

Structures of the glycosylphosphatidylinositol membrane anchors from *Aspergillus fumigatus* membrane proteins

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Glycosylphosphatidylinositol (GPI)-anchored proteins have been identified in all eukaryotes. In fungi, structural and biosynthetic studies of GPIs have been restricted to the yeast *Saccharomyces cerevisiae*. In this article, four GPI-anchored proteins were purified from a membrane preparation of the human filamentous fungal pathogen *Aspergillus fumigatus*. Using new methodology applied to western blot protein bands, the GPI structures were characterized by ES-MS, fluorescence labeling, HPLC, and specific enzymatic digestions. The phosphatidylinositol moiety of the *A. fumigatus* GPI membrane anchors was shown to be an inositol-phosphoceramide containing mainly phytosphingosine and monohydroxylated C_{24:0} fatty acid. In contrast to yeast, only ceramide was found in the GPI anchor structures of *A. fumigatus*, even for Gellp, a homolog of Gas1p in *S. cerevisiae* that contains diacylglycerol. The *A. fumigatus* GPI glycan moiety is mainly a linear pentomannose structure linked to a glucosamine residue: Man α 1-3Man α 1-2Man α 1-2Man α 1-6Man α 1-4GlcN.

Key words: *Aspergillus*/glycoprotein/glycosylphosphatidylinositol

Introduction

Numerous membrane glycoproteins of eukaryotic cells are anchored in the lipid bilayer by a glycosylphosphatidylinositol (GPI) membrane anchor. GPI-anchored proteins include receptors, enzymes, and protective cell surface coats. In fungi, GPI-anchored proteins are also involved in cell wall organization and biosynthesis. Indeed, gene disruption of numerous putative GPI-anchored proteins induces reduced growth, an altered cell wall, and a hypersensitivity to dyes interfering with cell wall polysaccharides (e.g., calcofluor white and congo red) (Rodriguez-Pena *et al.*, 2000). Sequence analysis and gene fusion strategies

with a reporter gene have suggested that more than half of the putative GPI proteins of *Saccharomyces cerevisiae* are covalently linked to the cell wall and are released by β -glucanase treatment (Caro *et al.*, 1997; Hamada *et al.*, 1998). Chemical evidence, at least for α -agglutinin and Tip1, showed these proteins are covalently associated to the β 1-6/1-3glucan complex, the main yeast cell wall component, through the glycan moiety of the GPI (Kollar *et al.*, 1997; Kapteyn *et al.*, 1996; Fujii *et al.*, 1999). In *Candida albicans*, the cell wall proteins present a similar organization (Kapteyn *et al.*, 2000). Other GPI proteins associated with the plasma membrane possess enzymatic activities, such as β -glucanase and β -glucanosyltransferase, able to modify cell wall polymers (Boone *et al.*, 1990, 1991; Cid *et al.*, 1995; Popolo and Vai, 1999). Genetic and biochemical analysis showed that the new family of GPI-anchored β 1-3glucanosyl-transferase, encoded by *GEL* in *Aspergillus fumigatus* and by the orthologous genes Gas1p in *S. cerevisiae* and Phr1p and Phr2p in *C. albicans*, is involved in cell morphology (Hartland *et al.*, 1996; Mouyna *et al.*, 2000a,b). Recently, a proteomic approach to identify GPI-anchored proteins of *A. fumigatus* lead to the identification of gene orthologs (CRH1, CRH2, ECM33) of genes known to play a role in yeast cell wall maintenance (Bruneau *et al.*, 2001).

All protein-linked GPI anchors have a conserved core structure of ethanolamine-*P*-Man₃GlcN-PI, and GPI biosynthetic pathways are also largely conserved between organisms. In fungi, structural and biosynthetic studies of GPI are mostly restricted to the model yeast *S. cerevisiae*. These GPI molecules have a glycan with mainly four mannose residues, Man α 1-2Man α 1-2Man α 1-6Man α 1-4GlcN α 1-6myo-inositol-1-PO₄, although some contain an additional α 1-2- or α 1-3-linked reducing terminal mannose residue (Frankhauser *et al.*, 1993). The lipid part differs from mammalian cells with the presence of ceramide or hydroxy-fatty acid-containing diacylglycerol (Conzelmann *et al.*, 1992; Frankhauser *et al.*, 1993).

To analyze the structure of the mature GPI anchors of a filamentous fungi, we purified several GPI proteins of *A. fumigatus*, a human pathogen, and analyzed the lipid and glycan moieties of their anchors.

Results

Purification of GPI-anchored proteins from A. fumigatus membrane

To analyze the GPI anchor structure, membrane proteins were solubilized in 50 mM Tris-HCl, 2 mM ethylenediamine tetra-acetic acid (EDTA), pH 8.8 in presence of protease inhibitors, 2% Triton X100, and 5 mM HgCl₂ to

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inhibit the endogenous GPI-phospholipase C (PLC). Two chromatographic steps were used to partially purify GPI-anchored proteins. All GPI-anchored proteins bound to the Q-Sepharose column at pH 8.8 and were eluted with 140 mM NaCl. This step allowed intact GPI-anchored proteins to be separated from phosphatidylinositol (PI)-PLC-cleaved GPI-anchored proteins, which were eluted with 25 mM NaCl. The Etienne Merck Darmstadt-DiEthyl Amino-Ethyl (EMD-DEAE) step allowed the purification of five GPI proteins (Figure 1). At pH 7.5, two GPI proteins were eluted with 50 mM NaCl (fraction F1: p56 and p60), one was eluted with 90 mM NaCl (fraction F2: p110), and two more were eluted with 190 mM NaCl (fraction F3: p70 and p80).

