Galactomannoproteins of *Aspergillus fumigatus*

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Galactomannennan (GM) is an essential molecule in the life of the human opportunistic fungal pathogen *Aspergillus fumigatus*. It is a polysaccharide that is an important structural component of the cell wall of *A. fumigatus* (10). GM is secreted during growth in culture (15) and has also been shown to exist in a glycosylphosphatidylinositol membrane-bound form (C. Costachel et al., unpublished data). The extracellular, membrane- or cell wall-linked GMs have a similar chemical organization composed of a linear α-mannan core with a repeating tetramannose unit (2M-6M-2M) with side chains of β-1,5-galactofuranose residues with an average degree of polymerization composed of a linear α-mannan core with a repeating tetramannose unit (2M-6M-2M) with side chains of β-1,5-galactofuranose residues with an average degree of polymerization of 4 attached to the α-1,2-linked mannose residues of the mannan chain (10, 15). GM is also produced in the host and is a well-known *A. fumigatus* antigen. Antibodies directed against this polysaccharide have been detected in patients with aspergilloma and in experimentally infected animals or in rabbits or mice hyperimmunized with total extracts of *A. fumigatus* (15, 26). In addition, it has been repeatedly shown that this molecule circulates in the biological fluids of patients with invasive aspergillosis. Indeed, serological diagnosis of this life-threatening fungal infection remains based on the detection of GM in the serum, urine, or bronchoalveolar lavage samples of infected patients (18, 29, 31) since heavily immunocompromised patients at risk for invasive aspergillosis are not able to mount an antibody response against *A. fumigatus*. MAbs or polyclonal antibodies directed against GM have been used in the search for circulating antigens. The only commercial kit available for detection of GM is a sandwich enzyme-linked immunosorbent assay (ELISA) based on the use of a rat monoclonal antibody (MAb), EB-A2, that reacts specifically with the galactofuranosyl (Galf)-containing moiety of the GM. In vitro, the best inhibition was obtained with a tetra-β-1,5-galactofuranosyloligosaccharide, suggesting that this oligosaccharide was the epitope recognized by the MAb (30).

Glycoproteins reacting positively with anti-galactofuranosyl MAb EB-A2 have been identified in culture filtrates or mycelial extracts by Western blotting experiments (11, 30). These results suggested that *A. fumigatus* can secrete both a polysaccharide GM and glycoproteins bearing a galactofuranosyl moiety. Recently, an α-galactosidase with a Galf-rich N-glycan was identified in *Aspergillus niger*. Analysis of the N-glycan moiety of the α-galactosidase showed that it was composed of a mixture of Hex$_x$HexNAc$_y$HexNAc$_z$ substituted with up to three β-Galf residues (36).

In this work, we have isolated and characterized two major galactomannoproteins (GMPs) secreted by *A. fumigatus* in a glucose-peptone-based medium. Biochemical and molecular analyses have shown that these GMPs were a phospholipase C (PLC) and a phytase. The chemical structure of the N-glycan has been characterized and shown to contain Galf in a terminal nonreducing position.

**MATERIALS AND METHODS**

**Strains and standard culture conditions.** A *fumigatus* strain CBS 144-89 was grown in Sabouraud’s liquid medium (2% glucose, 1% mycoprotein; Biokar, Beauvais, France). Cultures were performed in a 15-liter fermentor in Sabouraud’s liquid medium for 24 h at 25°C as previously described (15). *Paracoccidioides brasiliensis* strain B339 was grown in YPD at 37°C as previously described (2).

**Preparation of protein extracts and protein purification.** A culture filtrate was incubated for 24 h with 1 vol of 10% (w/v) sodium acetate buffer (pH 5.2) and then centrifuged (5,000 × g, 10 min). The supernatant (30 ml) was filtered through a 0.45-μm (Whatman) filter and dialyzed against 10 mM sodium acetate buffer (pH 5.2). Sample was loaded onto a Mono S HR 5/5 column (Pharmacia, Uppsala, Sweden) and eluted with a linear NaCl gradient (0 to 500 mM in 45
min) at a flow rate of 0.8 ml/min. Fractions were tested by Western blot assays with the Bio-Rad-anti GM EB-A2 MAb. Positive fractions were further purified by gel filtration chromatography on a Superdex 75 HR 10/30 column (Pharmacia) in the same acetate buffer supplemented with 120 mM NaCl at a flow rate of 0.4 ml/min. The last purification step was performed on the same Mono S HR 5/5 cation-exchange chromatography column equilibrated with acetate buffer (10 mM at pH 5.7). After dialysis against this buffer, positive anti-GM fractions obtained from the gel filtration column were loaded onto the column and eluted with a linear gradient of NaCl (100 to 250 mM in 30 min) at a flow rate of 0.5 ml/min. Purified proteins were kept at 4°C in the elution buffer.

