Glycosylphosphatidylinositol-anchored Fungal Polysaccharide in Aspergillus fumigatus*

Corina Costachel1, Bernadette Coddeville2, Jean-Paul Latgé3, and Thierry Fontaine1

From the 1Unité des Aspergillus, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris cedex 15, France and the 2Laboratoire de Chimie Biologique, Université des Sciences et Technologies de Lille, 59655 Villeneuve d’Ascq cedex, France

Glycosylphosphatidylinositol (GPI) glycan is a tetramannose structure linked to a glucosamine residue: Man \(1\to2\)Man \(1\to3\)Glcn. The galactomannan structure is shown to be GPI-anchored.

Aspergillus fumigatus has become over the past decade the most prevalent airborne fungal pathogen causing fatal invasive infections in immunocompromised patients (1). One of the characteristic components of this fungus is composed of a linear mannan chain with a tetra-\(\alpha\)-mannoside repeating unit and side chains of \(\beta1\to5\) galactofuranoside residues (2). The galactomannan from \(A.\ fumigatus\) has been described as a free polysaccharide found in the culture medium, and it is also covalently associated to the cell wall through the \(\beta1\to3\) glucan net and plays a role in the structural organization of the cell wall (2, 3). A monoclonal antibody directed against the galactofuranose side chain has been used to detect the presence of this polymer in the sera of patient with invasive aspergillosis (4, 5). Biosynthesis of the galactomannan is totally unknown in this fungus. During the search of lipid molecules that could be an anchor for the galactomannan, we isolated a membrane bound fraction that contained galactomannan. We investigated the nature of the membrane anchor of the galactomannan by GLC-MS2 (gas liquid chromatography-mass spectrometry) and ES-MS-MS (electroospray-tandem mass spectrometry) and found that the lipid anchor was a glycosylphosphatidylinositol.

1 To whom correspondence should be addressed. Tel.: 33-1-45-68-82-25; E-mail: tfontain@pasteur.fr.

2 The abbreviations used are: GLC-MS, gas liquid chromatography-mass spectrometry; ES-MS-MS, electrospray-tandem mass spectrometry; PI-PLC, phosphatidylinositol-specific phospholipase C; HPLC, high performance liquid chromatography; IBM, jack bean \(\alpha\)-mannosidase; LGM, lipogalactomannan; IPC, inositol phosphocheramide; GIPC, glycosylinositol phosphocheramide; ZGL, zwitieronic glycosphingolipid.

* This work was supported in part by a research and development grant awarded by Aventis-Pharma (to J.-P. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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DECEMBER 2, 2005 • VOLUME 280 • NUMBER 48

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FIGURE 1. Purification of a protease digest of a chloroform/methanol extract of a membrane preparation of A. fumigatus mycelium. The extract was added to an octyl-Sepharose column equilibrated with 5% propan-1-ol in 100 mM ammonium acetate. Bound products were eluted with a linear gradient of propan-1-ol (5–60%). Neutral sugars were detected with the phenol/sulfuric colorimetric assay (closed circles) (8), and galactofuranose epitope was detected by enzyme-linked immunosorbent assay (open circles) (2).

TABLE ONE
Table:<br>Molar ratio of methyl ethers obtained after permethylation of the native, HF-treated and HCl-treated lipogalactomannan isolated from A. fumigatus membrane preparations<br>Molar ratio was calculated by GLC-flame ionization detection.<br><br>Table:<br>| Methyl ethers     | Sugar linkages | Molar ratio | Native | HF-treated | HCl-treated |
|-------------------|----------------|-------------|--------|------------|-------------|
| 2,4,6-Man         | t-Man<sub>p</sub> | >0.1        | 0.2    | 1.0        |
| 3,4,6-Man         | 2-Man<sub>p</sub> | 2.7         | 3      | 1.5        |
| 2,3,4-Man         | 6-Man<sub>p</sub> | 1           | 1      | 1          |
| 3,4-Man           | 2-Man<sub>p</sub> | 1.3         | 0.4    | 0.3        |
| 4,6-Man           | 2,3-Man<sub>p</sub> | 0.4         | 0.3    |            |
| 2,3,5,6-Gal       | t-Gal<sub>i</sub> | 1.3         | 0.2    |            |
| 2,3,6-Gal         | 5-Gal<sub>i</sub> | 3.3         | >0.1   |            |
| 3,4,6-GlcN(Me)    | t-GlcN          |             | 0.8    |            |
| 3,6-GlcN(Me)      | 4-GlcN          |             | 0.4    |            |

oligosaccharides (Glucidex 19 from Roquette Frères, Lestrem, France) were used as molecular weight standards.

