The glycoinositol-phospholipids of *Phytomonas*

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The *Phytomonas* spp. are trypanosomatid parasites of plants. A polar glycolipid fraction of a *Phytomonas* sp., isolated from the plant *Euphorbia characias* and grown in culture, was fractionated into four major glycolipid species (Phy 1–4). The glycolipids were analysed by chemical and enzymic modifications, composition and methylation analyses, electrospray mass spectrometry and microsequencing after HNO₂ deamination and NaB³H₄ reduction. The water-soluble headgroup of the Phy2 glycolipid was also analysed by ¹H NMR. All four glycolipids were shown to be glycoinositol-phospholipids (GIPLs) with phosphatidylinositol (PI) moieties containing the fully saturated alkylacylglycerol lipids 1-*O*-hexadecyl-2-*O*-palmitoylglycerol and 1-*O*-hexadecyl-

2-*O*-stearoylglycerol. The structures of the Phy 1–4 GIPLs are: Man α 1-2Man α 1-6Man α 1-4GlcN α 1-6PI, Glc α 1-2(NH₂-CH₂CH₂-HPO₄-)Man α 1-2Man α 1-6Man α 1-4GlcN α 1-6PI, Glc α 1-2(NH₂CH₂CH₂-HPO₄-)Man α 1-2Man α 1-6Man α 1-4(NH₂-CH₂CH₂-HPO₄-)GlcN α 1-6PI and Glc α 1-2Glc α 1-2(NH₂CH₂-CH₂-HPO₄-)Man α 1-2Man α 1-6Man α 1-4(NH₂CH₂CH₂-HPO₄-)GlcN α 1-6PI. The *Phytomonas* GIPLs represent a novel series of structures. This is the first description of the chemical structure of cell-surface molecules of this plant pathogen. The *Phytomonas* GIPLs are compared with those of other trypanosomatid parasites and are discussed with respect to trypanosomatid phylogenetic relationships.

INTRODUCTION

The flagellated trypanosomatids of the genus *Phytomonas* are parasites of plants. In some cases they parasitize plants without apparent pathogenicity but they can also cause crop diseases of economic significance in plantations of coconut, oil palm, cassava and coffee [1,2]. The parasites live mostly in the xylem and phloem of the infected plants and are thought to be transmitted through the bite of phytophagous insects [1,2]. The successful culturing of *Phytomonas* species is a relatively recent advance [1–3] and consequently there are relatively few data on their biochemistry. The *Phytomonas* spp. have ultrastructural features typical of the family Trypanosomatidae. They contain a kinetoplast, a single tubular mitochondrion (with few cristae) [4,5] and glycosomes [6]. Studies of carbohydrate metabolism [6] have revealed that the parasite contains and secretes polysaccharide- and oligosaccharide-degrading enzymes and that energy production, like that in the bloodstream forms of the animal parasite *Trypanosoma brucei*, is via aerobic glycolysis. Nothing is known about the molecular architecture of the parasite surface.

The glycoinositol-phospholipids (GIPLs) are free glycolipids, based on a phosphatidylinositol (PI) phospholipid moiety, that are related to the glycosylphosphatidylinositol (GPI) protein anchors in that they share the structural motif Man α 1-4GlcN α 1-6myo-inositol-1-HPO₄-lipid [7]. The GIPLs, first fully described in the promastigote forms of *Leishmania major* [8], are the most abundant surface components of several trypanosomatids, including the promastigote and amastigote forms of all species of the *Leishmania* [8–15], the epimastigote forms of *Trypanosoma cruzi* [16–18], *Leptomonas samueli* [19,20], *Endotrypanum schaudinni* [21] and *Herpetomonas samuelpeessoai* [22]. The lipid components of the parasite GIPLs (reviewed in refs. [7,23]) are alkylacylglycerols in the case of *Leishmania* and ceramides in the case of *Herpetomonas*, *Leptomonas* and *Endotrypanum*. Both alkylacylglycerol and ceramide lipids have been found as com-

ponents of *T. cruzi* GIPLs [16–18]. The GIPLs can be subdivided into three types, according to their glycan structure beyond the common Man α 1-4GlcN α 1-6PI motif [7,12,22]. The type-1 GIPLs are more homologous to the GPI protein anchors and contain a core of Man α 1-6Man α 1-4GlcN α 1-6PI, the type-2 GIPLs contain a core of Man α 1-3Man α 1-4GlcN α 1-6PI and the hybrid-GIPLs contain a core of Man α 1-3(Man α 1-6)Man α 1-4GlcN α 1-6PI. A wide variety of glycosyl groups are found attached to the aforementioned cores, including α Man, α Galp, β Galp, α Glc, β Xyl, α Rha, α GlcA and D-Arap residues [7–22]. A number of phosphoryl substituents are also found, including ethanolamine phosphate [12,14,21], 2-aminoethylphosphonate (2-AEP) [16–20,22], Glc α 1-phosphate [12] and, in the case of the lipophosphoglycans (LPGs) of the *Leishmania*, linear repeating units of [6(R-*O*-3)Gal β 1-4Man α 1-HPO₄-] where R can be H, monosaccharide or oligosaccharide (reviewed in ref. [7]). The GIPLs, LPGs and/or GPI-anchored proteins constitute the major components of the cell surfaces of all trypanosomatid parasites characterized to date [7,24,25].

Here we report the identification and structural characterization of four abundant GIPLs (Phy1–4) extracted from a *Phytomonas* sp. isolated from the plant *Euphorbia characias* [3]. This is the first detailed chemical characterization of surface molecules from the *Phytomonas* spp. The structures of the Phy1–4 GIPLs are compared with those of the other trypanosomatids and are discussed in terms of phylogenetic relationships and life cycles.