To obtain the gp43 antigen, culture supernatants from a 5-day-old culture of *P. brasiliensis* were inactivated with 0.02% thimerosal for 2 h at 35°C and precipitated with 4 volumes of 100% ethanol. The precipitate was resuspended in 10 mM sodium acetate buffer (pH 5.0) and dialyzed against the same buffer.

**Protein analysis.** Protein samples were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (14) using 4% stacking and 10% separating gels. Proteins were stained with Coomassie blue or electrotansferred to nitrocellulose overnight at 30 V in 50 mM Tris HCl (pH 8.0) buffer containing 200 mM glycine and 20% ethanol. Blots were immunoblotted with anti-GM MAb EB-A2 (30). The MAb was used at a 1:10,000 dilution (0.27 μg immunoglobulin M [IgM] ml⁻¹). Immunoblots were air dried and the bands for the detection of GM in blood samples were visualized using a peroxidase anti-rat IgG (heavy and light chains) conjugate diluted 1:1,000 and the ECL detection method (Amersham). Internal peptide sequencing was performed by J. D’Alayer (Planta) in the same acetate buffer supplemented with 120 mM NaCl at a flow rate of 1 ml/min. Analysis of PLC and phytase activities.

**Phytase activity.** Phytase activity was determined as previously described (21) using phytic acid (Sigma) in the same acetate buffer supplemented with 120 mM NaCl at a flow rate of 1 ml/min. After addition of 500 μM of iodoacetate per mg protein (5 M excess over DTT), the sample was flushed again with argon and incubated at room temperature overnight in darkness. The sample was then dialyzed for 48 h against 50 mM ammonium hydrogen carbonate at 4°C and lyophilized. The reduced carbomethylated protein was digested with 1-tosylamide-2-phenylethylchloromethylketone bovine pancreas trypsin (EC 3.4.21.14; Sigma) with an enzyme-to-substrate ratio of 1:50 (by mass), and the mixture was incubated at 37°C for 2 h. After digestion of 500 μM of iodoacetate per mg protein (5 M excess over DTT), the sample was flushed again with argon and incubated at 37°C for 2 h. After addition of 500 μM of iodoacetate per mg protein (5 M excess over DTT), the sample was flushed again with argon and incubated at 37°C for 2 h. After addition of 500 μM of iodoacetate per mg protein (5 M excess over DTT), the sample was flushed again with argon and incubated at 37°C for 2 h. After addition of 500 μM of iodoacetate per mg protein (5 M excess over DTT), the sample was flushed again with argon and incubated at 37°C for 2 h. After addition of 500 μM of iodoacetate per mg protein (5 M excess over DTT), the sample was flushed again with argon and incubated at 37°C for 2 h. After addition of 500 μM of iodoacetate per mg protein (5 M excess over DTT), the sample was flushed again with argon and incubated at 37°C for 2 h. After addition of 500 μM of iodoacetate per mg protein (5 M excess over DTT), the sample was flushed again with argon and incubated at 37°C for 2 h. After addition of 500 μM of iodoacetate per mg protein (5 M excess over DTT), the sample was flushed again with argon and incubated at 37°C for 2 h.
jection at 120°C. The GC oven was held at 120°C for 1 min before increasing to 200°C at 2°C/min and then to 240°C at 15°C/min.

MALDI ionization-time of flight analysis of N-glycans. Reflectron spectra were performed on a VYORÆGER DE STR Pro instrument (Perseptive Biosystem, Framingham, MA). Desorption and ionization were obtained with a pulsed UV laser beam (nitrogen laser, \( \lambda = 337 \) nm). Irradiance was used slightly above the threshold of ion detection. Ion spectra resulted from positive ion mode analysis. Acceleration and reflector voltages were set up as follows: target voltage, 20 kV; first grid at 66% of target voltage; and delayed extraction at 200 ns. Spectra were obtained by accumulation of 100 shots with calibration according to the manufacturer’s recommendations. Sample were prepared by mixing directly on the target 1 \( \mu l \) of oligosaccharide solution (about 25 pmol) and 1 \( \mu l \) of 2.3-dihydroxybenzoic acid matrix solution (10 mg/ml dissolved in CH\(_3\)OH-H\(_2\)O [50:50, vol/vol]).

