Biosynthesis of the glycolipid anchor of lipophosphoglycan and the structurally related glycoinositolphospholipids from *Leishmania major*

Lorna PROUDFOOT,*†, Pascal SCHNEIDER,* Michael A. J. FERGUSON* and Malcolm J. McCONVILLE*†§

*Department of Biochemistry, University of Dundee, Dundee DD1 4HN, Scotland, U.K., and †Department of Biochemistry, University of Melbourne, Parkville 3050, Vic., Australia

The major macromolecule on the surface of the protozoan parasite *Leishmania major* is a lipophosphoglycan (LPG) which contains a glycosylphosphatidylinositol glycolipid anchor. This parasite also synthesizes a complex family of abundant low-molecular-mass glycoinositolphospholipids (GIPLs) which are structurally related to the LPG anchor. In this study, *L. major* promastigotes were metabolically labelled with [³H]GlcN, and the kinetics of incorporation into free glycolipids and the LPG anchor followed to elucidate the pathway of GIPL biosynthesis and possible precursor–product relationships between the GIPLs and LPG. Labelled GIPLs were identified by TLC and by liquid chromatography of the released headgroups, before and after enzymic and chemical cleavage. On the basis of the measured specific radioactivities of the GIPLs, and their kinetics of radiolabelling, we suggest the pathway GlcN-PI → Man₁GlcN-PI (M1) → Man₂GlcN-PI (iM2) → Gal₁Man₂GlcN-PI (GIPL-1) → Gal₁Gal₁Man₂GlcN-PI (GIPL-2) → Gal₂Gal₁Man₂GlcN-PI (GIPL-3). All of the GIPLs were shown to contain alkyl-

acylglycerol or *lyso*-alkylglycerol lipid moieties with the exception of the earliest intermediate, glucosaminylphosphatidylinositol (GlcN-PI), which contained both alkylacylglycerol and diacylglycerol. A significant proportion (approx. 50%) of GIPL-3 appeared to be selectively modified by the addition of a Glc-1-PO₄ residue to one of the mannose residues (P-GIPL-3). On the basis of the specific radioactivity and kinetics of labelling of GIPL-3 and P-GIPL-3 we suggest that both of these low-abundance species are rapidly utilized as LPG precursors. The turnover of LPG and the GIPLs was also studied by [³H]Gal pulse–chase labelling and cell-surface labelling experiments. Whereas LPG was rapidly shed from the cell surface, consistent with previous studies, the GIPLs (both the total cellular and cell-surface pools) had a much slower turnover. These results suggest that the majority of the GIPLs do not act as LPG precursors and indicate that the cellular levels of these molecules is determined, at least in part, by the rate at which they are shed from the cell surface.

INTRODUCTION

Protozoan parasites belonging to the genus *Leishmania* cause a number of widespread diseases in man. These parasites have a digenetic life cycle, alternating between a flagellated promastigote stage that resides within the digestive tract of the insect vector (phlebotomine sandflies) and an intracellular amastigote stage that proliferates within the phagolysosome of mammalian macrophages. The major macromolecule on the promastigote surface is a complex lipophosphoglycan (LPG) which is thought to be required for parasite survival in both the insect and mammalian hosts [1,2]. In this regard, it appears to form a protective barrier against digestive enzymes in the insect midgut [3,4] and also to mediate the attachment of promastigotes to midgut epithelial cells [5,6]. In the mammalian host, LPG may protect the sandfly-introduced promastigotes from complement-mediated lysis [7] and also have a role in facilitating the attachment of promastigotes to the host macrophage [1,2,8]. It is also thought to inhibit a number of macrophage microbicidal activities that are dependent on protein kinase C signal-transduction pathways [1,2].

The LPGs of all *Leishmania* appear to have a conserved backbone comprised of a linear phosphoglycan chain and an unusual glycosylphosphatidylinositol (GPI) glycolipid anchor [9–11]. The phosphoglycan chain is made up of repeating PO₄-6Galβ1–4Man units, which may be elaborated with species-specific glycan side chains, whereas the GPI anchor contains a

hexasaccharide core linked to a *lyso*-alkylphosphatidylinositol (PI) lipid moiety (Figure 1). This anchor diverges from the well-characterized GPI protein anchors, beyond the conserved core sequence Manα1-4GlcNα1-6myo-inositol-1-PO₄-lipid [2]. Recent studies on the biosynthesis of the LPG in microsomal preparations have shown that the disaccharide repeat units of the phosphoglycan chains can be built up by repeated cycles of addition of Man-1-PO₄ and Gal residues to an exogenously added glycolipid acceptor [12,13]. However, to date there is little information on the nature of the endogenous acceptor and its biosynthesis.

We have previously shown that the major glycolipids in *L. major* belong to a family of glycoinositolphospholipids (GIPLs) that are structurally related to the LPG anchor (for structures and nomenclature of these type-2 GIPLs see Figure 1) [14,15]. These glycolipids are abundant components on the cell surface where they are thought to cover a significant proportion of the plasma membrane [16]. Although these studies suggest that most of the GIPLs are metabolic end products, we have proposed, on the basis of their structural relatedness to the LPG anchor [2,15], that a small proportion of the more polar species may also act as precursors of the LPG.

In this study we have investigated the biosynthesis *in vivo* of the type-2 GIPLs and the LPG anchor in *L. major* promastigotes. These results provide evidence for a common biosynthetic pathway and are consistent with the notion that some of the more polar GIPL species may act as precursors of LPG. In

Abbreviations used: LPG, lipophosphoglycan; GIPL, glycoinositolphospholipid; P-GIPL, phosphorylated or Glc-1-PO₄-substituted GIPL; PI, phosphatidylinositol; GPI, glycosylphosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; AHM, 2,5-anhydromannitol; HPAE-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; HPTLC, high-performance TLC; Du, Dionex units.

