Intracellular trafficking of glycosylphosphatidylinositol (GPI)-anchored proteins and free GPIs in *Leishmania mexicana*

Julie E. RALTON[1], Kylie A. MULLIN[1] and Malcolm J. McCONVILLE[2]

Department of Biochemistry and Molecular Biology, University of Melbourne, Parkville, Victoria 3010, Australia

Free glycosylphosphatidylinositols (GPIs) are an important class of membrane lipids in many pathogenic protozoa. In this study, we have investigated the subcellular distribution and intracellular trafficking of an abundant class of free GPIs [termed glycosylinositolphospholipids (GIPLs)] in *Leishmania mexicana* promastigotes. The intracellular transport of the GIPLs and the major GPI-anchored glycoprotein gp63 was measured by following the incorporation of these molecules into sphingolipid-rich, detergent-resistant membranes (DRMs) in the plasma membrane. In metabolic-labelling experiments, mature GIPLs and gp63 were transported to DRMs in the plasma membrane with a $t_{1/2}$ of 70 and 40 min, respectively. Probably, GIPL transport to the DRMs involves a vesicular mechanism, as transport of both the GIPLs and gp63 was inhibited similarly at 10 °C. All GIPL intermediates were quantitatively recovered in Triton X-100-soluble membranes and were largely orientated on the cytoplasmic face of the endoplasmic reticulum, as shown by their sensitivity to exogenous phosphatidylinositol-specific phospholipase C. On the contrary, a significant proportion of the mature GIPLs ($\approx 50\,\%$ of iM4) were accessible to membrane-impermeable probes on the surface of live promastigotes. These results suggest that the GIPLs are flipped across intracellular or plasma membranes during surface transport and that a significant fraction may populate the cytoplasmic leaflet of the plasma membrane. Finally, treatment of *L. mexicana* promastigotes with myriocin, an inhibitor of sphingolipid biosynthesis, demonstrated that ongoing sphingolipid biosynthesis is not required for the plasma-membrane transport of either gp63 or the GIPLs and that DRMs persist even when cellular levels of the major sphingolipid are depleted by 70 %.

Key words: detergent-resistant membranes, endoplasmic reticulum, flippases, lipid rafts, sphingolipids.

INTRODUCTION

Single-celled protozoa from diverse phylogenetic groups such as the Apicomplexa, Trypanosomatidae and Entamoebidae are the cause of many serious diseases in human beings. The plasma membranes of these parasitic protozoa are characteristically covered with conspicuous surface coats that are dominated by glycosylphosphatidylinositol (GPI)-anchored glycoproteins [1,2]. The use of GPI anchors may be required to accommodate the high levels of expression of these proteins and also serve to insulate the cytoplasm from the external environment [1]. In addition, many of these protozoa also synthesize high levels of free (non-protein-linked) GPIs that can constitute the major glycolipid class [1,2]. The metabolic relationship between the GPI-protein-anchor precursors and these free GPIs remains unclear. Moreover, little is known about the function of these free GPIs or their distribution within intracellular organelles versus the plasma membrane. Although some of these glycolipids appear to be potent modulators of the host immune response [3,4], they are also synthesized by plant parasites and free-living protozoa [5,6], suggesting that they have a more general role(s).

The structure and biosynthesis of the GPI-anchored glycoproteins and free GPIs of *Leishmania* spp. have been extensively studied. These parasites alternate between a flagellated promastigote stage that proliferates in the midgut of the sandfly vector and a non-motile amastigote stage that invades mammalian macrophages and divides within the phagolysosome compartment. *Leishmania* promastigotes are coated with a number of GPI-anchored glycoproteins (gp63, PSA2 and a proteophosphoglycan), a hyperglycosylated GPI, termed lipophosphoglycan (LPG) and a family of abundant free GPIs, termed glycosylinositolphospholipids (GIPLs) [1,7]. These molecules are thought to form a protective surface glycocalyx and have specific roles in mediating the attachment of promastigotes to the sandfly midgut and establishment of an infection in the mammalian host [7]. The assembly and surface transport of the major surface glycoprotein gp63 have been the subject of several studies. Gp63 is initially translocated into the lumen of the endoplasmic reticulum (ER), where it is modified with N-linked glycans and a GPI anchor [8]. It is subsequently exported to the single Golgi apparatus at the anterior end of the parasite via a specialized transitional ER [9]. Gp63 passes through the Golgi apparatus without receiving any modifications to either the N-linked glycans or the GPI anchor and is delivered to the membrane of the flagellar pocket. The flagellar pocket is a specialized invagination in the plasma membrane that surrounds the flagellum and is the only part of the cell-body plasma membrane that is not subtended by a highly cross-linked array of microtubules. It is thus thought to be the sole site of exo- and endo-cytosis in these parasites [10,11]. Although gp63 does not receive any Golgi modifications, the N-terminal domain of this protein is removed during transport to the cell surface or upon reaching the plasma membrane [12]. Aspects of the assembly and surface transport of the LPG have also been investigated. The glycan core of the LPG anchor (having the structure Manα1-3Manα1-4GlcN-PI) is assembled in the same subcompartment of

Abbreviations used: BiP, immunoglobulin heavy-chain binding protein; DRMs, detergent-resistant membranes; GPI, glycosylphosphatidylinositol; GIPL, glycosylinositolphospholipid; ER, endoplasmic reticulum; HPTLC, high-performance thin layer chromatography; IPC, inositol phosphoceramide; LPG, lipophosphoglycan; PI-PLC, phosphatidylinositol-specific phospholipase C; Tos-Lys-CH_2Cl, tosyl-lysylchloromethane; TTBS, Tris-buffered saline containing 0.05 % Tween 20.

[1] These authors have contributed equally to this work.

[2] To whom correspondence should be addressed (e-mail malcolmm@unimelb.edu.au).

the ER as the protein anchors [13]. This GPI precursor is subsequently elongated with three galactose residues, the first of which appears to be added in the lumen of the Golgi apparatus [14]. The galactosylated LPG-anchor precursor is further extended with a linear phosphoglycan chain by Golgi glycosyltransferases [15], before being transported to the flagellar pocket and the plasma membrane. Thus both gp63 and LPG are transported to the cell surface via the classical secretory pathway.

On the contrary, comparatively little is known about the surface transport of the GIPLs. In *L. mexicana*, the GIPLs have the same glycan backbone as the protein anchors, but contain an additional α1-3 mannose residue that also occurs in the LPG anchor (hybrid-type GIPLs) [16]. The GIPL precursors are 10–100-fold more abundant than the protein-anchor precursors, but are assembled in the same ER subcompartment [13]. Although there is evidence that some of these hybrid-type GIPLs are expressed on the cell surface [17], it is not known whether surface transport occurs within the lumen of the secretory pathway, as suggested by a number of studies [18], or via a number of potential cytoplasmic routes. The extent to which GIPLs are transported to intracellular organelles is also unknown. These questions have important implications for understanding the function of these very abundant glycolipids.

In the present study, we have investigated whether the GIPLs are transported to the plasma membrane via the same vesicular transport pathway as gp63 in *L. mexicana* promastigotes. To measure the surface transport of these glycolipids, we have exploited the finding that both gp63 and the GIPLs become incorporated into sphingolipid-rich Triton X-100-insoluble domains upon reaching the plasma membrane. These domains have the same properties as the detergent-resistant membranes (DRMs) or lipid rafts of other eukaryotes [19–21]. The results suggest that most of the mature GIPL species reach the plasma membrane via the same temperature-sensitive vesicular transport pathway as gp63, although with slightly slower kinetics than gp63. Topological studies suggest that the mannosylated GIPLs are initially transported to the cytoplasmic leaflet of the ER before being flipped to the lumen of the secretory pathway or the exoplasmic leaflet of the plasma membrane. Finally, the surface transport of both gp63 and the GIPLs is not dependent on ongoing sphingolipid biosynthesis, in contrast with the situation in some other eukaryotes. These results support the notion that the GIPLs are major components of the surface glycocalyx and provide insights into the possible function of DRMs in trypanosomatids.

EXPERIMENTAL

Cell culture

Promastigotes of *L. mexicana* (strain MNYC/BZ/62/M379) and the *L. mexicana* ΔLPG2 mutant that lacks the GDP-Man transporter [22] were routinely cultivated in RPMI 1640 medium containing 10 % fetal bovine serum at 27 °C.

Metabolic labelling

Mid-log-phase *L. mexicana* promastigotes were labelled to steady state with [^{3}H]ethanolamine or [^{3}H]inositol (5 μCi/ml) over 16–20 h as previously described [16]. GPI intermediates and gp63 were also pulse–chase-labelled with [^{3}H]mannose, [^{3}H]serine and Tran-^{35}S label as described previously [16,23]. Briefly, promastigotes were incubated in glucose- and methionine-free media for 10 min at 27 °C before addition of [^{3}H]mannose (50 μCi/ml), [^{3}H]serine (50 μCi/ml) or Tran-^{35}S label (200 μCi/ml). After 10 min, promastigotes were resuspended at 2×10^7 cells/ml in conditioned RPMI-FBS medium and aliquots (2×10^7 cells) harvested by centrifugation (16000 ***g***, 15 s) at defined time intervals. Promastigotes were labelled with [^{3}H]myristic acid using the same protocol except that the [^{3}H]myristic acid (50 mCi/ml) was delivered as an equimolar complex with defatted BSA [16].

