Glycosylphosphatidylinositol (GPI) glycolipids are major cell surface constituents in the Leishmania parasites. Distinct classes of GPI are present as membrane anchors for several surface glycoproteins and an abundant lipophosphoglycan as well as being the major glycolipids (GIPs) in the plasma membrane. In this study we have identified putative precursors for the protein and lipophosphoglycan anchors and delineated the complete pathway for GIPL biosynthesis in Leishmania mexicana promastigotes. Based on the structural analyses of these GPI intermediates and their kinetics of labeling in vivo and in cell-free systems, we provide evidence that the GIPLs are the products of an independent biosynthetic pathway rather than being excess precursors of the anchor pathways. First, we show that the similar glycan head groups of the GIPL and protein/lipophosphoglycan anchor precursors are assembled on two distinct pools of PI corresponding to 1-O-(C18:0)-alkyl-2-stearoyl-PI and 1-O-(C24:0/C26:0)-2-stearoyl-PI, respectively. These PI species account for 20 and 1% of the total PI pool, respectively, indicating a remarkable specificity in their selection. Second, analysis of the flux of intermediates through these pathways in vivo and in a cell-free system suggests that the GIPL and anchor pathways are independently regulated. We also show that GIPL biosynthesis requires fatty acid remodeling, in which the sn-2 stearoyl chains are replaced with myristoyl or lauroyl chains. Fatty acid remodeling is dependent on CoA and ATP and occurs on pre-existing but not on de novo synthesized GIPLs. We suggest that the compartmentalization of different GPI pathways may be important in regulating the species and stage-specific expression of different GPI structures in these parasites.

GPI glycolipids are important membrane constituents in all eukaryotic cells. They anchor a variety of proteins to the outer leaflet of the plasma membrane and may also have a role in the intracellular trafficking, transmembrane signaling, and intercellular transport of these proteins (reviewed in Refs. 1–3). Non-protein-linked GIPLs may also be abundant in the plasma membrane, although the function and biosynthetic relationship between these glycolipids and the anchor GIPs in higher eukaryotes is unclear (4–6).

GPI glycolipids are particularly abundant in lower eukaryotes such as yeast and the parasitic protozoa where they are commonly utilized as membrane anchors for the major surface proteins (2). In the trypanosomatid parasites, non-protein-linked GIPs can also be the major cellular glycolipids (2). This is the case for the sandfly-transmitted Leishmania parasites which cause a range of important diseases in humans. Leishmania parasites alternate between a flagellated promastigote stage that resides within the digestive tract of the sandfly vector and a non-motile amastigote stage that proliferates within the phagolysosome of macrophages in the mammalian host. The major macromolecule on the promastigote surface is a complex lipophosphoglycan (LPG), which contains a polydisperse phosphoglycan chain and a novel GPI anchor with the structure Galα(Glc-1-PO4)4ManαGlcN-lyso-PI (Fig. 1) (2, 7). This anchor is distinct from the protein anchors of Leishmania and other eukaryotes, which have the core structure: ethanolamine-PO4-ManαGlcN-PI (Fig. 1) (2, 9). Interestingly, despite these differences in glycan structure, both the protein and LPG anchors contain a PI lipid moiety which is highly enriched for 1-O-alkylglycerols with very long (C24:0 or C26:0) alkyl chains (7–9). The LPGs are essential virulence factors which have been shown to mediate the attachment of promastigotes to the insect midgut and mammalian macrophages, protect the parasite surface in these hostile environments, and modulate signal transduction pathways involved in activating various microbialic processes in the host macrophage (2, 7). Intriguingly, the surface expression of both the LPG- and GPI-anchorated proteins is massively down-regulated in the intracellular amastigote stages of several species (10–12), suggesting that the surface expression of these macromolecules is either not required or not compatible for life in the macrophage phagolysosome.

4 This work was supported in part by the Australian National Health and Medical Research Council and the Wellcome Trust. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Wellcome Trust Senior Research Fellow. To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, University of Melbourne, Parkville 3052, Victoria, Australia. E-mail: m.mcconville@biochemistry.unimelb.edu.au

1 The abbreviations used are: GPI, glycosylphosphatidylinositol; PI, phosphatidylinositol; IPC, inositol phosphorceramide; GIPL, glycosyniotol phospholipid; HPAC, high pH anion exchange chromatography; LPG, lipophosphoglycan; TLCK, N-seryl-L-lysine chloromethyl ketone; HPTLC, high performance liquid chromatography; MALD-TOF-MS, matrix-assisted laser desorption time of flight mass spectrometry. The nomenclature used to describe the L. mexicana GIPLs and GPI precursors is as follows (see Fig. 13 for structures): the letter M and numbers 1–4 refer to the number of mannose residues in each structure. The prefix “i” indicates the presence of an α1–3-linked mannose branch on the mannose proximal to glucosamine (the iso series) (29). Structures without the i prefix lack this branch and have part or all of the structure Manα1–2Manα1–6Manα1–4GlcN-PI. The prefix “EP” indicates the presence of an ethanolamine-phosphate residue on the glycan backbone (13), while the subscript “VLAC” refers to GPI species which contain 1-O-alkylglycerols with very long alkyl chains (C24:0, C26:0). HPTLC-derived fractions used in this study are denoted by a band number and the major composite GPI species.
Leishmania spp. also synthesize glycoinositol phospholipids (GIPLs), a family of low molecular weight GPI glycolipids which are not linked to either protein or polysaccharide. Unlike the LPG- and GPI-anchored proteins, the GIPLs are major surface constituents (10^7 copies/cell) on both the promastigote and amastigote stages (11, 13–15). Depending on the species and developmental stage, the glycan moieties of the GIPLs may be homologous to the protein anchors (type I GIPLs), the LPG anchors (type 2 GIPLs), or contain elements of both anchors (hybrid type) (2). Despite having similar glycan moieties to the protein or LPG anchors, the GIPLs characteristically have distinct lipid compositions which are often enriched with alkenyl-sn-glycosyl-sn-phosphatidylinositol (C18:0-alkyl chains (11, 13). Less is known about the function of the GIPLs, but as the major cell surface constituents of the amastigote stage they may have specific role(s) in protecting the parasite surface and/or modulating signal transduction pathways in the macrophage (14, 16, 17). However, structurally related GIPLs are also abundant in the plasma membranes of other trypanosomatid organisms which have quite different hosts (ranging from humans to insects and plants) (2, 18), suggesting that these glycolipids may have a more general function in regulating the physico-chemical properties of cellular membranes.

The biosynthesis of protein anchor precursors has been extensively studied in a number of protozoan parasites such as Trypanosoma brucei and Toxoplasma gondii (19–21), and as GIPLs which are the major cell surface glycolipids (13). Galactofuranose.

**Fig. 1. Structures of the major GPI classes synthesized by L. mexicana promastigotes.** Distinct classes of GPI occur as membrane anchors for the surface glycoprotein, gp63 (9), the LPG (27), and as GIPLs which are the major cell surface glycolipids (13). Gαl, galactofuranose.