MATERIALS AND METHODS

Cell culture and glycolipid extraction

Phytomonas sp. [3] stabulates were obtained from Dr. Fred R. Opperdoes, Research Unit for Tropical Diseases, University of Louvain, Brussels, Belgium. The cells were grown in SDM-79

Abbreviations used: 2-AEP, 2-aminoethylphosphonate; AHM, 2,5-anhydromannitol; APAM, *Aspergillus phoenicis* α -mannosidase; ES-MS, electrospray mass spectrometry; GIPL, glycoinositol-phospholipid; GPI, glycosylphosphatidylinositol; GPI-PLD, GPI-specific phospholipase D; HPAEC, high-pH anion-exchange chromatography; HPTLC, high-performance TLC; JBAM, jack bean α -mannosidase; LPG, lipophosphoglycan; PI-PLC, PI-specific phospholipase C; QAE, quaternary aminoethyl; PI phosphatidylinositol.

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medium containing 10% heat-inactivated fetal calf serum and harvested at late exponential phase by centrifugation. Glycolipids were extracted and purified by the method of McConville and Bacic [26]. Briefly, the cell pellet (3×10^{10} cells) was washed with 50 ml of ice-cold PBS and extracted three times with 5 ml of chloroform/methanol/water (1:2:0.8, by vol.). After centrifugation, the solvent extract was converted into a biphasic mixture by the addition of water to give a final ratio chloroform/methanol/water of 1:2:1.4 (by vol.). The upper methanol/water-rich phase, containing polar glycolipids, was recovered and dried under a stream of N_2 . The extract was redissolved in 2 ml of 5% propanol-1-ol in 100 mM ammonium acetate (solvent A) and applied slowly (4 ml/h) to a column (15 cm $\times$ 1 cm) of octyl-Sepharose (Pharmacia) pre-equilibrated in solvent A. The column was eluted with a 100 ml gradient from 100% solvent A to 100% solvent B (60% propanol-1-ol in water) at 20 ml/h. Fractions (1 ml) were collected and 2 μ l aliquots were spotted on to silica gel 60 TLC plates and stained for carbohydrate using orcinol/ H_2SO_4 . A peak of carbohydrate-containing material eluted at about 40% propanol-1-ol was pooled, dried by rotary evaporation, redissolved in 1 ml of water and freeze-dried to remove ammonium acetate. The residue was redissolved in 40% propanol-1-ol and applied in a 14 cm-wide band to an aluminium-backed silica gel 60 high-performance TLC (HPTLC) plate (BDH-Merck). The plate was developed with chloroform/methanol/1 M ammonia (10:10:3, by vol). The edges and centre of the HPTLC were cut out and stained with orcinol/ H_2SO_4 to detect the four glycolipid bands. The four bands were then scraped from the remaining regions of the plate and eluted from the silica by sonication with chloroform/methanol/water (1:2:0.5, by vol.).

Chemical and enzymic treatments of intact glycolipids

Aliquots of HPTLC-purified glycolipids (approx. 4 μ g of carbohydrate) were incubated with and without *Bacillus thuringiensis* PI-specific phospholipase C (PI-PLC), human serum GPI-specific phospholipase D (GPI-PLD), jack bean α -mannosidase (JBAM) and HNO_2 and repartitioned into butan-1-ol for analysis by HPTLC as described previously [27]. Aliquots were also incubated in 20% propanol-1-ol, with and without 4 M NH_3 (mild base hydrolysis) at 37 °C for 16 h. The HPLC was performed as described above and the glycolipids were visualized by orcinol/ H_2SO_4 stain.

Composition analysis

Aliquots of the four HPTLC-purified glycolipids were analysed for monosaccharide, lipid and inositol content by gas chromatography-mass spectrometry and for hexosamine and ethanolamine content by amino acid analyser, as described previously [28].

Electrospray mass spectrometry (ES-MS)

Glycolipid samples were analysed after mild base hydrolysis (as described above) and drying or after digestion with *B. thuringiensis* PI-PLC [27] and partitioning of the water-soluble headgroups into the aqueous phase of a water/butan-1-ol mixture (1:1, v/v). Samples were introduced into the electrospray source of a VG Biotech Quattro triple-quadrupole mass spectrometer (Fisons Instruments) in 50% acetonitrile at 5 μ l/min. Spectra were recorded in negative-ion mode with a capillary voltage of 3.1 kV, high-voltage lens voltage of 0.35 kV and focus and skimmer voltages of 25 and 30 V respectively. For collision-induced dissociation daughter-ion spectra, the parent ion was accelerated through a potential difference of 20 or 30 V into a hexapole cell containing argon at 0.33 Pa. Multiple scans were collected and processed using MassLynx software.

HNO_2 deamination and NaB^3H_4 reduction

Aliquots of glycolipids (2 nmol, according to *myo*-inositol content) were deaminated and reduced [28]. The radiolabelled headgroup products, containing 2,5-[1- 3H]anhydromannitol (AHM) in place of the original GlcN residue, were purified by descending paper chromatography for 60 h with butan-1-ol/ethanol/water (4:1:0.6, by vol.) [29].

Analysis of the radiolabelled headgroups

Aliquots of radiolabelled headgroups were converted into neutral glycans by dephosphorylation with aq. 50% HF, desalted and analysed by Dionex high-pH anion-exchange chromatography (HPAEC) and Bio-Gel P4 gel-filtration in the presence of dextran oligomer internal standards [29] or by HPTLC alongside standards of [1- 3H]AHM and NaB^3H_4 -reduced dextran oligomers [30]. The HPTLC plates were developed once with propanol-1-ol/acetone/water (9:6:5, by vol.) and once with propanol-1-ol/acetone/water (5:4:1, by vol). The sizes of neutral glycans measured by Bio-Gel P4 chromatography are expressed in glucose units (Gu), relative to the dextran oligomer standards [29]. Similarly, the neutral glycan-elution positions on Dionex HPAEC are expressed in Dionex units (Du) relative to the dextran oligomer internal standards [29]. The migration positions of the neutral glycans on HPTLC are expressed in TLC units (Tu) relative to the migration of AHM (0 Tu) and the reduced dextran oligomers, where glucitol (Glc-ol) is 1 Tu, Glc α 1-6Glc-ol is 2 Tu, Glc α 1-6 Glc α 1-6Glc-ol is 3 Tu, etc. [30].