† Present address: Department of Immunology, University of Glasgow, Glasgow G11 6NT, Scotland, U.K.

§ To whom correspondence should be sent at the University of Melbourne address.

Figure 1 Structure of *Leishmania* LPG and type-2 GPIs

The structure of the LPG anchor is conserved in all species of *Leishmania* [9–11]. The type-2 GPIs have been mainly characterized from *L. major* where they are the major class of glycolipid [15,19]. Type-2 GPIs containing a Glc-1-PO₄ residue on the mannose distal to the GlcN have been found in various levels in both *L. major* and *Leishmania mexicana* promastigotes [17,19,20].

addition, we show that, although both the GPIs and the LPGs are expressed at the cell surface, the GPIs have a much slower turnover, which may account for the finding that these glycolipids are the major glycoconjugates of these parasites.

MATERIALS AND METHODS

Materials

Aluminium-backed silica-gel 60 high-performance TLC (HPTLC) plates were obtained from Merck (Darmstadt, Germany), glucose-free Schneider's *Drosophila* medium was from Life Technologies, and [6-³H]GlcN hydrochloride (40 Ci/mmol), [6-³H]Gal (34 Ci/mmol) and NaB[³H]₄ (9–11 Ci/mmol) were purchased from DuPont. Jack-bean α -mannosidase and coffee-bean α -galactosidase were from Boehringer (Mannheim, Germany) and calf intestine alkaline phosphatase was from Sigma. Phosphatidylinositol-specific phospholipase C (PI-PLC) from

Bacillus thuringiensis was generously given by Dr. Martin G. Low (Columbia University, New York, NY, U.S.A.). [³H]GlcN-labelled dimyristoyl-GlcN-PI from *Trypanosoma brucei* was a gift from Dr. Lucia Güther (University of Dundee). All other reagents were of analytical grade.

Cell culture

Promastigotes of *L. major* V121 strain were maintained in Schneider's *Drosophila* medium supplemented with 10% heat-inactivated fetal calf serum. Cells were subcultured every 3–4 days.

Metabolic labelling of promastigotes

Promastigotes in exponential phase of growth (2×10^7 – 3×10^7 cells/ml) were harvested by centrifugation (1000 *g*, 5 min), washed with labelling medium and resuspended at a density of 10^8 cells/ml in 5 ml of labelling medium (glucose-free Schneider's medium containing 2.5% BSA and, for GlcN labelling uniquely, 0.8 μ g/ml tunicamycin). Cells were preincubated for 30 min at 27 °C before the addition of [³H]GlcN (80 μ Ci/ml) or [³H]Gal (20–40 μ Ci/ml). In [³H]Gal pulse-chase experiments, the cell cultures were split into two after 1 h incubation and unlabelled galactose (2 mM final concentration) was added to one batch. Aliquots of the labelling reaction mixture (500 μ l) were removed at the indicated time and were added to 100 μ l of Schneider's medium containing 10 mM glucose. Cells were immediately recovered by centrifugation (6000 *g*; 30 s) and the pellet was extracted twice with 500 μ l of chloroform/methanol/water (1:2:0.8, by vol.) with vortex mixing and sonication at room temperature until the pellet was suspended as very fine particles. Insoluble material was removed by centrifugation (16000 *g*; 5 min) and the pooled supernatants dried under a stream of nitrogen. This fraction contained the GPIs. The pellet was briefly dried under nitrogen and re-extracted twice with 500 μ l of 9% butan-1-ol in water, with sonication. The pooled supernatants were taken to dryness. This fraction contained the LPG.

Isolation of individual glycolipid species

[³H]Glc-labelled glycolipids purified by octyl-Sepharose chromatography (see below) were applied to silica-gel 60 HPTLC (approx. 1.5×10^8 c.p.m./cm) and the plate was developed in solvent B (see below). Labelled glycolipids were localized with a radioactivity linear analyser (RITA; Raytest), individually scraped from the plate, eluted from the silica with chloroform/methanol/water (10:10:3, by vol.) (3×350 μ l, with sonication) and stored in 40% propan-1-ol. Isolated glycolipids were used for the deamination and reduction procedure or for deacylation (as described below).

Deacylation of GPI glycolipids

Deacylation of the GPIs and dimyristoyl-GlcN-PI standard from *T. brucei* was achieved in a mixture of 40% propan-1-ol/13 M ammonia (1:1, v/v; 100 μ l) for 16 h at 37 °C [17]. The samples were dried under nitrogen and analysed by HPTLC (solvent B) either directly or after partition between water (100 μ l) and water-saturated butan-1-ol (200 μ l).

Generation and purification of glycan headgroups from biosynthetically labelled GPIs

The processing of [³H]GlcN-labelled GPIs and LPG, which is summarized in Figure 2, was designed to identify unambiguously

Figure 2 Purification scheme for the analysis of $[^3\text{H}]\text{GlcN}$ -labelled GIPLs and LPG glycan core

Abbreviation: Du, Dionex units.