Materials—Materials were obtained as follows: ATP and coenzyme A were from Sigma. Jack bean α-mannosidase and Bacteria coccus phosphatidylinositol-specific phospholipase C were from Boehringer Mannheim. [2-^3H]Mannose (17 Ci/mmol), [6-^3H]Glucosamine (2 Ci/mmol), [6-^3H]Glucose (3.1 Ci/mmol), [9,10-^3H]Myristic acid (11.2 Ci/mmol), [9,10-^3H]Palmitic acid (35.9 Ci/mmol), [1-^14C]Stearic acid (55 Ci/mmol), and GDP-[2-^3H]Man (7 Ci/mmol) were from NEN Life Science Products Inc. [14C]Octadecanol (52 Ci/mmol) was from American Radiolabeled Chemicals and [3H]Ethanolamine (28 Ci/mmol) from Achemas.

**EXPERIMENTAL PROCEDURES**

**Metabolic Labeling of L. mexicana Promastigotes.—Promastigotes of L. mexicana (strain MYNC/BZ/62/M379) were routinely cultivated at 27 °C in RPMI medium supplemented with 10% fetal bovine serum. For labeling with [3H]Inositol or [3H]Ethanolamine, mid-log growth phase promastigotes were resuspended in RPMI-fetal bovine serum medium (15, 000 cell/ml) containing 10 μCi of [3H]Inositol or 20 μCi of [3H]Ethanolamine and cultivated for 12–20 h (steady state labeling) at 27 °C before harvesting.**
before being dried in a Speed-Vac (Savant) concentrator. GPI glycolipids recovered in the 1-butanol phase were reextracted in 40% 1-propanol for HPTLC analyses. In the experiment shown in Fig. 5, LPG was extracted from the digitonin pellet of HMan-labeled promastigotes in 0.5% aqueous 1-butanol (2 × 500 μl) and purified on a small column (1 ml) of an octyl-Sepharose column (28). The dephosphorylated LPG anchor was obtained by mild hydrolysis of this extract in 40 mM trifluoroacetic acid (12 min, 100 °C), digestion of the hydrolysate with alkaline phosphatase (20 units/50 μl) in 0.5 M ammonium bicarbonate, pH 8.5 (18 h, 37 °C), and separation of the anchor from labeled LPG repeat units on a mini octyl-Sepharose column as described above (28). Analysis of GPI Glycolipids in L. mexicana Cell-free Systems—Promastigotes were preincubated in RPMI medium containing 10% fetal bovine serum and 1 μg/ml tunicamycin for 30 min at 27 °C, then hypotonically permeabilized in water containing 2 mM EGTA, 0.1 mM TLCK, 0.1 μM leupeptin, 1 mM dithiorthioleucine (106 cells/ml) for 10 min at 0 °C. Permeabilized cells were pelleted by centrifugation (16,000 × g, 5 min, 4 °C) and resuspended in 106 cells/ml of GDP-[3H]Man (0.5 μCi) and UDP-GlcNAc (10 mM) to 50 μl of permeabilized cells. After 10 min, the label was chased by adding an equal volume of buffer A containing 1 mM UDP-GlcNAc and 1 mM GDP-Man. Aliquots (100 μl) of the 1-butanol phase were resuspended in 40% 1-propanol, 60% 1-propanol. Calibration was carried out with previously defined GIPL standards.

RESULTS

The Lipid Moieties of the Different GPI Classes Are Distinct from Each Other and from the Total Cellular Pool of Free Inositol Phospholipids

Previous analyses have shown that the GPI anchors and GIPLs of L. mexicana promastigotes contain alkylacyl-P1 (or lyso-alkyl-P1) with C18:0 in the GIPLs (25) and C24:0 and C26:0 in the LPG and protein anchors alkyl chains. The sn-2 fatty acyl composition of the GIPLs and protein anchors has not been examined in detail. To determine to what extent these distinctive PI moieties are represented in the total PI pool, we have analyzed the molecular species composition of promastigote PI and also re-examined the acyl chain composition of the GIPLs. After steady state labeling with [3H]inositol, PI accounted for 58% of the inositol lipids (Fig. 2A, first lane). Mild base hydrolysis of this fraction showed that approximately 80% of the PI contained a base-sensitive diacylglycerol lipid, while 20% comigrated with lyso-PI, indicating the presence of an alkylacylglycerol lipid (Fig. 2A, second lane). Analysis of HPTLC-purified PI by MALDI-TOF-MS produced one major pseudomolecular ion at m/z 864 and minor ions at m/z 836 and 850 (Fig. 2B). These ions corresponded to the [M-1] pseudomolecular ion of distearoyl-PI, stearyloyl-palmitoyl-PI, and 1-O-(C18:0)alkyl-2-O-stearyloyl-PI, respectively, as judged by GC-MS compositional analysis (data not shown). The presence of the latter species was confirmed by analysis of the base-treated fraction, which generated a single [M-1] pseudomolecular ion at m/z 584, the expected mass of 1-O-(C18:0)alkyl-2-lyso-PI (Fig. 2C). Alkylacyl-PI molecular species with C24:0 and C26:0 alkyl chains accounted for less than 5% of the plasmaloyl-PI, as determined by GC-MS, and were not detected by the less sensitive MALDI-TOF-MS analyses.

In contrast, the [3H]inositol-labeled GIPL species, iM2, iM3, and iM4, contained exclusively alkylacylglycerol lipids, as shown by their reduced HPTLC mobility after base treatment (Fig. 2A, second lane). GC-MS and MALDI-TOF-MS analyses confirmed the presence of exclusively C18:0 alkyl chains in these glycolipids, but also revealed the presence of a distinct sn-2 fatty acyl composition. For example, the MALDI-TOF mass spectrum of iM2 (Manα1–3Manα1–4GlcN-PI) contained one major [M-1] pseudomolecular ion at m/z 1281 and a minor ion at 1252 (Fig. 3A). After base treatment only a single pseudomolecular ion was detected at m/z 1071 (Fig. 3B), which is the correct mass for Hex2HexN-1-O-(C18:0)alkyl-2-lyso-PI.
These data suggest that iM2 contains either 1-O-(C18:0)alkyl-2-myristoylglycerol (90%) or 1-O-(C18:0)alkyl-2-lauroylglycerol, which accounted for 95 and 5% of the molecular species, respectively, as judged by GC-MS compositional analysis. Identical lipid compositions were found in the other two major GIPL species, iM3 and iM4 (Fig. 3, C and D). Thus the lipid moieties of the GIPLs differ from those of the major alkylacyl-PI pool in having predominantly myristate instead of stearate. These data suggest that the GIPLs are either assembled on a very minor pool of alkylacyl-PI or that the predominant alkylacyl-PI molecular species are utilized but then subjected to fatty acid remodeling.