Triton X-100 detergent extractions

L. mexicana cell pellets [(2–5) $\times 10^7$ cells] were washed once in ice-cold PBS and then extracted in 20–100 μl of ice-cold PBS containing 1 % Triton X-100, 0.2 mM tosyl-lysylchloromethane (Tos-Lys-CH_2Cl, 'TLCK'), 0.1 μM leupeptin, 0.1 mM PMSF, for 30 min on ice. After centrifugation (16000 ***g***, 5 min, 4 °C), the Triton X-100 insoluble (pellet) and soluble (supernatant) fractions were processed as follows. Metabolically labelled lipids were extracted from the pellet in chloroform/methanol/water (1:2:0.8, v/v; 300 μl) and then recovered from the dried organic extract by biphasic partitioning in a 1-butanol/water (1:1, v/v) mixture [24]. Detergent-soluble lipids were recovered directly by 1-butanol/water partitioning of the extract. [^{3}H]GPIs/[^{3}H]sphingolipids were resuspended in 40 % 1-propanol and resolved by chromatography on aluminium-backed silica-gel 60 HPTLC sheets (Merck) developed in chloroform/methanol/1 M ammonium acetate/13 M NH_4OH/water (180:140:9:9:23, v/v; solvent system A), unless otherwise stated. Incorporation of radiolabel into individual bands was measured using a Bertold LB 2821 automatic TLC linear scanner. GPIs were also detected by fluorography after spraying HPTLC sheets with EN^{3}HANCE spray (Dupont) and exposing them to Biomax MR (Kodak) film at −70 °C.

For extraction of gp63, the Triton X-100-insoluble pellets were resuspended in PBS containing 1 % Triton X-114 (0 °C, 30 min). After removal of insoluble material by centrifugation, the extract was phase-partitioned and the Triton X-114 detergent phase collected after two rounds of condensation and centrifugation through a sucrose cushion [25]. Gp63 was quantitatively recovered in the Triton X-114 detergent phase and precipitated with 9 vol. of cold acetone. Gp63 in the Triton X-100 soluble fractions was also recovered by Triton X-114 phase partitioning, after the extracts were diluted to give a final concentration of 1 % Triton X-114 and 0.02 % Triton X-100 in PBS. ^{35}S- and [^{3}H]ethanolamine-labelled proteins were separated by SDS/PAGE on 12 % gels and detected by fluorography after treating the gels with Amplify (Amersham) prior to drying, then exposing them to Biomax MR film (Kodak) at −70 °C.

Labelling of GPI glycolipids *in vitro*

L. mexicana promastigotes from mid-log-growth cultures were harvested by centrifugation (800 ***g***, 10 min, 25 °C), washed with ice-cold PBS, then hypotonically permeabilized in 1 mM Hepes/NaOH buffer, pH 7.4, containing 2 mM EGTA, 2 mM dithiothreitol, 0.2 mM Tos-Lys-CH_2Cl, 2 μM leupeptin, 0.1 mM PMSF (1×10^8 cells/ml) for 10 min on ice. Permeabilized cells were pelleted by centrifugation (2000 ***g***, 2 min, 4 °C), washed with 50 mM Hepes/NaOH buffer, pH 7.4, containing 100 mM KCl, 2 mM EGTA, 1 mM ATP, 2 mM dithiothreitol, 0.2 mM Tos-Lys-CH_2Cl, 0.1 μM leupeptin, 0.1 mM PMSF and ATP-regenerating system (10 mM creatine phosphate, 50 μg/ml creatine kinase), 1 mM UDP-GlcNAc (assay buffer), then resuspended in assay buffer at 1×10^9 cell equivalents/ml. The permeabilized cells were pulse-labelled by adding GDP-[^{3}H]Man (0.5 mCi to 1×10^7 cell equivalents) and then the label was chased after 10 min by addition of 1 mM GDP-Man to the mixture and further incubated at 27 °C for 1 h.

Sucrose-gradient centrifugation

L. mexicana promastigotes were extracted with 1% Triton X-100 as described above and the total extract was adjusted to 60% sucrose, before being overlaid with a discontinuous sucrose gradient consisting of 50% sucrose (4 ml), 45% sucrose (2 ml), 40% sucrose (2 ml), 30% sucrose (1 ml) and 5% sucrose (1 ml) in an ultracentrifuge tube. Gradients were centrifuged at 200000 ***g*** for 16–20 h at 4 °C in a Beckman SW41 rotor and fractions (600 μl) collected from the top. Proteins were solvent-precipitated [26] and resolved using 12% SDS/PAGE. Proteins were stained with Coomassie Blue or detected by immunoblotting with anti-gp63 antibodies after transfer to nitrocellulose. The plasma membrane marker 3′-nucleotidase was detected using known procedures [27,28]. ^{3}H-labelled lipids were recovered from the sucrose fractions by biphasic partitioning in 1-butanol/water (1:1, v/v) and analysed by high-performance thin layer chromatography (HPTLC).

Surface biotinylation

L. mexicana promastigotes (2×10^7 cells) were surface-biotinylated as previously described [23]. Biotinylated and non-biotinylated proteins were separated on streptavidin–agarose and acetone-precipitated prior to analysis on 12% SDS/PAGE and detection by fluorography. Protein was quantified by densitometry and the first time point at which ^{35}S-labelled gp63 was first detected was taken as $t = 0$ min for the calculation of surface-transport kinetics.

L. mexicana promastigotes were also pulse–chase-labelled with [^{3}H]mannose and purified plasma membranes obtained after surface biotinylation and cell lysis as follows. Biotinylated promastigotes were permeabilized in ice-cold Hepes/NaOH hypotonic buffer (10^8 cells/ml), then lysed by expulsion ($\times 15$) through a 27-gauge needle. The osmolarity of the cell lysate was adjusted by addition of 6× assay buffer and biotinylated plasma membranes were recovered by incubation of the lysate with streptavidin-Dynabeads (140 μl of bead suspension/2×10^7 promastigote equivalents) on a rotating wheel for 30 min at 4 °C. The beads were pelleted in a magnetic particle concentrator (Dynal) for 2 min, then washed with 2×PBS. Lipids were extracted from the beads in chloroform/methanol/water (1:2:0.8, v/v) and recovered by 1-butanol/water partitioning. Protein was eluted from the beads by boiling in Laemmli's reducing-sample buffer. Microsomes in the unbound fraction were recovered by centrifugation at 100000 ***g*** for 30 min at 4 °C. The membrane pellet was extracted in the same way as the bound fraction.

Trypsin-latency experiments

The latency of ER membranes in permeabilized promastigotes was examined by trypsin digestion [29]. Permeabilized promastigotes were incubated with trypsin (2 units, Worthington Biochem. Corp.) for 20 min at 25 °C and the digestions stopped by addition of soya bean trypsin inhibitor (64 μg) and a further 10 min incubation on ice. In control experiments, trypsin was either omitted or inactivated with soya bean trypsin inhibitor prior to addition. Trypsin digestion of all proteins was facilitated by inclusion of 1% Triton X-100 in the reaction mixture. Protein was recovered by solvent precipitation [26] and resolved by 12% SDS/PAGE, prior to transfer to nitrocellulose and immunoblotting with anti-(immunoglobulin heavy-chain binding protein) (BiP) antibodies.

Jack-bean α-mannosidase and phosphatidylinositol-specific phospholipase C (PI-PLC) digestions

Live ΔLPG2 promastigotes were treated with jack-bean α-mannosidase (Sigma) in RPMI, pH 6.0 containing 1% BSA (2×10^7 cells, 50 μl) with or without 1% Triton X-100. Live and permeabilized promastigotes were also treated with *Bacillus cereus* PI-PLC (ICN) in 50 mM triethanolamine/HCl buffer, pH 7.5, containing 10 mM EDTA, 0.25 M sucrose and 10 mM glucose (2×10^7 cells, 50 μl) with or without 1% Triton X-100. Enzyme digests were incubated at 25 °C for 3.5 h. Lipids were extracted as described above.

Immunoblotting

After separation by 12% SDS/PAGE, proteins were transferred to nitrocellulose membranes which were blocked with 5% powdered skim milk in Tris-buffered saline containing 0.05% Tween 20 (TTBS). For detection of gp63 and BiP, the nitrocellulose was probed with the monoclonal antibody Tü L3.8 [30] and a polyclonal antibody raised against the *Trypanosoma brucei* BiP [31] followed by anti-mouse horseradish peroxidase-conjugated antibody (1:10000 dilution; Silenus) and anti-rabbit horseradish peroxidase-conjugated antibody (1:10000 dilution; Silenus), respectively. All antibodies were diluted in 1% powdered skim milk/TTBS, except for anti-BiP which was diluted in TTBS. Detection of the secondary antibodies was by ECL Western detection system (Amersham).

Treatment with myriocin

Mid-log promastigotes were either incubated in the presence of 20 μg myriocin/ml (1:200 dilution of stock in methanol) or 0.4% methanol, as control, for 20 min or 20 h. The efficacy of myriocin treatment was monitored by pulse–chase-labelling promastigotes with [^{3}H]serine. Sphingolipids were extracted and analysed after base treatment [24] by HPTLC in chloroform/methanol (9:1, v/v) or solvent system A. Inositol phosphoceramide (IPC) was detected by spraying HPTLC sheets with 3% cupric acetate in 8% orthophosphoric acid, followed by charring at 140 °C.

