In Vitro Biosynthesis of Glycosylphosphatidylinositol in *Aspergillus fumigatus*†

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ABSTRACT: Glycosylphosphatidylinositol (GPI) represents a mechanism for the attachment of proteins to the plasma membrane found in all eukaryotic cells. GPI biosynthesis has been mainly studied in parasites, yeast, and mammalian cells. *Aspergillus fumigatus*, a filamentous fungus, produces GPI-anchored molecules, some of them being essential in the construction of the cell wall. An in vitro assay was used to study the GPI biosynthesis in the mycelium form of this organism. In the presence of UDP-GlcNAc and coenzyme A, the cell-free system produces the initial intermediates of the GPI biosynthesis: GlcNAc-PI, GlcN-PI, and GlcN-(acyl)PI. Using GDP-Man, two types of mannosylation are observed. First, one or two mannose residues are added to GlcN-PI. This mannosylation, never described in fungi, does not require dolichol phosphomannoside (Dol-P-Man) as the monosaccharide donor. Second, one to five mannose residues are added to GlcN-(acyl)PI using Dol-P-Man as the mannose donor. The addition of ethanolamine phosphate groups to the first, second, and third mannose residue is also observed. This latter series of GPI intermediates identified in the *A. fumigatus* cell-free system indicates that GPI biosynthesis in this filamentous fungus is similar to the mammalian or yeast systems. Thus, these biochemical data are in agreement with a comparative genome analysis that shows that all but 3 of the 21 genes described in the *Saccharomyces cerevisiae* GPI pathways are found in *A. fumigatus*.

Glycosylphosphatidylinositol (GPI)[1] anchors are present in organisms at most stages of eukaryotic evolution including protozoa, fungi, yeasts, plants, invertebrates, and vertebrates and are found on a diverse range of proteins (*1*−*4*). They are primarily responsible for the anchoring of cell surface proteins to the plasma membrane and are also involved in the intracellular transport of GPI-anchored proteins (*5*−*7*). The basic GPI core structure attached to the protein is composed of NH_2-CH_2-CH_2-PO_4H-6Manα1−2Manα1−6Manα1−4GlcNα1−6-*myo*-inositol-1-PO_4H-lipid, where the lipid can be a diacylglycerol, an alkylacylglycerol, or a ceramide. This common core glycan can be modified by additional sugars or molecules such as ethanolamine phosphate, glucose, galactose, *N*-acetylgalactosamine, or mannose (*2*, *4*).

Biosynthesis of GPI anchors has been reviewed recently (*2*, *8*−*10*). In all organisms, GPI biosynthesis occurs in the ER and involves the addition of GlcNAc to phosphatidylinositol (PI) to give GlcNAc-PI, which is de-N-acetylated to form GlcN-PI (*11*−*13*). The core mannoses are derived from dolichol phosphomannose (Dol-P-Man) (*14*). Ethanolamine phosphate residues are then added to the mannosyl core before transfer to the protein. Significant differences among parasite, yeast, and mammalian pathways have been observed. Acylation of the 2-hydroxyl group of the D-*myo*-inositol occurs before the mannose addition in yeast or mammalian cells (*15*−*17*) whereas it occurred after the first mannosylation in *Trypanosoma brucei* GPI intermediates (*18*). This acylation of D-*myo*-inositol can be inhibited by PMSF in *T. brucei* but not in HeLa cells (*19*). Differences have been also demonstrated by using synthetic substrate analogues. GlcN-PI analogues, having the 2-hydroxyl group of the inositol ring alkylated, are substrates or inhibitors in the parasite pathway whereas they have no effect in the mammalian system (*20*, *21*). The addition of an ethanolamine phosphate to the third mannose residue allows transfer of the GPI anchor to protein via an enzymatic transamination reaction. In mammalian and yeast cells, two additional ethanolamine phosphate residues are added to the trimannosyl core (*17*, *22*) whereas no such modifications are found in *T. brucei* or *Leishmania major*.

In fungi, biosynthetic studies of GPI are mostly restricted to the model yeast *Saccharomyces cerevisiae*. In this yeast, the GPI biosynthetic pathway is essential for growth, and 21 genes have been identified to be involved in this pathway

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[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; ATP, adenosine triphosphate; Co-A, coenzyme A; DFP, diisopropyl fluorophosphate; Dol-P-Man, Dolichol phosphate mannose; GDP-Man, guanosine diphospho-D-mannose; GlcN, D-glucosamine; GPI, glycosylphosphatidylinositol; GPI-PLD, GPI-specific phospholipase D; HPTLC, high-performance thin-layer chromatography; JBAM, Jack bean α-D-mannosidase; Man, D-mannose; PI-PLC, PI-specific phospholipase C; PI, phosphatidylinositol; UDP-GlcNAc, uridine diphospho-*N*-acetyl-D-glucosamine; Etn-P, ethanolamine phosphate; PNT, 1,10-phenanthroline; NEM, *N*-ethylmaleimide.

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(*9*, *23*, *24*). The yeast and mammalian GPI pathways are almost identical, but two main differences have been described: First, in yeast, the addition of a fourth α1−2-mannose residue to the glycan precedes the addition of the ethanolamine phosphate group to the third mannose residue (*23*). Second, the presence of ceramide in addition to diacylglycerol in mature yeast GPI-anchored proteins is never found in mammalian GPI proteins (*25*). The exchange of diacylglycerol with a ceramide (lipid remodeling) occurs in yeast on protein-linked GPI (*26*) in both the ER and Golgi apparatus (*27*−*29*).

This is the first investigation of the GPI biosynthesis in filamentous fungi. In silico and biochemical approaches have identified GPI-anchored proteins in *Aspergillus fumigatus* (*30*, *31*). Some of these GPI-anchored proteins are carbohydrate-active enzymes playing a role in cell wall biosynthesis (*32*). Analysis of the GPI structure from membrane proteins of *A. fumigatus* showed that the main structure corresponds to a linear pentomannoside linked to the GlcN-PI (*33*). The lipid moiety is exclusively a ceramide constituted by a C_{18}-phytosphingosine and a 2-hydroxy-$C_{24:0}$ fatty acid. Since GPI biosynthesis has not been investigated in filamentous fungi and since the structure of the GPI anchor is characterized in *A. fumigatus* and is different at least partially from the major one seen in yeast, we embarked on an investigation of the biosynthesis of GPI in *A. fumigatus*. For that purpose, we adapted the cell-free system assay originally described by Masterson et al. (*34*) and modified by Smith and colleagues (*21*, *35*).