**Early Intermediates in GPI Biosynthesis Contain Unremodeled Alkylacyl-PI**

We next identified early intermediates in these pathways to determine at what stage the GIPLs and GPI anchors acquire their distinct alkylacyl chain compositions. All these glycolipids contain the common core structure Manα1–4GlcNAc–6-myoinositol and are expected to be synthesized via the early intermediates, GlcNAc-PI, GlcN-PI, and Man,GlcN-PI (1, 30, 31). Attempts to metabolically label GlcNAc-PI and GlcN-PI with [3H]GlcN were unsuccessful, as this monosaccharide is
FIG. 4. Metabolic labeling of L. mexicana promastigotes GPI glycolipids. Promastigote lipids were labeled to steady state (24 h) with [3H]inositol (Ino, lanes 1 and 8), [14C]octadecanol (Oct, lanes 4 and 11), and [3H]ethanolamine (EtN, lanes 7 and 14), or in short term (5–30 min) labeling experiments with [3H]GlcN (lanes 2 and 9), [3H]Man (lanes 3 and 10), [3H]myristic acid (Myr, lanes 5 and 12), or [3H]phosphoric acid (Pal, lanes 6 and 13). Total lipid extracts were resolved on HPTLC before (lanes 1–7) and after (lanes 8–14) digestion with PI-PLC. The left hand column of labels refers to [3H]Man and reduction of the upper and lower bands of this doublet in (lanes 8–14) digestion with PI-PLC. The right hand column refers to [3H]GlcN. Bands 1–3 were isolated as PI-PLC-sensitive bands (Fig. 4, lanes 3 and 4). The lower bands of this doublet corresponded either to the mature GPI species (iM2, iM3, and iM4) or to unique intermediates in the pathways of GIPL, corresponding either to the mature GIPL species (iM2, iM3, and iM4, or to unique intermediates in the pathways of GIPL, i.e. previously characterized GIPL species iM2, iM3, iM4, and EPiM3. Bands 1–13 refer to GIPL species labeled with [3H]Man which were characterized in this study. Note that PI-PLC digests all inositol lipids except PIP.

Identification of Unremodeled GIPL Intermediates and Putative Anchor Precursors

Metabolic labeling of promastigotes with [3H]Man identified 13 PI-PLC-sensitive bands (Fig. 4, lanes 3 and 10), which corresponded either to the mature GIPL species (iM2, iM3, and iM4) or to unique intermediates in the pathways of GIPL, protein anchor, and LPG anchor biosynthesis. The [3H]Man-labeled species were HPTLC purified and further characterized to define the intermediates in these pathways and the steps involved in fatty acid remodeling. Man-GlcN-PI Complex—Bands 3/iM2VLAC and 4/iM2 had a faster HPTLC mobility than mature iM2 and were isolated as a mixture (Fig. 4, lane 3; Fig. 7, lane 5). HPTLC analysis of the deaminated/reduced glycan head groups from this mixture indicated that both species had the same head group as iM2 (i.e. Manα1–3Manα1–4GlcN) (Fig. 6, lane 4), suggesting that they differed from mature iM2 in having more hydrophobic lipid moieties. After mild base treatment, these bands showed a similar shift to a slower HPTLC mobility, indicating that the differences in the lipid moieties resided primarily in their alkyl chain composition (Fig. 7, lanes 5 and 6). Deacylated 3/iM2 comigrated with the lyso derivative of mature iM2 (Fig. 7, compare lanes 6 and 8), while deacylated 3/iM2VLAC had a significantly faster HPTLC mobility. It is likely that 3/iM2VLAC corresponds to a iM2 species with very long alkyl chains (presumably C24:0 and C26:0), while 4/iM2 corresponds to a iM2 species with the same alkyl chain composition as mature iM2 (i.e. C18:0) but with a more hydrophobic sn-2 fatty acid. Band 4/iM2 comigrated with a [14C]stearate-labeled band (data not shown), suggesting that the sn-2 fatty acyl chain may be stearate. These results are consistent with 3/iM2VLAC being the first committed precursor of the LPG anchor, and 4/iM2 being an unremodeled iM2 species with a 1-octadecyl-2-stearylglycerol lipid.

A third band in this complex, 5/M2iM2 migrated as a partially resolved doublet, with the same or slightly faster HPTLC mobility than mature iM2 (Fig. 7, lane 7). Nitrous acid deamination and reduction of this band released two glycan head groups that comigrated with Manα1–6Manα1–6AHM (M2) and Manα1–3Manα1–6AHM (iM2) on HPTLC (Fig. 6, lane 5), indicating that it contained two ManαGlcN-PI species with distinct glycan head groups. The lower band in this doublet was enriched for the iM2 head group (results not shown) and is likely to correspond to mature iM2 from its HPTLC mobility. Both species had a slower HPTLC mobility after base treatment suggesting that they contained alkylacylglycerol lipids (Fig. 7, lanes 7 and 8). However, the relative mobilities of the lyso species was inverted compared with the native species (data not shown), suggesting that the upper M2 species has a more hydrophobic fatty acid than the iM2 species. This was confirmed by HPTLC analysis of the GlcN-PI core obtained by jack bean α-mannosidase digestion. This treatment generated a doublet which comigrated with GlcN-[1-O-(C18:0)alkyl-2-stearoyl]PI (upper band) and GlcN-[1-O-(C18:0)alkyl-2-myristoyl]-PI (lower band) (Fig. 9, compare asterisked bands in lane 5 with GlcN-PI standards derived from the ManαGlcN-PI doublet in lane 3 and from mature iM4 in lane 9). After base treatment this doublet collapsed to a single band that comigrated with a standard GlcN-[1-O-(C18:0)alkyl-2-hydroxyl]-PI (results not shown). Taken together, these results suggest that 5/M2,iM2 contains mature iM2 (with 1-O-(C18:0)alkyl-2-myr-
istoylglycerol) and a Man$_1$–6Man$_1$–4GlcN-PI (M2) species with the same C18:0 alkyl chain composition but longer (probably C18:0) sn-2 fatty acids.

Man$_3$GlcN-PI Complex—Band 6/M3,iM3$_9$ had a slightly faster HPTLC mobility than mature iM3 (Fig. 4 and Fig. 7, upper band in lane 9) and contained Man$_n$GlcN-PI species from HPTLC analysis of the deaminated/reduced glycan head group (Fig. 6, lane 6). Because linear and branched Man$_n$AHM glycans are not resolved on HPTLC, the glycans obtained from a more highly purified fraction of band 6/M3,iM3$_9$ were further analyzed by Dionex HPAEC. Two glycans were resolved which coeluted with the linear glycan, M3 (Man$_a$1–2Man$_a$1–6Man$_a$1–4AHM) at 2.8 glucose units and the branched glycan, iM3 (Man$_a$1–6[Man$_a$1–3]Man$_a$1–4AHM) at 3.2 glucose units (data not shown), suggesting that band 6/M3,iM3$_9$ contained unbranched and branched Man$_3$GlcN-PI species in the ratio of 3:1. Both Man$_3$GlcN-PI species appeared to have the same lipid composition as they comigrated together with lyso-iM3 after base treatment (Fig. 7, lanes 9 and 10). Moreover, only one GlcN-PI molecular species was generated by α-mannosidase treatment of 6/M3,iM3$_9$ (Fig. 9, lane 7). The α-mannosidase-treated band comigrated with GlcN-[1-O-(C18:0)-2-stearoyl]-PI derived from 2/M1 (Fig. 9, compare asterisked bands in lanes 3 and 7), suggesting that both the M3 and iM3 species in this band have C18:0 alkyl chains and predominantly C18:0 fatty acids.