RESULTS

L. mexicana gp63 and GIPLs become incorporated into DRMs upon reaching the plasma membrane

We have previously found that a variety of surface-labelling approaches (periodate oxidation, amine-reactive reagents, PI-PLC digestion [32]) could not be used to measure the rate of surface transport of the major GIPL species of *L. mexicana* promastigotes. We therefore examined whether these glycolipids were incorporated into DRMs while in transit to or upon reaching the plasma membrane and whether this parameter could be used to follow intracellular transport. When *L. mexicana* promastigotes were extracted in ice-cold 1% Triton X-100, most of the gp63 and the GIPLs were recovered in the Triton X-100-insoluble fraction (Figures 1A and 1B). HPTLC analysis of the [^{3}H]inositol-labelled lipids revealed that the Triton X-100-insoluble fraction was highly enriched in IPC, the major sphingolipid of these parasites, and was depleted in glycerophospholipids such as phosphatidylinositol and phosphatidylinositol phosphate (Figure 1A). When the ice-cold Triton X-100 extract was subjected to flotation centrifugation in a sucrose gradient, essentially all of the gp63, GIPLs and IPC were recovered in a

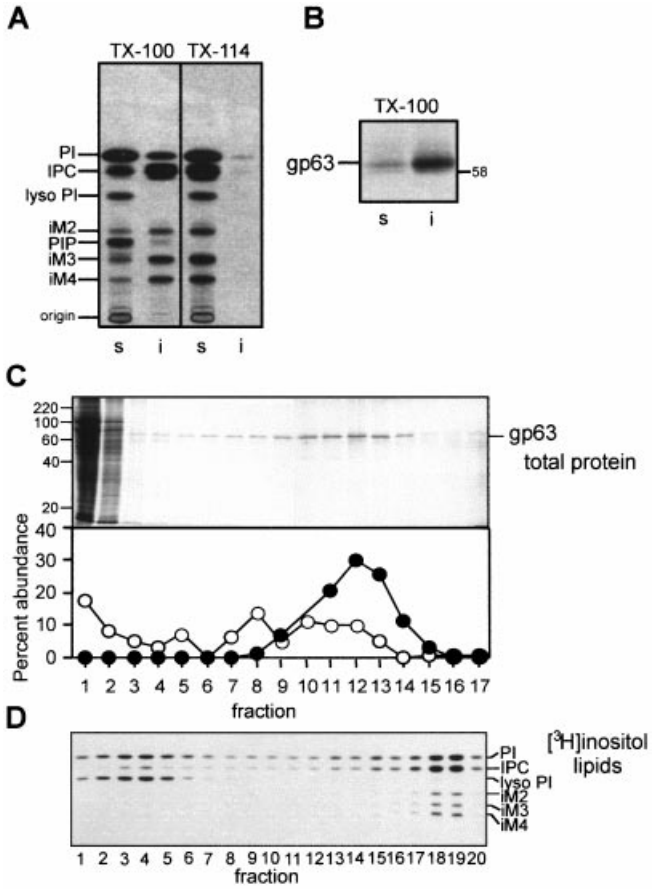

Figure 1 Steady-state pools of free GPIs and gp63 are primarily localized in sphingolipid-rich Triton X-100-insoluble membranes

(**A**) *L. mexicana* promastigotes were metabolically labelled to steady state with [^{3}H]inositol and then extracted in 1% (v/v) Triton X-100 or 1% (v/v) Triton X-114 at 0 °C for 30 min. [^{3}H]Inositol lipids in the detergent-soluble (s) and -insoluble (i) fractions were analysed by HPTLC. Most of the diacyl-PI, lyso-PI and phosphatidylinositol phosphate (PIP) were soluble in Triton X-100, but essentially all the GIPLs (iM2, −3, −4) and IPC were Triton X-100 insoluble. On the contrary, all the inositol lipids were solublized in Triton X-114. (**B**) *L. mexicana* promastigotes were labelled to steady state with [^{3}H]ethanolamine, and the distribution of labelled gp63 in the ice-cold 1% (v/v) Triton X-100-soluble (s) and -insoluble (i) fractions was determined after SDS/PAGE and fluorography. (**C**) An ice-cold 1% (v/v) Triton X-100 extract of *L. mexicana* promastigotes was overlaid with a 5–50% discontinuous sucrose gradient and subjected to flotation equilibrium centrifugation. Proteins in each fraction were resolved by SDS/PAGE and detected by staining with Coomassie Blue (top panel). The major band in fractions 9–13 was identified as gp63 by immunoblotting. Distribution of gp63 (●) and 3′-nucleotidase (○) were determined by immunoblotting and enzyme assay, respectively (bottom panel). (**D**) *L. mexicana* promastigotes were metabolically labelled with [^{3}H]inositol, and a Triton X-100 extract was subjected to flotation centrifugation. Labelled lipids were recovered from each fraction and analysed by HPTLC. Sucrose density decreases from left to right in the Figure. Gp63, the GIPLs and IPC were all found in fractions with the same sucrose density [fractions 12, 13 in (**C**); fractions 18, 19 in (**D**)].

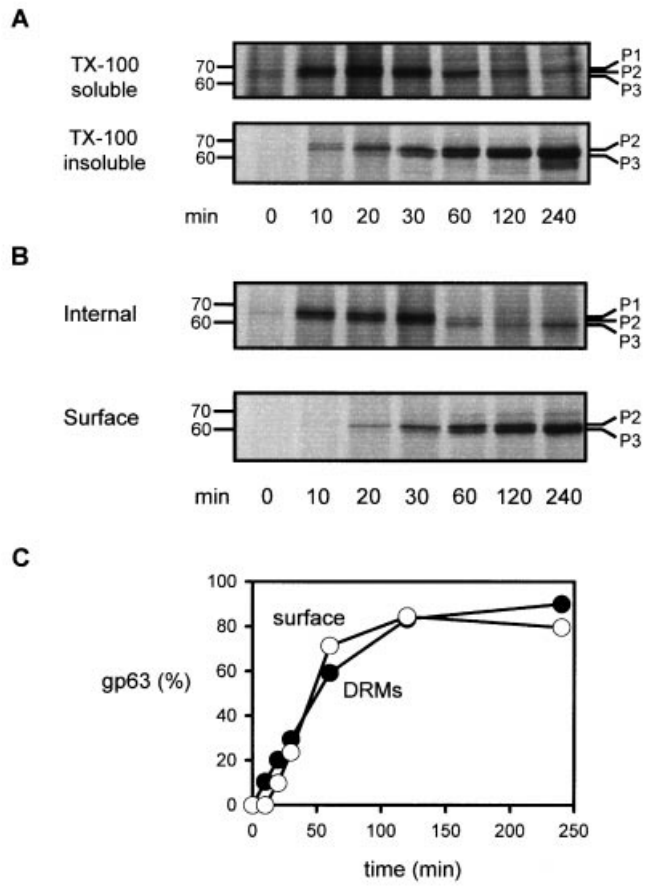

Figure 2 Gp63 associates with DRMs upon arrival at the cell surface

L. mexicana promastigotes were pulse–chase-labelled with [^{35}S]Met/Cys to follow the incorporation of gp63 into DRMs and cell-surface transport. (**A**) At different time points in the chase, promastigotes were extracted in ice-cold Triton X-100 and the distribution of gp63 in Triton X-100-soluble and -insoluble fractions was determined by SDS/PAGE. (**B**) A parallel aliquot of promastigotes was surface-biotinylated at each time point, and biotinylated and non-biotinylated gp63 were separated on streptavidin–agarose and detected by SDS/PAGE and fluorography. (**C**) The kinetics of incorporation of gp63 into DRMs and surface-biotinylated fractions was determined after quantification of the levels of gp63 in each fraction by densitometry. P1, a GPI-anchored proform of gp63; P2, a processed form of P1 and a second (major) proform of gp63; P3, the major isoform of gp63. Although P1 remains soluble in Triton X-100, P3 associates with DRMs with increasing chase times.

buoyant fraction near the top of the gradient (Figures 1C and 1D). The buoyant membrane fraction was remarkably depleted of other cellular proteins, which remained in the bottom two fractions (Figure 1C). We also tested whether other less-abundant cell-surface proteins were present in these buoyant membranes. Using a sensitive enzymatic assay, a significant proportion of the 3′-nucleotidase (a type-1 membrane protein) was recovered in this fraction (Figure 1C). These results suggest that the steady-state pools of gp63 and the GIPLs are associated with Triton X-100-insoluble membranes that have similar properties to the DRMs from other eukaryotic cells [19–21].

We next investigated at what point in the secretory pathway gp63 and the GIPLs associate with the DRMs. *L. mexicana* promastigotes were labelled with Tran^{35}S-label for 10 min, then resuspended in complete medium and parallel samples taken at various times for extraction with ice-cold Triton X-100 or surface biotinylation. Previous studies have shown that GPI-anchored proforms of gp63 are processed by an undefined protease activity [12]. When analysed by SDS/PAGE, three gp63 polypeptides were resolved. The upper band corresponds to the minor proform of gp63, termed P1. The middle band (P2) contains a mixture of processed P1 and a second major proform of gp63. The bottom band (P3) corresponds to the major (processed) isoform of gp63 (Figure 2A) (K. A. Mullin and M. J. McConville, unpublished work). The proforms of gp63 (i.e. P1, P2) were the major bands labelled in the 0–30 min time points and were quantitatively recovered in the Triton X-100-soluble