Samples were digested with *Aspergillus phoenicis* (Man α 1-2Man-specific) α -mannosidase (APAM) and JBAM, and subjected to acetolysis [30]. Liver α glucosidase I (generously given by Dr. Terry Butters, Oxford Glycobiology Unit, Oxford, U.K.) was used as described previously [31].

NMR spectroscopy

One-dimensional and two-dimensional (COSY) 1H -NMR spectra of PI-PLC-treated Phy2 glycolipid were recorded in 2H_2O at 30 °C [31].

RESULTS

The *Phytomonas* glycolipids are GIPL-like molecules

Four orcinol-staining glycolipid components were purified by preparative HPTLC. Glycolipids Phy1, Phy2 and Phy3 were purified to homogeneity, but the glycolipid Phy4 fraction contained approx. 50% Phy3. Each fraction was subjected to a variety of chemical and enzymic treatments and the products that partitioned into butan-1-ol were analysed by HPTLC and orcinol staining. A representative set of results, that for Phy2, is shown in Figure 1 and the results for all four glycolipids are summarized in Table 1. All of the glycolipids were sensitive to HNO_2 deamination, PI-PLC and GPI-PLD. These data define all four components as GIPL-like molecules containing a GlcN-*myo*-inositol-1-HPO $_4$ -lipid (GLcN-PI) motif. All four components could be modified by mild alkaline hydrolysis to yield more polar glycolipid species (Table 1, and see Figure 1, lanes 7 and 8), suggesting that they contain alkylacylglycerol lipid moieties. From compositional analysis, all four fractions were found to contain Man, GlcN, *myo*-inositol, 1-*O*-hexadecylglycerol and approximately equal amounts of palmitic and stearic acid. Phy2, Phy3 and Phy4 also contained ethanolamine and Phy3 and Phy4 contained Glc.

Figure 1 Chemical and enzymic treatments of native Phy2 glycolipid

Aliquots (4 μ g) of HPTLC-purified Phy2 were incubated with (+) and without (–) HNO_2 , *B. thuringiensis* PI-PLC, human serum GPI-PLD, mild base (OH^-) and JBAM as indicated. The products that partitioned into butan-1-ol were analysed by HPTLC and stained for carbohydrate with orcinol/ H_2SO_4 . The fast-moving components seen in lanes 3 and 4 and 9 and 10 are due to the charring of detergents (Triton X-100 and taurodeoxycholate respectively) that partition into butan-1-ol from the respective enzyme incubation buffers.

Structure of Phy1

Phy1 was shown to be sensitive to JBAM (Table 1), suggesting an unsubstituted α Man-containing glycan. The radiolabelled neutral glycan, prepared by deamination and NaB^3H_4 reduction, had chromatographic properties on Bio-Gel P4 and Dionex HPAEC (Table 2) that were indistinguishable from an authentic standard

Figure 2 Sequencing of the Phy1 neutral glycan

The purified deaminated and NaB^3H_4 -reduced neutral glycan from Phy1 was analysed by HPTLC and fluorography before (lane 1) and after APAM digestion (lane 2), acetolysis (Ac_2O) (lane 3) and JBAM digestion (lane 4). The positions of authentic standards of AHM, $\text{Man}\alpha 1\text{-4AHM}$ (Man_1AHM), $\text{Man}\alpha 1\text{-6 Man}\alpha 1\text{-4AHM}$ (Man_2AHM) and $\text{Man}\alpha 1\text{-2Man}\alpha 1\text{-6Man}\alpha 1\text{-4AHM}$ (Man_3AHM) are indicated on the left of the fluorograph. The TLC units (Tu) indicated on the right of the fluorograph are as described in the Materials and methods section.

of $\text{Man}\alpha 1\text{-2Man}\alpha 1\text{-6Man}\alpha 1\text{-4AHM}$ [29]. This sequence was confirmed by digestion of the Phy1 neutral glycan with the $\text{Man}\alpha 1\text{-2Man}$ -specific α -mannosidase APAM, $\text{Man}\alpha 1\text{-6Man}$ -selective acetolysis and the broad-specificity α -mannosidase JBAM. These treatments produced radiolabelled products that

Table 1 Properties of the native *Phytomonas* glycolipids

Sensitivities of the native glycolipids to HNO_2 , PI-PLC, GPI-PLD, JBAM and mild base hydrolysis (OH^-) are indicated by +; resistance is indicated by –. The first set of R_f values are of the native glycolipids on silica-gel HPTLC using chloroform/methanol/1 M ammonia (10:10:3, by vol.) as solvent. The mol % values were estimated from the relative amounts of *myo*-inositol in the HPTLC-purified Phy1–4 fractions. The second set of R_f values are of the mild-base-treated glycolipids on silica HPTLC using chloroform/methanol/1 M ammonia (10:10:3, by vol.) as solvent. The masses of the mild-base-treated glycolipids, as measured by negative-ion ES-MS, are given in the final column.

Glycolipid	R_f	<i>myo</i> -Inositol (mol%)	HNO_2	PI-PLC	GPI-PLD	JBAM	OH^-	R_f	Mass (Da)
Phy1	0.43	10	+	+	+	+	+	0.26	n.d.
Phy2	0.30	40	+	+	+	–	+	0.11	1492.6 ± 0.5
Phy3	0.14	40	+	+	+	–	+	0.03	1614.6 ± 0.5
Phy4	0.10	10	+	+	+	–	+	< 0.03	1776.8 ± 0.5

Table 2 Chromatographic properties of the neutral glycan fractions

The hydrodynamic volumes of the neutral glycans on Bio-Gel P4 are given in glucose units (Gu), relative to dextran oligomers. The elution positions of the neutral glycans on Dionex HPAEC are expressed in Dionex units (Du); see the Materials and methods section.