Analytical Methods
Phosphate was quantified according to Ames (9). Total hexoses were quantified by the phenol-sulfuric acid procedure using mannose and galactose as standards (8). Neutral hexoses were identified by GLC as aldito acetates (10) and/or as methyl glycosides (15). Methyl ethers were analyzed by GLC-MS as polyol acetates (14) and/or as methyl glycosides (15).

Chemical and Enzymatic Treatments
Phosphatidylinositol-specific phospholipase C (PI-PLC) Digestions—1.5 mg of purified lipogalactomannan was dissolved in 100 µl ofimidazole/acetate buffer, pH 7.5, containing 5 mM sodium azide and 0.1% Triton X-100 and incubated with 5 µl of PI-PLC (Glyko, 200–300 units/ml) at 37 °C for 4 h. Digested products were then eluted with 50% propan-1-ol in 100 mM ammonium acetate. Bound products were then eluted with 10% acetic acid/acetate buffer (pH 4.0, buffer containing 5 mM sodium azide and incubated with 10 µl of JBAM (Sigma) at 37 °C for 24 h. Released lipid moiety was extracted with water-saturated butanol and then purified on a small silica column.

Acetylation—500 µg of purified lipogalactomannan was peracetylated by treatment with formamide/acetate anhydride/pyridine (2:2:1, v/v/v) overnight at room temperature, then dialyzed against water and freeze-dried. The peracetylated products were treated with 200 µl of an acetic acid/acetate anhydride/sulfuric acid solution (10:10:1 v/v/v) at 37 °C for 7 h. The reaction was stopped by addition of 800 µl of an ice-cold pyridine/water mixture (1/3 v/v). Acetylated products were extracted with chloroform and the organic phase was washed with water and then concentrated to dryness. De-O-acetylation was performed in 300 mM NaOH containing NaBH₄ (10 mg/ml) overnight at room temperature. The excess of reagent was destroyed by addition of 10% acetic acid. Released lipid moiety was extracted by the butanol-water partitioning.

TLC Analysis—TLC was performed on precoated aluminum-backed silica 60 HPTLC plates (Merck). Plates were developed at room temper-
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Purification and Composition of the Lipogalactomannan—The crude membrane fraction of *A. fumigatus* mycelium was treated with a chloroform/methanol/water mixture, and the pellet was digested with a protease mixture. Soluble material was fractionated by a hydrophobic interaction. The major phenol-sulfuric acid-positive peak, bound to the octyl-Sepharose column, was also positive with the anti-galactofuranose antibody detection (Fig. 1). The purified fraction had an apparent molecular mass of 30 kDa on a Superose 12 column (data not shown) and represented 0.04 ± 0.01% of total mycelium dry weight.

Colorimetric assays and GLC analysis showed that the octyl-bound fraction was mainly composed of mannose and galactose residues in a ratio 3:2 and for this reason was called lipogalactomannan (LGM). The LGM fraction contains a small amount of glucosamine, phosphate, myo-inositol, 2-monohydroxy-C24:0 fatty acid, and C18-phytosphingosine (data not shown). The presence of these compounds suggested that the polysaccharide was anchored to the membrane by a GPI anchor (7). A PI-PLC- or HNO2-treated lipogalactomannan did not bind to the octyl-Sepharose column (data not shown). Taken all together, these results showed that the galactomannan moiety was bound to a GPI anchor.

Analysis of the Carbohydrate Structure of the Lipogalactomannan—To determine the monosaccharide linkages, the membrane-bound polysaccharide fraction was permethylated. Methyl ethers analysis revealed the presence of mannose residues monosubstituted in position 2 or 6 and disubstituted in position 2,3 or 2,6 as described for the secreted galactomannan isolated from *A. fumigatus* culture filtrate (TABLE ONE) (2). Terminal galactofuranose has also been identified. 2,3,6-Tri-O-methyl-1,4,5-tri-O-acetyl-galactitol indicated the presence of galactofuranose residues substituted in position 5 or galactopyranose in position 4. The specific detection with the anti-galactofuranosyl monoclonal antibody and the sensitivity of galactose residue to a mild acid analysis: 100–200 °C at 10 °C/min, 200–260 °C at 15 °C/min, and 260 °C for 13 min.