Antigenicity testing. Native or de-N-glycosylated GMPs were blotted onto nitrocellulose as described above. After blocking, blots were incubated with a pool of 17 sera from different aspergilloma patients and a pool of 7 control sera (kind gift of P. Recco, Toulouse, France). Human sera were diluted 1/1,000 and labeled with an anti-human peroxidase conjugate diluted 1/1,000. Peroxidase binding was verified with ECL chemiluminescence (Amersham) as described by the manufacturer. Quantification of the humoral response was done in an ELISA format. GMP1, GMP2, and GM (15) were coated onto ELISA plates (Greiner reference no. 762070) at a concentration of 2 \( \mu g/ml \) in 0.1 M carbonate buffer (pH 9.0). After coating, the wells were washed with phosphate-buffered saline (PBS)-0.1% Tween 20. Patient sera were diluted 1/500 in PBS containing 0.05% PBS–0.05% Tween 20 and 1% bovine serum albumin. The ELISA contained classically the following steps: incubation for 1 h at 37°C with 24 individual control and patient sera; five washings with PBS–0.05% Tween 20; incubation with a secondary anti-IgG (heavy and light chains) antibody conjugated to peroxidase (Sigma); and five washings and incubation with O-phenylenediamine dihydrochloride (OPD) for OD readings. Experiments were done in duplicate and repeated at least once. Statistical analysis of the data was done using the JMP software (SAS, Cary, NC). Variance analysis was used for mean comparisons and for bivariate analysis of the distribution of one continuous variable to another one.

RESULTS

Identification of GMPs. The two major proteins secreted in the culture filtrate (GMP1 and -2) that reacted with the anti-galactofuran antibody were purified. These proteins bound to the cation-exchange Mono S column. GMP1 was eluted at 140 to 180 mM NaCl at pH 6.2. The GMP1-containing fractions were collected, pooled, and further purified by gel filtration. On Superdex 75, GMP1 was found in fractions of 55 to 60 kDa. These fractions were pooled, dialyzed, and subjected to a second cation-exchange chromatography at pH 5.7. GMP1 was eluted at 150 to 160 mM NaCl. GMP2 was eluted initially from the cation-exchange Mono S column with a 320 to 340 mM NaCl solution at pH 6.2. A further purification step with the fractions containing GMP2 was realized on a Superdex 75 gel filtration column. GMP2 was recovered in the 55- to 65-kDa-containing fractions.

Figure 1 shows the apparent \( M_r \) of the native and N-deglycosylated GMPs. The molecular mass shift resulting from N-deglycosylation was between 10 and 15 kDa, indicating the presence of small N-glycans. N-deglycosylation was always associated with the loss of the reactivity with the anti-GM MAb.

Structural characterization of N-glycan of GMP1. (i) Native glycans released by PNGase F. GC analysis showed that the mixture of PNGase F-released oligosaccharides contained mannose, galactose, and N-acetylgalactosamine in a molar ratio of 6.4:2.3:2.

Data from MALD-MS of permethylated PNGase F-released glycans eluting in the 50% (vol/vol) aqueous acetonitrile fraction from a C\(_{18}\) Sep-Pak cartridge are shown in Fig. 2A. The data indicated that the glycoprotein contained glycans with a Hex\(_{3}\)HexNAc\(_2\) composition. The major molecular ion observed corresponded to Hex\(_3\)HexNAc\(_2\) (m/z 1,783). ELISAs showed that the mixture of native PNGase-released oligosaccharides reacted positively with the anti-GM EB-A2 MAb (lanes 3 and 4). Arrowheads indicate GMP1 (A) and GMP2 (B) before and after deglycosylation. The values on the left are molecular sizes in kilodaltons.