MATERIALS AND METHODS

Materials. GDP-[2-^{3}H]mannose (22.2 Ci/mmol), UDP-[6-^{3}H]GlcNAc (35 Ci/mmol), and En^{3}Hance were purchased from Dupont NEM. Jack bean α-mannosidase (JBAM) was purchased from Boehringer Mannheim and *Bacillus thuringensis* phosphatidylinositol-specific phospholipase C (PI-PLC) from Glyko (Novato). Whole human serum was used as a source of glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD). *n*-Octyl β-D-glucopyranoside was obtained from Calbiochem. Ion-exchange resins (AG50-X8 and AG1-X8, 200−400 mesh) were obtained from Bio-Rad. Nikkomycin Z was purchased from Sigma. Amphomycin was a gift from Ralph Schwarz (Marburg, Germany). All of the other reagents were purchased from Merck-BDH or Sigma. Radiolabeled GlcNAc-PI and GlcN-PI were obtained from the *T. brucei* cell-free system (*35*), and Man_{1-4}-[^{3}H]AHM (Man_{1-4}-[^{3}H]anhydromannitol) was obtained by partial acid hydrolysis of a glycan headgroup from *Trypanosoma cruzi* GPI glycolipid (*36*, *37*).

Synthetic Substrate Analogues. D-GlcNα1−6-D-*myo*-inositol-1-HPO_4-3-*sn*-1,2-dipalmitoylglycerol (GlcN-PI) was synthesized according to Cottaz et al. (*38*). D-GlcNα1−6-D-(2-*O*-methyl)-*myo*-inositol-1-HPO_4-3-*sn*-1,2-dipalmitoylglycerol [GlcN-(2-OMe)PI] was prepared according to Crossman et al. (*39*). These compounds were N-acetylated as described below for the radiolabeled glycolipids. The purity of the synthetic substrates was checked by negative ion electrospray mass spectrometry prior to use and measured by analysis of the *myo*-inositol content by GC-MS as described in Smith et al. (*35*).

Strain, Growth Conditions, and Cellular Lysate Preparation. A. fumigatus CBS 144-89 was grown in a 1.2 L fermenter in liquid medium containing 2% glucose and 1% mycopeptone (Biokar Diagnostics, Beauvais, France) as described previously (*40*). After 22 h of culture at 25 °C, the mycelium was collected by filtration, washed extensively with water, and dried between paper sheets. The resulting wet mycelium was then resuspended in a pH 7.4 buffer containing 100 mM Hepes, 50 mM KCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 20% glycerol, 0.1 mM sodium *p*-tosyl-L-lysine chloromethyl ketone (TLCK), and 1 μg/mL leupeptin. The mycelium was disrupted by using a CO_2-cooled glass bead cell homogenizer (B. Braun Melsungen AG; glass bead diameter 1 mm) for 2.5 min. The disrupted mycelial suspension was centrifuged at 10000*g* for 10 min to remove cell wall fragments. One hundred milliliters of the cellular lysate preparation was obtained. The protein concentration was estimated at 6.5 mg/mL with the BCA kit (Pierce) using bovine serum albumin as standard. Aliquots (1 mL) of the supernatant were snap frozen in liquid nitrogen and stored at −80 °C.

Cell-Free System Assay. Prior to use, 300 μL of cellular lysate was diluted with 500 μL of 300 mM Tris-HCl, pH 7.5, 12 mM $MgCl_2$, and 16 mM dithiothreitol containing 30 μM nikkomycin and 45 μg of tunicamycin (previously dissolved in dimethyl sulfoxide at 1 mg/mL). The mixture was sonicated in a water bath for 20 s and incubated at 30 °C for 5 min. Fifty microliters of this lysate mixture was added to 12.5 μL of water containing UDP-[6-^{3}H]GlcNAc (2 μCi) or GDP-[2-^{3}H]mannose (0.25 μCi). After a brief sonication, the reaction tube was incubated at 30 °C for 90 min. The reaction was stopped by addition of 417 μL of chloroform/methanol (1/1 v/v). The glycolipid products were recovered in the chloroform/methanol/water-soluble fraction, which was evaporated and partitioned between 100 μL of butan-1-ol and 100 μL of water, as previously described (*35*). Aliquots of the butan-1-ol phase containing the glycolipid products were subjected to HPTLC analysis both before and after enzymatic and chemical treatments. Several effectors were tested in the cell-free system assay: ATP (0−1 mM), coenzyme A (1 mM), palmitoyl-coenzyme A (0.1 mM), UDP-GlcNAc (1 mM), GDP-Man (1 mM), DFP (1 mM), PMSF (1 mM), *n*-octyl glucoside (0.3%, m/v), amphomycin (0.5 mM)/$CaCl_2$ (10 mM), acyl-CoA binding protein (*41*), GlcNAc-PI (0.1 mM), GlcN-PI (0.1 mM), GlcNAc-(2-OMe)-PI (0.1 mM), 1,10-phenanthroline (0.5 and 1 mM), and *N*-ethylmaleimide (10 mM).

Enzymatic and Chemical Treatment of Radiolabeled Glycolipids. Digestions with JBAM, PI-PLC, and GPI-PLD and processing of the products for analysis by HPTLC were performed as described previously (*19*, *35*).