Band 7/iM3 comigrated with mature iM3 on HPTLC and was prominently labeled with [3H]inositol, [3H]GlcN, [3H]Man, [14C]octadecanol, and [3H]myristate (Fig. 4, lanes 1–4). Glycan head group analysis indicated the presence of a Man$_n$GlcN head group (Fig. 6, lane 6). This was confirmed by HPAEC analysis of the glucan obtained from HPTLC-purified band 7/iM3. A single glycan was released which comigrated with Man$_a$1–6[Man$_a$1–3]Man$_a$1–4AHM at 3.2 glucose units (data not shown). Band 7/iM3 had a slower HPTLC mobility after base treatment, comigrating with authentic lyso-iM3 (Fig. 7, lane 9).

FIG. 5. [3H]Man pulse-chase labeling of L. mexicana GPI glycolipids. Promastigotes were labeled with [3H]Man for 5 min, then resuspended in fresh complete medium and analyzed by HPTLC in solvent A (Panel A). Ino, [3H]inositol-labeled lipids. Panels B–E show the kinetics of [3H]Man labeling of individual GPI species and the LPG anchor during the chase as described under "Experimental Procedures" (given as total counts/min). Panel B, incorporation into M1, M2, and M3. Panel C, incorporation into the mature GIP species, iM2, iM3, iM4, and EPiM3. Panel D, incorporation into unremodeled intermediates iM3, iM4, lyso-iM3, and lyso-iM4. The kinetics of incorporation into iM2' were identical to iM3' and are not shown for clarity. Panel E, incorporation into M1$_{VLAC}$, iM2$_{VLAC}$, EPiM3$_{VLAC}$, and the mature LPG anchor.

FIG. 6. Glycan head group analysis of [3H]Man-labeled GPI glycolipids. [3H]Man-labeled GPIs were purified by HPTLC and the glycan head groups (terminating in 2,5-anhydromannitol, AHM) obtained by nitrous acid deamination and reduction. The fractions are the same as those analyzed as native GPIs in Fig. 7 (see lane headings in Fig. 7). The major GPIs in each fraction are as follows; lane 2, 1/M1$_{VLAC}$; lane 3, 2/M1; lane 4, 3/M2$_{VLAC}$ and 4/M2; lane 5, 5/M2,iM2; lane 6, 6/M3,iM3 and 7/iM3; lane 7, 8/iM4 and 9/iM4; lane 8, 9/iM4 and 11/lyso-iM3; lane 9, 13/lyso-iM4. The migration position of glycans derived from the partial hydrolysis of the deaminated/reduced iM4 glycan are indicated in lane 1. HPTLC sheets were developed in solvent B.
FIG. 7. All the GPIs of *L. mexicana* contain an alkylacyl-PI lipid moiety. Promastigote GPIs labeled with \[3H\]Man were purified by HPTLC and reanalyzed before and after mild base (OH-') deacetylation. The assignment of structures in each fraction (shown at top of chromatogram) is discussed in the text. The HPTLC profiles of \([3H]\)inositol (Unso) and \([3H]\)Man (Man) labeled lipids are shown in the left-hand lanes. Note that lane 9 is cross-contaminated with sample from lane 10.

(Fig. 8). These data indicate that band 7/iM3 corresponds to mature iM3 with a 1-O-octadecyl-2-myristoylglycerol lipid. Band 11/lyso-iM3 also contained a iM3 head group (Fig. 6, lane 9). This species comigrated with lyso-iM3 (Fig. 4, compare lanes 8/iM4 and 9/iM4). Band 8/iM4 indicated that both 9/iM4 and 9/iM4 migrated just above mature iM4, whereas 9/iM4 comigrated with lyso-iM4 on HPTLC (Fig. 4, lanes 1, 4, and 5) correspond to mature iM4 with 2-O-(C18:0)alkyl-2-myristoylglycerol. Glycan analysis of band 10/lyso-iM4 indicated the presence of a third Man,GlcN-PI species (Fig. 6, lane 9). This species comigrated with lyso-iM4, was base resistant (Fig. 7, lanes 15 and 16), and is likely to correspond to the deacylated intermediate in the fatty acid remodeling of iM4.

FIG. 8. Negative ion MALDI-TOF mass spectrum of Man,GlcN-PI. The HPTLC-purified Man,GlcN-PI doublet was analyzed before (panel A) and after (panel B) base treatment.

(Ethanolamine-containing GPIs)—steady state labeling of *L. mexicana* promastigotes with \([3H]\)ethanolamine identified three PI-PLC-sensitive bands (Fig. 4, lanes 7 and 14). Two of these \([3H]\)ethanolamine-labeled species comigrated with the ethanolamine-phosphate-modified forms of iM3 (EPiM3, RP 0.1) and iM4 (EPiM4, RP 0.06). Previous analyses have shown that these species contain predominantly 1-O-(C18:0)alkyl-2-myristoylglycerol (13). The third GPI species, band 10/EPM3VLAC (asterisked band Fig. 4, lane 7) was a minor band in the \([3H]\)ethanolamine profile (Fig. 4, lane 7, RP 0.16), but was pulse-labeled with \([3H]\)Man more strongly than either EPiM3 or EPiM4 (Fig. 4, lane 3), indicating that it has a faster turnover than the ethanolamine-substituted GPIs. Band 10/EPM3VLAC was resistant to \(\alpha\)-mannosidase digestion (Fig. 9, lanes 8 and 9) and had a slower HPTLC mobility after mild base (data not shown), indicating that the ethanolamine may cap the terminal mannose residue and that this species has an alkylacyl-PI lipid. Although the glycan and lipid head group of this species was not further characterized, its faster HPTLC mobility relative to EPM3 is consistent with it having the structure; ethanolamine-PO4-Man,GlcN-PI with very long alkyl chains. This minor species is a putative precursor for the protein anchors.

Pulse-chase \([3H]\)Man labeling of GPI intermediates

Precursor-product relationships between the various GPI intermediates were further investigated with pulse-chase labeling experiments. Incorporation of \([3H]\)Man into each species was determined from the analysis of the labeled lipids as well as glycan head group analysis of individual HPTLC bands that contained more than one GPI species (see "Experimental Procedures"). \([3H]\)Man was incorporated into the mature GPI species, 5/iM2, 7/iM3, 9/iM4, and 12/EPM3 (after 40 min lag) throughout the chase, consistent with these species being metabolic end-products (Fig. 5, A and C). In contrast, all the other intermediates were depleted after the 4-h chase. The rapid labeling and chase kinetics of M1, M2, and M3 strongly suggest that these species form the biosynthetic series M1 → M2 → M3.
FIG. 9. Analysis of the GlcN-PI moiety derived from α-mannosidase-treated GPIs. [3H]Glc-labeled GPIs were purified by HPTLC then reanalyzed before and after jack bean α-mannosidase digestion to generate the respective GlcN-PI cores of each species (designated with an asterisk). Note that the amount of label in the core GlcN-PI species is very low, indicating that most of the label in the native GPIs was present as mannnose. The major species in selected fractions were as follows: lanes 2 and 3, 1/M1VLAC and 2/M1; lanes 4 and 5, 4/iM2 and 5/M2; iM2; lanes 6 and 7, 6/M3, iM3; lanes 8 and 9, 9/iM4 and 10/EPM3VLAC; lanes 10 and 11, total [3H]Man-labeled GPIs, lanes 12 and 13, total [3H]inositol-labeled lipids.