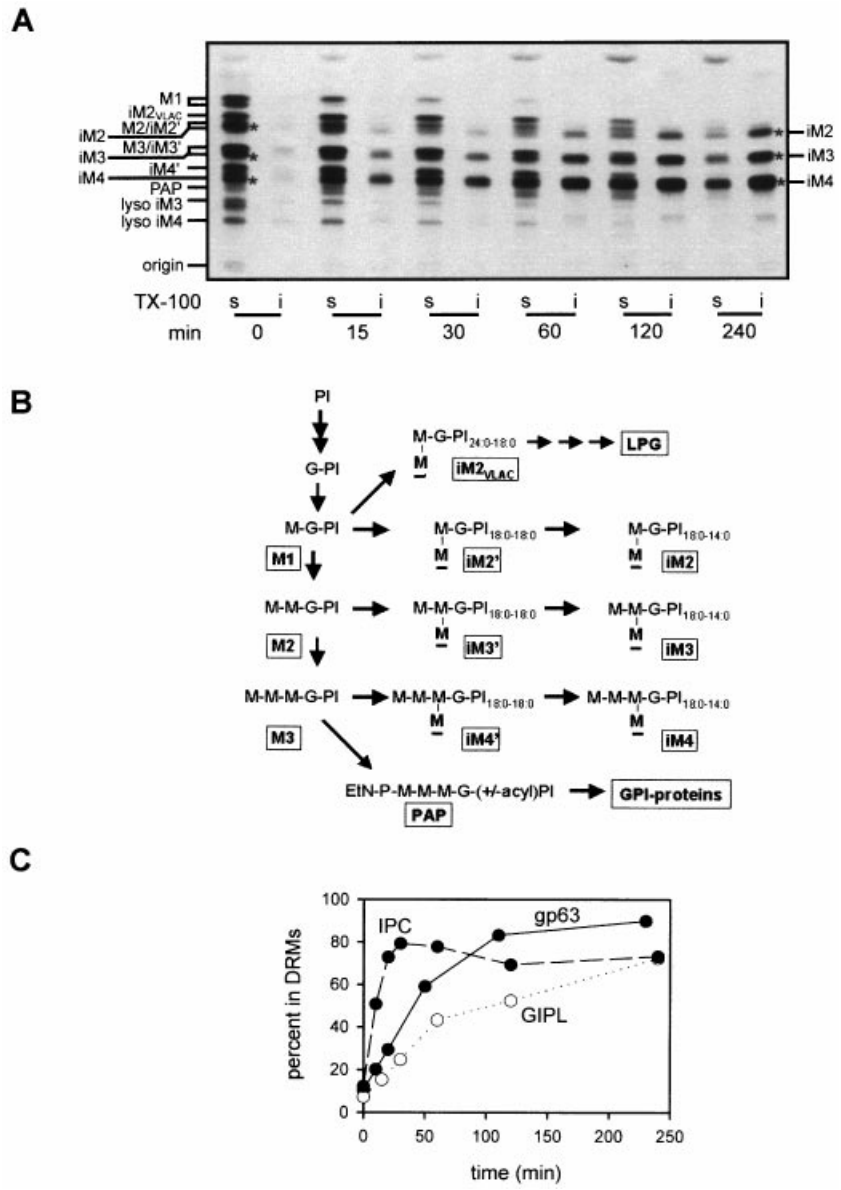

Figure 3 Only mature GIPL species associate with DRMs

(**A**) *L. mexicana* promastigotes were pulse-labelled with [^{3}H]Man, and extracted in ice-cold 1% Triton X-100 at the indicated chase times. Labelled GPIs in the Triton X-100-soluble and -insoluble fractions were analysed by HPTLC. GIPL intermediates containing unremodelled lipid moieties (see **B**) remained in the Triton X-100-soluble (s) fractions, but mature GIPLs (iM2, −3, −4; species marked by an asterisk) become incorporated into DRMs (i) with time. (**B**) Schematic showing relationship between intermediates in GIPL, protein anchor and LPG anchor biosynthesis. Intermediates in different pathways contain distinct alkylacyl-PI lipid moieties (denoted by subscript that lists the chain length of the major *sn*-1 alkyl and *sn*-2 acyl chains) and undergo fatty-acid remodelling reactions [16]. M, mannose; M, α1-3-linked mannose; G, glucosamine, PI, phosphatidylinositol. (**C**) Kinetics of transport of gp63, GIPLs and the major sphingolipid IPC into DRMs.

fraction (Figure 2A). The mature forms of gp63 (i.e. P3) were first detected in the 30 min chase and were primarily recovered in the Triton X-100-insoluble fraction (Figure 2A). Separate aliquots of promastigotes were surface-biotinylated in parallel, and the non-biotinylated (internal) and biotinylated (surface) gp63 was analysed by SDS/PAGE. The P1 proform was present exclusively in internal membranes, but the mature P3 band was primarily located at the cell surface (Figure 2B). The kinetics of transport of gp63 into DRMs and the cell surface were very similar ($t_{1/2} \approx 40$ min; representative of three experiments) (Figures 2A and 2B), suggesting that gp63 becomes incorporated into DRMs in a late-secretory compartment or upon reaching the plasma membrane. Interestingly, a proportion of the mature P3 band always appeared in the non-biotinylated fraction at later time points, possibly reflecting some internalization via endocytosis (Figures 2B and 2C). This was not associated with a detectable redistribution of P3 into the Triton X-100-soluble fraction (Figures 2A and 2C), suggesting that the internalized protein may remain in DRMs.

We next investigated the kinetics of GIPL incorporation into DRMs. *L. mexicana* promastigotes were pulse-labelled with [^{3}H]mannose, then resuspended in complete medium to follow the incorporation of label into GPI intermediates and mature GIPLs (Figure 3B). All the intermediates in the pathways of

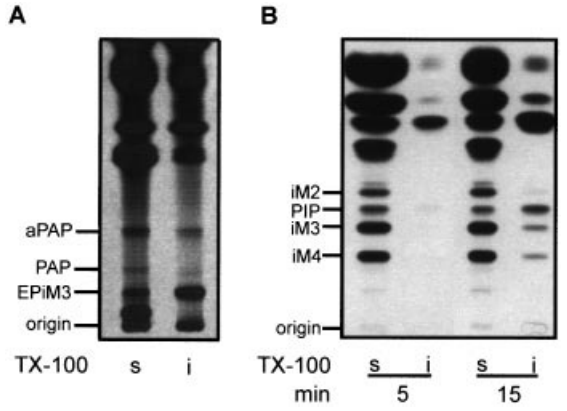

Figure 4 Incorporation of GIPLs into DRMs is not dependent on their lipid composition

(**A**) HPTLC analysis of [^{3}H]ethanolamine steady-state-labelled lipids that had been partitioned between Triton X-100-soluble (s) and -insoluble (i) fractions. The GPI protein-anchor precursors, PAP and aPAP (an inositol acylated form of PAP) that contain very long ($C_{24:0}$) alkyl chains are primarily present in the Triton X-100-soluble fraction. In contrast, the ethanolamine-phosphate-modified iM3 species EPiM3 with shorter ($C_{18:0}$) alkyl chains, is primarily present in the Triton X-100-insoluble fraction. (**B**) *L. mexicana* promastigotes were pulse–chase-labelled with [^{3}H]myristic acid for 5 min (5), then resuspended in fresh medium containing unlabelled myristic acid for 10 min (15). Fatty-acid-remodelled GPIs (containing myristic acid at the *sn*-2 position) were extracted in ice-cold 1% Triton X-100, and soluble (s) and insoluble (i) fractions analysed by HPTLC and fluorography. The GIPL intermediates are myristylated before they become incorporated into DRMs, demonstrating that fatty-acid remodelling is not responsible for the acquisition of Triton X-100 insolubility.

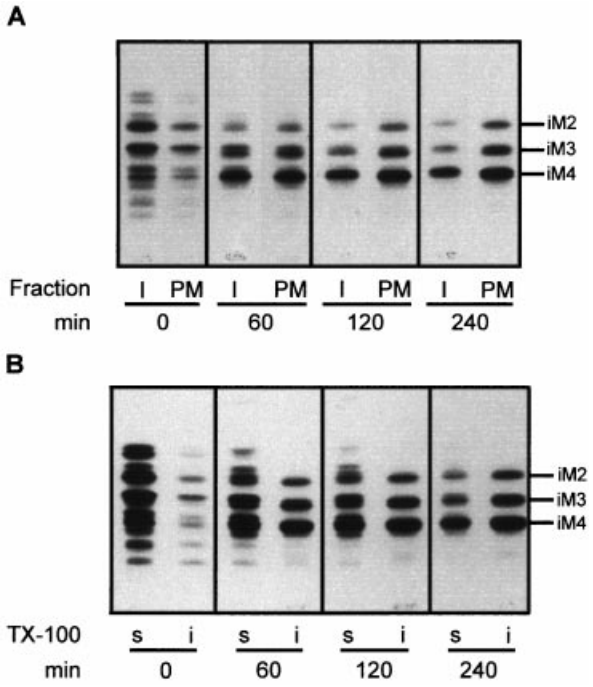

Figure 5 Free GPIs are transported to DRMs and the plasma membrane with the same kinetics

(**A**) *L. mexicana* promastigotes were pulse–chase-labelled with [^{3}H]Man and nascent GIPLs in affinity-purified plasma membrane (PM) and internal membranes (I) analysed by HPTLC and fluorography. (**B**) An aliquot of the metabolically labelled *L. mexicana* promastigotes from (**A**) was extracted in ice-cold 1% Triton X-100, and labelled GIPLs in the soluble (s) and insoluble (i) fractions were analysed as in (**A**).

GIPL, protein-anchor and LPG-anchor biosynthesis were labelled after a 5 min pulse and quantitatively extracted in Triton X-100 (Figure 3A). A number of mature GIPL species were also labelled at this time point (species marked by an asterisk) and were detergent-soluble (Figure 3A, $t = 0$ min). The label was chased progressively into these mature GIPLs, which gradually accumulated in the Triton X-100-insoluble fraction (Figure 3A, see $t = 240$ min). On the contrary, none of the precursor GIPLs (i.e. M2/iM2′, $iM2_{VLAC}$ and iM4′) partitioned into the insoluble fraction, even after a 2 h chase when they could still be readily detected and most of the mature GIPLs were insoluble in Triton X-100 (Figure 3A).