and quantify the different glycan headgroups of neutral and phosphorylated GIPLs and the glycan core of LPG. GIPLs were resuspended in 0.1 M ammonium acetate, pH 7.0, containing 5% propan-1-ol and loaded on to a column (1 ml) of octyl-Sepharose equilibrated in the same buffer. The column was washed with 3 ml of buffer, followed by 40% propan-1-ol (1 ml). Lipids eluted with 40% propan-1-ol were freeze-dried to remove volatile salts, then treated with PI-PLC in 20 mM Tris/acetate, pH 7.4, containing 0.1% Triton X-100 (10 μl ; 60 m-units; 37 $^{\circ}\text{C}$; 16 h). Undigested lipids, including dolichol-containing species, were removed by phase partitioning in a two-phase mixture containing 100 μl of water and 200 μl of water-saturated butan-1-ol. The delipidated glycans recovered in the aqueous phase were taken to dryness, redissolved in sodium acetate buffer, pH 4.0 (0.4 M; 15 μl) and NaNO_2 (1 M; 7.5 μl) and the mixture was deaminated for 2.5 h at 40 $^{\circ}\text{C}$. Boric acid (5 μl ; 0.4 M) was added and the pH adjusted to pH 9–10 with 2 M NaOH. The deaminated glycans were immediately reduced with NaBH_4 (1 M; 10 μl ; 30 min; room temperature). Excess reductant was destroyed with acetic acid and the mixture was passed down a column of AG50 (X12; H^+ form) (200 μl each) and fractionated into two classes by high-performance anion-exchange (HPAE) chromatography using a CarboPac PA1 column on a Dionex HPLC column equipped with a pulsed ampero-

metric detector (PAD). The column was eluted with 0.15 M NaOH/12.5 mM sodium acetate for 1 min, then with a linear gradient of sodium acetate (12.5–55 mM) in 0.15 M NaOH over 60 min at a flow rate of 0.6 ml/min. The elution time of the labelled species was determined relative to a mixture of co-injected dextran oligomers (detected by PAD) and expressed as Dionex units (Du). Labelled glycans eluted between 0.8 and 2.5 Du (i.e. glycans from GlcN-PI, M1 and iM2) and 5.5 and 7.0 Du (i.e. glycans from GIPL-1, -2, -3) were pooled, desalted on a mixed-bed ion-exchange column and analysed by HPTLC in solvent system A (see below). Glycans batch-eluted from the QAE-Sephadex column were freeze-dried twice and treated with alkaline phosphatase (4 units in 20 μl of 0.1 M ammonium bicarbonate). The mixture was desalted and analysed by HPAE-PAD. Glycans eluted between 5.5 and 7.0 Du were pooled, desalted and analysed by HPTLC. In some instances, the total mixture of deaminated and reduced glycolipid headgroups was directly fractionated by silica-gel HPTLC using solvent system A. The plate was scraped in 2 mm fractions and glycans were eluted from the silica with 40% propan-1-ol. Radioactivity migrating in the false front contained the P-GIPL-3 headgroup [18,19], which was digested with alkaline phosphatase, desalted and analysed by HPAE-PAD as described above.

Generation and isolation of biosynthetically labelled LPG core

The LPG fraction was subjected to mild acid hydrolysis (40 mM trifluoroacetic acid; 100 $^{\circ}\text{C}$; 10 min) [9]. Acid was removed by evaporation and the released glycolipid anchor was separated from the hydrophilic repeat units by octyl-Sepharose chromatography. The bound fraction was deaminated and reduced,

fractionated on QAE-Sephadex, treated with alkaline phosphatase and analysed by HPTLC as described above for the phosphorylated GIPLs.

Composition of the total GIPL pool

For the characterization of unlabelled GIPLs, a mixture of cellular GIPLs was deaminated and reduced with $\text{NaB}[\text{H}_4]$ [15]. The labelled glycans were purified from radiochemical impurities by descending paper chromatography in butan-1-ol/ethanol/water (4:1:0.6, by vol.) or HPTLC using solvent system A. The ratio of the various GIPLs was determined by counting the radioactivity incorporated into 2,5-anhydromannitol (AHM) after the GIPL headgroups were separated by HPTLC and HPAE as described above.

Microsequencing of the GIPL headgroups

Desalted neutral glycans were sequenced by digestion with jack-bean α -mannosidase (0.8 unit in 30 μl of 0.1 M sodium acetate buffer, pH 5.0; 16 h; 37 °C), coffee-bean α -galactosidase (0.8 unit in 30 μl of 0.1 M sodium citrate/phosphate buffer, pH 6.0; 16 h; 37 °C) or by mild acid hydrolysis under conditions known to hydrolyse specifically hexofuranosidic linkages (40 mM trifluoroacetic acid; 100 μl ; 1 h; 100 °C) [15]. Enzyme reactions were stopped by heating for 5 min at 100 °C and reaction mixtures desalted by passage through a column of AG50 (X12; H^+ form) over AG3 (X4; OH^- form) (200 μl of each). The products of enzyme and chemical treatments were analysed by HPTLC in solvent system A.

Surface labelling of promastigote GIPLs and LPG

Cell-surface glycoconjugates containing galactopyranose residues with a free C-6 hydroxyl were labelled by the galactose oxidase/ $\text{NaB}[\text{H}_4]$ procedure as previously described [16]. Surface-labelled promastigotes were maintained in full Schneider's *Drosophila* medium. Samples of cells were removed at the indicated times and labelled GIPLs and LPG were extracted and purified by octyl-Sepharose chromatography as described above.

TLC

Neutral oligosaccharides were separated using solvent system A [propan-1-ol/acetone/water (9:6:5, by vol.) or (9:6:4, by vol.)], followed by an optional second development in the same dimension using propan-1-ol/acetone/water (5:4:1, by vol.) [18]. Glycolipids were resolved using solvent B [chloroform/methanol/0.25% KCl (10:10:3, by vol.)]. Radioactivity was detected by fluorography, after the HPTLC sheets were sprayed with En^3Hance . For quantification purposes, the exposed film was used to locate precisely the position of the radioactive bands of interest. Silica from the corresponding areas was scraped into scintillation counting vials and mixed with water before the addition of scintillant.