The labeling and chase kinetics of 1/M1VLAC were consistent with it being a precursor to 3/iM2VLAC and 10/EPM3VLAC which were maximally labeled after 40 min (Fig. 5E). Labeling of iM2VLAC and lyso-iM4 occurred with essentially the same kinetics as the unremodeled acylated species, although in some cases maximum labeling of the lyso species occurred just before its putative precursor. This was probably due to the presence of low levels of labeled Man5GlcNAc2-P-Dol and Man6GlcNAc2-P-Dol in the early time points which comigrate with lyso-iM3 and lyso-iM4.2 The presence of these species reflects a low level of dolichol-oligosaccharide synthesis in the presence of tunicamycin.

The labeling and chase kinetics of 1/M1VLAC were consistent with it being a precursor to 3/iM2VLAC and 10/EPM3VLAC which were maximally labeled after 40 min (Fig. 5E). Labeling of iM2VLAC preceeded that into the mature LPG anchor and was effectively chased over 2–3 h, while label in the LPG anchor increased over 2 h before leveling off (Fig. 5, Panel E), presumably due to the loss of newly synthesized LPG from the cell surface (25). A precursor-product relationship between 10/EPM3VLAC and the protein anchors was not determined from these analyses because of the difficulty of detecting significant and specific [3H]Man incorporation into the anchor moiety of newly synthesized proteins. Low levels of incorporation were not surprising given that GPI-anchored promastigote proteins are approximately 10-fold less abundant than the LPGs. Finally, the distinct differences in the turnover of the two Man5GlcN-PI bands, 1/M1VLAC and 2/M1, suggested that the relative flux of intermediates into the GIPs is more than twice as fast as the flux of intermediates into combined anchor pathways (Fig. 5A, B, E).

Fatty Acid Remodeling of Pre-existing Pools of GIP and Anchor Intermediates in Vivo

The specificity of the fatty acid remodeling reactions was analyzed by continuous labeling of L. mexicana promastigotes with [3H]myristic, [3H]palmitic, or [14C]stearic acids. [3H]Myristic acid was rapidly incorporated into all the inositol lipids (PI, PIP, and IPC) as well as into the mature GIPs (Fig. 10A).

After 4 h labeling the majority of the label (>95%) was still present as myristate (data not shown). [3H]Myristate was also rapidly incorporated into 10/EPM3VLAC with similar kinetics to the GIPs (Fig. 4, lanes 5 and 12; Fig. 10A). Importantly, the specific activity of the [3H]myristic acid-labeled GIPs was comparable to that of the total PI pool at the earliest time point of 5 min (Fig. 10B), suggesting that the myristic acid was being incorporated directly into pre-existing GIP pools rather than by de novo synthesis of GIPs from [3H]myristate-labeled PI. These kinetics were quite distinct from those found when promastigotes were metabolically labeled with [3H]inositol. Labeling of GIPs with [3H]inositol was only detected after several hours, following strong labeling of the PI, PIP, and IPC pools (data not shown). Consequently, the GIPs have a very low relative specific activity at early time points (Fig. 10B). The kinetics of incorporation of [3H]Palmitic and [14C]Stearic acids into the GIPs were similar to those for [3H]inositol, suggesting that these fatty acids were also being incorporated via PI (Fig. 10B). Thus [3H]myristic acid is unusual in that it is specifically incorporated into pre-existing GIP pools, while fatty acids that are characteristic of unremodeled GIP species are incorporated via [3H]labeled PI. Interestingly, the [3H]myristic acid-labeled GIPs had a very slow turnover in pulse-chase experiments (Fig. 10C), suggesting that the myristoylated GIPs are not extensively remodeled.

Fatty Acid Remodeling of GIPs and Anchor Precursors in a Cell-free System

The fatty acid remodeling reactions were further investigated in a cell-free system. When permeabilized cells were incubated with CoA and either [3H]myristic or [3H]palmitic acids, only myristic acid was incorporated into the GIPs (Fig. 11A). This labeling occurred in the absence of nucleotide sugar additives (GDP-Man or UDP-GlcNAc) suggesting that labeling was not dependent on continued GPI biosynthesis (Fig. 11A). Myristate labeling was dependent on the addition of ATP and was markedly stimulated by CoA (Fig. 11B), consistent with the major acyl donor being myristoyl-CoA. These data suggest that fatty acid remodeling of pre-existing pools of GIP precursors can be reconstituted in vitro.

To investigate whether de novo synthesized (as distinct from pre-existing) GIP pools were being remodeled in vitro, permeabilized cells were pulsed with GDP-[3H]Man and UDP-[14C]GlcNAc for 10 min, then chased in the presence of unlabeled GDP-Man, myristic acid, and CoA. As shown in Fig. 12, most of

2 R. Ralton and M. J. McConville, unpublished data.
the GPI intermediates including the unremodeled and lysoglycoproteins were synthesized in the permeabilized cells. However, synthesis of fully remodeled GIPLs was very inefficient. As the remodeling enzymes are known to be active using these assay conditions (see above) it is possible that the de novo synthesized GIPLs must be translocated to a different compartment for myristoylation in a step that has not been reconstituted in vitro. Note that some of the label in the lyso species at early time points is due to the presence of variable amounts of comigrating dolichol-P-P-oligosaccharides (i.e. Man₅₋₆GlcNAc₂-P-P-dol).²

**FIG. 10.** In vivo labeling of GPIs with [³H]myristic, [³H]palmitic, and [¹⁴C]stearic acids. Panel A, promastigotes were metabolically labeled with [³H]myristic acid and aliquots removed at designated time intervals for HPTLC analysis of the labeled lipids. Panel B, the specific activity of the GIPLs and PI (cpm/nmol) was determined after metabolic labeling with [³H]inositol (45 min), [³H]myristic acid (5 min), [³H]palmitic acid (15 min), and [¹⁴C]stearic acid (15 min). The specific activities of the GIPLs are given relative to PI (100%) which varied with each isotope ([³H]inositol-PI, 3100 cpm/pmol; [³H]myristoyl-PI, 62,000 cpm/pmol; [³H]palmitoyl-PI, 61,000 cpm/pmol; [¹⁴C]stearyl-PI, 1,500 cpm/pmol). Panel C, incorporation of [³H]myristic acid into iM4 during continuous labeling (filled squares) and after a 5-min pulse and 2-h chase in full medium (open squares).

**FIG. 11.** Reconstitution of fatty acid remodeling in permeabilized cells. Permeabilized promastigotes were incubated with [³H]fatty acids, ATP, and CoA, and the labeled lipids analyzed by HPTLC. Panel A, permeabilized cells were labeled with either [³H]myristic (lanes 1 and 2) or [³H]palmitic (lanes 3 and 4) acids, with (lanes 2 and 4) or without (lanes 1 and 3) nucleotide sugars. Panel B, permeabilized cells were incubated with [³H]myristic acid with or without ATP or CoA. Ino refers to the HPTLC profile of [³H]inositol lipids.

The putative anchor intermediates, 1/M1VLAC, 3/iM2VLAC, and 10/EPM3VLAC, were also labeled in these cell-free assays, although their turnover during the chase was very slow (Fig. 12, A and B). The slow turnover is most likely due to the depletion in suitable sugar donors (i.e. the galactofuranose donor for the next step in LPG anchor biosynthesis) or protein acceptors, which are required for further processing of these intermediates, respectively. In contrast, the labeling and turnover of the GIPL intermediates was comparable to that observed in vivo, suggesting that GIPL biosynthesis can be uncoupled from that of the protein and LPG anchor biosynthesis.