For JBAM digestion, glycolipids were dissolved in 20 μL of 50 mM NaOAc, pH 4.0, containing 0.1% *n*-octyl glucoside and incubated with 10 μL of enzyme solution (95 units/mL; Sigma) at 37 °C for 24 h. For PI-PLC, glycolipids were dissolved in 20 μL of 50 mM imidazole/acetate, pH 7.4, containing 0.1% Triton X-100 and incubated with 0.25 μL of PI-PLC (200−300 units/mL; Glyko) in at 37 °C for 24 h. For GPI-PLD, glycolipids were dissolved in 20 μL of 50 mM Tris-HCl, 10 mM NaCl, and 2.6 mM $CaCl_2$, pH 7.4, containing 0.1% Nonidet-P40 and incubated with 3 μL of human serum as the GPI-PLD source at 37 °C for 24 h.

Base treatment was carried out in 150 μL of 50% propan-1-ol and 150 μL of 30% aqueous ammonia at 50 °C for 5 h.

Resistant glycolipids were extracted into butan-1-ol for analysis by HPTLC. Nitrous acid deamination of glycolipids was carried out in 20 μL of 0.1 M sodium acetate, pH 4.0, containing 0.01% Zwittergent 3-16. Aliquots (10 μL) of freshly prepared 0.5 M $NaNO_2$ were added at hourly intervals with incubation at 50 °C for 3 h.

Lipidic Products Were Extracted into Butan-1-ol for Analysis by HPTLC. Glycolipids were N-acetylated at 0 °C in 100 μL of saturated $NaHCO_3$ solution by the addition of three aliquots (2.5 μL) of acetic anhydride over 20 min. The reaction mixture was warmed to room temperature, and the N-acetylated glycolipids were extracted into butan-1-ol. Residual salts were removed by washing the butan-1-ol phase with water.

Mild acid hydrolysis (80 mM TFA at 100 °C for 1 h) was used to selectively hydrolyze sugar−1-phosphate linkages and to destroy Dol-P-Man.

Glycan Analysis. Radiolabeled glycolipids from the *A. fumigatus* assay were purified by preparative HPTLC. They were eluted from the excised silica with chloroform/methanol/water (10/10/3 v/v/v), partitioned between butan-1-ol and water, and delipidated by base treatment as described above. The radiolabeled soluble glycan products were recovered in the aqueous phase of the butan-1-ol/water partition and were treated by the following procedure: nitrous deamination, reduction with $NaBH_4$, aqueous HF dephosphorylation, and desalting by passage through AG50-X8 (H^+) over AG1-X8 (OH^-) ion-exchange resins to yield neutral glycans (*42*). Neutral glycans were analyzed by HPTLC. To identify the presence of an aqueous HF labile group on glycans, a JBAM digestion was done before and after aqueous HF treatment during headgroup analysis.

HPTLC. Glycolipid samples and standards were applied to 10 cm aluminum-backed silica gel 60 HPTLC (Merck) which were developed with choloroform/methanol/1 M ammonium acetate/13 M ammonium hydroxide/water (180/140/9/9/23 v/v). HPTLC of neutral glycans released from GPI intermediates were developed with propan-1-ol, acetone, and water (9/6/5 v/v) as previously described (*37*). Radiolabeled components were detected by fluorography at −70 °C using Kodak XAR-5 film and an intensifying screen after the plate was sprayed with En^{3}Hance.

RESULTS

Production of GlcNAc-PI, GlcN-PI, and GlcN-(acyl)PI in the Presence of UDP-[^{3}H]GlcNAc. Incubation of the *A. fumigatus* lysate with UDP-[^{3}H]GlcNAc produced three main glycolipids (Figure 1). The major product, glycolipid A, migrating with a R_f of 0.38, was resistant to GPI-PLD, PI-PLC, and mild acid and base treatments, indicating that it is neither a glycerolipid, a Dol-P-sugar, nor a GPI intermediate (Figure 1). The nature of glycolipid A remains unknown. Glycolipid B, migrating with the same R_f as the GlcN-PI standard (R_f 0.41) was sensitive to GPI-PLD, PI-PLC, and base treatments (data not shown). Glycolipid C, migrating with the same R_f as GlcNAc-PI (R_f 0.43), was resistant to GPI-PLD and sensitive to PI-PLC and base treatment (data not shown). The addition of Co-A to the lysate induced the formation of a small amount new glycolipid (glycolipid D) characterized by the fastest migration of all radiolabeled products (R_f 0.49). This glycolipid

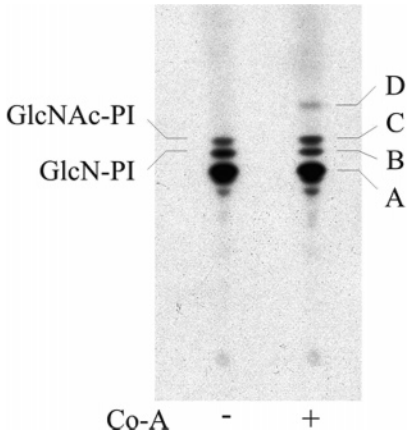


FIGURE 1: Radiolabeled glycolipids produced by *A. fumigatus* lysate in the presence of UDP-[^{3}H]GlcNAc. *A. fumigatus* mycelium lysate was incubated with 10 mM DTT, 30 μM nikkomycin, 45 μg/mL tunicamycin, and 2 μCi of UDP-[^{3}H]GlcNAc with or without coenzyme A (Co-A, 1 mM) at 30 °C for 90 min. Reactions were terminated by addition of $CHCl_3/CH_3OH$ (1/1), allowing extraction of glycolipids. These were partitioned between butan-1-ol/water and subsequently separated by HPTLC and visualized by fluorography. The migration of GlcNAc-PI and GlcN-PI standards, obtained from the *T. brucei* cell-free system, is indicated in the left of the panel.