Identification of GPI proteins

Immunodetection with anti-Gellp antibody and tryptic mass fingerprint data identified p56 as Gellp. Edman sequencing of a purified tryptic peptide from p60 provided the sequence SFSAPXLTETTGDVF, which matches with ECM33p of *A. fumigatus* (Chabanne, personal communication). The p70 and p80 proteins gave similar tryptic mass fingerprints, and Edman sequencing of a purified tryptic peptide from both proteins provided the sequence TGYV-NYD. This sequence matched with PhoAp, an acid phosphatase. In addition, p70 and p80 reacted positively with a specific antibody against PhoAp, indicating that p70 and p80 correspond to two forms of the same protein (Bernard *et al.*, 2002). Edman sequencing of a purified tryptic peptide of p110 provided the sequence QDFDAAGYI that was already obtained from a GPI-anchored protein of 114 kDa of *A. fumigatus* that shares homology with fungal phospholipases (Bruneau *et al.*, 2001).

Analysis of PIs

PI moieties from partially purified GPI proteins were isolated from western blots on polyvinylidene difluoride (PVDF) membrane after nitrous acid deamination and butanol extractions. After purification on a silica column, PIs were characterized by negative ion electrospray mass spectrometry (ES-MS) and electrospray tandem mass spectrometry (ES-MS-MS) (Figure 2). The same data were

obtained from all GPI-anchored proteins. The ES-MS spectra revealed one principal $[M-H]^-$ pseudomolecular ion at m/z 924 and three minor ions at m/z 910, 938, and 952. The fragmentation of each PI compound produced only two daughter ions at m/z 241 [inositol-1,2-cyclic phosphate] $^-$ and m/z 259 [inositol-monophosphate] $^-$. The presence of these daughter ions and the absence of fatty acid carboxylate ions and/or 1-O-alkylglycerol-2,3-cyclic phosphate ions is characteristic of inositol-phosphoceramide (IPCs) (Treumann *et al.*, 1998). From the measured mass of the main component (925 Da), we can deduce the molecular weight of the ceramide component (683 Da). By analogy with typical ceramide structures in yeasts and fungi (Frankhauser *et al.*, 1993; Lester and Dickson, 1993), this suggests that the ceramide is made of a C18-phytosphingosine and a 2-monohydroxylated C_{24:0} fatty acid. The minor components that differ by 14 Da or 28 Da presumably reflect variability of the size of one or other of the aliphatic chains.

Analysis of the GPI anchor glycans

The amino-bonded phase high-performance liquid chromatography (HPLC) analyses of the 2-aminobenzamide (2-AB)-labeled GPI glycan moieties isolated from all five GPI-anchored proteins are shown in Figure 3. The proteins p56, p60, p70, and p80 all produced similar HPLC profiles with a single major peak, whereas p110 produced two peaks.

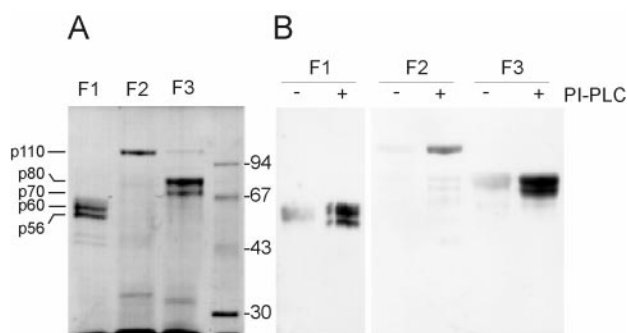


Fig. 1. SDS-PAGE and immunoblot of GPI-anchored proteins from *A. fumigatus* isolated after Q-Sepharose and EMD-DEAE chromatographic purification steps. (A) Coomassie blue-stained 10% SDS-PAGE; (B) immunoblot using the anti-CRD epitope detection with (+) or without (-) PI-PLC treatment of isolated fractions.

