The Candida albicans Phospholipomannan Is a Family of Glycolipids Presenting Phosphoinositolmannosides with Long Linear Chains of \( \beta,1,2 \)-Linked Mannose Residues

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In a series of studies, we have shown that Candida albicans synthesizes a glycolipid, phospholipomannan (PLM), which reacted with antibodies specific for \( \beta,1,2 \)-oligomannosides and was biosynthetically labeled by \([\text{H}^3] \text{mannose}, [\text{H}^3] \text{palmitic acid}, \) and \([\text{P}^32\text{P}] \text{phosphorus}. PLM has also been shown to be released from the C. albicans cell wall and to bind to and stimulate macrophage cells. In this study, we show by thin layer chromatography scanning of metabolically radiolabeled extracts that the C. albicans PLM corresponds to a family of mannose and inositol co-labeled glycolipids. We describe the purification process of the molecule and the release of its glycan fraction through alkaline hydrolysis. Analysis of this glycan fraction by radiolabeling and release of its glycan fraction through alkaline hydrolysis has been shown to be protective against C. albicans in rodent models of systemic and vaginal candidosis (5, 12). \( \beta,1,2 \)-oligomannosides derived from C. albicans phosphoplipomannan have also been shown to induce TNF-\( \alpha \)-synthesis from cells of the macrophase lineage through a phosphytosine kinase-dependent pathway (13) and to bind to macrophage cell membranes (14, 15).

In previous studies, we have shown by use of specific monoclonal antibodies (16) that \( \beta,1,2 \)-oligomannosides are present (in the absence of accessible \( \alpha \)-linked mannose residues) on a polydispersed low molecular weight antigen and that this antigen is a glycolipid. This glycolipid has been named a phospholipomannan (PLM) on the basis of its composition (17). The PLM is a strong TNF-\( \alpha \)-inducer in vitro and in vivo (18). When C. albicans comes into contact with macrophages, large amounts of PLM are rapidly shed by C. albicans, which trigger intense signaling and secretory responses from these target cells (19). Similar signaling events induced in host cells have been described as induced by GPI-related glycolipids from pathogens of the genera Leishmania, Trypanosoma, and Mycobacteria (20–23). In this study, we have further purified and chemically analyzed the C. albicans PLM to establish the relationship of PLM with these microbioglycolipids and to provide a structural basis for the understanding of the immunochernical and immunomodulatory properties of PLM.

**MATERIALS AND METHODS**

**Strain, Culture Conditions, and Metabolic Labelings**—C. albicans VV32 strain (serotype A) was used throughout this study. For germ tubes preparation, C. albicans was first grown in Sabouraud dextrose agar for 18 h at 28 °C and then transferred to RPMI 1640 medium (Roche Molecular Biochemicals) for 4 h at 37°C with mild shaking. Metabolic labelling with \([\text{H}^3] \text{mannose and } [\text{H}^3] \text{inositol was done in 50 ml of RPMI 1640 medium containing either 10-fold less glucose and 200 } \mu\text{Ci of } [\text{H}^3] \text{mannose (Isotopchim, Ganagobie-Peyruis, France) or 10-}

**Cell Extracts**—Whole cell extracts, designated as Fr. A, were ob-

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French press extract of *C. albicans* (Fr. A)

Chloroform/Methanol extract (2:1)

**Pellet**

Chloroform/Methanol/water extract (10:10:1)

**Butanol/water partitions → butanol phase**

**Water phase (Fr. C)**

Chromatographies (Phenyl)-sepharose; Silicagel 60

Purified PLM (Fr. D)

**Alkaline treatment (KOH 3 3°C 90h)**

Lipid extract

Octyl-sepharose

**Bio-gel P2**

Glycan moiety of PLM (Fr. Dg)

**Chemical analysis**

**NMR analysis**

**Mass spectrometry analysis**

![Diagram](Image)

**FIG. 1.** Purification scheme of *C. albicans* phospholipomannan. Fr. A–D refer to PLM-containing fractions with improved purification. Fr. Dg corresponds to the glycan moiety of PLM released by alkaline hydrolysis of Fr. D.