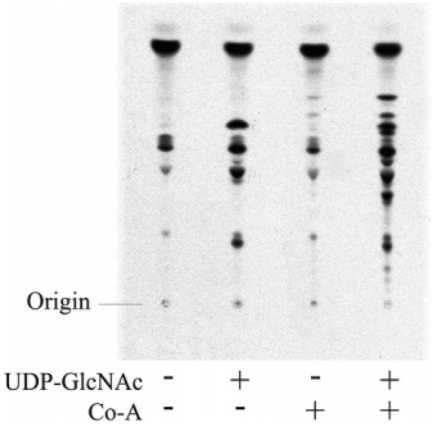
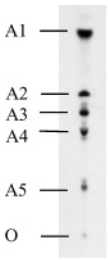


FIGURE 2: Radiolabeled glycolipids produced by *A. fumigatus* lysate in the presence of GDP-[^{3}H]Man. *A. fumigatus* mycelium lysate was incubated with 10 mM DTT, 30 μM nikkomycin, 45 μg/mL tunicamycin, and 0.25 μCi of GDP-[^{3}H]Man with or without UDP-GlcNAc (1 mM) and coenzyme A (Co-A, 1 mM) at 30 °C for 90 min. Reactions were terminated by addition of $CHCl_3/CH_3OH$ (1/1), allowing extraction of glycolipids. These were partitioned between butan-1-ol/water and subsequently separated by HPTLC and visualized by fluorography.

D was sensitive to GPI-PLD and base treatments but resistant to PI-PLC (data not shown). All of these data allowed identification of compounds B, C, and D as GlcN-PI, GlcNAc-PI, and GlcN-(acyl)PI, respectively.

Production of Man-GlcN-PI and Etn-P-Man-GlcN-PI in the Presence of GDP-[^{3}H]Man and UDP-GlcNAc. The addition of radiolabeled GDP-[^{3}H]Man in the presence of UDP-GlcNAc to the lysate mixture resulted in the synthesis of five radiolabeled glycolipids (Figure 2, Table 1). Radiolabeled glycolipids purified by preparative HPTLC were tested for their sensitivity to GPI-PLD, PI-PLC, nitrous deamination, mild acid hydrolysis, base treatment, N-acetylation, and Jack bean α-D-mannosidase (JBAM) digestion. As described in Table 1, the product A1 migrated as the

Table 1: Analysis of Radiolabeled Mannosylated Glycolipids Obtained in the Presence of UDP-GlcNAc and GDP-[^{3}H]Man[a]



Glycolipids	Enzymatic and chemical treatments								
	GPI-PLD	PI-PLC	JBAM	base	HNO_2	N-acetyl	TFA	Man nb	Proposed structure
A1	-	-	-	-	-	-	+	-	Dol-P-Man
A2	+	+	+	+	+	+	-	1	Man-GlcN-PI
A3	-	-	+	-	-	-	-	-	non-GPI
A4	+	+	-	+	+	+	-	1	Etn-P-Man-GlcN-PI
A5	+	+	-	+	+	+	-	2	Etn-P-Man-(Etn-P)Man-GlcN-PI

[a] Key: (−), resistant; (+), sensitive; HNO_2, nitrous acid deamination; TFA, mild acid hydrolysis; Man nb, number of mannose residues obtained by the headgroup analysis (see Materials and Methods for details); O, origin.

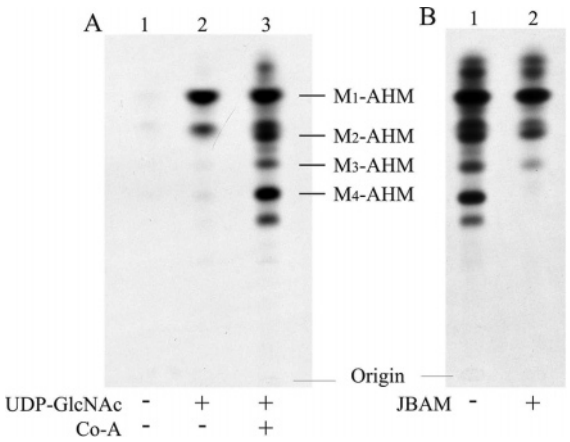
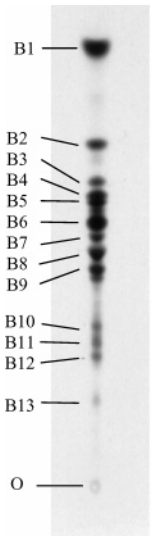


FIGURE 3: Headgroup analysis of radiolabeled glycolipids produced by *A. fumigatus* lysate in the presence of GDP-[^{3}H]Man. *A. fumigatus* mycelium lysate was incubated with 10 mM DTT, 30 μM nikkomycin, 45 μg/mL tunicamycin, and 0.25 μCi of GDP-[^{3}H]Man with or without UDP-GlcNAc (1 mM) and coenzyme A (Co-A, 1 mM) at 30 °C for 90 min. Reactions were terminated by addition of $CHCl_3/CH_3OH$ (1/1), allowing extraction of glycolipids. These were partitioned between butan-1-ol/water, and labeled GPI intermediates were submitted to base treatment, nitrous deamination, reduction with a borohydride, and aqueous HF dephosphorylation. Released headgroup glycans were desalted by passing through a AG50-X8 (H^+) and AG1-X8 (OH^-) column and separated by HPTLC. The presence of Etn-P in the glycan core was analyzed by submitting these glycolipids to a JBAM digestion at 37 °C for 24 h. Glycans were separated by HPTLC using the solvent system propan-1-ol/acetone/water (9/6/5 v/v/v) and visualized by fluorography. The migration of the Man_{1-4}-AHM standard is indicated in the center of the panel.

Dol-P-Man standard, was resistant to GPI-PLD, PI-PLC, JBAM, N-acetylation, and nitrous deamination, but is sensitive to the mild acid hydrolysis. We concluded that compound A1 was Dol-P-Man. Compounds A2, A4, and A5 were sensitive to GPI-PLD, PI-PLC, and nitrous deamination, indicating that these glycolipids were GPI intermediates without an acyl group on the inositol ring. In contrast, A3 product, which was not sensitive to GPI-PLD, PI-PLC, and nitrous deamination, was not a GPI structure. When the whole glycolipid fraction was treated successively with base, $NaNO_2$, $NaBH_4$, and HF to analyze the glycan headgroup, two products were released (Figure 3A, lane 2). One corresponded to Man_1-AHM (mannose anhydromannitol), indicating the presence of one mannose residue linked to the glucosamine. The other product was larger and did not comigrate with any of the Man_{1-4}-AHM GPI headgroup structures prepared similarly from *T. cruzi* GPI. Similar treatment performed on purified A2 and A4 produced only Man_1-AHM, indicating that the two glycolipids contained one mannose residue (Table 1). Taking into account the results presented in the Table 1 (i.e., sensitivity to JBAM), A2 corresponded to Man-GlcN-PI and A4 to Etn-P-Man-GlcN-PI. Consequently, headgroup analysis done on the whole glycolipid fraction indicated that A5 contained two mannose residues (Figure 3A, lane 2). However, the R_f is different of Man_2-AHM from *T. brucei*, suggesting that A5 corresponds to Etn-P-Man-(Etn-P)Man-GlcN-PI, where the glycosidic linkage between the two mannose residues may not be $\alpha 1-6$ (*37*).