FIG. 1. Analysis of native (lanes 1 and 3) and deglycosylated (lanes 2 and 4) forms of GMP1 (A) and GMP2 (B) on a 10% SDS-PAGE gel stained with Cooomassie blue (lanes 1 and 2) or transferred onto nitrocellulose and revealed by Western blot assay with the EB-A2 anti-galactofuran MAb (lanes 3 and 4). Arrowheads indicate GMP1 (A) and GMP2 (B) before and after deglycosylation. The values on the left are molecular sizes in kilodaltons.
FIG. 2. MALD-MS spectra of permethylated N-glycans from *A. fumigatus* GMP1. The N-glycans were released from tryptic glycopeptides by digestion with PNGase F, separated from peptides by Sep-Pak purification, and permethylated. The derivatized glycans were purified by Sep-Pak, and the 50% (vol/vol) aqueous acetonitrile fraction was screened by MALD-MS. (A) Intact N-glycans. (B) N-glycans treated with HF. Major ions are indicated.

### TABLE 1. GC-MS analysis of partially methylated alditol acetates obtained from the PNGase F-released N-glycans of GMP1

| Retention time (min) | Characteristic fragment ions | Assignment       | Relative abundance | No treatment | After HF treatment |
|----------------------|------------------------------|------------------|--------------------|--------------|-------------------|
| 26.48                | 102, 118, 129, 145, 161, 162, 205 | Terminal mannose | 1.00               | 1.00         |                   |
| 26.82                | 89, 102, 118, 162, 205, 278     | Terminal galactose| 0.31               | ND           |                   |
| 30.99                | 129, 130, 161, 190             | 2-Linked mannose | 0.76               | 0.41         |                   |
| 32.03                | 99, 102, 118, 129, 162, 189, 233 | 6-Linked mannose | 0.17               | 0.14         |                   |
| 36.10                | 129, 130, 189, 190            | 2,6-Linked mannose | 0.28              | 0.23         |                   |
| 36.62                | 118, 129, 189, 234            | 3,6-Linked mannose | 0.25              | 0.24         |                   |
| 41.25                | 117, 159, 233                 | 4-Linked GlcNAc  | 0.48               | 0.52         |                   |

*The 50% acetonitrile fractions from Sep-Pak purifications of permethylated glycans were hydrolyzed, reduced, acetylated, and analyzed by GC-MS. ND, not detected.*
ture of Hex$_x$HexNAc$_z$ without Galf residues was also present in the original native mixture of N-glycans.

GC-MS data showed that after HF treatment, 2-linked Man was the only linked mannose residue which decreased significantly (Table 1), suggesting that terminal Galf residues were attached to the 2 position of Man prior to HF treatment. This result was in agreement with Western blot data showing the absence of reactivity of a culture filtrate of \textit{P. brasiliensis} with EB-A2 (data not shown). This extract contained the major glycoprotein gp43 that was characterized by the presence of β-1,6-Galf linked in β-1,6 to one of the mannosyl residues of the N-glycan mannan core (2).

When native glycan pools were subjected to α-mannosidase digestion prior to HF treatment, the reactivity of the resulting N-glycans digested with α-mannosidase against the anti-GM MAb was identical to the reactivity of the native N-glycans, indicating that this exomannosidase treatment did not remove any Galf residues (data not shown). When the HF-treated N-glycans were digested with α-mannosidase, they were trimmed to HexHexNAc$_2$ (m/z 763), Hex$_2$HexNAc$_2$ (m/z 967), and Hex$_3$HexNAc$_2$ (m/z 1,171).

The chemical and immunochemical data obtained above showed that GMP1 of \textit{A. fumigatus} contained a heterogeneous mixture of N-glycans (Hex$_{a_{11}}$HexNAc$_z$). The N-glycans reacting with the anti-GM MAb contained only one Galf residue at the terminal nonreducing position attached to the 2 position of the mannose residues.

**Structural characterization of N-glycan of GMP2.** N-glycans of GMP2 were released by PNGase treatment as described for GMP1. ELISAAs showed that the mixture of native PNGase-released oligosaccharides reacted positively with the anti-galactofuran EB-A2 MAb (data not shown). These experiments indicated the presence of Galf residues in the mixture of N-glycans. The MALD-MS data indicated that the glycoprotein contained glycans with a Hex$_{a_{11}}$HexNAc$_z$ composition. The major native molecular ions observed corresponded to Hex$_{a_{11}}$HexNAc$_z$ (m/z 1,581) and Hex$_{a_{12}}$HexNAc$_z$ (m/z 1,743), indicating that GMP2 N-glycans were similar to GMP1 N-glycans in size (Fig. 3). After HF treatment removing Galf residues, the oligosaccharides had an average size of 7.2 hexose units whereas the native oligosaccharides showed an average size of 8.0 hexose units. These data suggested the presence of one galactofuranosyl unit per average oligosaccharide. GC-MS data showed that the Galf was only present at the nonreducing position attached to the 2 position of the mannose residue.