Purification scheme of *C. albicans* phospholipomannan. Fr. A–D refer to PLM-containing fractions with improved purification. Fr. Dg corresponds to the glycan moiety of PLM released by alkaline hydrolysis of Fr. D. 2 mg of purified PLM (Fr. D) estimated by its sugar content was successively washed with 1, 5, and 10% ethanol. PLM elution was crystallized onto the target at room temperature. The electrospray (ESI) ionization mass spectrometry was carried out on a Quattro II triple-quadrupole mass spectrometer (Micromass). Fr. Dg was dissolved in 50% methanol at an approximate concentration of 50 pmol/μl, and the solution was infused into the electrospray ion source by a Harvard syringe pump. The voltage difference between the needle tip and the source electrode was ~3.2 kV.

**RESULTS**

TLC Analysis of *C. albicans* Extracts Showed that PLM Corresponds to a Family of Glycolipids Labeled with [3H]Mannose and [3H]Inositol—Preliminary characterization of *C. albicans* PLM was made through chloroform/methanol/water extraction procedure and Western blot analysis (30). When the same extraction procedure was applied to [3H]mannose-labeled Fr. A and analyzed by thin layer chromatography (Fig. 2, a–c), PLM corresponded to an heterogeneous peak with an average relative migration (R, of 0.147, which was present only in the more polar extract of the last extraction step (Fr. B) (Fig. 2c). This was confirmed by dot-blot analysis, which revealed its reactivity with mAb DF9-3 (data not shown). Subsequent butanol/water partitions of Fr. B resulted in the presence of PLM in the water phase (Fr. C) (Fig. 2d) in contrast to other peaks that were separated in the butanol phases (Fig. 2e).

The heterogeneity of the PLM preparation was clearly demonstrated by thin layer chromatography analysis of [3H]mannose-labeled Fr. C using 0.1% KCl instead of water in the solvent system (Fig. 3a). Moreover, the profiles of [3H]mannose- and [3H]inositol-labeled Fr. C (Fig. 3, a and b) were strikingly superimposed, with the exception of the peak at R, 0.69, which was observed only with the inositol labeling. Analysis by dot-blot procedure of the reactivity of the peaks with...
mAb DF9-3 (Fig. 3c) confirmed that peaks 1–5, observed in both profiles, were related to a family of glycolipids expressing β-1,2-oligomannosidic epitopes. Peaks 6 and 7 did not react with the antibody. Moreover, these two peaks displayed properties quite different from the others when tested on octyl-Sepharose column (data not shown) and thus appeared unrelated to PLM.

**Alkaline Hydrolysis of Purified PLM Released a Soluble Fraction Containing Mannose Residues and Inositol**—To obtain large quantities of PLM for physicochemical analysis, Fr. C was prepared from *C. albicans* grown in a bioreactor. Orcinol staining of this fraction also revealed the main contaminant previously observed with [3H]inositol labeling (Fig. 3b). Successive chromatographic purifications of Fr. C on phenyl-Sepharose and silica gel 60 columns were thus performed and resulted in a PLM preparation (Fr. D) that produced a single spot on TLC (Fig. 4a, lane 1). Fr. D displayed a low solubility in most solvent systems, leading to opalescent solutions. This suggested a micellar conformation of PLM at a high concentration. This hypothesis was confirmed by the exclusion of concentrated Fr. D from the Ultrogel AcA34 column (Biosepra, Cergy-Pontoise, France), for which the exclusion limit was 750 kDa (data not shown). According to these properties, mass spectrometry

**Fig. 2.** Thin layer chromatography analysis of *C. albicans* extracts metabolically labeled with [3H]mannose. A [3H]Man-labeled French press extract of *C. albicans* VW32 was successively extracted with chloroform/methanol (2:1, by volume) (panel a), chloroform/methanol/water (10:10:1, by volume) (panel b) and chloroform/methanol/water (10:10:3, by volume) (Fr. B) (panel c). The last extract was then partitioned between water (Fr. C) (panel d) and water-saturated butanol (panel e). Solvent system, C/M/W3. O, origin; F, solvent front.