Production of Intermediates with Acylated Inositol in the Presence of GDP-[^{3}H]Man, UDP-GlcNAc, and Coenzyme A. The addition of coenzyme A with UDP-GlcNAc and radiolabeled GDP-Man in the lysate mixture induced the synthesis of the highest number of labeled glycolipids (Figure 2). To investigate the structure of each mannosylated glycolipid, a preparative HPTLC was done, and 13 products (B1−B13) were purified and tested with different chemical and enzymatic treatments. All of them, with the exception of B1 and B6, were GPI-PLD sensitive, indicating that addition of Co-A increased the synthesis of GPI intermediates (Table 2). Compound B1 presented the same chromatographic and mild acid susceptibility as compound A1 and was identified as Dol-P-Man. Compound B2 was sensitive to GPI-PLD and nitrous deamination and resistant to PI-PLC. The resistance to PI-PLC showed that it contains an acyl group linked to the C-2 of the inositol ring. Headgroup analysis (Table 2) showed the presence of one mannose residue. According to these results and the migration in HPTLC, compound B2 was identified as Man-GlcN-(acyl)PI. Sensitivity of B3 to the treatments was similar to B2, but the glycan contained two mannose residues, indicating that B3 was Man-Man-GlcN-(acyl)PI. Glycolipid B4 showed similar properties to B2 except that it was sensitive to PI-PLC, suggesting that B4 is Man-GlcN-PI. Glycolipid B5 showed similar properties to B2 except that it was resistant to JBAM, suggesting that B5 is Etn-P-Man-GlcN-(acyl)PI. Glycolipid B6 was resistant to GPI-PLD and PI-PLC and is, therefore, a non-GPI metabolite. Glycolipid B7 showed similar properties to B3, except for a much lower R_f, and JBAM digestion converted it to B5. These data suggest that B7 is Man-(Etn-P)Man-GlcN-(acyl)PI. Glycolipid B8 showed similar properties to B4 except that it was resistant to JBAM, suggesting that B8 is Etn-P-Man-GlcN-PI. Glycolipid B9 showed similar properties to B7, in that JBAM digestion converted it to B5, but headgroup analysis revealed the presence of four mannose residues,

Table 2: Analysis of Radiolabeled Mannosylated Glycolipids Obtained in the Presence of UDP-GlcNAc, Coenzyme A, and GDP-[^{3}H]Man[a]



Glycolipids	An	Enzymatic and chemical treatments								
		GPI-PLD	PI-PLC	JBAM	base	HNO2	N-acetyl	TFA	Man nb	Proposed Structure
B1	A1	-	-	-	-	-	-	+	-	Dol-P-Man
B2		+	-	+	+	+	+	-	1	Man-GlcN-(acyl)PI
B3		+	-	+	+	+	+	-	2	Man_2-GlcN-(acyl)PI
B4	A2	+	+	+	+	+	+	-	1	Man-GlcN-PI
B5		+	-	-	+	+	+	-	1	EtN-P-Man-GlcN-(acyl)PI
B6	A3	-	-	+	-	+	-	-	-	non-GPI
B7		+	-	+	+	+	+	-	2	Man-EtN-P-Man-GlcN-(acyl)PI
B8	A4	+	+	-	+	+	+	-	1	EtN-P-Man-GlcN-PI
B9		+	-	+	+	+	+	-	4	Man_3-EtN-P-Man-GlcN-(acyl)PI
B10		+	-	+	+	nd	nd	-	nd	
B11		+	-	+	+	nd	nd	-	nd	
B12	A5	+	+	-	+	nd	nd	-	nd	Etn-P-Man-(Etn-P)Man-GlcN-PI
B13		+	-	+	+	nd	nd	-	nd	

[a] Key: (−), resistant; (+), sensitive; nd, not determined; HNO_2, nitrous acid deamination; TFA, mild acid hydrolysis; Man nb, number of mannose residues obtained by the headgroup analysis (see Materials and Methods for details); An, comigration of glycolipids (An described in Table 1); O, origin.

suggesting that B9 is Man-Man-Man-(Etn-P)Man-GlcN-(acyl)PI. Other GPI intermediates (B10−B13), were sensitive to the GPI-PLD treatment but were in too low amount to be identified. Their low R_f suggested that they can be GPI intermediates with a glycan group with a higher number of mannose and/or phosphoethanolamine residues. Indeed, glycan headgroup analysis of the pool of GPI intermediates showed the presence of up to Man_5-AHM (Figure 3, lane A3). JBAM digestion, prior to the glycan headgroup analysis, removed the Man_5-AHM and Man_4-AHM (Figure 3, panel B). The resistance of Man_1-AHM, Man_2-AHM, and Man_3-AHM indicated that ethanolamine phosphate groups were present on the first, second, and third mannose residues of some GPI intermediates.

Influence of Effectors on the in Vitro GPI Biosynthesis. (A) Effect of Amphomycin and N-Ethylmaleimide (NEM). Amphomycin in the presence of calcium is an inhibitor of the Dol-P-Man synthase (*43*). In the *A. fumigatus* cell-free system, at 0.5 mM final concentration, amphomycin inhibited the synthesis of Dol-P-Man (Figure 4A). In the presence of UDP-GlcNAc without Co-A, amphomycin did not affect the production of glycolipids A2, A3, and A4 (see Discussion). In the presence of UDP-GlcNAc and Co-A, amphomycin inhibited the production of all GPI intermediates with an acyl group linked to the inositol ring (Figure 4A, lanes 3 and 4). Thus, as was shown for the GPI biosynthetic pathway in yeast and mammalian cells, Dol-P-Man is essential for the mannosylation of GlcN-(acyl)PI.