Electrospray-Mass Spectrometry—All electrospray-mass measurements were carried out in negative-ion mode on a triple quadrupole instrument (Micromass Ltd., Altrincham, UK) fitted with an atmospheric pressure ionization electrospray source. A mixture of polypropylene glycol (range 30–2,000 daltons) was used to calibrate the quadrupole mass spectrometer. The samples were dissolved in chloroform/methanol (1/2) at a concentration of 1–10 pmol/μl. Solutions were infused using a Harvad syringe pump at a flow rate of 3 μl/min. Quadrupole was scanned from 400 to 2000 Da with a scan duration of 3–5 s and a scan delay of 0.1 s. The samples were sprayed using 3.25 kV needle voltage, and the declustering (cone) was set at 70 V. For collision-induced dissociation experiments, the pressure of argon in the cell was set at 2.7·10⁻³ mbar, and the collision energy was set to values ranging from 40 to 70 V depending on the studied daughter ions.

Matrix-assisted Desorption Ionization Time-of-Flight Mass Spectrometry—Mass spectra were measured on a reflectron-type Vision 2000 time of flight mass spectrometer (Finnigan MAT, Bremen, Germany). Samples were mounted on an x,y movable stage allowing irradiation of selected areas. A nitrogen laser with an emission wavelength of 337 nm and 3-ns pulse duration was used. The spectrum was recorded in the positive ion mode and accelerated to an energy of 10 keV before entering the flight tube. Ions 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/10 mM NaCl (80:20 v/v).

RESULTS

Purification and Composition of the Lipogalactomannan—The crude membrane fraction of *A. fumigatus* mycelium was treated with a chloroform/methanol/water mixture, and the pellet was digested with a protease mixture. Soluble material was fractionated by a hydrophobic interaction. The major phenol-sulfuric acid-positive peak, bound to the octyl-Sepharose column, was also positive with the anti-galactofuranose antibody detection (Fig. 1). The purified fraction had an apparent molecular mass of 30 kDa on a Superose 12 column (data not shown) and represented 0.04 ± 0.01% of total mycelium dry weight.

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hydrolysis and/or aqueous HF treatment (TABLE ONE) indicated that all galactose residues were in furanic configuration similar to those described in the secreted galactomannan (2).

To confirm the similarity of structures, acetolysis was performed on the lipogalactomannan and secreted galactomannan. Analysis of released products by gel filtration (Fig. 2), matrix-assisted desorption ionization time-of-flight mass spectrometry, and GLC analysis revealed the same pattern of degraded products such as mainly galactose residue and a tetra-α1–2-mannoside from both polysaccharide fractions. All these data indicated that the membrane-bound galactomannan from A. fumigatus has the same structure as the soluble polymer.

Analysis of the Lipid Anchor of the Lipogalactomannan—The lipid moiety, released by nitrous acid deamination from the lipogalactomannan, purified by butanol/water partition and by chromatography on a small silica column, was analyzed by ES-MS-MS as described previously (7). The ES-MS spectrum of the PI fraction (Fig. 3A) revealed one principal [M–H]− pseudomolecular ion at m/z = 924 and four minor ions at m/z = 908, 910, 938, and 952. The fragmentation of each PI compounds produced only two daughter ions at m/z = 241 [inositol 1,2-cyclic phosphate]− and at m/z = 259 [inositol monophosphate]− (Fig. 3B). The presence of these daughter ions and the absence of the fatty acid carboxylate ion and/or 1-O-alkylglycerol-2,3-cyclic phosphate ion is characteristic of inositol phosphoceramides (IPCs) (17). From the measured mass and taking into account the identified lipid compounds, we concluded that the main PI structure is made of a C18-phytosphingosine and a 2-monohydroxylated C24:0 fatty acid. The minor components that differ by a mass of 14, 16, or 28 reflected the variability of size of fatty acid or the absence of hydroxyl group that was observed by the fatty acid analysis (data not shown).

To confirm the position of glucosamine residue, the lipogalactomannan fraction was submitted to a mild HCl acid hydrolysis followed by a JBAM digestion. The ES-MS spectrum of the released lipid moiety revealed one principal [M–H]− pseudomolecular ion at m/z = 1086 (Fig. 4A). Except for minor ions due to the variability of the aliphatic chain (m/z = 1072, 1100, and 1114), two minor [M–H]− pseudomolecular ions at m/z = 1248 and 1410 were observed and corresponded to an increase of 162 and 324 suggesting the presence of 1 and 2 hexose residues, respectively. The fragmentation of the ion at m/z = 1086 produced two daughter ions at m/z = 402 [hexosamine-inositol-1,2cyclic phosphate]− and at m/z = 420 [hexosamine-inositol monophosphate]− (Fig. 4B). Since glucosamine has been identified in the LGM fraction, and since the ion mass at m/z = 1086 corresponds to the presence of an
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FIGURE 4. ES-MS analysis of the butanol-soluble products released by mild HCl hydrolysis and jack bean α-mannosidase digestion from the purified lipogalactomannan of A. fumigatus. A, negative ion ES-MS spectrum; B, daughter ion ES-MS-MS spectrum of the ion at 1086; C, daughter ion ES-MS-MS spectrum of the ion at 1248. P, phosphate group; hexagon represents the inositol ring.