RESULTS

Identification of early intermediates in GIPL and LPG biosynthesis

L. major promastigotes were metabolically labelled with $[\text{H}]\text{GlcN}$ to identify the early intermediates in the biosynthesis of the GIPLs and the LPG anchor. This monosaccharide is a constituent of all GPI glycolipids and is not significantly metabolized to other monosaccharides present in the GIPLs (see Figure 4), providing a quantitative measure of *de novo* GPI synthesis.

When the total pool of $[\text{H}]\text{GlcN}$ -labelled GPIs was extracted after a 4 h incubation and analysed by HPTLC, a complex pattern of at least eight bands was observed (Figure 3a, lane labelled Tot). These GPI species were purified as six bands by preparative HPTLC and characterized as described below. Information on the lipid moiety was obtained by HPTLC analysis of the products of mild base treatment, and the identity of GPI species in each band was determined by analysis of the glycan headgroups which were released by HNO_2 deamination and NaBH_4 reduction. The released glycans were identified from their co-migration with authentic glycan standards on both HPTLC and Dionex HPLC and from the HPTLC analysis of the products of exoglycosidase digestion and mild acid hydrolysis.

Band 1 (Figure 3a) migrated as a doublet on HPTLC, close to a GlcN-PI standard from *T. brucei*, and yielded only AHM after HNO_2 deamination (Figure 3b, lane 1). Three distinct products were generated by mild base hydrolysis. The major product had a lower R_f value than the native species (Figure 3a) but still partitioned into the butan-1-ol phase (results not shown). It is likely that this band corresponds to GlcN-*lyso*-alkyl-PI. The two minor products had a much lower R_f value (Figure 3a) and no longer partitioned into the butan-1-ol phase (results not shown). The lower product co-migrated with an authentic standard of GlcNAc1-6*myo*-inositol-1- PO_4 -glycerol, suggesting that a portion of the original band-1 doublet is GlcN-diacyl-PI. The nature of the middle band is not clear, although it may correspond to the glycan headgroup of GlcNAc-diacyl-PI, which would not be detected in the analysis of deaminated/reduced headgroups.

Band 2 contained a major GPI species, which migrated as a more polar species after base treatment, and a minor contamination with band 1. HNO_2 deamination yielded a jack-bean α -mannosidase-sensitive glycan that co-migrated with Man α 1-4AHM (Figure 3b, lane 2). These data suggest that band 2 corresponds to M1 (see Figure 1 for nomenclature) with an alkylacyl-PI lipid moiety.

Band 3 contained a single GPI species which co-migrated with authentic GIPL-1 on HPTLC and was susceptible to base treatment. HNO_2 deamination released a major glycan which co-migrated with the authentic GIPL-1 glycan on HPTLC (Figure 3b, lane 3). The terminal monosaccharide of this glycan was resistant to α -mannosidase digestion, but was removed after mild acid hydrolysis under conditions known to cleave hexofuranosidic linkages [15]. The acid-hydrolysis product co-migrated on HPTLC with Man α 1-3Man α 1-4AHM and was susceptible to α -mannosidase treatment giving a product that co-migrated with AHM (Figure 4). These data suggest that band 3 corresponds to GIPL-1 with an alkylacyl-PI lipid moiety.

Band 4 contained a complex of four GPI species. The major components in the upper, middle and lower bands in this complex were base-stable and co-migrated with the *lyso* derivatives of M1, iM2 and GIPL-1 respectively (Figure 3a). A minor base-sensitive component was also present which co-migrated with a GIPL-2 on HPTLC and after base treatment with a *lyso*-GIPL-2 standard containing $\text{C}_{24:0}$ alkyl chains. The presence of these four species was confirmed by HPTLC analysis of the glycan headgroups (Figure 3b, lane 4), and by sequencing of the individual HPAE-purified glycans (Figure 4 and Table 1).

Band 5 contained a single base-resistant band which co-migrated with *lyso*-GIPL-2 containing $\text{C}_{24:0}$ alkyl chains (Figure 3a). HNO_2 deamination released a glycan that co-migrated with the authentic GIPL-2 glycan on HPTLC (Figure 3b), supporting the assignment of this species as *lyso*-GIPL-2.

At least three other GPI species were identified by scraping the silica of the preparative HPTLC between the origin and Band 5. HNO_2 deamination of this fraction released three glycan species

Figure 3 HPTLC analysis of the lipid moieties and glycan headgroups of isolated [³H]GlcN-labelled GIPLs

(a) [³H]GlcN-labelled GIPLs were partially purified by HPTLC, and bands 1–5 reanalysed in solvent B before (–) and after (+) mild base treatment. Tot, total GIPL mixture from 4 h time point. The migration positions of authentic lyso-GIPL-2 (lyso-2) with C_{24:0} and C_{18:0} alkyl chains is indicated. GIPG indicates the migration position of authentic GlcNAc1-6-*myo*-inositol-1-PO₄-glycerol. o, Origin. (b) The mixture of GIPLs and the HPTLC-purified bands were deaminated and reduced. The total mixture was further treated with (Tot JBAM) or without (Tot) jack-bean α -mannosidase. Released glycans were analysed by HPTLC in solvent system A. The migration positions of authentic standards are indicated along the left-hand margin: M1, Man α 1-4AHM; iM2, Man α 1-3Man α 1-4AHM; M2, Man α 1-6Man α 1-4AHM; GIPL-1, Gal β 1-3Man α 3 Man α 1-4AHM; GIPL-2, Gal α 1-3Gal β 1-3Man α 1-3Man α 1-4AHM; GIPL-3, Gal α 1-6Gal α 1-3Gal β 1-3Man α 1-3Man α 1-4AHM. Band > 5 represents the pool of glycolipids migrating slower than band 5 in solvent B [see (a)]. Dex, NaB³H₄-reduced dextran hydrolysate standard.

which co-migrated with the deaminated glycans of GIPL-2, GIPL-3 and GIPL-A (an isomer of GIPL-3 in which the terminal Gal is in β 1-3 rather than α 1-6 linkage) (Figure 3b). The putative GIPL-3 glycan was digested by coffee-bean α -galactosidase to give a product that co-migrated on HPTLC with the GIPL-1 glycan, consistent with it having this structure (Figure 4).