The acquisition of Triton X-100 insolubility could reflect changes in the lipid composition of the GIPLs as a result of fatty-acid remodelling. In *L. mexicana* promastigotes, these remodelling reactions involve the removal of a long (predominantly $C_{18:0}$) fatty acid from the *sn*-2 position in GIPL precursors and its replacement with a shorter $C_{14:0}$ or $C_{12:0}$ acyl chain [16]. These remodelling reactions result in a decrease in the hydrophobicity of the lipid moiety and are thus unlikely to contribute to the decrease in the detergent solubility of these glycolipids. Moreover, the mature GPI-protein-anchor precursor (PAP) was also found to remain primarily in the Triton X-100-soluble fraction despite containing a very long ($C_{24:0}/C_{26:0}$) *sn*-1 alkyl chain (Figure 4A). Finally, when *L. mexicana* promastigotes were pulse–chase-labelled with [^{3}H]myristic acid (which is only incorporated into GIPLs via the remodelling reactions), label was initially incorporated into GIPLs in the Triton X-100-soluble fraction and these glycolipids were subsequently chased into the insoluble fraction (Figure 4B). These results show that the GIPLs are subjected to fatty-acid remodelling reactions before they become incorporated into DRMs and suggest that the acquisition of detergent insolubility is not due to fatty-acid remodelling.

This metabolic-labelling approach was extended to examine whether the GIPLs were associating with the same DRMs that contain gp63. *L. mexicana* promastigotes were pulse–chase-labelled with [^{3}H]Man and either extracted in ice-cold Triton X-100 or surface-biotinylated to obtain affinity-purified plasma-membrane preparations at each time point. At the beginning of the chase, newly synthesized GIPLs were largely present in Triton-X-100-soluble intracellular membranes (Figures 5A and 5B). However, with increasing chase times the mature GIPLs partitioned into DRMs and gp63-containing plasma membranes with very similar kinetics (Figures 5A and 5B).

These findings suggest that the acquisition of Triton X-100 insolubility can be used to compare the rates of plasma-membrane transport of gp63 and GIPLs. As shown in Figure 3(C), the GIPLs were transported to the plasma membrane with slightly slower initial kinetics than gp63 ($t_{1/2}$ 70 versus 40 min). Interestingly, the disparity between the rate of transport of the GIPLs and gp63 increased at later time points in the chase. These biphasic kinetics may reflect the fact that [^{3}H]Man is incorporated into two pools of GPI precursors: pre-existing GPI precursors (i.e. M2, M3 and mature GIPLs) and *de novo* synthesized GPIs (i.e. M1) [16]. Although the pre-existing GIPL precursors appear to be efficiently transported to the DRMs, *de novo* synthesized GIPLs were detected up to the 2 h chase time point and remained in the Triton X-100-soluble fraction (see Figure 3A). Finally, the transport of both gp63 and GIPLs into DRMs was substantially slower than that of the major sphingolipid species, IPC ($t_{1/2} < 10$ min). Recent studies in yeast [33] and *Leishmania* (K. A. Mullin and M. J. McConville, unpublished work) suggest

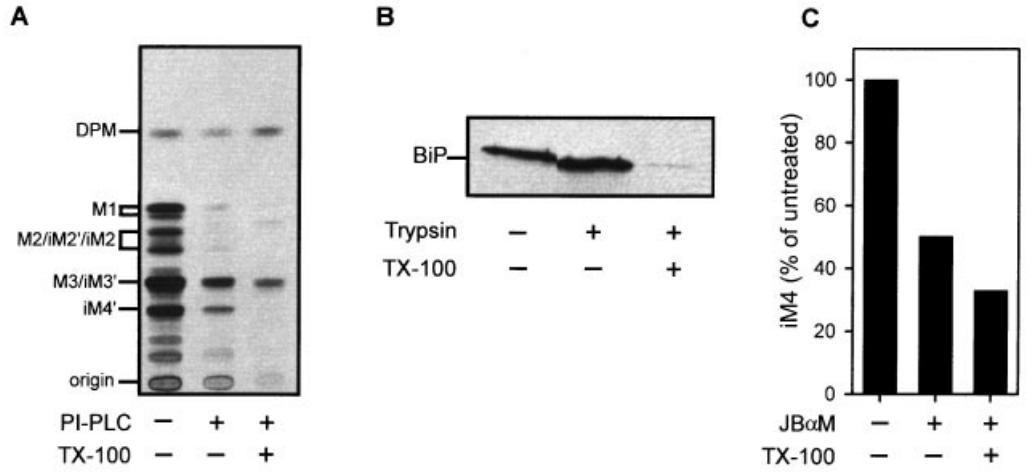

Figure 6 Analysis of the topology of newly synthesized and steady-state GIPL pools

(**A**) Permeabilized *L. mexicana* promastigotes were pulse–chase-labelled with GDP-[^{3}H]Man to label GIPL intermediates with mature glycan headgroups (see Figure 3B for structures). The permeabilized promastigotes were subsequently treated with buffer control (lane 1), PI-PLC (lane 2) or PI-PLC in Triton X-100 (lane 3) for 90 min at 15 °C. ^{3}H-labelled GIPLs were extracted and analysed by HPTLC. (**B**) ER membrane latency in permeabilized promastigotes was examined by treating permeabilized promastigotes with inactive or active trypsin in the absence or presence of Triton X-100. Levels of the ER marker BiP were determined by SDS/PAGE and immunoblotting. (**C**) ΔLPG2 promastigotes were metabolically labelled to steady state with [^{3}H]inositol and then treated with buffer control, jack-bean α-mannosidase or jack-bean α-mannosidase in Triton X-100. [^{3}H]Inositol lipids were analysed by HPTLC and the levels of iM4 quantified using a linear scanner.

that IPC is synthesized in the Golgi apparatus. As newly synthesized IPC is soluble in Triton X-100, these results support the notion that the *L. mexicana* DRMs form in a late or post-Golgi compartment.

Newly synthesized GPIs are expressed on the cytoplasmic face of the ER, whereas the major steady-state pool of iM4 is present in the exoplasmic leaflet of the plasma membrane

We next investigated whether the GIPLs are restricted to the lumen of the secretory pathway. *L. mexicana* promastigotes were hypotonically permeabilized and then pulse–chase-labelled with GDP-[^{3}H]Man to label all mannosylated GIPL intermediates prior to the fatty-acid remodelling steps (Figure 6A) [16]. The membrane topology of the newly synthesized GIPLs was then probed by incubating the permeabilized promastigotes with bacterial PI-PLC in the absence or presence of the detergent. All of the GIPL intermediates were hydrolysed by the exogenous PI-PLC regardless of whether the detergent was present or not (Figure 6A). The ER membranes were intact in the absence of the detergent, as the ER luminal marker, BiP, was resistant ($> 90\,\%$) to trypsin digestion (Figure 6B). These results suggest that all the mannosylated GIPL intermediates become transiently or stably orientated on the cytoplasmic face of the ER.

To probe whether the GIPLs retain a cytoplasmic orientation after being transported to the plasma membrane, we investigated the surface topology of the major GIPL species. As previous studies have shown that the surface coat of LPG molecules may prevent macromolecular probes such as PI-PLC from reaching the plasma membrane [34], we used the LPG-deficient *L. mexicana* mutant ΔLPG2 for these studies [22]. Cellular levels of gp63 and GIPLs in the ΔLPG2 mutant are the same as in wild-type *L. mexicana* promastigotes (results not shown). Unexpectedly, when ΔLPG2 promastigotes were incubated with exogenous PI-PLC, the steady-state pools of these molecules were not digested. However, they were quantitatively cleaved if the promastigotes were fixed in glutaraldehyde, or permeabilized in hypotonic media or detergent ($0.1\,\%$ Triton X-100) (results not shown). The reason for the resistance of surface pools of gp63 to exogenous PI-PLC remains unclear, but these results suggest that it only occurs on live parasites. We therefore used a second macromolecular probe, jack-bean α-mannosidase, to examine the surface topology of the GIPLs. In the absence of the detergent, this enzyme removes the terminal α1-2-mannose from the major GIPL species, iM4. As shown in Figure 6(C), α-mannosidase digestion of live ΔLPG2 promastigotes resulted in a $40\,\%$ decrease in the cellular levels of iM4. This compared with a maximum of $70\,\%$ digestion in the presence of detergent (Figure 6C), suggesting that a minimum of $57\,\%$ of the steady-state pool of iM4 was exposed on the cell surface. The incomplete digestion of the iM4 in the live and detergent-permeabilized promastigotes reflected the short incubation time and comparatively high pH used to ensure that the live parasites remained viable throughout these experiments. In two other independent experiments, values of 50 and $53\,\%$ surface exposure were obtained. These results suggest that newly synthesized iM4 must be translocated (flipped) across either the ER/Golgi or plasma membrane to reach the cell surface.