**Fig. 3.** Comparative TLC analysis of [3H]mannose-labeled (panel a) and [3H]inositol-labeled (panel b) Fr. C of *C. albicans*. Fr. C were obtained as described in the legend to Fig. 1 from metabolically labeled cells and were analyzed on the same TLC plate. Following radioactivity scanning on a Berthold scanner, areas 1–7 of the silica gel corresponding to the main peaks were scraped off the plates, extracted with solvents and their reactivity to the mAb DF9-3 specific for β-1,2-oligomannosides was analyzed by a dot-blot procedure (panel c). Solvent system, chloroform/methanol/KCl 0.1% (10:15:5, by volume). O, origin; F, front.

**Fig. 4.** TLC analysis of the alkali-released fraction of PLM (Fr. Dg). In panel a, Fr. Dg (lane 2) obtained following alkaline hydrolysis of PLM was compared with Fr. D (unhydrolysed PLM) (lane 1) and β-1,2-linked oligomannosides released from *C. albicans* phosphopentomannan with DP = 13 (lane 4) and 14 (lane 3) used as controls. In panel b, radiolabeled Fr. Dg (lane 1) was prepared from *C. albicans* metabolically labeled with [3H]inositol and compared with the unlabeled Fr. Dg (lane 3) used for the physicochemical analyses. Unhydrolyzed radiolabeled PLM (Fr. Dg) (lane 2) and β-1,2-linked oligomannosides with a DP = 14 (lane 4) were used as controls. All lanes were revealed by an orcinol stain with the exception of lanes 1 and 2 of panel b, which were revealed by fluorography. Solvent system, butanol/acetic acid/water (20/8/17, by volume). Panels a and b correspond, respectively, to one and five runs of migration in this solvent system.
and NMR spectrometry analyses of Fr. D were unsuccessful, and assays were therefore performed to obtain a soluble derivative from Fr. D. Treatment of Fr. D in 1N KOH in 30% methanol for 90 h at 37 °C was found to gradually increase its solubility through the release of a highly water-soluble fraction that no longer interacted with an octyl-Sepharose column. TLC analysis of this fraction (Fr. Dg) was dissolved in D$_2$O (99.95%) and analyzed in a 400-MHz spectrometer in which acetone was used as the internal standard. The H-1 and H-2 signals of the proton-NMR spectrum (panel a) and the heteronuclear two-dimensional spectrum (panel b) evidenced linear chains of β-1,2-linked mannose residues.

**Table I**

| Sugar residue | Chemical shifts (δ) |
|---------------|---------------------|
|               | H-1    | H-2    | H-3    | H-4    | H-5    | H-6    | C-1    | C-2    | C-3    | C-4    | C-5    | C-6    |
| Man(β-1,2) 1  | 4.908  | 4.29   | 3.73   | 3.59   | 3.41   | 3.93   | 100.4  | 80.9   | 73.2   | 68.8   | 77.4   | 61.9   |
| Man(β-1,2) 2  | 4.934  | 4.416  | 3.70   | 3.47   | 3.41   | 3.93   | 102.8  | 80.6   | 73.2   | 68.8   | 77.4   | 61.9   |
| Man(β-1,2) 3 to (n−1) | 5.046  | 4.384  | 3.70   | 3.47   | 3.41   | 3.93   | 102.6  | 80.6   | 73.2   | 68.8   | 77.4   | 61.9   |
| Man(β-1,2) n | 5.050  | 4.155  | 3.63   | 3.59   | 3.41   | 3.93   | 102.3  | 71.6   | 74.2   | 68.8   | 77.4   | 61.9   |