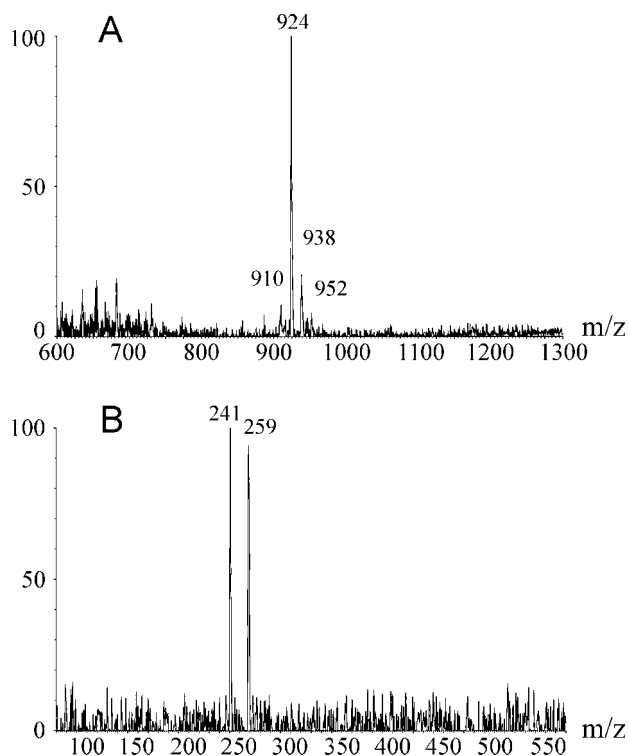


Fig. 2. ES-MS analysis of PI released by nitrous deamination from p80 on PVDF membrane. (A) Negative ion ES-MS spectrum of PI; (B) daughter ion ES-MS-MS spectrum of the ion at m/z 924.

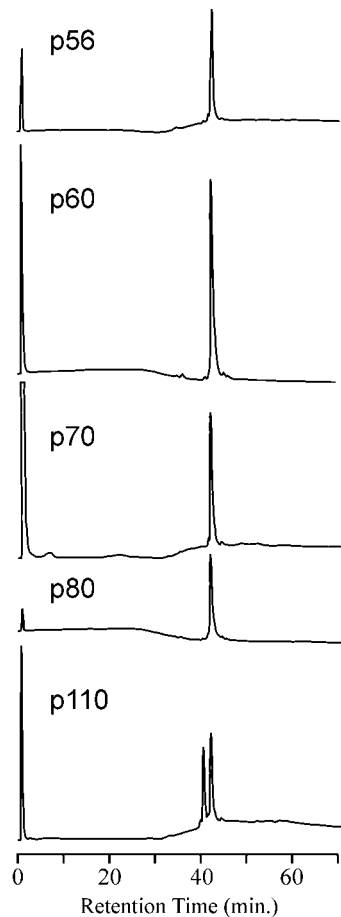


Fig. 3. HPLC chromatography on amino-bonded phase column of 2-AB-labeled GPI anchor glycans prepared from p56, p60, p70, p80, and p110 proteins. 2-AB compounds were detected with a fluorometer.

The peak fraction from the chromatogram of the p80 2-AB-labeled GPI glycan was analyzed by MS. MALDI-TOF analysis revealed a major ion at m/z 1115.3, corresponding to $[\text{Hex}_5\text{-AHM-2-AB} + \text{Na}]^+$ (data not shown), whereas ES-MS revealed a major ion at m/z 1093.4, corresponding to $[\text{Hex}_5\text{-AHM-2-AB} + \text{H}]^+$ (Figure 4A). The daughter ion ES-MS-MS spectrum of m/z 1093.4 is consistent with this assignment (Figure 4B). However, the daughter ion spectrum of native material cannot readily distinguish linear from branched glycan species; therefore, the sample was analyzed further following permethylation (Dell *et al.*, 1993). The daughter ion spectrum of the $[\text{permethyl-Hex}_5\text{-AHM-2-AB} + \text{Na}]^+$ ion at m/z 1409.6 is shown in Figure 4C. In this case, the absence of daughter ions 14, 28, or 32 Da smaller than those at m/z 987.5, 783.4, and 579.3 implies a purely linear glycan structure.

The primary structures of the 2-AB-labeled GPI glycans were further characterized by HPLC before and after enzymatic digestions with exo- α -mannosidases (Figures 5 and 6). All structures were degraded with jackbean α -mannosidase to produce anhydromannose (AHM)-2-AB, indicating the exclusive presence of α -mannopyranose residues. Specific α 1-2 and α 1-6 mannosidases did not degrade the structure from p80 (Figure 5), but the α 1-2,3 mannosidase partly degraded it to form species cochromatographing with