Neutral glycan fraction	Hydrodynamic volume (Gu)	Elution position (Du)	Proposed structure
Phy1	4.2	2.5	$\text{Man}\alpha 1\text{-2Man}\alpha 1\text{-6Man}\alpha 1\text{-4AHM}$
Phy2	5.1	3.6	$\text{Glc}\alpha 1\text{-2Man}\alpha 1\text{-2Man}\alpha 1\text{-6Man}\alpha 1\text{-4AHM}$
Phy3	5.1	3.6	$\text{Glc}\alpha 1\text{-2Man}\alpha 1\text{-2Man}\alpha 1\text{-6Man}\alpha 1\text{-4AHM}$
Phy4	6.0	3.9	$\text{Glc}\alpha 1\text{-2Glc}\alpha 1\text{-2Man}\alpha 1\text{-2Man}\alpha 1\text{-6Man}\alpha 1\text{-4AHM}$

Figure 3 Partial acid hydrolysis and JBAM digestion of the Phy1-4 neutral glycan fractions

The neutral glycans of Phy1-4 and a Man α 1-2Man α 1-2Man α 1-6Man α 1-4AHM standard (Man₄AHM) were subjected to partial acid hydrolysis (Partial H⁺) followed by JBAM digestion as indicated. The products were analysed by HPTLC and fluorography. The positions of authentic standards of AHM, Man α 1-4AHM (Man₁AHM), Man α 1-6Man α 1-4AHM (Man₂AHM), Man α 1-2Man α 1-6Man α 1-4AHM (Man₃AHM) and Man α 1-2Man α 1-2Man α 1-6Man α 1-4AHM (Man₄AHM) are indicated on the right of the fluorograph. The lane marked Dex contains NaB³H₄-reduced dextran oligomers. The TLC units (Tu) indicated on the left of the fluorograph are as described in the Materials and methods section.

Figure 4 Negative-ion ES-MS daughter-ion spectrum of the Phy1 PI-PLC product

The $[M-2]^{2-}$ pseudomolecular ion (m/z 452.6) of the Phy1 PI-PLC product was subjected to collision-induced dissociation and the daughter-ion spectrum was recorded. The inset shows the interpretation of the spectrum; the numbers in parentheses indicate the charge state (1⁻ or 2⁻) of fragment ions.

co-migrated with authentic Man α 1-6Man α 1-4AHM, Man α 1-4AHM and AHM respectively (Figure 2). The Phy1 neutral glycan was also subjected to partial acid hydrolysis and analysed by HPTLC before and after JBAM digestion (Figure 3, lanes 2 and 3). As expected, the partial hydrolysis produced a ladder of four bands that co-migrated with the upper four of the five bands generated by the partial acid hydrolysis of a Man α 1-2Man α 1-2Man α 1-6Man α 1-4AHM standard obtained from a *T. cruzi* GIPL [17] (Figure 3, lane 14). The three lower bands (Figure 3,

lane 2) were all converted into AHM on digestion with JBAM (Figure 3, lane 3).

A sample of PI-PLC-treated Phy1 produced a negative-ion ES-MS spectrum containing pseudomolecular ions at m/z 906.2 and 452.6 (results not shown) consistent with $[M-1]^{-}$ and $[M-2]^{2-}$ ions respectively of a species of composition Hex₃, HexN, inositol, (HPO₄)₁ (measured mass 907.2 ± 0.2 Da, theoretical monoisotopic mass 907.23 Da). Collision-induced dissociation of the doubly charged ion produced the daughter-ion

Table 3 Methylation-linkage analyses of dephosphorylated and *N*-acetylated glycan fractions

Partially methylated alditol acetates	Origin	Dephosphorylated <i>N</i> -acetylated glycan fraction			
		Phy1	Phy2	Phy3	Phy4
1,5-Di- <i>O</i> -acetyl-2,3,4,6-tetra- <i>O</i> -methylglucitol	Terminal Glc	—	+	+	+
1,2,5-Tri- <i>O</i> -acetyl-3,4,6-tri- <i>O</i> -methylglucitol	2- <i>O</i> -Substituted Glc	—	—	—	+
1,5-Di- <i>O</i> -acetyl-2,3,4,6-tetra- <i>O</i> -methylmannitol	Terminal Man	+	—	—	—
1,2,5-Tri- <i>O</i> -acetyl-3,4,6-tri- <i>O</i> -methylmannitol	2- <i>O</i> -Substituted Man	+	+	+	+
1,5,6-Tri- <i>O</i> -acetyl-1,2,4-tri- <i>O</i> -methylmannitol	6- <i>O</i> -Substituted Man	+	+	+	+
1,4,5-Tri- <i>O</i> -acetyl-3,6-di- <i>O</i> -methyl-(2-methylacetamido)-2-deoxyglucitol	4- <i>O</i> -Substituted GlcNAc	+	+	+	+
6- <i>O</i> -Acetyl-1,2,3,4,5-penta- <i>O</i> -methyl- <i>myo</i> -inositol	6- <i>O</i> -Substituted Inositol	+	+	+	+

Figure 5 Negative-ion ES-MS daughter-ion spectrum of the Phy2 PI-PLC product

The $[M-2]^{2-}$ pseudomolecular ion of the Phy2 PI-PLC product was subjected to collision-induced dissociation and the daughter-ion spectrum was recorded. The inset shows the interpretation of the spectrum; the numbers in parentheses indicate the charge state of the fragment ions.

spectrum shown in Figure 4. The ES-MS data confirmed the linear Hex-Hex-Hex-HexN-Ino- HPO_4 sequence and showed that there were no unidentified substituents on the inositol ring.