Homo- and heteronuclear two-dimensional NMR spectra of Fr. Dg are shown in Fig. 5 and summarized in Table I. The proton resonance region (Fig. 5a) is dominated by two major signals at δ 5.046 and 4.384 ppm, which were assigned, respectively, to the H-1 and H-2 atom resonances of β-mannose. Three other resonances at δ 4.908, 4.934, and 5.05 ppm also possessed the characteristics of β-mannose anameric protons. The set of these H-1 and H-2 resonances was identical to the NMR data obtained for the β-1,2-Man homopolymers released from *C. albicans* phosphopeptidomannan by mild acid hydrolysis (24) and allowed us to ascertain the mannose units on the spectrum from 1 to n. However, the absence of reducing terminal α-Man can be notified in Fr. Dg and results in the modification of H-1 and H-2 resonances of Man 1 to Man 3. The integration value of the main peak at δ 5.046, which included the additive contributions of Man 3 to Man n−1, also revealed a higher degree of polymerization of the β-1,2-oligomannosides from PLM with an average DP of 14 mannose residues. Signals relative to inositol and glycerol units were not detected, and the two minor atom resonances at δ 5.557 and 5.140 ppm, respectively, seemed to possess the characteristics of the anameric protons of α- or β-Man-1-phosphate (J$_{H,P}$ ≥ 7.5), but these correlations remain to be verified. Moreover, the heteronuclear NMR spectrum (Fig. 5b) clearly indicates that all C-2 atoms resonate at δ 80.6–80.9 ppm, with the exception of the C-2 atom of the mannose unit n (δ 71.66 ppm) in the terminal nonreducing position, and definitively confirms the presence of a linear chain of β-1,2-linked mannose residues in PLM.

**Mass Spectrometry Revealed that the Heterogeneity of PLM Alkali-labile Fraction Was Due to Different Chain Lengths of**
**FIG. 6.** MALDI-TOF and ES mass spectra of Fr. Dg generated by alkaline hydrolysis of PLM. The MALDI-TOF spectrum (panel a) revealed three families of peaks (i.e. m/z 2527, 2607, and 2621). The m/z increment between two consecutive peaks of the same family is always 162. The ES mass spectrum (panel b) contains five families of peaks (i.e. m/z 1303, 1310, 1314, 1345, and 1375) with, for two consecutive peaks of the same family, a m/z increment of 81 resulting from the formation of double-charged pseudo-molecular ions and consequently corresponding to an effective mass increment of 162 (see Table III).

| m/z | Pseudo-molecular ion | M | Mass increment | Composition | Δm |
|-----|-----------------------|---|---------------|-------------|-----|
| 2527 | (M - H) | 2528 | 14 | 1 | 1 | +162 |
| 2607 | (M - H) | 2608 | 14 | 1 | 2 | +162 |
| 2621^a | (M - H) | 2689 | 15 | 1 | 1 | -18^a |
| 2851 | (M - H) | 2952 | 16 | 1 | 1 | +162 |

^a The composition of the molecule corresponding to this pseudo-molecular ion was not determined.

| m/z | Pseudo-molecular ion | M | Mass increment | Composition | Δm |
|-----|-----------------------|---|---------------|-------------|-----|
| 1263 | (M - 2H)/2 | 2528 | 14 | 1 | 1 | -18^a |
| 1294 | (M - 2H)/2 | 2590 | 14 | 1 | 2 | -18^a |
| 1303 | (M - 2H)/2 | 2608 | 14 | 1 | 2 | -18^a |
| 1314.5^b | (M - 3H + Na)/2 | 2608 | 14 | 1 | 2 | -18^a |
| 1354 | (M - 2H)/2 | 2770 | 14 | 1 | 2 | -18^a |
| 1485 | (M - 2H)/2 | 2952 | 16 | 1 | 2 | -18^a |

^a The negative shift probably results from the formation of a cyclic phosphodiester bridge during alkaline hydrolysis.

^b The composition of the molecule corresponding to this pseudo-molecular ion was not determined.
Inositol and β-1,2-Man Linkages in C. albicans Phospholipomannan

Fig. 7. Proposed structure for the family of glycolipids found in PLM of C. albicans VW32, serotype A. The structure of the glycan moiety was deduced from our results, and its linkage to the lipid moiety was postulated both from our results and from the usual structures of inositol and phosphorus containing glycolipids; n may vary from 5 to 15. (a), the position of this branch in the molecule and the percentage of molecules displaying this branch are still unknown.