The surface transport of GIPLs is reduced at low temperature

These analyses raised the possibility that the *L. mexicana* GIPLs could be transported to the plasma membrane on either the cytoplasmic face of secretory pathway organelles or delivered directly to the plasma membrane via a non-vesicular pathway (i.e. glycolipid transfer proteins or direct ER plasma-membrane continuities). To distinguish between these possibilities, *L. mexicana* promastigotes were treated with brefeldin A, ilimaquinone or monensin, which inhibit vesicular transport in many other eukaryotes. However, these compounds had no effect on the rate of surface transport of either gp63 or the GIPLs (results not shown). We therefore investigated the effect of temperature on the transport of these molecules, as studies in *T. brucei* bloodstream forms have shown that transport of GPI-

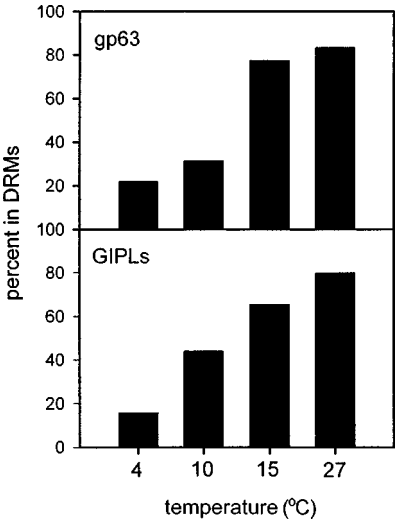

Figure 7 Transport of both gp63 and free GPIs is inhibited at low temperature

L. mexicana promastigotes were pulse-labelled with either [^{35}S]Met/Cys or [^{3}H]Man at 27 °C for 10 min, then resuspended in complete medium and aliquots incubated at the indicated temperatures. Incorporation of [^{35}S]gp63 or [^{3}H]GIPL into DRMs was measured after a 2 h chase. A similar pattern of inhibition was observed when cells were chased for only 1 h.

anchored variant surface glycoprotein ('VSG') from the Golgi to the flagellar pocket is inhibited at low temperature [35]. *L. mexicana* promastigotes were pulse-labelled with either Tran^{35}S-label or [^{3}H]Man at 27 °C and then incubated in complete medium for 2 h at a range of temperatures. The rate of transport of gp63 was only slightly decreased at 20 and 15 °C, but was significantly decreased when promastigotes were incubated at 10 or 4 °C (Figure 7, top panel). The plasma-membrane transport of the GIPLs was similarly affected by decreasing the temperature (Figure 7, bottom panel), suggesting that a vesicular transport mechanism may be involved. The incorporation of gp63 into DRMs at 10 and 4 °C correlated with their appearance at the cell surface, as shown by surface biotinylation experiments (results not shown), indicating that the intracellular site of DRM formation remained the same over a wide range of temperatures.

Sphingolipid biosynthesis is not required for surface transport of GPI-anchored proteins and free GPIs or the formation of DRMs

Ongoing sphingolipid synthesis is required for efficient transport of GPI-anchored proteins from the ER to the Golgi apparatus in yeast [36,37]. Sphingolipids are also thought to be essential for the formation of DRMs in many eukaryotes [19,21]. To examine whether sphingolipids fulfil similar functions in *Leishmania*, the plasma-membrane transport of gp63 and GIPLs and the presence of DRMs was measured after a short- or long-term incubation of *L. mexicana* promastigotes with myriocin, a potent inhibitor of the first enzyme in sphingolipid biosynthesis, serine palmitoyltransferase [36]. Sphingosine and ceramide synthesis was completely inhibited after 20 min preincubation of promastigotes with myriocin (Figure 8A). After a long-term incubation in myriocin (20 h), a substantial decrease in the cellular levels of IPC (by $> 70\%$) was observed, as determined by both HPTLC analysis of total lipids (Figure 8B) and GC–MS mass analysis (results not shown). In contrast with the situation in yeast [36], the surface transport of gp63 was not inhibited in the absence of ongoing sphingolipid biosynthesis (Figure 8C). Moreover, the

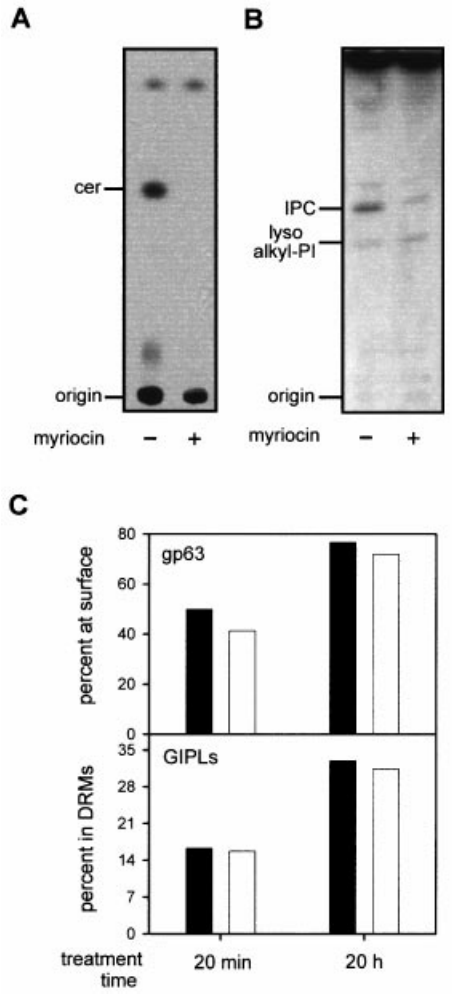

Figure 8 Ongoing sphingolipid synthesis is not required for the surface transport of gp63 or free GPIs

(**A**) *L. mexicana* promastigotes were pulse–chase-labelled with [^{3}H]serine with or without prior myriocin treatment (20 min), and base-resistant lipids analysed by HPTLC. The migration position of ceramide standard is indicated. Short-term myriocin treatment results in the complete inhibition of ceramide synthesis. (**B**) After long-term (20 h) incubation of promastigotes with myriocin, the cellular levels of the two major base-resistant lipids, IPC and lyso-alkyl-PI, were quantified by HPTLC. Long-term myriocin treatment results in a 70% decrease in the cellular levels of IPC but not lyso-alkyl-PI. (**C**) *L. mexicana* promastigotes were incubated in the absence (shaded bars) or presence (empty bars) of myriocin for either 20 min (short term) or 20 h (long term) before being pulse-labelled with either [^{35}S]Met/Cys or [^{3}H]Man. The cell-surface transport of [^{35}S]gp63 and incorporation of [^{3}H]GIPLs into DRMs was measured after a 1 h chase. Myriocin treatment does not affect the rate at which gp63 reaches the cell surface or the extent to which the GIPLs become incorporated into DRMs.

extent to which both the GIPLs (Figure 8C) and gp63 associated with DRMs was unaffected by the long-term depletion of IPC levels (Figure 8C). Thus high levels of IPC are not essential for the formation of DRMs in *L. mexicana* promastigotes.

DISCUSSION

Free GPIs are the major glycolipids of *Leishmania* spp. and a number of other pathogenic trypanosomatids [1,2,11]. We have used the finding that gp63 and the GIPLs of *L. mexicana* promastigotes become incorporated into Triton X-100-insoluble membranes just before or upon reaching the plasma membrane to examine the intracellular transport of these molecules. Our

results suggest that the GIPLs are transported to the plasma membrane via a similar temperature-sensitive (vesicular) pathway as gp63, but that the kinetics of transport of these glycolipids is significantly slower than gp63. GIPL transport also appears to involve one or more transmembrane-flipping steps, as all the mannosylated intermediates in this pathway are initially orientated on the cytoplasmic leaflet of the ER, whereas most of the mature GIPLs appear to be equally distributed between the inner and exoplasmic leaflet of the plasma membrane. Finally, the plasma-membrane transport of both the GIPLs and gp63 does not require ongoing sphingolipid biosynthesis, in contrast with the situation in some other unicellular eukaryotes. These findings support the notion that the GIPLs are major components in the surface glycocalyx of *Leishmania* promastigotes, but also raise the possibility that cytoplasmically orientated pools of these glycolipids may be functionally important.

The temperature sensitivity of GIPL plasma-membrane transport suggests that they transit through the single Golgi apparatus. This is supported by findings that the type-2 GIPLs of *L. major* may be partly assembled in the lumen of the Golgi apparatus [14]. Similarly, excess GPI protein-anchor precursors in *T. brucei* bloodstream forms (the equivalent of the leishmanial GIPLs) can be modified with glycan side chains in the Golgi [38]. Finally, recent studies by Menon and co-workers [32,39] suggest that a small pool of free GPIs in animal cells is transported to the plasma membrane in a Brefeldin A- and temperature-sensitive manner. Collectively, these results suggest that free GPI species in both the protozoa and animal cells that are not utilized as protein anchors can be transported to the plasma membrane via the Golgi apparatus.

The GIPLs are transported to the plasma membrane more slowly than gp63 ($t_{1/2} = 70$ versus 40 min). The different transport kinetics might reflect (1) the selective incorporation of gp63 into ER transport vesicles while the GIPLs are transported at the rate of bulk flow or (2) the partial retention or slow rate of maturation of GIPLs in the ER. Evidence for the selective incorporation of GPI-anchored proteins into ER transport vesicles has been obtained from studies using both yeast [40] and *T. brucei* [11,41,42]. In yeast, the selective incorporation of Gas1p into ER transport vesicles is mediated by members of the p24 family of cargo receptors [43]. It is possible that similar cargo receptors in *Leishmana* may facilitate the rapid export of gp63 from the ER. Alternatively, the *L. mexicana* GIPLs may take longer than gp63 to mature and/or be exported from the ER at a rate that is slower than bulk flow. A slow rate of maturation is suggested by the finding that [^{3}H]Man is chased out of early GIPL precursors (i.e. M1) with very slow kinetics (approx. hours). Moreover, GIPL intermediates with un-remodelled lipid moieties appear to be selectively retained within Triton X-100-soluble membranes, as only mature GIPLs are incorporated into DRMs. The slow maturation and export of these GIPL intermediates may account for the biphasic transport kinetics of the GIPLs into DRMs.