Taken together with the methylation-linkage analysis of the aq. HF-dephosphorylated *N*-acetylated headgroup (Table 3), Phy1 can be defined as: $\text{Man}\alpha 1\text{-}2\text{Man}\alpha 1\text{-}6\text{Man}\alpha 1\text{-}4\text{GlcN}1\text{-}6\text{myo-inositol-1-HPO}_4$ (*sn*-1-hexadecyl-2-acylglycerol). The compositional data suggest that the acyl group can be either palmitate ($\text{C}_{16:0}$) or stearate ($\text{C}_{18:0}$).

Structure of Phy2

Phy2 was shown to be resistant to JBAM (Figure 1, lanes 9 and 10), suggesting that any α Man-containing glycan was blocked by an α -mannosidase-resistant moiety. Negative-ion ES-MS of deacylated Phy2 showed a prominent pseudomolecular ion at m/z 745.3, consistent with a $[M-2]^{2-}$ ion of a species of composition (ethanolamine)₁, Hex₄, HexN, inositol, (HPO_4)₂,

hexadecylglycerol (measured mass 1492.6 ± 0.5 Da, theoretical monoisotopic mass 1492.59 Da). A sample of PI-PLC-treated Phy2 produced a negative-ion ES-MS spectrum containing a prominent pseudomolecular ion at m/z 595.1 (results not shown), consistent with a $[M-2]^{2-}$ ion of a species of composition (ethanolamine)₁, Hex₄, HexN, inositol, (HPO_4)₂ (measured mass 1192.2 ± 0.2 Da, theoretical monoisotopic mass 1192.29 Da). Collision-induced dissociation of this doubly charged ion produced the daughter-ion spectrum shown in Figure 5. The singly charged daughter ion at m/z 1111 is due to a loss of a phosphomonoester group and the singly charged daughter ion at m/z 140 corresponds to $[\text{NH}_2\text{CH}_2\text{CH}_2\text{-HPO}_3]^-$. The latter ion shows that the ethanolamine residue is attached to the rest of the structure via a phosphodiester bridge.

The radiolabelled neutral glycan, prepared by deamination and NaB^3H_4 reduction, had chromatographic properties on Bio-Gel P4 and Dionex HPAEC (Table 2) that were indistinguishable from an authentic standard of $\text{Glc}\alpha 1\text{-}2\text{Man}\alpha 1\text{-}$

Table 4 ^1H -NMR assignments for PI-PLC-treated Phy2

NMR data were recorded in $^2\text{H}_2\text{O}$ and chemical-shift values are referenced to acetone. The H-2 protons were correlated with H-1 (anomeric) protons from the COSY spectrum. The proposed structure of PI-PLC-treated Phy2 is: $\text{Glc}\alpha 1\text{-}2(\text{NH}_2\text{CH}_2\text{CH}_2\text{-HPO}_4)\text{Man}\alpha 1\text{-}2\text{Man}\alpha 1\text{-}6\text{Man}\alpha 1\text{-}4\text{GlcN}\alpha 1\text{-}6\text{myo-inositol-1-HPO}_4$. The αMan residues are numbered 1–3 from the reducing end to the non-reducing end.

Residue	H-1	H-2	$J_{1,2}$ (Hz)
$\alpha\text{Man}1$	5.39	4.10	—
$\alpha\text{Man}2$	5.11	4.02	—
$\alpha\text{Man}3$	5.26	4.17	—
αGlc	5.14	3.55	3.85
αGlcN	5.65	3.36	4.39

$2\text{Man}\alpha 1\text{-}6\text{Man}\alpha 1\text{-}4\text{AHM}$ [31]. The ^1H -NMR and ^1H - ^1H -NMR (COSY) spectra of PI-PLC-cleaved Phy2 (Table 4) were similar to those recorded for the *Torpedo* acetylcholinesterase GPI anchor [31] which has the structure: $\text{Glc}\alpha 1\text{-}2(\text{Ser-ethanolamine-HPO}_4\text{-}6)\text{Man}\alpha 1\text{-}2\text{Man}\alpha 1\text{-}6(\text{ethanolamine-HPO}_4\text{-}2)\text{Man}\alpha 1\text{-}4\text{GlcN}\alpha 1\text{-}6\text{myo-inositol-1-HPO}_4$. In particular, the spectra of both samples contained H-1 anomeric proton and H-2 proton resonances (δ 5.14 p.p.m., $J_{1,2}$ 3.85 Hz, and δ 3.55 p.p.m. respectively) consistent with the presence of an terminal αGlc residue [31].

The location of the αGlc residue was confirmed by analysis of the partial acid hydrolysate of the Phy2 neutral glycan before and after JBAM digestion (Figure 3, lanes 5 and 6). In this case, the lowest band of the ladder of five bands was unaffected by JBAM, whereas the other bands were converted into AHM. This results shows that the α -mannosidase-resistant residue (αGlc) must be attached to the non-reducing terminal αMan residue of the underlying $\text{Man}\alpha 1\text{-}2\text{Man}\alpha 1\text{-}6\text{Man}\alpha 1\text{-}4\text{AHM}$ core.