Identification of polar intermediates in GIPL and LPG biosynthesis

We have shown previously that some *Leishmania* strains synthesize highly polar type-2 GIPLs in which one of the core mannose residues is substituted by a Glc-1-PO₄ residue (P-GIPLs, see Figure 1) [17,19,20]. This type of substitution occurs on the LPG anchor [10,11,20] raising the possibility that the P-GIPLs

may be the immediate precursors of LPG. GIPLs with the expected properties of the P-GIPLs were identified as very minor components (less than 0.6% of the total pool) in the *L. major* strain used in this study. The glycan headgroups of these species were obtained by deamination and NaB³H₄ reduction of the total GPI pool and fractionation on QAE-Sephadex chromatography to remove neutral glycan headgroups. The labelled glycans migrated in the false front on HPTLC (a characteristic of charged species), but were converted into their corresponding neutral glycans by alkaline phosphatase treatment and identified by their co-migration with standards on HPTLC or HPAE. Their susceptibility to alkaline phosphatase arises because the capping glucose residue is removed during HNO₂ deamination leaving a phosphate monoester group [19]. The same protocol

Figure 4 Microsequencing of the glycan headgroups of [³H]GlcN-labelled GIPLs

The deaminated and reduced [³H]GlcN-labelled glycan headgroups were purified by HPTLC and Dionex HPLC. They were sequenced by using one or several of the following treatments, as indicated at the bottom of the figure: mild acid hydrolysis for 1 h in 40 mM trifluoroacetic acid (TFA), jack-bean α -mannosidase (JBAM), alkaline phosphatase (APase) and coffee-bean α -galactosidase (CBAG). When two treatments were performed on the same sample, TFA preceded JBAM and APase preceded CBAG. The products were analysed by HPTLC using solvent system A. The migration positions of authentic standards are shown along the left-hand margin (for detailed structures, see Figure 3 legend). The main glycan headgroup present in each purified fraction is indicated at the top of the figure. o, Origin.

Table 1 Composition of [³H]GlcN-labelled glycolipid bands

Bands are numbered as shown in Figure 3.

Band	GIPLs	Schematic structure
Band 1	GlcN-PI	GlcN-alkylacyl-PI
	GlcN-PI	GlcN-diacyl-PI
	GlcNAc-PI	GlcNAc-diacyl-PI
Band 2	M1	Man ₁ GlcN-alkylacyl-PI
Band 3	iM2	Man ₂ GlcN-alkylacyl-PI
Band 4	GIPL-1	Gal ₁ Man ₂ GlcN-alkylacyl-PI
	<i>lyso</i> -M1	Man ₁ GlcN- <i>lyso</i> -alkyl-PI
	GIPL-2	GalGal ₁ Man ₂ GlcN-alkylacyl-PI*
	<i>lyso</i> -iM2	Man ₂ GlcN- <i>lyso</i> -alkyl-PI
	<i>lyso</i> -GIPL-1	Gal ₁ Man ₂ GlcN- <i>lyso</i> -alkyl-PI
Band 5	<i>lyso</i> -GIPL-2	GalGal ₁ Man ₂ GlcN- <i>lyso</i> -alkyl-PI*
Band > 5	<i>lyso</i> -GIPL-A	Gal β 1-3GalGal ₁ Man ₂ GlcN- <i>lyso</i> -alkyl-PI
	<i>lyso</i> -GIPL-3	Gal α 1-6GalGal ₁ Man ₂ GlcN- <i>lyso</i> -alkyl-PI

* GIPL-2 and *lyso*-GIPL-2 contained mainly C_{24:0} alkyl chains (and little C_{18:0} alkyl chains), as shown by co-migration with defined standards.

was used (except that NaBH₄ was used instead of NaB³H₄) to identify [³H]GlcN-labelled P-GIPLs (Figure 2). As shown in Figure 5(a), three glycan headgroups were identified that bound

to QAE-Sephadex and co-migrated with the headgroups of GIPL-1, GIPL-2 and GIPL-3 respectively after alkaline phosphatase treatment. The identity of the putative P-GIPL-3 was supported by the susceptibility of the derived neutral glycan to α -galactosidase treatment (Figure 4). This fraction was highly enriched for the GIPL-3 glycan and did not contain the glycan headgroups of M1 or iM2, suggesting that it was not contaminated with the neutral glycan headgroups of the major GIPLs.

The same approach was used to measure [³H]GlcN incorporation into the glycolipid anchor of LPG. The depolymerized LPG was fractionated on octyl-Sepharose to obtain the GPI anchor which was then deaminated and reduced, fractionated on QAE-Sephadex, and dephosphorylated before analysis by HPTLC. This procedure gave a highly purified LPG core that was the only radiolabelled component (Figure 5a).