N-Ethylmaleimide (NEM) is a sulfhydryl alkylating reagent which inhibits GlcNAc-PI formation (*44*) and inositol acylation in the yeast and mammalian GPI biosynthetic pathway (*45*). In the *A. fumigatus* cell-free system, the addition of 10 mM NEM did not inhibit the production of Dol-P-Man or the production of nonacylated GPI intermediates (Figure 4B). But the synthesis of acylated inositol intermediates was substantially inhibited by NEM, suggesting that NEM inhibits inositol acylation in *A. fumigatus*. However, in contrast to other species, NEM did not inhibit GlcNAc-PI formation in *A. fumigatus*.

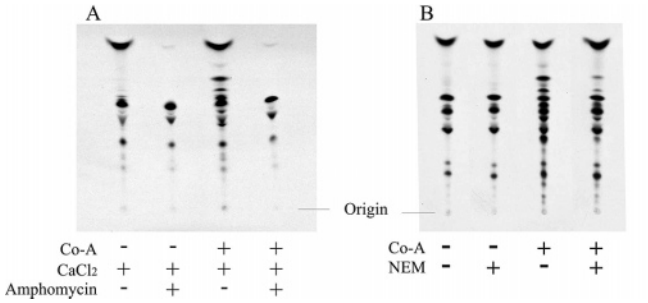


FIGURE 4: Effect of drug on the production radiolabeled glycolipids produced by *A. fumigatus* lysate in the presence of GDP-[^{3}H]Man: (A) effect of amphomycin; (B) effect of *N*-ethylmaleimide (NEM). *A. fumigatus* mycelium lysate was incubated with 10 mM DTT, 30 μM nikkomycin, 45 μg/mL tunicamycin, and 0.25 μCi of GDP-[^{3}H]Man with or without UDP-GlcNAc (1 mM) and coenzyme A (Co-A, 1 mM) at 30 °C for 90 min. Preincubation was done in the absence or presence of amphomycin (0.5 mM), $CaCl_2$ (10 mM), and NEM (10 mM). Reactions were terminated by addition of $CHCl_3/CH_3OH$ (1/1), allowing extraction of glycolipids. These were partitioned between butan-1-ol/water and subsequently separated by HPTLC and visualized by fluorography.

(B) Analysis of GPI Production in the Presence of an Exogenous Acceptor, GlcNAc-PI. Addition of exogenous GlcNAc-PI to a mixture of lysate containing GDP-[^{3}H]Man induced the production of one glycolipid with a migration and sensitivity to various treatments similar to the one of Man-GlcN-PI (compound A2 or B4) (Figure 5). The addition of palmitoyl-Co-A to the mixture induced the production of two other glycolipids. Sensitivity to GPI-PLD, base treatment, HNO_2, JBAM digestion, the resistance to PI-PLC, and the headgroup analysis showed that, in the presence of palmitoyl-Co-A, Man-GlcN-(acyl)PI and Man-Man-GlcN-(acyl)PI were formed (Figure 5). These results indicated that the exogenous GlcNAc-PI can be de-N-acetylated prior to mannosylation and that acylation of the inositol can be obtained with exogenous palmitoyl-Co-A.

GlcNAc-(2-OMe)PI is an analogue of GlcNAc-PI with a methyl group linked on position 2 of the inositol ring that prevents O-acylation of the inositol. GlcNAc-(2-OMe)PI was

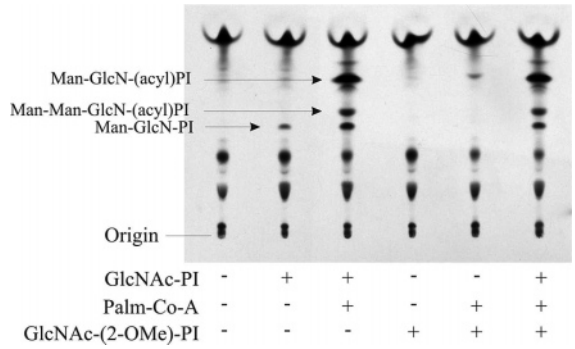


FIGURE 5: Effect of exogenous putative acceptor [GlcNAc-PI and GlcNAc-(2-OMe)PI] on the production of radiolabeled glycolipids produced by *A. fumigatus* lysate in the presence of GDP-[^{3}H]Man. *A. fumigatus* mycelium lysate was incubated with 10 mM DTT, 30 μM nikkomycin, 45 μg/mL tunicamycin, and 0.25 μCi of GDP-[^{3}H]Man in the presence of 0.3% *n*-octyl glucopyranoside (*n*-OG) in the absence or presence of GlcNAc-PI (0.1 mM), GlcNAc-(2-OMe)PI (0.1 mM), and palmitoyl-coenzyme A (Palm-Co-A, 1 mM) at 30 °C for 90 min. Reactions were terminated by addition of $CHCl_3/CH_3OH$ (1/1), allowing extraction of glycolipids. These were partitioned between butan-1-ol/water and subsequently separated by HPTLC and visualized by fluorography.

tested in the *A. fumigatus* cell-free system as a substrate or as an inhibitor of the mannosylation of GlcN-(acyl)PI (Figure 5). Following the addition of GlcNAc-(2-OMe)PI, no Man-GlcN-(2-OMe)PI was detected, indicating that, in contrast to the *T. brucei* system, in *A. fumigatus* GlcNAc-(2-OMe)PI is not used as a substrate. When GlcNAc-(2-OMe)PI is added to a reaction mixture containing GlcNAc-PI, no difference was observed in the production of GPI intermediates (Figure 5), indicating that GlcNAc-(2-OMe)-PI is not an inhibitor of the GPI de-N-acetylation, inositol acylation, or mannosylation (Figure 5).