hexosamine residue linked to the identified PI, the presence of the two daughter ions is characteristic of the glucosamine linked to the inositol phosphoceramide. The fragmentation of the ion at \( m/z = 1248 \) produced also two daughter ions at \( m/z = 564 \) [hexose-hexosamine-inositol-1,2cyclic phosphate] and at \( m/z = 582 \) [hexose-hexosamine-inositol-monophosphate] (Fig. 4C). Since only mannose has been identified as hexose in the butanol phase after the HCl-JBAM treatment, these daughter ions are characteristic of the Man-GlcNAc linked to the inositol phosphoceramide.

The LGM fraction was submitted to acetylation, which cleaves preferentially the 1–6 linkage. The ES-MS of the butanol-soluble-resistant products revealed two main \([M - H]^-\), pseudomolecular ions at \( m/z = 965 \) and at \( m/z = 1290 \) (Fig. 5A). Minor ions at \( m/z = 803 \) and at \( m/z = 1128 \) correspond to the loss of 162 from the two main ions, respectively, suggesting the loss of an hexose residue. The fragmentation of the ion at \( m/z = 1290 \) produced two main daughter ions at \( m/z = 606 \) [hexose-N-acetylhexosamine-inositol-1,2cyclic phosphate] and at \( m/z = 624 \) [hexose-N-acetyl-hexosamine-inositol monophosphate] (Fig. 5B). Since only mannose was identified as hexose in this fraction, and since acetylation resulted in the N-acetylation of the glucosamine residue, the daughter ions are characteristic of the Man-GlcNAc linked to the inositol phosphoceramide. The fragmentation of the ion at \( m/z = 965 \) produced three daughter ions at \( m/z = 606 \), at \( m/z = 624 \), and at \( m/z = 420 \) (Fig. 5C). The ions at \( m/z = 606 \) and at \( m/z = 624 \) are similar to those obtained by the fragmentation of the parental ion at \( m/z = 1290 \), indicating a same structure of the glycan part in both parental ions. The mass difference between this both parental ions corresponds to the substitution of the 2-monohydroxyl C24:0 fatty acid by an acetyl group, indicating that the acetylation cleaved the acetamide linkage. The presence of one mannose residue linked to the N-acetylg glucosamine after acetylation indicated that this first mannose residue is substituted in position 6.

After mild acid hydrolysis (50 mM HCl for 15 h at 100°C) without JBAM digestion, the ES-MS spectrum of HPLC-purified glycolipids revealed 10 main pseudomolecular ions at \( m/z = 1086 \), 1248, 1410, 1572, 1734, 1896, 2058, 2220, 2382, and 2544 (Fig. 6). The ion at \( m/z = 1086 \) corresponds to the GlcN-IPC described in the legend to Fig. 4. The increase of ion mass of 162, 324, 486, 648, 810, 972, 1134, 1296, and 1458 indicates the presence of a mixture of oligosaccharides containing 0–9 hexoses linked to the GlcN-IPC. Monosaccharide linkage analysis, done after peracylation, permethylation, and methanolysis, revealed the presence of terminal glucosamine, 4-substituted glucosamine, terminal mannos, 2-substituted mannos, 6-substituted mannos residues (TABLE ONE). According to these ES-MS data, the terminal glucosamine residue came from GlcN-IPC. The presence of the 4-substituted glucosamine residue indicates that the first mannose residue is linked in position 4 to the glucosamine. Taking into account the results from acetylation experiment, the 6-substituted mannos corresponds to the first mannose residue in the Manm-GlcN-IPC structures. The quantity of 2-substituted mannos, higher than the one of 6-substituted mannos, indicated that at least the two following mannos residues (third and fourth) are substituted in position 2. All of these mannos residues, removed by a JBAM digestion, are linked in α-anomeric configuration (data not shown). After mild acid hydrolysis, the presence of structures containing more than 5 mannos residues linked to GlcN-IPC and the absence of phosphoethanolamine group excluded the putative contamination from GPI-anchored proteins (7). The sensitivity of A. fumigatus LGM to the PI-PLC indicated that the linkage between GlcN and inositol is not α1–2 (18). Taken all together, these results were in agreement with the following GPI structure: galactomannan-Manα1–2Manα1–2Manα1–6Manα1–4GlcN-inositol-P-ceramide.