The neutral GIPLs belong to a biosynthetic series

Given the complexity of the native GIPL HPTLC profile, where several glycolipid species co-migrate, further experiments were performed by analysing the ³H-labelled GIPL headgroups rather than the intact GIPLs. The protocol used to detect and quantify the GIPLs is summarized in Figure 2. This procedure allows us to purify, unambiguously identify and quantify the [³H]GlcN-labelled LPG core and the [³H]GlcN-labelled GIPL species,

Figure 5 Kinetics of incorporation of $[^3\text{H}]\text{GlcN}$ into the GIPLs and LPG glycan core

Headgroups of neutral GIPLs, phosphorylated GIPLs and the LPG core (a) were obtained as described in Figure 2 and in the Materials and methods section. Neutral GIPL headgroups were fractionated into a 0.8–2.5 Du (a) (lanes A) and a 5.5–7.0 Du (a) (lanes B) fractions by Dionex HPLC. Migration positions of authentic glycans are shown on the sides (see Figure 1 for their detailed structures), and the time of labelling (in min) is indicated at the bottom. The HPTLC plates were developed in solvent system A. (b) and (c) Quantification of the HPTLCs shown in (a). Note that the LPG core is shown in both (b) and (c). In (b): $\square$, GlcN-PI; $\triangle$, M1; $\bullet$, iM2; $\circ$, GIPL-1; $\blacksquare$, GIPL-2; $\blacktriangle$, LPG core. In (c): $\triangle$, GIPL-3; $\circ$, P-GIPL-1; $\blacksquare$, P-GIPL-2; $\square$, P-GIPL-3; $\blacktriangle$, LPG core.

GlcN-PI, M1, iM2, GIPL-1, GIPL-2, GIPL-3, P-GIPL-1, P-GIPL-2 and P-GIPL-3, with no distinction being made with regard to the nature of the lipid portion.

The time course of continuous biosynthetic labelling with $[^3\text{H}]\text{GlcN}$ is shown in Figure 5. Over the time of the experiment (2 h), only two glycolipids were labelled to steady state: GlcN-PI after about 30 min, and M1 after about 1 h; all the other glycolipid species and the LPG core were still incorporating the label at this time. Attempts to label other GIPL species to steady state, by labelling for longer periods, were not successful because the rate of $[^3\text{H}]\text{GlcN}$ incorporation fell significantly after 2 h, probably because of reduced cell metabolism in the glucose-free medium. These data indicate that GlcN-PI and M1 are early glycolipid intermediates in the pathway, as expected from their structure. Precursor-product relationships between the other

neutral GPI species were deduced from the kinetics of $[^3\text{H}]\text{GlcN}$ incorporation (Figure 5b) and the specific radioactivities of each species at 2 h (Table 2). These data support the following biosynthetic sequence for the neutral GIPLs: GlcN-PI $\rightarrow$ M1 $\rightarrow$ iM2 $\rightarrow$ GIPL-1 $\rightarrow$ GIPL-2 $\rightarrow$ GIPL-3.

Candidate precursor GPIs for the LPG anchor

On the basis of their structures, GIPL-3 and P-GIPL-3 are the most likely candidate precursor species for the LPG anchor. This is consistent with the finding that both had the same (or higher) specific radioactivity as the LPG core after 2 h of labelling (Table 2). However, incorporation of label into these GIPLs always lagged behind the LPG core. For example, whereas incorporation into the LPG core was detected after 10 min, labelled GIPL-3

Table 2 Structures and specific activities of [³H]GlcN-labelled GIPL species from *L. major* promastigotes

The elution positions on Dionex of the deaminated and reduced glycan headgroups (and dephosphorylated in the case of the P-GIPLs) are shown. Unlabelled GIPL was determined by the amount of radioactivity incorporated into AHM after deamination/ NaB^3H_4 reduction of a pool of unlabelled glycolipids extracted from the same parasites as used for the biosynthetic labelling with GlcN. Specific activity is given relative to M1, which was given an arbitrary specific radioactivity of 100. +, present; ND, not determined.

GIPL	Schematic structure	Glycerolipid composition (% of total)			Elution position on Dionex (Du)	Unlabelled GIPL (mol %)	Specific activity (at 2 h)
		Diacyl	Alkylacyl	/iso-Alkyl			
GlcN-PI	GlcN-PI	23	77	—	1.0	0.4*	100*
M1	Man ₁ GlcN-PI	—	62	38	1.1	3	100
iM2	Man ₂ GlcN-PI	—	8	92	2.1	7	40
GIPL-1	Gal ₁ Man ₂ GlcN-PI	—	56	44	6.3	26	5
GIPL-2	GalGal ₁ Man ₂ GlcN-PI	—	63	37	6.1	37	1.8
GIPL-3	Gal α 1-6GalGal ₁ Man ₂ GlcN-PI	—	—	+	5.9	2	1.4
GIPL-A	Gal β 1-3GalGal ₁ Man ₂ GlcN-PI	—	—	+	8.0	6	ND
P-GIPL-1	Gal[Glc-PO ₄] ₁ Man ₂ GlcN-PI†	—	ND	—	~ 6.3	< 0.2	> 6
P-GIPL-2	GalGal ₁ [Glc-PO ₄] ₁ Man ₂ GlcN-PI	—	ND	—	~ 6.0	< 0.2	> 7
P-GIPL-3	Gal α 1-6GalGal ₁ [Glc-PO ₄] ₁ Man ₂ GlcN-PI	—	ND	—	5.9	0.2	13
LPG core	-PO ₄ -Gal α 1-6GalGal ₁ [Glc-PO ₄] ₁ Man ₂ GlcN-PI	—	ND	—	ND	20	1.2

* Derived from values for M1, assuming that these two glycolipids have the same specific radioactivity at steady state.

† The presence of the acid-labile Glc residue has not been shown in this study but it is likely to be present on all phosphorylated species.

Figure 6 Pulse-chase labelling of GIPLs and LPG with [³H]galactose

Labelling was performed as described in the Materials and methods section. The chase was initiated in half of the culture at 1 h. ●, Continuous labelling; ○, chase. (a) Kinetics of labelling of the galactosylated GIPL pool (i.e. HPTLC-purified GIPL-1, GIPL-2 and their respective /iso derivatives); (b) kinetics of labelling of LPG (values refer to radioactivity eluted in the LPG fraction after octyl-Sepharose purification).