**DISCUSSION**

In this study we have delineated the pathway for GIPL biosynthesis in *L. mexicana* promastigotes and identified several putative precursors for the protein and LPG anchors. We propose that the anchor pathways share a set of common early intermediates, whereas the GIPLs are the products of a separate pathway. We also show that intermediates in the GIPL, and possibly also the protein and LPG anchor pathways undergo highly specific fatty acid remodeling reactions that result in the incorporation of short chain fatty acids, primarily myristate into these glycolipids. These findings are incorporated into a new model for GPI biosynthesis in *L. mexicana* promastigotes which is summarized in Fig. 13, and described in more detail below.

The major GPI intermediates labeled in vivo and in the cell-free system were intermediates in the pathway of GIPL biosynthesis based on the characterization of their glycan and lipid moieties and their kinetics of labeling in the [³H]Man pulse-chase experiments. The initial steps in this pathway appear to be the same as for the assembly of the protein anchors except that all these precursors contain a PI lipid moiety with C18:0 rather than C24:0 or C26:0 alkyl chains which are characteristic of mature protein anchors (Fig. 13). The earliest intermediates in this pathway, GlcNAc-PI and GlcN-PI, were not detected in the in vivo [³H]GlcN labeling experiments because of conversion of the label to comigrating PI and IPC lipids, but have been identified after incubation of cell-free systems with UDP-[^³H]GlcNAc. These species are mannosylated to form the unbranched GPI intermediates M1, M2, and M3 which are subsequently modified with a
Manα1–3 branch to form the unremodeled GIPL species, iM2′, iM3′, and iM4′ (Fig. 13). Although this appears to be the main pathway for the assembly of the GIPL head groups, it is possible that some of the Manα1–3 branched GIPLs may be further extended on their α1–6 mannose arm (see ? arrows in Fig. 13). The sn-2 fatty acid (predominantly stearate) of these unremodeled intermediates is removed to form the corresponding lyso derivative which is reacylated with myristate (or laurate) to form the mature GIPL species (Fig. 13). The remodeling reactions appear to be specific for short chain fatty acids as neither [3H]palmitic nor [14C]stearic acids are incorporated into the GIPLs, either in vivo or in vitro. The remodeling reactions require both CoA and ATP, essential factors in acyl-CoA synthesis, suggesting that the fatty acids are transferred from myristoyl- or lauroyl-CoA. Interestingly, while [3H]myristic acid was specifically incorporated into pre-existing GIPL pools in the cell-free systems, remodeling of de novo synthesized GIPLs was very inefficient even in the presence of CoA and fatty acids. This raises the possibility that a transport step is required before newly synthesized GIPLs can be accessed by the remodeling enzymes. Finally, some of the iM3 and iM4 species are elaborated with an ethanolamine-phosphate residue which is attached to the α1–6-linked mannose residue (13, 14). From the kinetics of in vivo labeling of EPM3 it is likely that this ethanolamine-phosphate is only added to fatty acid remodeled GIPL species. Only a minor pool of GIPLs are modified with ethanolamine-phosphate in L. mexicana promastigotes, while the converse is true in the amastigote stage (14). This modification appears to be unique to the GIPLs and may be mediated by a second ethanolamine-phosphotransferase or in a compartment from which GPI-anchored proteins are excluded.

A number of GIPI intermediates were identified which are likely to be precursors to the protein and LPG anchors. These included (i) the Manα1Glcn-Pi molecular species with very long alkyl chains (M1VLAC), which may be precursors for both anchor pathways, (ii) iM2VLAC which is likely to be the first committed intermediate in the LPG anchor pathway; and (iii) EPM3VLAC which may represent the final precursor in the protein anchor pathway. These analyses allow several conclusions to be made regarding the anchor pathways. First, they strongly suggest that the distinctive alkyl chain composition of both the protein and LPG anchors is acquired by the selection of specific alkylacyl-Pi molecular species rather than by alkyl chain remodeling later in the pathway. This is consistent with the finding that neither LPG nor the GIPLs are substrates for the L. donovani glycerol ether monoxygenase that is capable of cleaving 1-O-alkylglycerols (32). The exclusive use of PIVLAC in the protein and LPG anchors indicates the presence of a highly efficient selection mechanism as these species account for less than 1% of the total PI pool. However, it should be noted that there are still 2 × 10⁶ molecules of PI VLAC per cell as judged by GC-MS mass analysis. This is comparable to the pool size of all GPI-anchored macromolecules (~6 × 10⁶ molecules/cell), suggesting that PI VLAC will not be limiting for protein and LPG anchor biosynthesis. Why very long alkyl chains are selectively incorporated into the protein and LPG anchors is unknown. However, it is a highly conserved feature and may be required to stabilize the association of GPI-anchored macromolecules with the plasma membrane (25) or to modulate transmembrane signal transduction pathways in the mammalian host (Ref. 33 and references therein).

Second, it is likely that the protein anchor precursors are fatty acid remodeled in the same way as the GIPLs. This is suggested by the rapid labeling of 10EPM3VLAC with [3H]myristic acid and the finding that the protein anchors of L. major are highly enriched for myristic acid (9). Whether the same fatty acid remodeling reactions also generate the unusual lyso-alkyl-Pi lipid moiety of the LPG anchors is unclear. This type of lipid could be generated if the fatty acid remodeling reactions did not proceed beyond removal of the sn-2 fatty acid. Even though the final LPG anchor precursors are synthesized in the Golgi apparatus (34), it is possible that earlier intermediates (such as iM2VLAC) are partially remodeled and that these lyso intermediates are selectively utilized as LPG anchors. Indeed a significant proportion of the steady state pool of LPG anchor precursors and type-2 GIPLs contain a lyso-alkyl-Pi anchor (15, 25). Alternatively, a second Golgi-located phospholipase A₂ activity that only acts on LPG anchor intermediates (or type-2 GIPLs) may be responsible for modifying the LPG anchor.

Third, it is of interest that iM2VLAC was the only dedicated precursor in the LPG anchor pathway to be detected in short term in vivo labeling experiments. This species is probably made in the endoplasmic reticulum, whereas the next step in the pathway, involving the addition of a galactofuranose residue, occurs in the Golgi apparatus (34). The absence of...

---

3 K. Mullin, S. Ilgoutz, J. Ralton, and M. J. McConville, unpublished data.
detectable levels of more polar LPG anchor intermediates suggests that the transport of iM2VLAC from the endoplasmic reticulum to the Golgi apparatus may be a rate-limiting step in this pathway. However, we have previously shown that the more polar intermediates are relatively abundant in *L. mexicana* promastigotes (13). It is possible that these GPIs represent excess LPG anchor intermediates which have escaped the site of LPG phosphoglycan synthesis and been transported to the cell surface. Depending on their rate of accumulation these species may not be labeled significantly in the short term labeling experiments used in this study.