Although most of the latter steps in GPI-anchor biosynthesis occur in the lumen of the ER [39], there is evidence that some of these intermediates may be flipped to the cytosolic leaflet of the ER. Menon and co-workers [29,32,44] showed that newly synthesized protein-anchor precursors were partially orientated on the cytoplasmic leaflet of *T. brucei* and animal-cell microsomal membranes. Similarly, ectopic expression of a cytosolically localized GPI-PLC in *Leishmania* spp., down-regulated levels of gp63, suggesting that this enzyme may access cytosolic pools of protein-anchor precursors [18,45]. On the contrary, the cellular levels of GIPLs were not decreased in *Leishmania* spp. expressing the GPI-PLC, suggesting that these glycolipids may have a different topology from the protein-anchor precursors [18,45]. However, in the present study we provide evidence that newly synthesized *L. mexicana* GIPLs are quantitatively exposed on the cytoplasmic leaflet of the ER *in vitro*. Other studies also support this conclusion. Specifically, we have shown that the α1–3-linked mannose branch in the hybrid-type GIPLs is added directly from GDP-Man rather than dolichol-phosphate-mannose [45]. As there is no evidence that the eukaryote ER contains a resident GDP-Man transporter, it is likely that this reaction occurs on the cytoplasmic leaflet of the ER [46]. Moreover, targeted deletion of the *Leishmania* Golgi GDP-Man transporter has no effect on the synthesis of the hybrid-type GIPLs [22]. Collectively, these results suggest that all the GIPLs are transported to the cytosolic face of the ER during their synthesis. As approximately half of the steady-state pool of iM4 is orientated in the exoplasmic leaflet of the plasma membrane, a significant proportion of these glycolipids must be translocated back to the lumen of secretory-pathway organelles, or flipped across the plasma membrane. As summarized above, the transbilayer movement of mannosylated GPIs seems to occur in both the protozoa and animal cells [39]. Why this should occur in trypanosomes or animal cells that do not modify their GPIs in the same way as *Leishmania* is unclear. One possibility is that it represents the non-selective action of the putative flippase that translocates early GPI intermediates (i.e. GlcN-PI) into the ER lumen [46].

Our results extend another recent study [47] suggesting that DRMs exist in a number of trypanosomatids. As in other eukaryotes, the DRMs of *Leishmania* promastigotes are highly enriched in the major cellular sphingolipid (IPC), sterols (ergosterol), and float in sucrose-density gradients (this study and [47]). The *L. mexicana* DRMs are resistant to extraction with ice-cold 1% (v/v) Triton X-100, but are completely extracted with ice-cold 1% (v/v) Triton X-114, consistent with early studies showing that gp63 is soluble in this detergent [46]. Gp63 is by far the most abundant protein in these domains, although at least one other cell-surface protein (i.e. 3′-nucleotidase) was found to partially localize to these domains. Collectively, GPI-anchored macromolecules and GIPLs are thought to cover $> 80\%$ of the cell surface of *L. mexicana* promastigotes [48], suggesting that DRMs may constitute the dominant phase in the plasma membrane.

By analogy with the situation in other eukaryotes, DRMs could play a role in protein and glycolipid sorting in the *Leishmania* secretory pathway [11,19,21]. Several recent studies suggest that a number of integral membrane proteins are targeted to an unusual tubular lysosome in *Leishmania* promastigotes, particularly when expressed at high levels [9,11,23,49]. Replacement of the transmembrane domain with a GPI attachment signal results in the efficient transport of these proteins to the cell surface [9,49]. Sorting of proteins to the lysosome could occur in either the Golgi apparatus or the flagellar pocket and could potentially involve the segregation of GPI-anchored proteins into DRMs that form in the late secretory pathway in *L. mexicana* promastigotes. However, it is important to note that the physical properties of DRMs differ markedly in different trypanosomatids, based on their sensitivity to Triton X-100. For example, the GIPLs and gp63 of *L. major* promastigotes are present in DRMs after short-pulse-labelling experiments, suggesting that most of the membranes in the secretory pathway are resistant to Triton X-100 [47] (J. E. Ralton and M. J. McConville, unpublished work). On the contrary, steady-state pools of *T. brucei* variant surface glycoprotein are readily extracted in ice-cold 1% (v/v) Triton X-100 and DRM-like membranes can only be detected using lower concentrations of Triton X-100 [47].

These comparative analyses strongly suggest that DRM-based-sorting mechanisms in trypanosomatids, if they occur, are unlikely to depend on the presence of membrane domains with a common lipid composition. Incorporation of gp63 and the GIPLs into DRMs may also increase the resistance of these molecules to exogenous lipases in the sandfly midgut and phagolysosome compartment of mammalian macrophages. This hypothesis is supported by the finding that surface pools of gp63 and iM4 were completely resistant to exogenous bacterial PI-PLC and other lipases (results not shown) in the ΔLPG2 mutant. Lipase sensitivity was restored if promastigotes were fixed or osmotically permeabilized, suggesting that the presence of an intact plasma membrane and possibly DRMs confer resistance.

Given that sphingolipids are highly enriched in the *L. mexicana* DRMs and are supposed to be crucial for the formation of these domains in other eukaryotes [50,51], we investigated whether ongoing sphingolipid biosynthesis is required for the surface transport of gp63 and the formation of Triton X-100-insoluble membranes. Although myriocin was found to inhibit ceramide synthesis in *L. mexicana* promastigotes, this drug had no effect on the plasma membrane transport of either gp63 or the GIPLs. This is in contrast with the situation in yeast, where inhibition of sphingolipid biosynthesis with myriocin selectively inhibits ER-Golgi transport of the major GPI-anchored protein Gas1p, but not other integral membrane proteins [36]. In yeast, ongoing sphingolipid biosynthesis is supposed to be required for the fusion of COPII vesicles with *cis*-Golgi and possibly also for the formation of DRMs in the ER [37,52]. These functions are apparently less important or are fulfilled by other lipids in *Leishmania*, and highlight further differences in the molecular mechanisms that underlie secretory transport in the evolutionarily divergent protozoa. Long-term incubation of *L. mexicana* promastigotes with myriocin resulted in a pronounced reduction ($> 70\,\%$) in the steady-state concentration of the only major sphingolipid in these parasites. However, depletion of IPC did not affect the overall growth rate of the promastigotes, the rate of surface transport of gp63, or the extent to which gp63 and the GIPLs were incorporated into the DRMs. Although the residual pool of IPC in myriocin-treated promastigotes may play a role in maintaining the DRMs, the results indicate that other lipids are important for DRM formation.

We thank Thomas Ilg (Max Planck Institute, Tübingen, Germany) for generously providing the ΔLPG2 cell line and the Tü L3.8 antibody and Merck for generously providing myriocin. This work was supported by grants from the Australian Research Council and the Australian National Health and Medical Research Council. J.E.R. is a recipient of University of Melbourne Research Fellowship. M.J.M. is a Howard Hughes International Research Scholar.