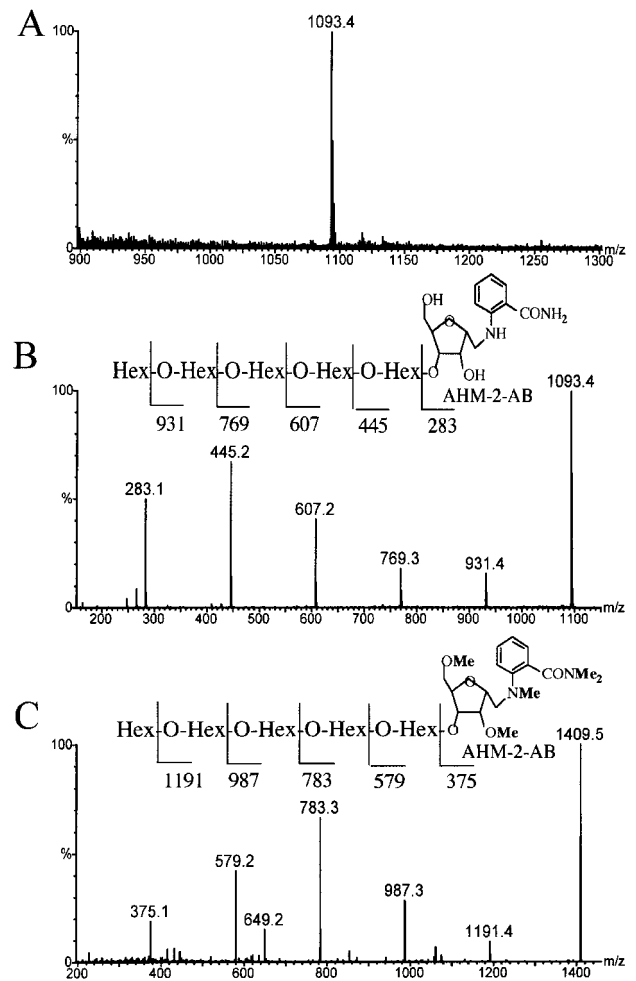


Fig. 4. ES-MS analysis of the 2-AB-GPI anchor glycan prepared from p80 protein. (A) Positive ion ES-MS spectrum of the native material; (B) daughter ion ES-MS-MS spectrum of the native material; (C) daughter ion ES-MS-MS spectrum of the permethylated material.

authentic $\text{Man}_4\text{-AHM-2-AB}$ ($\text{Man}\alpha 1\text{-2Man}\alpha 1\text{-2Man}\alpha 1\text{-6Man}\alpha 1\text{-4AHM-2-AB}$) and $\text{Man}_2\text{-AHM-2-AB}$ ($\text{Man}\alpha 1\text{-6Man}\alpha 1\text{-4AHM-2-AB}$) standards. Similar results were obtained from 2-AB-labeled GPI glycans isolated from p56, p60, and p70 (data not shown). The two 2-AB-labeled GPI glycans ($\text{Man}_4\text{-AHM-2-AB}$ and $\text{Man}_5\text{-AHM-2-AB}$) from p110 were resistant to the α 1-6 mannosidase, but the α 1-2 mannosidase degraded the $\text{Man}_4\text{-AHM-2-AB}$ structure to yield $\text{Man}_2\text{-AHM-2-AB}$. The α 1-2,3 mannosidase activity degraded both structures slowly to form $\text{Man}_2\text{-AHM-2-AB}$. Taken together with the MS data, these results are consistent with 2-AB-labeled glycan structures of $\text{Man}\alpha 1\text{-3Man}\alpha 1\text{-2Man}\alpha 1\text{-2Man}\alpha 1\text{-6Man}\alpha 1\text{-4AHM-2-AB}$ and $\text{Man}\alpha 1\text{-2Man}\alpha 1\text{-2Man}\alpha 1\text{-6Man}\alpha 1\text{-4AHM-2-AB}$. These glycan structures are combined with the PI structure already described to yield suggested structures for the *A. fumigatus* GPI anchors (Figure 7).

Discussion

In yeast, many GPI-anchor proteins are known to be involved in cell wall structural organization and biosynthesis

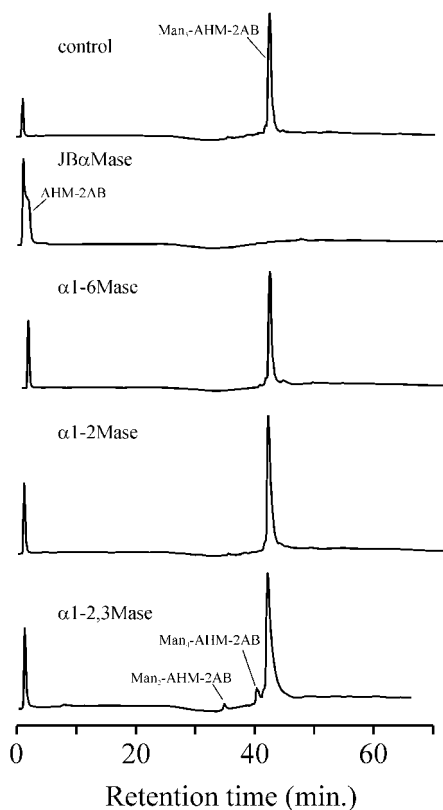


Fig. 5. HPLC chromatography on amino-bonded phase column of 2-AB-labeled GPI anchor glycans prepared from p80 protein, after enzymatic digestions with exo- α -mannosidase. JB α Mase: jackbean α -mannosidase; α 1-6Mase, α 1-6mannosidase; α 1-2Mase: α 1-2mannosidase, α 1-2,3Mase: α 1-2, α 1-3mannosidase.

(Kapteyn *et al.*, 1999, 2000). In filamentous fungi, cell wall organization is less extensively studied (Fontaine *et al.*, 2000). Recent studies on the human pathogenic fungus *A. fumigatus* suggest that at least nine GPI-anchored proteins are common to filamentous fungi and yeast (Bruneau *et al.*, 2001). However, in contrast to *S. cerevisiae*, nothing is known about structure and biosynthesis of GPI-anchor in filamentous fungi. GPI proteins can be selectively hydrolyzed by PI-PLC or GPI-PLC and detected with an anti-cross-reacting determinant (CRD) antibody. We took advantage of this selective detection to isolate five GPI-anchored proteins from *A. fumigatus* membrane preparations. Mass fingerprinting and/or Edman sequencing of these five *A. fumigatus* proteins revealed that they are Gellp (p56), Ecm33p (p60), two species of PhoAp (an acid phosphatase) (p70 and p80), and a phospholipase (p110) that have been previously identified in our proteomic study (Bruneau *et al.*, 2001).