The Phosphoinositolmannosides—The MALDI-TOF mass spectrum (Fig. 6), recorded in the negative mode, exhibited a series of main peaks with an alternatively spaced m/z ratio of 80 and 82 Da, which may be clustered in different families (Table II). The first family of pseudo-molecular ions with m/z increments of 162 from 745 to 3499 Da corresponded to molecules containing n mannoses + 1 inositol + 1 phosphate group, with n varying from 3 to 19, whereas the second series with the same m/z increments of 162 Da from 663 to 3417 Da corresponded to molecules containing n mannoses + 1 inositol + 2 phosphate groups, n also varying from 3 to 19. A third series of minor peaks close to the second one (Δm = +14) was also observed, but at the moment their masses did not fit to any reasonable molar composition. This analysis revealed the distribution and high degrees of polymerization of β-Man residues from PLM. These DP mainly comprised between 8 and 18 sugar units because phosphoinositolmannosides with a mass lower than 1200 Da seemed to be overestimated by this method according to the TLC analysis of the chloroform/methanol/water extracts (Figs. 2 and 3) and NMR results. It also evidenced the absence of glucosamine. The absence of this residue is consistent with the PLM resistance to nitrous acid treatment (29) and its unlabeling with [3H]glucosamine (unpublished data). Glucosamine linking inositol to the sugar moiety is a common feature of GPI and GPI-related glycolipids (39) of eukaryotic cells. Its absence has only been reported to date in lipoarabinomannan, a GPI-like structure from prokaryotes of the genus Mycobacteria. Another peculiarity of PLM lies in the exclusive presence of β-1,2-linked mannose residues in its sugar moiety, which were found to be organized in linear chains with degrees of polymerization ranging from 8 to 18.

Confirmation of the probable presence of a Man-1-phosphate linkage in the molecule, as deduced from NMR spectrum, will require further studies.

The average mass of PLM may be estimated, from the present study, to be about 4 kDa. This mass is different from the former description of the PLM as corresponding to a C. albicans 14–18-kDa antigen in Western blotting (16). By using more reticulated gels (7–20% acrylamide) and migration conditions favoring the progressive blockage of the molecules in the gel rather than their migration speed, we observed that the PLM relative molecular mass upon SDS-PAGE decreased to 7 kDa (data not shown).

In Fig. 7 we suggest a structural model for the PLM glycan moiety, based on the first chemical evidence for the presence of β-1,2-linked oligomannosides in a glycoconjugate other than the yeast phosphopentidomannan. Very little is known about β-1,2-mannosyltransferases of C. albicans, their activation and substrate specificity, but the presence of such linear chains of up to 18 mannose residues represent quite unusual structures (24). It has been suggested that a consequence of coating parasite surfaces with long sugar chains is the triggering of host effector mechanisms at a distance too great for efficient antimicrobial activity on the parasite. The recent demonstration for the presence of PLM at the C. albicans cell wall surface (19) suggests that these mechanisms may play a role during host-C. albicans interaction. Moreover, β-1,2-oligomannosides have been shown to act as C. albicans adhesins for the macrophage membrane (15) and to stimulate macrophages to produce high levels of TNF-α (13, 18). The stimulating activity of β-1,2-
oligomannosides was found to depend on the length of the mannose chain and maximum activity was observed for DP's of 8 or higher (13). Interestingly, these high DP's are present mainly in the C. albicans PLM but correspond to minor components among the β-1,2-oligomannosides released from the mannans of the same species (24).

In conclusion, we have shown that the pathogenic yeast C. albicans synthesizes inositol-labeled glycolipids that have glycan moieties devoid of glucosamine. These C. albicans glycolipids are thus structurally more similar to lipooarabinomannans of Mycobacteria than to the glycosylinositolphospholipids of parasitic protozoa or the lipophosphoglycan of Leishmania. Recently, as well as being B cell antigens, mannose sequences of lipooarabinomannan have been implicated in the presentation to T cells by CD1b nonclassical major histocompatibility complex molecules (40). Whether or not this property is shared by C. albicans PLM remains to be investigated. An important PLM structural peculiarity lies in the presence of long chains of β-1,2-linked mannose residues. There is now considerable experimental evidence that these sugar residues are involved in virulence and immunomodulation and can elicit protective antibody responses. Therefore, PLMs are molecules that must be considered for a comprehensive analysis of host-C. albicans relationships. A complete elucidation of their structure and biosynthetic pathways will be necessary to provide a structural basis for understanding their immunomodulatory properties and some aspects of the pathogenesis of C. albicans infections.

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