REFERENCES

1 McConville, M. J. and Ferguson, M. A. J. (1993) The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes. Biochem. J. **294**, 305–324
2 Guha-Niyogi, A., Sullivan, D. R. and Turco, S. J. (2001) Glycoconjugate structures of parasitic protozoa. Glycobiology **11**, 45R–59R
3 Tachado, S. D., Gerold, P., Schwarz, R., Novakovic, S., McConville, M. and Schofield, L. (1997) Signal transduction in macrophages by glycosylphosphatidylinositols of *Plasmodium*, *Trypanosoma*, and *Leishmania*: activation of protein tyrosine kinases and protein kinase C by inositolglycan and diacylglycerol moieties. Proc. Natl. Acad. Sci. U.S.A. **94**, 4022–4027
4 Almeida, I. C., Camargo, M. M., Procopio, D. O., Silva, L. S., Mehlert, A., Travassos, L. R., Gazzinelli, R. T. and Ferguson, M. A. (2000) Highly purified glycosylphosphatidylinositols from *Trypanosoma cruzi* are potent proinflammatory agents. EMBO J. **19**, 1476–1485
5 Redman, C. A., Schneider, P., Mehlert, A. and Ferguson, M. A. J. (1995) The glycoinositol-phospholipids of *Phytomonas*. Biochem. J. **311**, 495–503
6 Azzouz, N., Striepen, B., Gerold, P., Capdeville, Y. and Schwarz, R. T. (1995) Glycosylinositol-phosphoceramide in the free-living protozoan *Paramecium primaurelia*: modification of core glycans by mannosyl phosphate. EMBO J. **14**, 4422–4433
7 Turco, S. J., Spath, G. F. and Beverley, S. M. (2001) Is lipophosphoglycan a virulence factor? A surprising diversity between *Leishmania* species. Trends Parasitol. **17**, 223–226
8 Schneider, P., Ferguson, M. A., McConville, M. J., Mehlert, A., Homans, S. W. and Bordier, C. (1990) Structure of the glycosyl-phosphatidylinositol membrane anchor of the *Leishmania major* promastigote surface protease. J. Biol. Chem. **265**, 16955–16964
9 Weise, F., Stierhof, Y. D., Kuhn, C., Wiese, M. and Overath, P. (2000) Distribution of GPI-anchored proteins in the protozoan parasite *Leishmania*, based on an improved ultrastructural description using high-pressure frozen cells. J. Cell Sci. **113**, 4587–4603
10 Landfear, S. M. and Ignatushchenko, M. (2001) The flagellum and flagellar pocket of trypanosomatids. Mol. Biochem. Parasitol. **115**, 1–17
11 McConville, M. J., Mullin, K. A., Ilgoutz, S. C. and Teasdale, R. D. (2002) The secretory pathway of trypanosomatid parasites. Microbiol. Mol. Biol. Rev. **66**, 122–154
12 McGwire, B. S. and Chang, K. P. (1996) Posttranslational regulation of a *Leishmania* HEXXH metalloprotease (gp63). The effects of site-specific mutagenesis of catalytic, zinc binding, N-glycosylation, and glycosyl phosphatidylinositol addition sites on N-terminal end cleavage, intracellular stability, and extracellular exit. J. Biol. Chem. **271**, 7903–7909
13 Ilgoutz, S. C., Mullin, K. A., Southwell, B. R. and McConville, M. J. (1999) Glycosylphosphatidylinositol biosynthetic enzymes are localized to a stable tubular subcompartment of the endoplasmic reticulum in *Leishmania mexicana*. EMBO J. **18**, 3643–3654
14 Ha, D. S., Schwarz, J. K., Turco, S. J. and Beverley, S. M. (1996) Use of the green fluorescent protein as a marker in transfected *Leishmania*. Mol. Biochem. Parasitol. **77**, 57–64
15 Hong, K., Ma, D., Beverley, S. M. and Turco, S. J. (2000) The *Leishmania* GDP-mannose transporter is an autonomous, multi-specific, hexameric complex of LPG2 subunits. Biochemistry **39**, 2013–2022
16 Ralton, J. E. and McConville, M. J. (1998) 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. J. Biol. Chem. **273**, 4245–4257
17 Winter, G., Fuchs, M., McConville, M. J., Stierhof, Y. D. and Overath, P. (1994) Surface antigens of *Leishmania mexicana* amastigotes: characterization of glycoinositol phospholipids and a macrophage-derived glycosphingolipid. J. Cell Sci. **107**, 2471–2482
18 Mensa-Wilmot, K., LeBowitz, J. H., Chang, K. P., Al-Qahtani, A., McGwire, B. S., Tucker, S. and Morris, J. C. (1994) A glycosylphosphatidylinositol (GPI)-negative phenotype produced in *Leishmania major* by GPI phospholipase C from *Trypanosoma brucei*: topography of two GPI pathways. J. Cell Biol. **124**, 935–947
19 Brown, D. A. and London, E. (2000) Structure and function of sphingolipid- and cholesterol-rich membrane rafts. J. Biol. Chem. **275**, 17221–17224
20 Simons, K. and Ikonen, E. (2000) How cells handle cholesterol. Science **290**, 1721–1726
21 Holthuis, J. C., Pomorski, T., Raggers, R. J., Sprong, H. and Van Meer, G. (2001) The organizing potential of sphingolipids in intracellular membrane transport. Physiol. Rev. **81**, 1689–1723
22 Ilg, T., Demar, M. and Harbecke, D. (2001) Phosphoglycan repeat-deficient *Leishmania mexicana* parasites remain infectious to macrophages and mice. J. Biol. Chem. **276**, 4988–4997
23 Mullin, K. A., Foth, B., Ilgoutz, S. M., Callaghan, J., McFadden, G. M. and McConville, M. J. (2001) Regulated degradation of ER membrane proteins in a novel tubular lysosome in *Leishmania mexicana*. Mol. Biol. Cell. **12**, 2364–2377
24 McConville, M. J. and Blackwell, J. M. (1991) Developmental changes in the glycosylated phosphatidylinositols of *Leishmania donovani*. Characterization of the promastigote and amastigote glycolipids. J. Biol. Chem. **266**, 15170–15179
25 Bordier, C. (1981) Phase separation of integral membrane proteins in Triton X-100 solution. J. Biol. Chem. **256**, 1604–1607
26 Wessel, D. and Flugge, U. I. (1984) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal. Biochem. **138**, 141–143
27 Dwyer, D. M. and Gottlieb, M. (1984) Surface membrane localization of 3′- and 5′-nucleotidase activities in *Leishmania donovani* promastigotes. Mol. Biochem. Parasitol. **10**, 139–150

28 Rathbun, W. B. and Betlach, M. V. (1969) Estimation of enzymatically produced orthophosphate in the presence of cysteine and adenosine triphosphate. Anal. Biochem. **28**, 436–445
29 Vidugiriene, J. and Menon, A. K. (1994) The GPI anchor of cell-surface proteins is synthesized on the cytoplasmic face of the endoplasmic reticulum. J. Cell Biol. **127**, 333–341
30 Medina-Acosta, E., Karess, R. E., Schwartz, H. and Russell, D. G. (1989) The promastigote surface protease (gp63) of *Leishmania* is expressed but differentially processed and localized in the amastigote stage. Mol. Biochem. Parasitol. **37**, 263–273
31 Bangs, J. D., Uyetake, L., Brickman, M. J., Balber, A. E. and Boothroyd, J. C. (1993) Molecular cloning and cellular localization of a Bip homologue in *Trypanosoma brucei*; divergent ER retention signals in a lower eukaryote. J. Cell Sci. **105**, 1101–1113
32 Baumann, N. A., Vidugiriene, J., Machamer, C. E. and Menon, A. K. (2000) Cell surface display and intracellular trafficking of free glycosylphosphatidylinositols in mammalian cells. J. Biol. Chem. **275**, 7378–7389
33 Levine, T. P., Wiggins, C. A. and Munro, S. (2000) Inositol phosphorylceramide synthase is located in the Golgi apparatus of *Saccharomyces cerevisiae*. Mol. Biol. Cell **11**, 2267–2281
34 Karp, C. L., Turco, S. J. and Sacks, D. L. (1991) Lipophosphoglycan masks recognition of the *Leishmania donovani* promastigote surface by human kala-azar serum. J. Immunol. **147**, 680–684
35 Duszenko, M., Ivanov, I. E., Ferguson, M. A., Plesken, H. and Cross, G. A. (1988) Intracellular transport of a variant surface glycoprotein in *Trypanosoma brucei*. J. Cell Biol. **106**, 77–86
36 Horvath, A., Sutterlin, C., Manning-Krieg, U., Movva, N. R. and Riezman, H. (1994) Ceramide synthesis enhances transport of GPI-anchored proteins to the Golgi apparatus in yeast. EMBO J. **13**, 3687–3695
37 Sutterlin, C., Doering, T. L., Schimmoller, F., Schroder, S. and Riezman, H. (1997) Specific requirements for the ER to Golgi transport of GPI-anchored proteins in yeast. J. Cell Sci. **110**, 2703–2714
38 Mayor, S., Menon, A. K. and Cross, G. A. (1992) Galactose-containing glycosylphosphatidylinositols in *Trypanosoma brucei*. J. Biol. Chem. **267**, 754–761
39 McConville, M. J. and Menon, A. K. (2000) Recent developments in the cell biology and biochemistry of glycosylphosphatidylinositol lipids. Mol. Membr. Biol. **17**, 1–16
40 Muniz, M., Nuoffer, C., Hauri, H. P. and Riezman, H. (2000) The emp24 complex recruits a specific cargo molecule into endoplasmic reticulum-derived vesicles. J. Cell Biol. **148**, 925–930
41 Bangs, J. D., Ransom, D. M., McDowell, M. A. and Brouch, E. M. (1997) Expression of bloodstream variant surface glycoproteins in procyclic stage *Trypanosoma brucei*: role of GPI anchors in secretion. EMBO J. **16**, 4285–4294
42 McDowell, M. A., Ransom, D. M. and Bangs, J. D. (1998) Glycosylphosphatidylinositol-dependent secretory transport in *Trypanosoma brucei*. Biochem. J. **335**, 681–689
43 Muniz, M., Morsomme, P. and Riezman, H. (2001) Protein sorting upon exit from the endoplasmic reticulum. Cell **104**, 313–320
44 van't Hof, W., Rodriguezboulan, E. and Menon, A. K. (1995) Nonpolarized distribution of glycosylphosphatidylinositols in the plasma membrane of polarized Madin–Darby canine kidney cells. J. Biol. Chem. **270**, 24150–24155
45 Ilgoutz, S. C., Zawadzki, J. L., Ralton, J. E. and McConville, M. J. (1999) Evidence that free GPI glycolipids are essential for growth of *Leishmania mexicana*. EMBO J. **18**, 2746–2755
46 Menon, A. K. (1998) Lipids: more than just membrane fabric. Trends Cell Biol. **8**, 374–376
47 Denny, P. W., Field, M. C. and Smith, D. F. (2001) GPI-anchored proteins and glycoconjugates segregate into lipid rafts in Kinetoplastida. FEBS Lett. **491**, 148–153
48 McConville, M. J., Collidge, T. A. C., Ferguson, M. A. J. and Schneider, P. (1993) The glycoinositol phospholipids of *Leishmania mexicana* promastigotes. Evidence for the presence of three distinct pathways of glycolipid biosynthesis. J. Biol. Chem. **268**, 15595–15604
49 Ghedin, E., Debrabant, A., Engel, J. C. and Dwyer, D. M. (2001) Secretory and endocytic pathways converge in a dynamic endosomal system in a primitive protozoan. Traffic **2**, 175–188
50 Stevens, V. L. and Tang, J. (1997) Fumonisin B1-induced sphingolipid depletion inhibits vitamin uptake via the glycosylphosphatidylinositol-anchored folate receptor. J. Biol. Chem. **272**, 18020–18025
51 Ledesma, M. D., Brugger, B., Bunning, C., Wieland, F. T. and Dotti, C. G. (1999) Maturation of the axonal plasma membrane requires upregulation of sphingomyelin synthesis and formation of protein-lipid complexes. EMBO J. **18**, 1761–1771
52 Bagnat, M., Keranen, S., Shevchenko, A. and Simons, K. (2000) Lipid rafts function in biosynthetic delivery of proteins to the cell surface in yeast. Proc. Natl. Acad. Sci. U.S.A. **97**, 3254–3259

Received 27 July 2001/21 November 2001; accepted 1 February 2002