Gellp is a member of a new family of GPI-anchored β 1-3 glucanosyltransferases involved in cell wall β -glucan modeling (Mouyna *et al.*, 2000a,b). Ecm33p is a homolog of a putative yeast GPI-anchored protein thought to be involved in cell wall biosynthesis because Δ ECM33 mutants show hypersensitivity to zymolyase (endo β 1-3glucanase), hygromycin B, and K1 killer toxin and have an altered glucose/mannose ratio (monosaccharides mainly found in the cell wall) (Caro *et al.*, 1997).

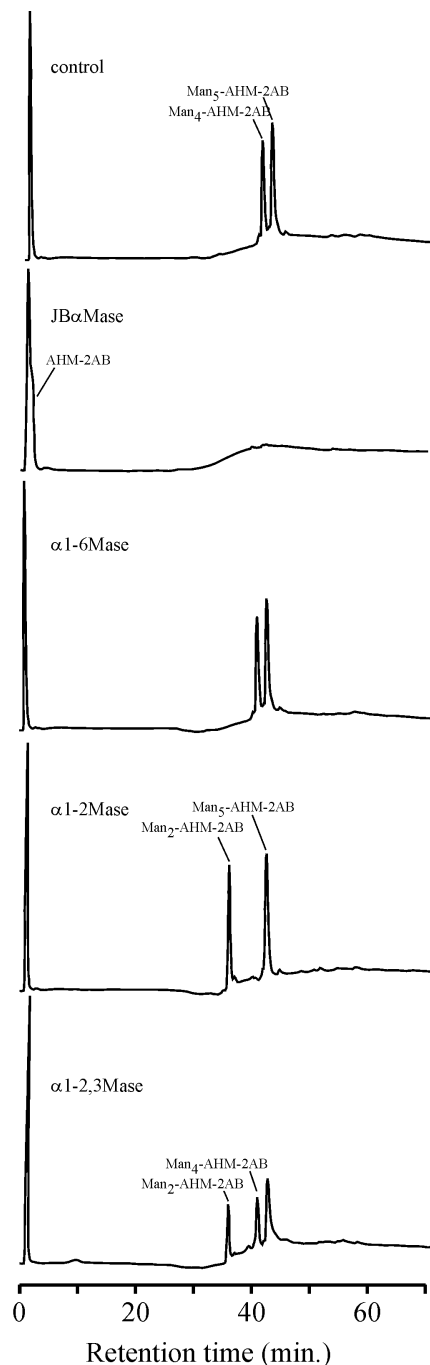


Fig. 6. HPLC chromatography on amino-bonded phase column of 2-AB-labeled GPI anchor glycans prepared from p110 protein, after enzymatic digestions with exo- α -mannosidase. JB α Mase: jackbean α -mannosidase; α 1-6Mase: α 1-6mannosidase; α 1-2Mase: α 1-2mannosidase, α 1-2,3Mase: α 1-2, α 1-3mannosidase.

PhoAp, an acid phosphatase purified as two protein bands on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) at 70 and 80 kDa, was identified by sequence homology to PhoAp from *Penicillium chrysogenum* and *Aspergillus niger* (Haas *et al.*, 1992; Ehrlich *et al.*, 1994; Bernard *et al.*, 2002). This protein, with 46% sequence identity to the *A. niger* homolog, is the major GPI-anchored

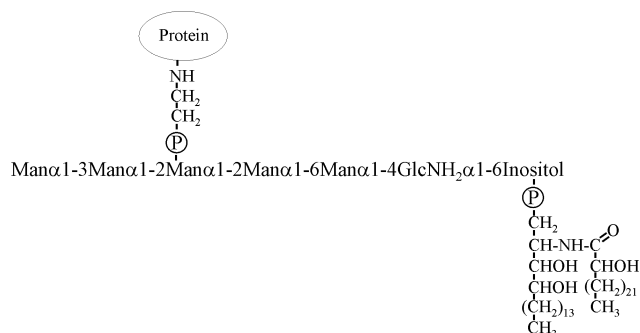


Fig. 7. Deduced GPI anchor structure isolated from GPI-anchored proteins of *A. fumigatus*. The position of phosphoethanolamine linked to the protein is suggested by studies in other eukaryotes (McConville and Ferguson, 1993; Ferguson *et al.*, 1999).

protein in the *A. fumigatus* membrane preparation. To date, PhoAp is the only protein in *A. fumigatus* found to be associated with the cell wall β 1-3 glucan as it is released with an endo- β 1-3 glucanase digestion. In *Kluyveromyces marxianus* cell wall β 1-3 glucanase digestion also released an acid phosphatase (Yoda *et al.*, 2000). Covalent linkage of GPI-anchored proteins to cell wall polymer has been mainly described in yeast, where β 1-6 glucan constitutes a bridge between protein and β 1-3 glucan, the main cell wall polysaccharide (Kapteyn *et al.*, 1996, 1999; Kollar *et al.*, 1997). However, no chemical proof of the covalent cross-linking for GPI-anchored PhoAp to cell wall β 1-3 glucan has been established to date.