Figure 7 Turnover of surface-labelled LPG and GIPLs

Promastigotes surface-labelled by the galactose oxidase/ NaB^3H_4 procedure were resuspended in Schneider's *Drosophila* medium containing 1% BSA. Aliquots were removed at the indicated times and the amount of radioactivity in the octyl-Sepharose-purified LPG (○) and in the galactosylated GIPL pool (●) are shown as a percentage of the time-zero levels (2.3×10^5 c.p.m./ 8×10^8 cells for LPG and 1.3×10^5 c.p.m./ 8×10^8 cells for GIPLs).

and P-GIPL-3 were only detected after 20–30 min (Figure 5c). None of these species reached steady state over 2 h. This pattern of labelling could be explained if most of the newly synthesized GIPL-3 and P-GIPL-3 species were rapidly utilized as LPG precursors, preventing their accumulation to detectable levels until after 20–30 min.

The minor P-GIPL-1 and P-GIPL-2 species were labelled with the same kinetics as the P-GIPL-3 (Figure 5c). Interestingly, there are marked differences in the extent to which GIPLs1–3 become phosphorylated. Although only 5% and 10% of the

labelled GIPL-1 and GIPL-2 pools were phosphorylated, approx. 50% of the GIPL-3 pool contained this substituent, despite being the least abundant species. These data suggest that newly synthesized GIPL-3 is preferentially modified by the addition of Glc-1-PO₄, compared with either GIPL-1 or -2.

Turnover of *in vivo* and surface-labelled GIPLs and LPG

The rates of turnover of GIPLs and LPG were estimated in two different experiments. In the first experiment, promastigotes were pulse-labelled with [³H]galactose for 60 min and then chased with unlabelled galactose (Figures 6a and 6b). In contrast with [³H]GlcN, this monosaccharide was incorporated without a lag into pre-existing GIPL and LPG pools. This was shown by the finding that most of the galactose label in the LPG molecule was found in the galactosylated phosphosaccharide repeats rather than the LPG core. As compared with the control without chase, the amount of label in the cell-associated LPG fell rapidly, suggesting that the LPG pool is turned over either by degradation or (more likely) by release into the medium, as has been previously documented [21,22]. In contrast, no detectable turnover of [³H]Gal-labelled GIPLs was observed under the same conditions. Most of the [³H]Gal label was present in the Gal-containing GIPLs (GIPL-1, GIPL-2 and *lyso*-GIPL-2 in the ratio 3:4:2) (results not shown) suggesting that these GIPLs are stable cell-associated end products.

In the second experiment, cell-surface LPG and GIPLs were labelled using galactose oxidase and NaB³H₄, and the loss of labelled LPG and GIPLs was monitored over time (Figure 7). In this case, LPG showed relatively rapid loss from the cell surface (with almost all the label lost by 12 h) whereas the GIPLs displayed a much lower rate of loss, with about half of the label still retained after 24 h of culture. The ratio of label in the individual cell-surface-labelled GIPLs (GIPL-2, *lyso*-GIPL-2 and GIPL-3, in the ratio, 65:30:5 respectively) remained unchanged throughout the chase period (results not shown), suggesting that there is no selective loss of any one species. Taken together, these experiments suggest that the overall turnover of the LPG and GIPLs is markedly different and that this is due, at least in part, to the rate at which they are removed from the cell surface.

DISCUSSION

The major GPI species to be labelled with [³H]GlcN in *L. major* promastigotes appeared to belong to the type-2 GIPL and LPG anchor biosynthetic pathway. This pathway can be delineated as follows, GlcN-PI → M1 → iM2 → GIPL-1 → GIPL-2 → GIPL-3, on the basis of the kinetics of incorporation into these species and their specific radioactivities after 2 h of labelling. Interestingly, although most of these species contained the expected alkylacyl- or *lyso*-alkyl-PI lipid moieties [14,15], some of the early intermediates appeared to contain diacyl-PIs. These molecular species either do not enter the GIPL/LPG pathway or are first remodelled to alkylacyl species by some kind of headgroup-exchange reaction. Although there is no precedent for this type of remodelling, it would be analogous to that described for the diacyl-PI to ceramide-PI exchange in yeast [23,24]. Finally, the tentative identification of GlcNAc-diacyl-PI in *L. major* is consistent with the route, PI → GlcNAc-PI → GlcN-PI, as described in *T. brucei* and other eukaryotes for GPI biosynthesis [25–28].

It is not possible from the present study to conclude which GIPLs act as precursors for the LPG anchor and to what extent.

The most likely candidates are GIPL-3 and the partially characterized P-GIPL-3, which probably contains a Glc-1-PO₄ residue on the core mannose distal to the GlcN residue (Figure 1). Although the specific radioactivities of these GIPLs are consistent with them being LPG precursors, the level of [³H]GlcN incorporation into both these species lagged behind that of the LPG anchor. These results could be explained if nearly all the newly synthesized GIPL-3 and P-GIPL-3 were rapidly utilized as LPG anchors. Thus the accumulation and detection of these intermediates would depend on the extent to which the supply of precursors exceeded demand. According to this view, the supply of GIPL-3 and/or P-GIPL-3 in this *L. major* strain is closely matched by their rate of utilization as LPG anchors. A similar situation may occur in other *Leishmania* species and strains where these GIPL species are present in very low or undetectable levels [29]. However, in some *Leishmania* strains, GIPL-3 and/or P-GIPL-3 may be present at relatively high levels [14,15,17], possibly due to the up-regulation of a putative α 1-6-galactosyl-transferase that converts GIPL-2 into GIPL-3. These strains synthesize the same amount of LPG, and it is likely that most of the accumulated polar GIPLs are end products rather than intermediates, consistent with the finding that they are expressed at the cell surface [14,16]. In summary, the [³H]GlcN-labelling data suggest that either GIPL-3 or P-GIPL-3 may be precursors of LPG. As some LPGs lack the Glc-1-PO₄ residue on their core [9,30], the most likely situation is that this residue is added to the GIPL-3 precursor just before or at the same time as the phosphorylated oligosaccharide repeat units.