Fourth, in contrast to the situation in mammalian cells (30, 31), yeast (35, 36), and some protozoa (19, 21–23), none of the GPI anchor intermediates in *Leishmania* appear to share a set of common early intermediates that contain PI molecular species with VLAC and long (primarily stearic) sn-2-fatty acids. These pathways bifurcate with the addition of the second (α-3- or α-6-linked) mannose residue. In contrast, the GIPLs are assembled on PI molecular species that contain predominantly 1-octadecyl-2-stearoylglycerol. None of these pathways utilize the more abundant diacyl-PI and IPC lipids. Based on the chase kinetics of the Man,GlcN-PI molecular species the flux of intermediates through the GIPL pathway is approximately twice as high as the combined anchor pathways. The mannosylated GPI species characterized in this study are named and the acyl/alkyl chain compositions of each intermediate are indicated schematically. Putative intermediates in the LPG anchor pathway beyond iM2VLAC have been characterized previously (13, 25), but only the final precursor is shown for clarity.

The presence of distinct PI lipid moieties in the GIPL and GPI anchor precursors as well as the marked differences in the flux of intermediates through these pathways provides strong evidence that the GIPLs are the products of a separate pathway rather than being excess precursors of the anchor pathways. The selective incorporation of PI with C18:0 and C24:0/C26:0 alkyl chains into the GIPL and anchor GPIs, respectively, is striking and may reflect differences in the substrate specificity of the early enzymes in each pathway or the subcellular compartmentalization of the GIPL and anchor pathways into different membranes with distinct alkylacyl-PI compositions. At present, there is no evidence that early enzymes, such as the N-acetylgalactosaminyl transferase show a

---

**FIG. 13.** Model for GPI biosynthesis in *L. mexicana* promastigotes. Intermediates from three distinct pathways of GPI biosynthesis have been characterized. The protein and LPG anchor precursors appear to share a set of common early intermediates that contain PI molecular species with VLAC and long (primarily stearic) sn-2-fatty acids. These pathways bifurcate with the addition of the second (α-3- or α-6-linked) mannose residue. In contrast, the GIPLs are assembled on PI molecular species that contain predominantly 1-octadecyl-2-stearoylglycerol. None of these pathways utilize the more abundant diacyl-PI and IPC lipids. Based on the chase kinetics of the Man,GlcN-PI molecular species the flux of intermediates through the GIPL pathway is approximately twice as high as the combined anchor pathways. The mannosylated GPI species characterized in this study are named and the acyl/alkyl chain compositions of each intermediate are indicated schematically. Putative intermediates in the LPG anchor pathway beyond iM2VLAC have been characterized previously (13, 25), but only the final precursor is shown for clarity.
strong selectivity for specific PI molecular species in either mammalian (Ref. 30 and references therein) or parasite systems. In this regard, we have recently shown that diacyl-PI can be incorporated into some leishmanial GPI classes in vivo, demonstrating a lack of specificity by early GPI enzymes for alkylacyl-PI. The mannosyltransferases involved in the assembly of the glycan head groups also lack a pronounced specificity for GPI molecular species with different lipid compositions as synthetic GlcN-PI acceptors containing short (C8) and long (C16:0) acyl chains are utilized by early mannosyltransferases in L. major (26), African trypanosome (38), and mammalian (37) cell-free systems. Moreover, as shown in this study, GPI precursors with different alkyl chain compositions (i.e. M1 and M1VLAC) can be used by enzymes such as the α1–3- and α1–6-mannosyltransferases (Fig. 13). In contrast, several lines of evidence support the notion that there may be some degree of subcellular compartmentalization of the GIP and GPI anchor biosynthetic pathways. First, it is clear from this study that a α1–3 mannose branch can be added to GPI precursors with the same backbone sequence as the protein anchors. However, this branch is completely absent from the protein anchors (and conversely the α1–6-linked mannose is absent from the LPG anchor) suggesting that distinct enzyme complexes are involved in the biosynthesis of the three classes of leishmanial GIPs. Second, the marked differences in the flux of intermediates through each pathway, notably the 2-fold higher flux through the GIP pathway compared with the combined anchor pathways may reflect the localization of these enzyme complexes to membranes with different concentrations of substrates such as PI and Dol-P-Man. Third, from the kinetics of labeling of GPI intermediates in the cell-free assays, the pathway of GIP biosynthesis appears to be uncoupled from those of protein and LPG anchor biosynthesis (Fig. 12). The independent regulation of these pathways is also supported by studies on the in vivo expression of these molecules in different developmental stages of the parasite. In particular, the transformation of promastigotes to intracellular amastigotes is associated with a dramatic down-regulation in the surface expression of GPI-anchored proteins and LPG, whereas the GIPs are synthesized in high copy number in both developmental stages (10–12, 14). Collectively, these observations support the notion that there are three distinct pathways of GIP biosynthesis in L. mexicana promastigotes and that the regulation of these pathways requires a degree of compartmentalization of overlapping enzymes. It is possible that a similar degree of compartmentalization of GIP biosynthetic enzymes in other eukaryotes may explain why many parasite and mammalian GPI protein anchors contain alkylacylglycerols which are relatively minor components of the total PI pool (30, 39).

The requirement for fatty acid remodeling in the synthesis of the GIPs and possibly also the protein and LPG anchors was an unexpected finding. However, it may be important in other Leishmania spp, as the structurally distinct type-2 GIPs of L. major also contain predominantly C14:0 and C12:0 fatty acids (15). These fatty acid remodeling reactions are similar to those that occur in the African trypanosomes, and which were previously thought to be unique to these parasites. As in Leishmania, fatty acid remodeling of the trypanosome GIPs occurs mainly after assembly of the glycan head group and involves the exchange of the sn-2 fatty acid (predominantly stearate) by myristoyl chains that are transferred from a myristoyl-CoA donor (19, 40). However, in African trypanosomes the sn-1 fatty acid is also replaced with myristate to generate a dimyristoylglycerol lipid in the mature protein anchor precursors (19). It is possible that L. mexicana contains the same enzymatic machinery but that the presence of an ether-linked aliphatic chain at the sn-1 position of the L. mexicana GIPs prevents this chain from being remodelled. There are two other differences between the remodeling reactions of L. mexicana and T. brucei. First, the turnover of myristate in the leishmanial GIPs is very slow (Fig. 10C), suggesting that remodelled species are not subjected to further rounds of remodeling. In contrast, myristoylated-GPI precursors and protein-linked GIPs are extensively remodelled in African trypanosomes in a series of “proof-reading” reactions that can be biochemically and topologically distinguished from the initial remodeling reactions (41). Second, shorter fatty acids than myristate (i.e. C12:0) are incorporated into the leishmanial but not the trypanosome GIPs, suggesting that there may be subtle differences in the substrate specificity of the acyltransferases of these parasites. The reason why both the trypanosomes and Leishmania incorporate myristate into their GPI glycolipids is unknown. However, it appears to be important as the trypanosomes will divert scarce myristate into GPI remodeling when exogenous supplies of this fatty acid are low (42) and myristate analogues have been found to have potent trypanocidal activity and cause disruption of the endomembrane system (43, 44). By analogy, it is possible that the incorporation of relatively short acyl chains into the abundant leishmanial GIP species is crucial for maintaining the physical properties of the plasma membrane and that this process could be a target for anti-trypanosomal drugs.

Acknowledgments—We thank Gavin Reid (The Ludwig Institute, Melbourne Branch) for assistance with the MALDI-TOF analyses, Dr. Claire Masterson who was involved in early experiments, and Dr. Steven Ilgoutz for critical reading of the manuscript.