Based on our chemical and enzymatic analysis, we can suggest that the main GPI anchor structure in *A. fumigatus* is as shown in Figure 7. For all four proteins analyzed, the lipid part is exclusively IPC, most likely containing a C18-phytosphingosine long-chain base amide-linked to a monohydroxylated C_{24:0} fatty acid, as judged by its mass and by analogy with the *S. cerevisiae* structure. Inositol-phosphoceramides are common in yeast GPI structures and have also been described in *Dictyostellium discoideum* (Stadler *et al.*, 1989; Haynes *et al.*, 1993), plants (Oxley and Bacic, 1999), and trypanosomatid parasites (McConville and Ferguson, 1993; Serrano *et al.*, 1995; Ferguson *et al.*, 1999). To date, IPCs have not been found in mammalian GPI structures. In fungi, IPCs are also found as free phospholipids and as mannosylated IPC containing 2-mono-hydroxylated long chain fatty acids (Lester and Dickson, 1993). However, their biosynthesis seems to be independent from that of GPI anchors (Reggiori and Conzelmann, 1998). The major glycan component of the *A. fumigatus* GPI anchors is Man α 1-3Man α 1-2Man α 1-2Man α 1-6Man α 1-4GlcN (Man₅GlcN), which conforms to the minimal Man α 1-2Man α 1-6Man α 1-4GlcN (Man₃GlcN) core GPI glycan structure common to all protein-linked (type 1) GPI structures (McConville and Ferguson, 1993) and is identical to one of the two Man₅GlcN glycoforms found in a minority of GPI structures in *S. cerevisiae* (Frankhauser *et al.*, 1993).

Although on-blot GPI glycan labeling and off-blot release methods have been described previously (Zitzmann and Ferguson, 1999; Zitzmann *et al.*, 2000), as have GPI PI-fraction analyzed by ES-MS and ES-MS-MS (Serrano *et al.*,

1995; Rudd *et al.*, 1997; Treumann *et al.*, 1998; Almeida *et al.*, 2000), this is the first report to describe a novel off-blot method for GPI PI-fraction isolation prior to ES-MS and ES-MS-MS analysis. Thus, the entire GPI structure analysis described in this article was performed on protein bands transferred to PVDF membrane. The approach was effective and sensitive. However, the aqueous Hydrofluoric acid (HF) dephosphorylation step, necessary to release the 2-AB-labeled GPI glycan from the PVDF membrane-bound protein component, results in the loss of all ethanolamine phosphate substituents. Thus, the ethanolamine phosphate bridge to the protein C-terminus shown in Figure 7 is drawn by analogy with other known GPI anchor structures (Ferguson *et al.*, 1999). Whether or not the *A. fumigatus* GPI anchor has additional ethanolamine phosphate substituents on the first and second mannose residues, which are common in higher-eukaryote GPI anchors, is unknown. The absence of additional ethanolamine phosphate groups in mature GPI anchors of *S. cerevisiae* (Frankhauser *et al.*, 1993) has been questioned recently, and it appears that at least some mature yeast GPI anchors retain the additional ethanolamine phosphate groups that are added during GPI biosynthesis (Canivenc-Gansel *et al.*, 1998; Flury *et al.*, 2000).

We considered the possibility that the *A. fumigatus* GPI anchors might contain galactofuranose substituents because this sugar occurs linked to mannan chains with similarity to the GPI structure in *A. fumigatus* galactomannans (Latgé *et al.*, 1994) and because it has been described in the N- and O-linked oligosaccharides of *Aspergillus* glycoproteins (Buitrago *et al.*, unpublished data; Takayanagi *et al.*, 1994; Haido *et al.*, 1998; Wallis *et al.*, 2001). The acid-labile galactofuranose residues are known to be labile to the full 48% aqueous HF dephosphorylation conditions used in this study (Turco *et al.*, 1989). Therefore, a mild aqueous HF treatment (using 25% aqueous HF for 24 h, data not shown) that retains a substantial proportion of galactofuranose residues was also investigated. However, the amino-bonded phase HPLC profiles of 2-AB-labeled GPI glycans prepared in this way were identical to those shown in Figure 3, suggesting that galactofuranose is not a component of *A. fumigatus* GPI anchors. This is also in agreement with the lack of immunoreactivity of the five GPI-anchored proteins with a galactofuranose-specific mono-clonal antibodies (data not shown) (Stylen *et al.*, 1992).

The data provided here on the mature GPI anchor structures of *A. fumigatus* provide a framework for probing the pathway of GPI biosynthesis in *A. fumigatus* that is presently unknown. From a structural point of view, it seems likely that the *A. fumigatus* GPI pathway will be similar to that of *S. cerevisiae* in that the GPI glycans contain a minimum of four α Man residues and the principal lipid is a ceramide instead of a glyceride.

Materials and methods

Fungal culture and membrane preparation

A. fumigatus, strain CBS 144-89, was grown in a 15-L fermenter in 2% glucose, 1% mycopeptone (Biokar

Diagnostics, Pantin, France) at 25°C for 24 h as described previously (Hartland *et al.*, 1996). The mycelium was collected by filtration under vacuum, washed with water, and then disrupted in 200 mM Tris-HCl, 20 mM EDTA, pH 8.0, 1 mM phenylmethylsulfonyl fluoride buffer with 5 mg/ml bovine serum albumin (BSA) at 4°C by passing through a Dyno mill homogenizer (W. A. Bachofen AG, Basel, Switzerland) in presence of glass beads (0.5–0.75 mm diameter). Cell wall was removed by centrifugation at 10,000 × *g* for 10 min at 4°C. Total membranes were then collected by ultracentrifugation at 125,000 × *g* for 60 min at 4°C. The membrane pellet was resuspended in the disruption buffer without BSA with a Dounce homogenizer and then recentrifuged at 125,000 × *g* for 60 min at 4°C. The pellet was resuspended again in 20 mM Tris-HCl, 2 mM EDTA, pH 8.0, and stored at –80°C.