The ethanolamine phosphate moiety was located to the non-reducing terminal αMan residue of the underlying $\text{Man}\alpha 1\text{-}2\text{Man}\alpha 1\text{-}6\text{Man}\alpha 1\text{-}4\text{AHM}$ core using the method described in

[30]. The reactions are summarized in Figure 6(a). The deaminated and NaB^3H_4 -reduced glycan from Phy2 was subjected to partial acid hydrolysis and split into aliquots. On aq. HF dephosphorylation, this fraction produced a ladder of five radioactive components (Figure 6b, lane 2). These co-migrated with (from bottom to top) the untreated dephosphorylated glycan (Figure 6b, lane 1), and authentic $\text{Man}\alpha 1\text{-}2\text{Man}\alpha 1\text{-}6\text{Man}\alpha 1\text{-}4\text{AHM}$, $\text{Man}\alpha 1\text{-}6\text{Man}\alpha 1\text{-}4\text{AHM}$, $\text{Man}\alpha 1\text{-}4\text{AHM}$ and AHM standards respectively. Digestion of this dephosphorylated mixture with JBAM destroyed all of the bands except for the lowest one (Figure 6b, lane 4). This control experiment is similar to that described above in Figure 3 (lanes 5 and 6). However, if the partial acid hydrolysate mixture was treated with JBAM before dephosphorylation, then the band corresponding to $\text{Man}\alpha 1\text{-}2\text{Man}\alpha 1\text{-}6\text{Man}\alpha 1\text{-}4\text{AHM}$ was also observed (Figure 6b, lane 3). This result implies that the phosphoryl substituent (ethanolamine phosphate) was attached to the non-reducing terminal αMan residue of the underlying $\text{Man}\alpha 1\text{-}2\text{Man}\alpha 1\text{-}6\text{Man}\alpha 1\text{-}4\text{AHM}$ core (see Figure 6a).

The methylation-linkage analysis of the aq. HF-dephosphorylated *N*-acetylated headgroup (Table 3) of Phy2 confirms the presence of the terminal Glc residue, and further suggests that it is linked to the 2-position of the penultimate α -Man residue. Taken together, these data suggest that Phy2 has the structure: $\text{Glc}\alpha 1\text{-}2(\text{NH}_2\text{CH}_2\text{CH}_2\text{-HPO}_4)\text{Man}\alpha 1\text{-}2\text{Man}\alpha 1\text{-}6\text{Man}\alpha 1\text{-}4\text{GlcN}\alpha 1\text{-}6\text{myo-inositol-1-HPO}_4\text{-(sn-1-hexadecyl-2-acylglycerol)}$. The compositional data suggest that the acyl group can be either palmitate ($\text{C}_{16:0}$) or stearate ($\text{C}_{18:0}$).

Structure of Phy3

Phy3 was also shown to be resistant to JBAM (Table 1). Negative-ion ES-MS of deacylated Phy3 showed a prominent pseudo-molecular ion at m/z 806.3 (results not shown), consistent with a $[M-2]^{2-}$ ion of a species of composition (ethanolamine) $_2$, Hex $_4$, HexN, inositol, $(\text{HPO}_4)_3$, hexadecylglycerol (measured mass 1614.6 ± 0.5 Da, theoretical monoisotopic mass

Figure 6 Location of the ethanolamine phosphate substituent of Phy2

(a) Summary of the reactions used to locate the ethanolamine phosphate group. The products analysed in (b) (lanes 1–4) are indicated. [box with diagonal lines], Ethanolamine phosphate; $\triangle$, αGlc ; $\bullet$, αMan ; $\leftarrow$, $[1\text{-}^3\text{H}]\text{AHM}$. (b) The deaminated and NaB^3H_4 -reduced Phy2 headgroup was subjected to partial acid hydrolysis (Partial H^+), aq. HF dephosphorylation (HF), JBAM digestion followed by aq. HF dephosphorylation (JBAM/HF) or vice versa (HF/JBAM) as indicated. The neutral glycan products were analysed by HPTLC and fluorography. The suggested identities of the bands, indicated on the left of the fluorograph, are: AHM, $\text{Man}\alpha 1\text{-}4\text{AHM}$ (Man_1AHM), $\text{Man}\alpha 1\text{-}6\text{Man}\alpha 1\text{-}4\text{AHM}$ (Man_2AHM), $\text{Man}\alpha 1\text{-}2\text{Man}\alpha 1\text{-}6\text{Man}\alpha 1\text{-}4\text{AHM}$ (Man_3AHM) and $\text{Glc}\alpha 1\text{-}2\text{Man}\alpha 1\text{-}2\text{Man}\alpha 1\text{-}6\text{Man}\alpha 1\text{-}4\text{AHM}$ ($\text{Glc}_1\text{Man}_3\text{AHM}$). The TLC units (Tu) indicated on the right of the fluorograph are as described in the Materials and methods section.

Figure 7 Location of the ethanolamine substituents in Phy3

The negatively charged components of partial acid hydrolysates of the deaminated and NaB^3H_4 -reduced Phy2 and Phy3 headgroups were recovered from QAE-Sephadex A25, desalted and dephosphorylated with aq. HF. The resulting neutral glycans were analysed by HPTLC and fluorography. The suggested identities of the bands, indicated on the left of the fluorograph, are: AHM, $\text{Man}\alpha 1\text{-4AHM}$ (Man_1AHM), $\text{Man}\alpha 1\text{-6Man}\alpha 1\text{-4AHM}$ (Man_2AHM), $\text{Man}\alpha 1\text{-2Man}\alpha 1\text{-6Man}\alpha 1\text{-4AHM}$ (Man_3AHM) and $\text{Glc}\alpha 1\text{-2Man}\alpha 1\text{-2Man}\alpha 1\text{-6Man}\alpha 1\text{-4AHM}$ ($\text{Glc}_1\text{Man}_3\text{AHM}$).

1614.60 Da). A sample of PI-PLC-treated Phy3 produced a negative-ion ES-MS spectrum containing a prominent pseudomolecular ion at m/z 656.6 (results not shown), consistent with a $[M-2]^{2-}$ ion of a species of composition (ethanolamine)₂, Hex₅, HexN, inositol, (HPO₄)₃ (measured mass 1315.2 ± 0.2 Da, theoretical monoisotopic mass 1315.28 Da). The radiolabelled neutral glycan, prepared by deamination and NaB^3H_4 reduction, also had chromatographic properties on Bio-Gel P4 and Dionex HPAEC (Table 2) that were indistinguishable from an authentic standard of $\text{Glc}\alpha 1\text{-2Man}\alpha 1\text{-2Man}\alpha 1\text{-6Man}\alpha 1\text{-4AHM}$. Partial acid hydrolysis and JBAM digestion confirmed that the Phy3-derived glycan had a $\text{Man}\alpha 1\text{-2Man}\alpha 1\text{-6Man}\alpha 1\text{-4AHM}$ core and an α -mannosidase-resistant non-reducing terminal residue (Figure 3, lanes 8 and 9). The methylation-linkage analysis of the aq. HF-dephosphorylated *N*-acetylated headgroup (Table 3) of Phy3 confirmed the presence of a terminal Glc residue, linked to the 2-position of the penultimate α Man residue. These data suggested that Phy3 was identical with Phy2 except that it contained an additional ethanolamine phosphate group.