**Characterization of GMPS. (i) GMP1.** The internal peptide sequence (NDPPDHAYGNIE) obtained from the purified GMP1 belongs to a protein encoded by an open reading frame (ORF) of 1,418 bp with an intron with a size of 50 bp in positions 562 to 612 (confirmed by sequencing the 270 bp obtained by PCR surrounding the intron with cDNA as a template). The ORF (70.m15708 in the TIGR database [http://tigrblast.tigr.org/USR/local/db/euk/private/aspergillus/annotation_dbs/ASP.pep]) encoded a putative protein of 456 amino acids with five predicted N-glycosylation sites, a predicted molecular mass of 49.8 kDa, and a pI of 6.12. A stretch of hydrophobic amino acids was seen at the amino terminus, and a putative signal peptide at the NH$_2$ terminus was predicted by the program SignalP (http://www.cbs.dtu.dk/services/SignalP) and in agreement with the (−3 −1) rule of Von Heijne (35). The putative cleavage site was between A18 and I19.

BLAST analysis of the deduced amino sequence of the \textit{A. fumigatus} protein showed a high degree of similarity with bacterial PLCs (47% and 38% similarities with \textit{Mycobacterium tuberculosis} and \textit{Pseudomonas aeruginosa} PLCs) and a putative PLC from \textit{Arabidopsis thaliana} (37% similarity). No homologies were seen with other fungal phospholipases present in the database, but BLAST against the TIGR \textit{A. fumigatus} database, however, showed the presence of two other homologous sequences (clones 65.m07340 and 52.m03766 in the TIGR database).

TLC analysis showed that upon incubation of the purified GMP1 with $^{14}$C-labeled PC, diacylglycerol was released, showing that GMP1 had PLC activity (Fig. 4). The enzymatic capacity to degrade different PLC substrates (PC, PE, SM, and PI) was investigated. Under the experimental conditions tested, the most efficiently degraded substrate was SM. PE was not cleaved. At the same substrate concentration, the amounts of radioactivity released from PC and PI were, respectively, 71 and 31% of the amount released from SM. Using NPCC as a substrate, the enzyme had a $K_m$ value of 3.85 ± 0.7 mM, with a specific activity for the batch analyzed of 30 ± 8 pmol/min/μg.

(ii) GMP2. An internal peptide sequence (KALARSVVPF IRASGS) obtained from the 70-kDa GMP2 protein matched PhyAp, a secreted phytase from \textit{A. fumigatus} (16, 21, 24, 34). The mature \textit{PHYA}-encoded enzyme consisted of 439 amino acids with a theoretical $M_r$ of 48,270 (GenBank accession no. U59804 [58.m07761 in the TIGR database]). The protein possessed seven putative N-glycosylation sites. It had high homologies with other phytases or acid phosphatases: 66%, 61%, and 48% identity to phytases of \textit{A. niger}, \textit{A. terreus}, and \textit{Mycelothora thermophila}, respectively (21). In the genome of \textit{A. fumigatus}, it belong to a cluster of three phytase genes, including the ORFs 52.m04104 and 66.m04644, and it also had homologies with two acid phosphatase genes, 69.m15283 and 56.m02370. The phytase activity of the GMP2 isolated from the culture filtrate was shown as described in Materials and Methods by the release of phosphate from phytic acid. The enzymatic activity was not further analyzed since this activity has been extensively studied previously (6, 21, 38). Using phytic acid as a substrate, the batch of enzyme analyzed had a specific activity of 100 ± 50 pmol/min/μg.