Protein solubilization and purification

Membrane preparations (30 ml at 20 mg protein/ml) were centrifuged at 50,000 × *g* for 40 min at 4°C. The membrane pellet was first resuspended with a Dounce homogenizer in 40 ml of 50 mM Tris-HCl, 2 mM EDTA, 20% glycerol, pH 8.8, with 5 mM HgCl₂, two anti-protease pastilles (Complete anti-protease cocktail, Roche, Meylan, France) for 10 min on ice. The mixture was made 2% (v/v) with respect to Triton X100 and mixed in the homogenizer to solubilize membrane proteins for 10 min. Insoluble material was removed by centrifugation (50,000 × *g* for 40 min at 4°C). Soluble material was applied to a Q-Sepharose Fast Flow column (20 × 1.6 cm, Pharmacia, Orsay, France) equilibrated in 50 mM Tris-HCl, 2 mM EDTA, 20% glycerol, pH 8.8, with 0.5 mM HgCl₂, 0.05% Triton X100 at a flow rate of 20 ml/h. After washing the unbound compounds, proteins were eluted with a NaCl gradient (0–600 mM, 300 ml) in the starting buffer. Proteins were detected with the BCA protein assay kit (Pierce, PerbioSciences, Bezons, France). Fractions from Q-Sepharose chromatography containing GPI proteins were pooled and dialyzed against 20 mM Tris-HCl, 1 mM EDTA, pH 7.5, with 0.5 mM HgCl₂, 0.05% Triton X100. Proteins were then applied onto an EMD-DEAE-650(S) column (Fractogel-Superformance 150 × 10 mm, Merck), equilibrated with the same buffer, at 1 ml/min. Bound proteins were eluted with a linear NaCl gradient (0–400 mM over 60 min).

SDS-PAGE and western blot

Protein samples were analyzed by SDS-PAGE (Laemmli, 1970) using a 10% separating acrylamide gels. Protein bands were visualized by staining with Coomassie blue. GelIp and PhoAp (acid phosphatase) were detected on western blot using specific mouse or rabbit antibodies (Mouyna *et al.*, 2000a; Bernard *et al.*, 2002). GPI proteins were detected with an antiserum directed against soluble form of variant surface glycoproteins of *Trypanosoma brucei* that recognize the CRD epitope formed by the action of (G)PI-PLC on GPI proteins (Zamze *et al.*, 1988). Protein samples (40 µl) were incubated with 10 µl 200 mM imidazole/acetic acid, pH 7.4, 5 mM dithiothreitol, and with or without 0.2% (v/v) PI-PLC from *Bacillus thuringiensis* (200–300 U/ml, Glyko)

for 2 h at 30°C. GPI proteins were subsequently visualized by western blot with the anti-CRD serum.

Isolation of PIs from GPI proteins

Protein samples (1–2 nmol) were separated by large-scale SDS-PAGE, and proteins were transferred onto PVDF membrane and visualized with Ponceau Red staining. GPI proteins were cut out with a razor blade. PVDF membrane strips were washed in Eppendorf tubes with methanol and water. The samples were deaminated by submerging the blot strips in 200 µl of 300 mM sodium acetate, pH 4.0, and an equal volume of freshly prepared 1 M NaNO₂ for 2–3 h at 37°C. The strips were washed three times with water, then PIs were extracted three times with 0.5 ml water-saturated butan-1-ol for 20 min with vortex agitation and for 10 min in an ultrasonic bath. The butanol phases were concentrated under vacuum, and the PIs were repartitioned between 200 µl each butan-1-ol and water. The butanol phase was re-extracted twice with 200 µl butanol-saturated water and dried. The PI extracts were purified by adsorption chromatography on a 100–200 µl silica column (Kieselgel 60, 70–230 mesh, Merck, Fontenay-sous-bois, France): PI extracts were dissolved in chloroform:methanol (4:1, v/v) and applied to the small silica column equilibrated in the same solvent. After extensive washing, the PIs were eluted with 10 column volumes of chloroform:methanol (1:4 v/v). The eluate was dried and redissolved in chloroform:methanol (2:3 v/v) immediately prior to ES-MS analysis.

Isolation of the GPI glycan moiety

Protein samples (around 0.5 nmol) were separated by SDS-PAGE, transferred onto a PVDF membrane, and visualized with Ponceau Red. After cutting out the protein bands of interest, the labeling and release of GPI glycans was performed as previously described (Zitzmann and Ferguson, 1999; Zitzmann *et al.*, 2000). Briefly, samples were deaminated and the free reducing end 2,5-AHM residue thus formed was labeled by reductive amination with 2-AB (Signal Labeling Kit, Glyko, Bicester, UK). The 2-AB-labeled glycans were released from the protein (and thus from the PVDF membrane) by dephosphorylation with ice-cold 48% aqueous HF treatment for 3 days. The aqueous HF reagent was removed by freeze-drying (three times).