REFERENCES
1. Englund, P. T. (1993) Annu. Rev. Biochem. 62, 121–138
2. McConville, M. J., and Ferguson, M. A. J. (1993) Biochem. J. 294, 305–324
3. Medof, M. E., Nagurjan, S., and Tykocinski, M. L. (1996) FASEB J. 10, 574–586
4. van’t Hof, W., Rodriguez-Boulan, E., and Menon, A. (1995) J. Biol. Chem. 270, 24150–24155
5. Sevlever, D., Humphrey, D. R., and Rosenberry, T. L. (1995) Eur. J. Biochem. 233, 384–394
6. Singh, N., Liang, L.-N., Tykocinski, M. L., and Tartakoff, A. M. (1996) J. Biol. Chem. 271, 12879–12884
7. Tummino, S. J., and Desouza, P. K. (1992) Annu. Rev. Microbiol. 46, 65–94
8. McConville, M. J., Schnur, L. F., Jaffe, C., and Schneider, P. (1995) Biochem. J. 310, 807–818
9. Schneider, P., Ferguson, M. A. J., McConville, M. J., Melhert, A., Homans, S. W., and Border, C. (1990) J. Biol. Chem. 265, 16955–16964
10. Medina-Acosta, E., Karess, R. R., Schwartz, H., and Russell, D. (1989) Mol. Parasitol. 37, 263–273
11. McConville, M. J., and Blackwell, J. M. (1991) J. Biol. Chem. 266, 15170–15179
12. Bahr, V., Stierhof, Y.-D., Ilg, T., Demar, M., Quinlin, M., and Overath, P. (1993) Mol. Biochem. Parasitol. 58, 107–121
13. McConville, M. J., Collidge, T. A. C., Ferguson, M. A. J., and Schneider, P. (1993) J. Biol. Chem. 268, 15595–15604
14. Winter, G., Fuchs, M., McConville, M. J., Stierhof, Y.-D., and Overath, P. (1994) J. Cell Sci. 107, 2471–2482
15. McConville, M. J., Homans, S. W., Thomas-Oates, J. E., Dell, A., and Bacie, A. (1990) J. Biol. Chem. 265, 7385–7394
16. Proudfout, L., O’Donnell, C. A., and Liew, F. Y. (1995) Eur. J. Immunol. 25, 745–750
17. Tschado, S. D., Gerold, P., Schwarz, R., Novakovics, S., McConville, M., and Schofield, L. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4022–4027
18. Redman, C. A., Schneider, P., Melhert, A., and Ferguson, M. A. J. (1995) Biochem. J. 311, 495–503
19. Masterson, W. J., Raper, J., Doering, T. L., Hart, G. W., and Englund, P. T. (1996) Cell 82, 73–80
20. Menon, A. K., Schwarz, R. T., Mayor, S., and Cross, A. M. (1990) J. Biol. Chem. 265, 9033–9042
21. Guth, M. L., and Ferguson, M. A. J. (1995) EMBO J. 14, 3080–3093
22. Heise, N., Raper, J., Buxbaum, L. U., Pernovich, T. M., and Almeida, M. L. (1996) J. Biol. Chem. 271, 16877–16887
23. Gerold, P., Diekmann-Schuppert, A., and Schwarz, R. T. (1994) J. Biol. Chem. 269, 2597–2606
24. Tomavo, S., Dubravetz, J.-F., and Schwarz, R. T. (1992) J. Biol. Chem. 267, 21446–21458
25. Proudfout, L., Schneider, P., Ferguson, M. A. J., and McConville, M. J. (1995)

4 J. Zawadzki, C. Scholz, G. Currie, G. Coombs, and M. J. McConville, submitted for publication.
26. Smith, T. K., Milne, F. C., Sharma, D. K., Crossman, A., Brimacombe, J. S., and Ferguson, M. A. J. (1997) Biochem. J. 328, 393–400
27. Ilg, T., Etges, R., Overath, P., McConville, M. J., Thomas-Oates, J., Homans, S. W., and Ferguson, M. A. J. (1992) J. Biol. Chem. 267, 6834–6840
28. McConville, M. J., Thomas-Oates, J. E., Ferguson, M. A. J., and Homans, S. W. (1990) J. Biol. Chem. 265, 19611–19623
29. Schneider, P., Ralton, J. E., McConville, M. J., and Ferguson, M. A. J. (1993) Anal. Biochem. 210, 106–112
30. Stevens, V. L. (1995) Biochem. J. 310, 361–370
31. Takeda, J., and Kinoshita, T. (1995) Trends Biochem. Sci 20, 367–371
32. Ma, D., Beverley, S., and Turco, S. J. (1996) Biochem. Biophys. Res. Commun. 227, 885–889
33. Giordione, J., Turco, S. J., and Epand, R. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11634–11639
34. Ha, D. S., Schwartz, J. K., Turco, S. J., and Beverley, S. M. (1996) Mol. Biochem. Parasitol. 77, 57–64
35. Costello, L. C., and Orlean, P. (1992) J. Biol. Chem. 267, 8599–8603
36. Sipos, G., Potti, A., and Conzelmann, A. (1994) EMBO J. 13, 2789–2796
37. Doerrler, W. T., Ye., J., Falck, J. R., and Lehrman, M. A. (1996) J. Biol. Chem. 271, 27031–27038
38. Smith, T. K., Cottez, S., Brimacombe, J. S., and Ferguson, M. A. J. (1996) J. Biol. Chem. 271, 6476–6482
39. Butokofer, P., Zollinger, M., and Brodbeck, U. (1992) Eur. J. Biochem. 208, 677–683
40. Doering, T. L., Pessin, M. S., Hart, G. W., Raben, D. M., and Englund, P. T. (1994) Biochem. J. 299, 741–746
41. Bauxbaum, L. U., Raper, J., Opperdoes, F. R., and Englund, P. T. (1994) J. Biol. Chem. 269, 30212–30220
42. Doering T. L., Pessin, M. S., Hoff, E. F., Hart, G. W., Raben, D. M., and Englund, P. T. (1993) J. Biol. Chem. 268, 9215–9222
43. Doering, T. L., Raper, J., Buxbaum, L. U., Adams, S. P., Gordon, J. I., Hart, G. W., and Englund, P. T. (1991) Science 252, 1851–1854
44. Doering, T. L., Lo, T., Werbovetz, K. A., Gokel, G. W., Hart, G. W., Gordon, J. I., and Englund, P. T. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9735–9739
Delineation of Three Pathways of Glycosylphosphatidylinositol Biosynthesis in *Leishmania mexicana*: PRECURSORS FROM DIFFERENT PATHWAYS ARE ASSEMBLED ON DISTINCT POOLS OF PHOSPHATIDYLINOSITOL AND UNDERGO FATTY ACID REMODELING

Julie E. Ralton and Malcolm J. McConville

*J. Biol. Chem.* 1998, 273:4245-4257.
doi: 10.1074/jbc.273.7.4245

Access the most updated version of this article at [http://www.jbc.org/content/273/7/4245](http://www.jbc.org/content/273/7/4245)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 44 references, 29 of which can be accessed free at [http://www.jbc.org/content/273/7/4245.full.html#ref-list-1](http://www.jbc.org/content/273/7/4245.full.html#ref-list-1)