It was surprising that most GIPLs, including those believed to be predominantly biosynthetic intermediates, were unable to reach steady-state labelling after 2 h. Moreover, newly synthesized LPG was not released into the medium after 2 h of labelling whereas surface LPG was rapidly shed (results not shown). These observations suggest that the pools of GIPLs and LPG in the biosynthetic compartment(s) are large and that the turnover of the intermediates is rather slow. This is supported by earlier findings showing that a large proportion of the GIPLs (about 30–60%) and of the LPG (about 20%) in *L. major* are inaccessible to surface-labelling experiments and are therefore believed to be intracellular [16].

Taken together, these data suggest that the type-2 GIPLs and LPG anchor share the common biosynthetic pathway outlined in Figure 8. This pathway is identical with the well-characterized pathway of protein-anchor biosynthesis up to M1 [25–28], after which it diverges. The precise role of the minor P-GIPLs, P-GIPL-1 and P-GIPL-2 in this pathway is not known. These species represent a very small proportion of their respective neutral species and consequently may be side products rather than true intermediates in the formation of the LPG core. It is notable that, although all species of *Leishmania* appear to have this biosynthetic pathway (as indicated by the presence of LPG with the same conserved core), high levels of type-2 GIPLs have only been found in strains of *L. major* [2]. We speculate that several enzymes along the LPG-anchor-biosynthetic pathway have been up-regulated in *L. major* and that this has led to the production of excess intermediates which accumulate within the biosynthetic compartment and eventually reach the cell surface. The up-regulation of enzymes involved in GlcN-PI metabolism (i.e. those involved in both protein-anchor and LPG-anchor biosynthesis) may be a general phenomenon in the *Leishmania* and have led to a remarkable variety of glycolipid compositions observed in different species, strains and developmental stages. Thus GIPLs which are structurally related to the protein anchors (type-1 GIPLs) or both the protein and LPG anchors (hybrid-type GIPLs) have been described in other *Leishmania* (reviewed

Figure 8 Proposed pathway of biosynthesis of type-2 GIPLs and LPG anchor

Neutral type-2 GIPLs are synthesized by sequential addition of monosaccharides to GlcN-PI and accumulate at the cell surface. It is proposed that most of the GIPL-3 in the *L. major* strain used in this study functions as a precursor for LPG, either before or after it is substituted with a Glc-1-PO₄ residue (see the Discussion section). Other polar GIPLs are also modified with this residue but not to the same extent and are thought to be metabolic side products. The cell-surface expression of GIPLs1–3 has been shown previously by labelling studies with periodate and galactose oxidase [16]. It is not known whether or not iM2 is expressed on the cell surface.

in ref. [2]). These glycolipids can be further modified by enzymic activities that are not part of the GPI protein anchor or LPG core biosynthetic pathways, giving rise to extra variety (e.g. the ethanolamine-phosphate-substituted hybrid-type GIPLs of *Leishmania mexicana* [17,31]). Thus the *Leishmania* would seem to have used a rather simple strategy to generate a great variety of surface glycolipids. Whether this diversity has direct relevance to the different life cycles of the various *Leishmania* species remains to be determined.

In the second part of this study we show that there is a marked difference in the turnover of the major GIPLs compared with the LPG. Whereas the *in vivo*- and cell surface-labelled LPG had a relatively rapid turnover, the GIPLs appeared to be stable cell surface constituents with a very low turnover. These data are consistent with the [³H]GlcN-labelling experiments suggesting that most of the GIPL species are metabolic end products, and also indicate that the accumulation of the GIPLs in these parasites (> 10⁷ copies/cell [16]), at least partially, reflects their slow turnover. The high turnover of LPG has been reported previously and shown to be due to the rapid shedding of molecules from the cell surface [21,22]. The shedding of LPG appears to be a stochastic biophysical event, based on monomers of LPG molecules leaving the plasma membrane spontaneously. In favour of this model is the observation that the shed molecules have (on average) shorter alkyl chains and thus are more polar than the

cell-associated LPGs [10]. The stability of the GIPLs containing alkylacyl-PI lipid moieties is consistent with this model as they will be much less polar than the LPGs. Presumably the *lyso*-GIPL species are also sufficiently apolar (by virtue of their relatively small glycan headgroups compared with LPG) to be stably associated with the plasma membrane. The difference in turnover of these two classes of surface molecules may reflect their different functions. For example, the high turnover of LPG molecules may be required to allow expression of new LPG structures on the parasite cell surface during their development within the sandfly midgut [30]. This turnover is thought to be important in regulating the attachment and subsequent detachment of promastigotes from epithelial cells along the midgut [5,6]. In contrast, the slow turnover of the GIPLs may be critical in maintaining a protective surface glycocalyx during and after the differentiation of promastigotes to amastigotes when the levels of LPG and GPI-anchored proteins are drastically diminished [29,31–33].

We thank Dr. M. Low for providing PI-specific phospholipase C. This work was supported by the British Medical Research Council and the Wellcome Trust. P.S. is a long-term EMBO Fellow, M.A.J.F. is a Howard Hughes International Research Scholar and M.J.M. is a Wellcome Trust Senior Research Fellow. The contributions of L.P. and P.S. to this study were equivalent.

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Received 9 August 1994/13 December 1994; accepted 23 December 1994