Others effectors such as 1,10-phenanthroline, PMSF, DFP, and acyl-Co-A binding protein under the concentrations tested have no effect on GPI synthesis by the cell-free system of *A. fumigatus* (data not shown).

(C) Influence of ATP. ATP has been described to be essential for the synthesis of yeast acyl-Co-A (*46*). In the *A. fumigatus* cell-free system, the addition of exogenous ATP was not necessary to obtain GlcN-(acyl)PI (Figure 1) or mannosylated GlcN-(acyl)PI (Figure 2). To confirm the requirement for ATP in the assay, an ATP privation system was set up. The preincubation of the lysate with a hexokinase in the presence of glucose for ATP depletion did not affect the production of either the acylated or nonacylated GPI intermediate in the presence of Co-A (data not shown). The addition of increasing concentrations of ATP up to 1 mM to the cell-free system with or without UDP-GlcNAc and Co-A caused a decrease of the amount of radiolabeled glycolipids (Figure 6). HPTLC analysis of radiolabeled glycolipids showed mainly that the Dol-P-Man quantity decreased with the increase of ATP concentration (Figure 6). This inhibition was gradual from 0.1 to 1 mM, suggesting that the addition of ATP was associated to a reduction in the production of Dol-P-Man and consequently a decrease in Dol-P-Man-dependent mannosylation. In the *A. fumigatus* cell-free system, the inositol acylation needed the presence of Co-A, indicating the requirement of acyl-Co-A. This observation suggests that ATP is not required for the acyl-Co-A formation in our in vitro system.

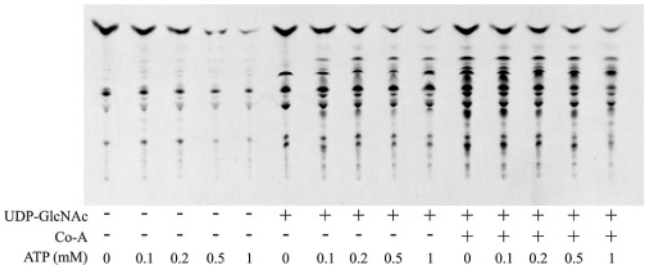


FIGURE 6: Analysis of the effect of various ATP concentrations on the production of radiolabeled glycolipids produced by *A. fumigatus* lysate in the presence of GDP-[^{3}H]Man. *A. fumigatus* mycelium lysate was incubated with 10 mM DTT, 30 μM nikkomycin, 45 μg/mL tunicamycin, and 0.25 μCi of GDP-[^{3}H]-Man with or without UDP-GlcNAc (1 mM), coenzyme A (Co-A, 1 mM), and varying concentrations of ATP at 30 °C for 90 min. Reactions were terminated by addition of $CHCl_3/CH_3OH$ (1/1), allowing extraction of glycolipids. These were partitioned between butan-1-ol/water and subsequently separated by HPTLC and visualized by fluorography.

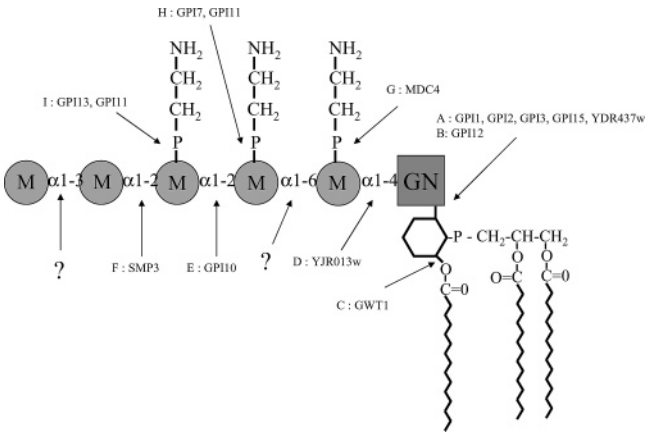


FIGURE 7: Yeast orthologues involved in the GPI biosynthetic pathway found in *A. fumigatus* genome: (A) GlcNAc-PI synthesis (*60*−*66*); (B) GlcNAc-PI de-N-acetylation (*67*); (C) inositol acylation (*68*, *69*); (D) first mannose addition (*70*); (E) third mannose addition (*71*, *72*); (F) fourth mannose addition (*23*); (G) addition of Etn-P to the first mannose residue (*73*, *74*); (H) addition of Etn-P to the second mannose residue (*75*, *76*); (I) addition of Etn-P to the third mannose residue (*75*−*77*).

DISCUSSION

GPI biosynthesis of the filamentous fungus, *A. fumigatus*, has been analyzed in vitro. The initial steps of biosynthesis, i.e., the formation of GlcNAc-PI, GlcN-PI, and GlcN-(acyl)-PI, were observed in the presence of UDP-GlcNAc and Co-A. Two different mannosylation reactions were seen in the *A. fumigatus* cell-free system (Figure 8).

First, there is mannosylation of GlcN-PI without acylation of the inositol ring. This has been observed in *T. brucei* (*2*, *18*). However, in *A. fumigatus*, this reaction did not require Dol-P-Man as the donor substrate nor did it use GlcN-(2-OMe)PI as substrate, indicating that this mannosylation event is not related to any other described GPI synthetic pathway. These GPI structures contained one or two mannose residues, partially substituted by one aqueous HF labile group, suggesting the presence of an ethanolamine phosphate (or some other phosphoryl substituent) on the first mannose. The synthesis of these nonacylated GPI structures was independent of the formation of acylated GPI structures. It indicates that a GDP-Man-dependent mannosyltransferase activity is able to use GlcN-PI as an acceptor in the mycelium lysate