The GPI anchor was linked to the α-mannos chain of the galactomannan, which contains the same repeat unit. The small amount of 2,6-disubstituted mannos (TABLE ONE) suggests the putative presence of a mannos as a side chain in this structure.

DISCUSSION

GPI structures have been observed in all eukaryotic cells. In mamalian and plant cells, GPI are involved in the anchoring of proteins in the lipid bilayer by a common core Manm-GlcN-PI. In parasite cells, in addi-
tion to GPI-anchored proteins, oligosaccharides, and polysaccharides have also been described to be GPI-anchored. To date, in fungi only proteins had been found to be GPI-anchored (7, 19). Herein, we described the first fungal polysaccharide to be linked to a GPI. Since many other ascomycetes have a galactomannan with similar structures (20–23), it can be expected now that other fungi also possess GPI-anchored polysaccharide. The role of these polysaccharides in fungal life remains to be understood. The lipid moiety of the LGM and proteins GPIs of *A. fumigatus* is the same ceramide (7). However, anchoring of the GPI to the protein and polysaccharide is different; no phosphoethanolamine group has been identified on the LGM anchor, and the fifth α1–3linked mannose residue of the GPI structure of anchored proteins is replaced by the galactomannan in the LGM structure. Nevertheless, in contrast to the *Leishmania* lipophosphoglycan and to the *Crithidia*
fasciculata liporarbinogalactan, where the second mannose residue is α1–3-linked (24, 25), it is the first time that a polysaccharide is linked to a GPI type 1 structure, as described for GPI-anchored proteins.

IPC structures from the LGM are similar to the glycosylinositol phosphoceramide (GIPC) described in A. fumigatus (26). Numerous GIPCs have been described at the cell membrane of other filamentous fungi and yeast cells but are absent from mammalian cells. The ceramide structure of these glycosphingolipids seems conserved between species with the presence of a phytosphingosine, associated to a saturated fatty acid, mainly the 2-hydroxylignoceric acid (2OH-C24:0) (27–35). In contrast to the lipid moiety, the glycan moiety is heterogenous between fungal species with the presence of mannose, galactose in a pyranic or furanic configuration, and even fucose residue in higher mushrooms (36). Usually, glycols of GIPC contain 2–6 hexose residues. Up to 18 furanic configuration, and even fucose residue in higher mushrooms. (ii) ZGL (zwitterionic glycosphingolipid), where the glycan moiety is linked to the IPC through a glucosamine residue (35, 37). In contrast to LGM structure, in this latter ZGL structure only described in three fungal species, the glucosamine residue is α1–2-linked to the inositol ring.

The galactomannan from A. fumigatus is mainly constituted by the polymerization of a tetramannoside unit containing two side chains of β-1-galactofuranose residues (2). The presence of a repeat unit suggests a biosynthesis of the tetramannoside on a acceptor prior to the polymerization of the mannan chain. Our data suggest that the acceptor could be a GPI moiety. Enzymes involved in this biochemical pathway are a role in this biochemical event. This activity, independent of the dolichol-phosphomannose synthesis and of the acylation of the inositol ring, is not involved in the biosynthesis of GPI intermediates of GPI-anchored proteins (38). These data suggest that GPI anchor of LGM could be synthesized in an independent biosynthetic pathway using GDP-Man as substrate donor, as it was speculated for ZGL in Acremonium and Trichoderma sp. (35).

The A. fumigatus galactomannan has now been described in three different forms: (i) soluble and recovered in the culture medium (2), which does not contain a lipid anchor (data not shown), (ii) covalently linked to the cell wall through the β1–3 glucan network (3), and (iii) GPI-anchored to the membrane. These forms present the same carbohydrate chemical structure, suggesting a common biosynthetic pathway. The galactomannan seems secreted to the plasma membrane with a GPI anchor. At this stage, the galactomannan could be transfered to β1–3 glucan chain as it has been proposed for some yeast GPI-anchored protein (39), or the galactomannan could be released in the culture medium by a specific glycosidase. Enzymes putatively involved in such in block transfer are actively searched.

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