**Antigenicity of the GMPS.** The two GMPS were recognized in Western blot assays by sera from patients with aspergilloma. After N-deglycosylation, they were no longer labeled with patient antibodies, indicating that the immunogenic moiety of these GMPS was the N-glycan whereas the peptide moiety of these two glycoproteins was not antigenic (Fig. 5A). ELISA data showed that the GMPS were differentially recognized by patient and control sera ($P < 0.01$ for both antigens and a df of 23) (Fig. 5B). In addition, a significant linear fit was seen between the OD values obtained with the different antigens and GM in ELISA (data not shown); this result confirmed the exclusive antigenicity of the N-glycans of the phytase and phospholipase molecules.
Galf-containing molecules have been repeatedly shown to be important antigens among human fungal pathogens. This is true for Aspergillus species but also in P. brasiliensis (2), Sporothrix schenckii, and dermatophytes (17, 33). The major exocellular antigens of P. brasiliensis used to detect the presence of antibodies in patients with paracoccidioidomycosis is a 43-kDa glycoprotein which contains an N-linked oligosaccharide terminated by a β-(1→6) galactofuranosyl residue linked to the mannosyl residues. However, in contrast to A. fumigatus GMPs, the antigenicity of gp43 was due to the peptide moiety rather than to the Galf-containing N-glycan.

Galf is not present in the human host, and galactofuran determinants could play a role in the host immune reactions during Aspergillus infections. Antibodies directed against the galactofuran moiety of the GM have already been identified in immunocompetent patients infected with A. fumigatus (15; J. Sarfati et al., unpublished data). In addition, molecules bearing Galf could help the host to recognize the fungus as non-self and induce cytokine synthesis to activate cellular immunity. This issue has not been investigated yet. Molecules recognized
by the EB-A2 MAb circulate in the biological fluids of patients with invasive aspergillosis. Indeed, serological diagnosis of this life-threatening fungal infection remains based on the detection of GM in the biological fluids of infected patients (18, 29, 31). Initially, it was thought that only polysaccharides were released and circulated during growth in the host. The findings described here show that glycoproteins could also circulate during infection and that the so-called “circulating antigen” is not a single molecule but a family of molecules for which expression could be modulated by the immediate fungal environment.

The characterization of the N-glycan of the \( \text{Aspergillus} \) GMPs is in agreement with the general composition of oligosaccharides of \( \text{Aspergillus} \) glycoproteins (19). (i) An average oligosaccharide size of \( \text{Hex}_{5-10}\text{GlcNAc}_2 \) has been reported. Large mannan chains (\( \text{Hex}_{50-200} \)) present in \( \text{Saccharomyces cerevisiae} \) and other yeasts have never been identified in filamentous fungi. (ii) Galf has been shown in several \( \text{Aspergillus} \) glycoproteins such as the \( \alpha \)-glucosidase and \( \beta \)-galactosidase of \( \text{A. niger} \) or the \( \beta \)-galactosidase of \( \text{A. oryzae} \) (32, 36, 37). (iii) When present, Galf is at the nonreducing terminal position. (iv) N-glycan with or without Galf has also been shown to be present simultaneously on the same glycoprotein. Galactose residues found at the nonreducing end of N-glycans can serve as a stop signal for further mannose addition, similar to the role proposed for \( \alpha \)-1,3 mannose in \( \text{S. cerevisiae} \) (8).

The structure of the N-glycan of the PLC of \( \text{A. fumigatus} \) looked similar to the N-glycan of \( \alpha \)-galactosidase A from \( \text{A. niger} \). The mannan oligosaccharides of the N-glycan of \( \alpha \)-galactosidase of \( \text{A. niger} \) were of a slightly higher molecular weight, with most abundant components being \( \text{Hex}_{10-15}\text{HexNAc}_2 \) whereas they were \( \text{Hex}_{6-8}\text{HexNAc}_2 \) in \( \text{A. fumigatus} \) PLC and phytase, respectively. The chemical organization of the N-glycan has not been precisely determined since N-glycans of \( \text{A. fumigatus} \) and \( \text{A. niger} \) were analyzed as a mixture due to the limited amounts of purified protein. Data obtained with different GMP batches have shown that the oligosaccharide mixtures obtained are often heterogeneous. For example, treatment of native N-glycans from GMP1 with \( \alpha \)-mannosidase often identified a population of N-glycans without Galf residues that were trimmed to \( \text{HexHexNAc}_2 \) (m/z 763), \( \text{HexHexNAc}_3 \) (m/z 967), and \( \text{HexHexNAc}_4 \) (m/z 1,171) by the \( \alpha \)-mannosidase treatment. Other batches contained two Galf units that were in the terminal nonreducing position, as shown by GC-MS analysis (data not shown). These variations could be due to the action of a galactofuranosidase that could trim the terminal reducing galactose of the exocellular proteins. Exogalactofuranosidases were shown to be secreted by various \( \text{Aspergillus} \) species (23, 36). Studies with both \( \text{A. niger} \) and \( \text{A. fumigatus} \) have shown that N-glycans from these two species shared a common essential features: Galf is present as single residues linked to C-2 of the nonreducing terminal mannose residues of N-glycan. The structure of the Galf-containing N-glycans of \( \text{A. fumigatus} \) is shown in Fig. 6.

