gene family, one deleted in Xp22.1-Xp21.3 mental retardation" (Jin, H., Gardner, R. J., Viswesvaraiah, R., Muntoni, F., and Roberts, R. G. (2000) Eur. J. Hum. Genet. 8, 87–94). We wish to apologize for our oversight.
Specific binding of a 14-3-3 protein to autophosphorylated WPK4, an SNF1-related wheat protein kinase, and to WPK4-phosphorylated nitrate reductase.

Yoshihisa Ikeda, Nozomu Koizumi, Tomonobu Kusano, and Hiroshi Sano

Pages 31698 and 31699: Unrevised versions of Figs. 5 and 7 were published inadvertently. The correct figures are shown below.

**FIG. 5.** Binding of TaWIN1 to a WPK4-phosphorylated NR peptide. A 1-μg aliquot of GST, GST-HMGR, or GST-NR (lanes 1 through 3) was incubated with 50 ng of GST-WPK4, separated by SDS-PAGE, stained (A), dried, and exposed to x-ray film (B). As the control, GST-NR without phosphorylation was used (lane 4). Far Western blotting analysis showing direct interaction of TaWIN1 with phosphorylated NR (C). Substrate proteins were phosphorylated by GST-WPK4 in the presence of cold ATP, separated by SDS-PAGE, and electroblotted on polyvinylidene difluoride. The membrane was incubated with His-TaWIN1, washed thoroughly, and detected using an anti-Penta-His antibody. Asterisks indicate phosphorylated samples. CBB, Coomassie Brilliant Blue.

**FIG. 7.** Proposed pathway for the regulation of NR by WPK4. WPK4 is transcriptionally activated by carbon deprivation (8). As a result, WPK4 autophosphorylates its own C-terminal region, thereby creating the TaWIN1 binding site. At the same time, TaWIN1 binds, WPK4-phosphorylated NR, to which TaWIN1 is transferred from WPK4. This transfer forms an enzymatically inactive NR complex. Thus, carbon deficiency can coordinate the suppression of a nitrogen assimilation machinery of a cell.