HPLC of 2-AB-labeled GPI glycans

2-AB-labeled GPI glycans were analyzed on an amino-bonded phase column (3 cm × 2.1 mm, Kromasil, Hitchrom, Theale, UK) and detected with a Gilson (model 121) fluorescence detector. Oligosaccharides were separated using the following conditions: flow 0.2 ml/min; buffer A, 95% acetonitrile; buffer B, 15 mM ammonium acetate in 5% acetonitrile. Gradient: 0–5 min, isocratic (100% buffer A); 5–25 min, linear gradient (100% A, 0% B to 70% A, 30% B); 25–70 min, linear gradient (70% A, 30% B to 30% A, 70% B). 2-AB-labeled GPI glycans isolated from several *T. brucei* variant surface glycoproteins were used as standards.

Enzymatic digestion of 2-AB-labeled GPI glycans

Enzymatic digestions were done with exo- α -mannosidase activities. Samples were dried and resuspended in 10 μ l of 100 mM ammonium acetate buffer at the optimum pH of each respective enzyme: nonspecific α -mannosidase from jackbeans (0.5 mU, optimum pH 5.5, Calbiochem, Nottingham, UK), α 1-2-mannosidase from *Aspergillus saito* (2 μ U, optimum pH 5.0, Glyko), α 1-2,3-mannosidase from *Xanthomonas manihotis* (10 U, optimum pH 6.0 with 5 mM CaCl₂, New England Biolabs, Hitchin, UK), or α 1-6-mannosidase from *Xanthomonas manihotis* (10 U, optimum pH 5.0, New England Biolabs). Reactions (37°C, overnight) were stopped by addition of 60 μ l of ethanol, dried under vacuum, dissolved in 6 μ l of water, and analyzed by HPLC on the amino-bonded phase column, as already described.

Permethylation of 2-AB-labeled GPI glycans

Samples were dried, redissolved in 50 μ l dimethylsulfoxide, and treated with 50 μ l of a 120 mg/ml slurry of NaOH in dimethylsulfoxide (20 min). Two aliquots (10 μ l) of methyl iodide were added at 10-min intervals. The reaction was quenched by the addition of 15 μ l acetic acid, 0.25 ml chloroform, and 1 ml water. The lower chloroform phase was washed four times with 1 ml water, dried, and redissolved in 80% acetonitrile, 0.5 mM sodium acetate.

ES-MS

PI samples were introduced into the electrospray source of a Micromass Quatro triple-quadrupole mass spectrometer at 6 μ l/min in chloroform:methanol (2:3, v/v). Negative ion ES-MS spectra were recorded with a capillary voltage of 3.0 kV and a cone voltage of 50 V. Negative daughter ion ES-MS-MS spectra of [M-H]⁻ pseudomolecular ions of PIs were recorded with argon as the collision gas (2.5 \times 10⁻³ Torr) and 60 V collision energy.

Native 2-AB-labeled GPI glycans were introduced into the nano-electrospray source of a Micromass Q-ToF2 mass spectrometer via a nanotip in 50% acetonitrile, 1% formic acid. Positive ion ES-MS and ES-MS-MS spectra were recorded with a tip voltage of 950 V and cone voltage of 40 V. The collision energy for ES-MS-MS was 40 V.

Permethylated 2-AB-labeled GPI glycans were introduced into the nano-electrospray source of a Micromass Q-ToF2 mass spectrometer via a nanotip in 80% acetonitrile, 0.5 mM sodium acetate. Positive ion ES-MS and ES-MS-MS spectra were recorded with a tip voltage of 950 V and cone voltage of 40 V. The collision energy for ES-MS-MS was 80 V.

Peptide analysis

Coomassie blue-stained GPI protein bands, isolated by SDS-PAGE, were excised from the gel. In-gel digests were done with bovine trypsin. Samples were desalted, and peptide masses were analyzed by MALDI-TOF MS (Shevchenko *et al.*, 1996). In addition, internal peptides obtained by reverse-phase HPLC after tryptic digestion were sequenced on an Edman microsequencer (Applied Biosystem 470 gas-phase sequencer) following the

procedure described in Beauvais *et al.* (1997). Analyses were performed by J. D'Alayer (Laboratoire de micro-sequencage, Institut Pasteur, Paris). MALDI-TOF and Edman sequencing data were matched against the NCBI nr and the Genome Therapeutics Corporation Pathogenome databases using the MS-Fit and MS-Edman programs (assembled into the Protein Prospector package, Baker, and Clauser), respectively. These protein identifications were performed by J. M. Bruneau (Aventis-Hoechst Marion Roussel, Romainville, France).

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Abbreviations

2-AB, 2-aminobenzamide; AHM, anhydromannose; BSA, bovine serum albumin; CRD, cross-reacting determinant; EDTA, ethylenediamine tetra-acetic acid; ES-MS, electrospray mass spectrometry; ES-MS-MS, electrospray tandem mass spectrometry; GPI, glycosylphosphatidyl inositol; HPLC, high-performance liquid chromatography; IPC, inositol-phosphoceramide; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PI, phosphatidylinositol; PLC, phospholipase C; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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