The recognition of N-glycans of these \( \text{Aspergillus} \) glycoproteins by MAb EB-A2 questioned the previously established identity of the epitope recognized by this MAb. Using chemically synthesized galactofurans of different sizes, we showed...
that the best-recognized oligosaccharide was a tetra-β,1,5,1,5-galactofuran (30). The absence of β,1,5-galactofuran chains in the N-glycans of *Aspergillus* GMPs suggests that an oligomannan substituted with a Galp on its terminal nonreducing end can be also recognized by this MAb. Although the Galp-Man epitope from the GMP N-glycan has not been identified yet, our data showed that the linkage between the Galp and the oligomannan is critical: a 1,2 linkage allows IgM binding, whereas a 1,6 linkage, as in gp43 of *P. brassiciflora*, prevents antigen recognition.

Besides being a main component of members of fungal genera such as *Aspergillus* and *Penicillium*, Galp is also present in the cell walls of mycobacteria, in the lipopolysaccharide O antigens of a variety of gram-negative bacteria, and in cell envelope components of eukaryotic parasites. The broad distribution of Galp in critical structures of pathogenic microorganisms and its absence in higher eukaryotes make the biosynthesis of Galp an attractive target for the development of new antimicrobial drugs. The first enzyme involved in this biosynthetic pathway has been well characterized in the genus *Mycobacterium*. It is a UDP-galactopyranose mutase that converts UDP-galactopyranose to UDP-Galf (25). This enzyme has not been identified in *A. fumigatus*, and no homologous sequence was found in the last release of the *A. fumigatus* TIGR database. The first step of the Galp pathway in *A. fumigatus* remains unknown, as well as the enzyme(s) responsible for the elongation of the galactofuran side chains or the transferases adding the Galp at the terminal nonreducing end of the mannan of the N-glycan.

A third GMP was isolated from the culture filtrate of *A. fumigatus* (GMP3) (data not shown). GMP3 was eluted with GMP2 during the first step of purification and further purified on the cation-exchange Mono S column at pH 6.5 at 250 mM NaCl. Although its N-glycan could not be analyzed due to the very small amount of protein purified, this 85-kDa protein was a GMP since de-N-glycosylation abolished the reactivity of GMP3 with the anti-galactofuran MAb. Two internal peptide sequences (FPVLGHQMTMSD and ALGQLDDTLII VTA) obtained from this protein matched the singleton 59,80496 of the TIGR database. This protein had high homologies with alkaline phosphatases (62% with alkaline phosphatase of *Neurospora crassa*). It possessed seven putative sites of N-glycosylation and an N-glycan of 12 kDa (estimated by SDS-PAGE after de-N-glycosylation). The alkaline phosphatase activity was confirmed using p-nitrophenylphosphate as a substrate in a 100 mM Tris-HCl (pH 8.0) buffer (10 ng protein was incubated for 30 min at 37°C with 200 μg PNPP, and the assay was stopped by addition of 3 volumes of 6% Na2CO3) (data not shown).

The three GMPs of *A. fumigatus* studied here are secreted enzymes that can be an essential part of a phosphate-scavenging system. For example, the main function of the PLC of *A. fumigatus* could be to retrieve phosphate from phospholipids. Such a role has been shown for the *P. aeruginosa* PLC that is regulated at the transcriptional level by Pi. In *A. fumigatus*, the PLC is also submitted to phosphate repression: it is absent in two defined media containing a high concentration of phosphate (73 mM) and repressed in a Sabouraud medium supplemented with 75 mM phosphate (data not shown). The other two enzymes, alkaline phosphatase and phytase, are directly responsible for phosphate retrieval from the external medium. *A. fumigatus* growth is highly dependent on free phosphate availability to grow: a concentration of around 1 mM phosphate is found in serum, and *A. fumigatus* requires 10 times the amount available in human biological fluids for optimal growth (unpublished data). The results presented here and the identification of an acid phosphatase as the major cell wall protein (5) suggest that phosphate availability and scavenging may be a significant determinant of *A. fumigatus* growth in the host. Strategies to interfere with such pathways could lead to useful therapeutics.

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