To locate the ethanolamine phosphate groups, partial acid hydrolysates of deaminated and NaB^3H_4 -reduced Phy2 and Phy3 were passed through a column of QAE-Sephadex A25 and the negatively charged (phosphorylated) components were eluted with 1 M ammonium acetate. After freeze-drying and aq. HF dephosphorylation, fragments that originally contained negative phosphoryl substituents were identified by HPTLC. As expected, the Phy2 sample showed that only the Man_3AHM and $\text{Glc}_1\text{Man}_3\text{AHM}$ fragments were originally phosphorylated (Figure 7, lane 1). However, all of the fragments were negatively charged in the partial acid hydrolysate of Phy3 (Figure 7, lane 2),

suggesting that the AHM residue must contain a phosphoryl substituent.

Taken together, these data suggest that Phy3 has the structure: $\text{Glc}\alpha 1\text{-2}(\text{NH}_2\text{CH}_2\text{CH}_2\text{-HPO}_4\text{-})\text{Man}\alpha 1\text{-2Man}\alpha 1\text{-6Man}\alpha 1\text{-4}(\text{NH}_2\text{CH}_2\text{CH}_2\text{-HPO}_4\text{-})\text{GlcN1-6myoinositol-1-HPO}_4\text{-(sn-1-hexadecyl-2-acylglycerol)}$. The compositional data suggest that the acyl group can be either palmitate ($\text{C}_{16:0}$) or stearate ($\text{C}_{18:0}$).

Structure of Phy4

Phy4 was also shown to be resistant to JBAM (Table 1). Negative-ion ES-MS of the deacylated Phy4 fraction showed pseudomolecular ions at m/z 806.3 (from contaminating Phy3) and at m/z 887.4. The latter ion is consistent with an $[M-2]^{2-}$ ion of a species of composition (ethanolamine)₂, Hex₅, HexN, inositol, (HPO₄)₃, hexadecylglycerol (measured mass 1776.8 ± 0.4 Da, theoretical monoisotopic mass 1776.65 Da). A sample of PI-PLC-treated Phy4 produced a negative-ion ES-MS spectrum containing a prominent pseudomolecular ion at m/z 737.6 (results not shown), consistent with a $[M-2]^{2-}$ ion of a species of composition (ethanolamine)₂, Hex₅, HexN, inositol, (HPO₄)₃ (measured mass 1477.2 ± 0.2 Da, theoretical monoisotopic mass 1477.36 Da).

The radiolabelled neutral glycan, prepared by deamination and NaB^3H_4 reduction, had unique chromatographic properties on Bio-Gel P4 and Dionex HPAEC (Table 2). Partial acid hydrolysis and JBAM digestion suggested that the Phy4-derived glycan had a $\text{Man}\alpha 1\text{-2Man}\alpha 1\text{-6Man}\alpha 1\text{-4AHM}$ core and a mannosidase-resistant non-reducing terminal residue (Figure 3, lanes 11 and 12). The methylation-linkage analysis of the aq. HF-dephosphorylated *N*-acetylated Phy4 headgroup fraction (Table 3) confirmed the presence of a terminal Glc residue and showed the presence of a 2-*O*-substituted Glc residue. The Phy4 6.0 Gu neutral glycan was digested with liver $\text{Glc}\alpha 1\text{-2Glc}$ -specific α -glucosidase I and produced a species of 5.1 Gu (results not shown).

Taken together, these data suggest that Phy4 has the structure: $\text{Glc}\alpha 1\text{-2Glc}\alpha 1\text{-2}(\text{NH}_2\text{CH}_2\text{CH}_2\text{-HPO}_4\text{-})\text{Man}\alpha 1\text{-2Man}\alpha 1\text{-6Man}\alpha 1\text{-4}(\text{NH}_2\text{CH}_2\text{CH}_2\text{-HPO}_4\text{-})\text{GlcN1-6myoinositol-1-HPO}_4\text{-(sn-1-hexadecyl-2-acylglycerol)}$. The compositional data suggest that the acyl group can be either palmitate ($\text{C}_{16:0}$) or stearate ($\text{C}_{18:0}$).

DISCUSSION

The major glycolipids of the *Phytomonas* sp. isolated from the rubber plant *Eu. characias* and grown in culture (Figure 8) are type-1 GIPLs that would appear to be part of a biosynthetic series where $\text{Phy1} \rightarrow \text{Phy2} \rightarrow \text{Phy3} \rightarrow \text{Phy4}$. All four GIPLs contain the sequence $\text{Man}\alpha 1\text{-2Man}\alpha 1\text{-6Man}\alpha 1\text{-4GlcN}\alpha 1\text{-6PI}$ and Phy2, Phy3 and Phy4 contain an ethanolamine phosphate group on the non-reducing terminal α Man residue. These features make the Phy2, Phy3 and Phy4 GIPLs very similar to GPI protein anchors, except that they are not linked to protein. Although it is possible that Phy2–4 could serve as GPI anchor precursors for *Phytomonas* surface proteins, their abundance (a minimum of 5×10^6 GIPLs per cell, based on myo-inositol content) would suggest that they are mostly end products in their own right.