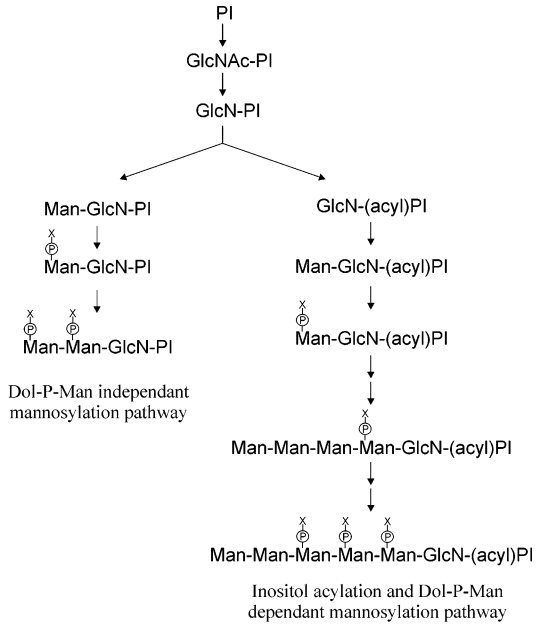


FIGURE 8: Biosynthetic pathways of glycosylphosphatidylinositol in *A. fumigatus* deduced from the in vitro cell-free assay. (X-P = phosphoryl substituent, which could be the ethanolamine phosphate group).

of *A. fumigatus*. This type of activity, never described in fungi, may be involved in the synthesis of the GPI-anchored galactomannan recently identified in *A. fumigatus* (Costachel, unpublished results) or some fungal glycosphingolipid that has a mannan moiety bound to inositol through a glucosamine residue (*47*, *48*). The use of GDP-Man as a donor in GPI biosynthesis in *Leishmania mexicana* has also been described (*49*), but in this case, it was restricted to the second mannosylation event (i.e., GDP-Man:Man_1-GlcN-PI $\alpha1-3$ mannosyltransferase) that leads to the formation of type 2 GPI structures containing the Man$\alpha1-3$Man-GlcN$-$PI motif.

Second, in the presence of UDP-GlcNAc, Co-A, and GDP-Man, the *A. fumigatus* cell-free system produced several GPI intermediates with an acyl group on position 2 of the inositol ring. Thus, many of the steps of the biosynthetic pathway described in yeast and mammalian cells have been observed: GlcNAc-PI synthesis, GlcNAc-PI de-N-acetylation, acylation of GlcN-PI, mannosylation using Dol-P-Man as the substrate donor, and ethanolamine phosphate addition. Mannose addition goes up to five residues, in agreement with the GPI structure identified in membrane GPI-anchored proteins from *A. fumigatus* (*33*). In the cell-free system, major GPI intermediates contained one to four mannose residues, and the ethanolamine phosphate group has been added to the second and third mannose but mainly to the first residue (Figure 3) as has been described in GPI intermediates of yeast and mammalian cells. These GPI structures and the inhibitory effects of compounds such as amphomycin, PMSF, NEM, and GlcNAc-(2-OMe)PI indicate a biosynthetic pathway close to the yeast/mammalian system and clearly different from the *T. brucei* pathway.

Most of the genes involved in the GPI biosynthesis have been identified in eukaryotic systems (*8*). In the yeast, *S. cerevisiae*, 21 genes have been described to be essential for the complete GPI synthesis (Figure 7), GPI anchoring of the protein (*50*$-$*54*), and inositol de-O-acylation (*24*). Comparative Blast analysis has shown that all genes of *Schizosaccharomyces pombe* and *S. cerevisiae* associated with GPI biosynthesis have homologues in the genome sequence of *A. fumigatus* (available at hppt:\\tigrblast.tigr.org), indicating a conserved biosynthetic pathway between all of these three species. However, genomic analysis has shown that DNA sequences of GPI biosynthetic enzymes are more closely related to *S. pombe* and human than *S. cerevisiae*, particularly for GPI15, YDR437w, GPI11, and DPM1 genes, as was observed for DPM1 of *Trichoderma reesei*, a filamentous fungi (*55*).

In *A. fumigatus*, at least five mannosyltransferase activities are required for the complete synthesis of the GPI anchor. Three homologues of yeast mannosyltransferase genes (YJR013w, GPI10, SMP3) involved in the GPI biosynthesis have been found in the *A. fumigatus* genomic sequence. These three *A. fumigatus* sequences contain the two, long and short, conserved peptide motifs of PIG-M, PIG-B, and SMP3, respectively (*56*). These conserved motifs in *A. fumigatus*, characteristic of glycosyltransferases using Dol-P monosaccharide as the donor substrate, are more closely related to *S. pombe* genes rather than *S. cerevisiae* genes. At least two other unknown genes are required for the complete GPI synthesis in *A. fumigatus*.

During the mannosylation process, the addition of ethanolamine phosphate groups (Etn-P) occurs on the mannose residues of GPI intermediates. In yeast and mammalian cells, Etn-P transfer can be inhibited (*57*, *58*). Indeed, 1,10-phenanthroline (PNT) partially blocks the Etn-P transfer to the first mannose residue and induces the accumulation of GPI structure without the Etn-P group. In contrast, in the *A. fumigatus* cell-free system, PNT did not inhibit the Etn-P transfer to the first mannose. Interestingly, the *A. fumigatus* cell-free system produced Etn-P-Man-GlcN-PI, suggesting that the Etn-P transfer activity does not recognize the acyl group of the GlcN-(acyl)PI.

The transamidation reaction transfers en block the GPI structure to the anchor attachment site of the protein (ω-site) with a concomitant release of a C-terminal peptide. In yeast, after the transfer to the GPI protein, a lipid remodeling occurs. Since the GPI anchor of *A. fumigatus* membrane GPI-anchored proteins contains a ceramide constituted by a C18-phytosphingosine and a 2-monohydroxylated $C_{24:0}$ fatty acid (*33*), a lipid exchange must occur to substitute the base-sensitive diacyl lipid moiety of the GPI intermediate. No proteins or genes have been described to be responsible for this lipid remodeling. However, this step seems to be important for the GPI transport to the plasma membrane and cellular trafficking (*5*, *7*, *59*). In contrast to yeast, the membrane GPI anchor of *A. fumigatus* contains only ceramide, suggesting that a lipid remodeling is more efficient or essential for the cellular traffic of GPI protein to the plasma membrane.

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