Within the trypanosomatid GIPL family, only Phy1 has an identical headgroup to a previously characterized GIPL (the M3 GIPL of *Leishmania donovani* amastigotes [10]). The presence of one and two α Glc residues in Phy2–4 (Figure 8) are novel structural features for GIPLs, although the single α Glc residue of Phy3 is also found in the GPI anchor of *Torpedo californica*

Figure 8 Proposed structures of the major GPIs (Phy1-4) of *Phytomonas*

The acyl chain can be either palmitate ($n = 14$) or stearate ($n = 16$).

acetylcholinesterase [31]. The presence of ethanolamine phosphate groups (or the corresponding phosphonyl analogue, 2-AEP) on GPI molecules is quite common [7,22]. Specifically, the presence of ethanolamine phosphate attached to the GlcN residue (cf. Phy3 and Phy4) has been observed previously in the GPIs of *E. schaudinni* [21], and 2-AEP has been found in the same location in the GPIs of *T. cruzi* [16–18], *L. samueli* [19,20] and *H. samuelpessoai* [22]. However, although the presence of ethanolamine phosphate or 2-AEP groups attached to α Man residues has been observed previously (cf. Phy2–4), these have been limited to type-2 GPIs of *E. schaudinni* [21] and *L. samueli* [19,20] and hybrid GPIs of *Leishmania mexicana* [14]. The *Phytomonas* type-1 GPIs therefore represent a novel subset of the GIPL family.

The lipid moieties of the *Phytomonas* GPIs are relatively homogeneous (i.e. 1-*O*-hexadecyl-2-*O*-palmitoylglycerol and 1-*O*-hexadecyl-2-*O*-stearoylglycerol). The exclusive use of a hexadecyl ($C_{16:0}$) alkyl chain is also observed in some *T. cruzi* GPIs [18] and *T. cruzi* GPI membrane anchors [29,32–35]. This is in contrast with the situation in the *Leishmania* where the GPIs contain $C_{18:0}$, $C_{24:0}$ and $C_{26:0}$ alkyl chains and where the LPG and protein GPI anchors contain $C_{24:0}$ and $C_{26:0}$ alkyl chains [7]. The apparent homogeneity of the *Phytomonas* and *T. cruzi* GPI alkyl chains may reflect the way in which these organisms obtain fatty alcohols for the synthesis of 1-*O*-alkylglycerol 3-phosphate. For example, if the fatty alcohols were derived by conventional means (i.e. by the conversion of acyl-CoAs into fatty aldehydes and subsequent reduction to fatty alcohols [36]), then the alkyl chains would be expected to be a mixture of chain lengths. If, on the other hand, the fatty alcohols were derived from the conversion of sphinganine 1-phosphate into palmitaldehyde [36], and subsequent reduction to hexadecanol, then only this form of fatty alcohol would predominate. The possibility that *Phyto-*

monas and *T. cruzi* utilize the latter, less common, pathway deserves further study.

Overall, the *Phytomonas* GPIs are most similar to GIPL A of *T. cruzi* epimastigotes [18], i.e. they are type-1 GPIs with 1-*O*-hexadecyl-2-*O*-acylglycerol lipid components. A recent analysis of trypanosomatid large- and small-subunit rRNA sequences [37] suggests that (i) *T. cruzi* is more closely related to *Phytomonas* than to *Leptomonas*, *Endotrypanum* and *Leishmania*, (ii) *Leptomonas*, *Endotrypanum* and *Leishmania* are more closely related to each other than to *Phytomonas* and *T. cruzi* and (iii) *T. brucei* is the most ancient and distantly related of all the trypanosomatids. In this context, it is worth noting that (i) although *T. brucei* expresses very high levels of protein-linked GPI anchors, it does not make significant quantities of GPIs, (ii) *T. cruzi* and *Phytomonas* appear to make only type-1 GPIs (the type most closely related to protein-linked GPI anchors) and (iii) *Leptomonas*, *Endotrypanum* and *Leishmania* make predominantly type-2 and/or hybrid GPIs. Thus one could suggest that the GPIs have arisen through adaptations of GPI anchor biosynthesis (ubiquitous in the eukaryotes) and that the type-1 GPIs preceded the type-2 GPIs, and related structures such as the *Leishmania* LPGs. There does not appear to be any correlation between the presence or types of GPIs expressed by trypanosomatids and their monogenetic (single host) or digenetic (host and vector) life cycles. For example, the digenetic parasite *T. brucei* does not express GPIs and the monogenetic parasite *Leptomonas* expresses high levels of complex type-2 GPIs [19,20].

The function of cell-surface GPIs in trypanosomatid parasites is unknown. Their abundance suggests that they may play a general protective role by providing a dense negatively charged glycocalyx close to the surface of the plasma membrane through which other macromolecules project [7]. However, this function

alone does not explain the species-specific nature of GIPL structures. In this study we have used a *Phytomonas* sp. isolated from a plant but subsequently adapted to growth in a medium that is normally used for growing the insect stage (procyclic form) of *T. brucei*. Thus it is not clear how closely this cultured form of *Phytomonas* resembles the plant-dwelling form in terms of cell-surface biochemistry. Nevertheless, it is worth noting that the *Phytomonas* GIPLs, unlike some *Leishmania* and *Endotrypanum* GIPLs [7], do not contain any Gal residues. This may be significant since the *Phytomonas* sp. studies here lives in the latex of the lactiferous tubules of *Eu. characias*, which also contains a bivalent Gal-specific lectin [38] that might otherwise agglutinate the parasite. It is conceivable, therefore, that the plant host range of different *Phytomonas* species may be restricted by a combination of parasite surface glycoconjugate structure and plant xylem or phloem (or in this case latex) lectin specificity.

This work was supported by the MRC and The Wellcome Trust. P.S. was an EMBO long-term fellow and M.A.J.F. is a Howard Hughes International Research Scholar. We thank Malcolm McConville for helpful advice, Fred Opperdoes for supplying *Phytomonas* stabilates and Terry Butters for providing liver α -glucosidase I.

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