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Evidence that free GPI glycolipids are essential for growth of *Leishmania mexicana*

Steven C.Ilgoutz, Jody L.Zawadzki, Julie E.Ralton and Malcolm J.McConville[1]

Department of Biochemistry and Molecular Biology, The University of Melbourne, Parkville, Victoria 3052, Australia

[1]Corresponding author
e-mail: m.mcconville@biochemistry.unimelb.edu.au

The cell surface of the parasitic protozoan *Leishmania mexicana* is coated by glycosylphosphatidylinositol (GPI)-anchored glycoproteins, a GPI-anchored lipophosphoglycan and a class of free GPI glycolipids. To investigate whether the anchor or free GPIs are required for parasite growth we cloned the *L.mexicana* gene for dolichol–phosphate–mannose synthase (DPMS) and attempted to create DPMS knockout mutants by targeted gene deletion. DPMS catalyzes the formation of dolichol–phosphate mannose, the sugar donor for all mannose additions in the biosynthesis of both the anchor and free GPIs, except for a α1-3-linked mannose residue that is added exclusively to the free GPIs and lipophosphoglycan anchor precursors. The requirement for dolichol–phosphate–mannose in other glycosylation pathways in *L.mexicana* is minimal. Deletion of both alleles of the DPMS gene (*lmdpms*) consistently resulted in amplification of the *lmdpms* chromosomal locus unless the promastigotes were first transfected with an episomal copy of *lmdpms*, indicating that *lmdpms*, and possibly GPI biosynthesis, is essential for parasite growth. As evidence presented in this and previous studies indicates that neither GPI-anchored glycoproteins nor lipophosphoglycan are required for growth of cultured parasites, it is possible that the abundant and functionally uncharacterized free GPIs are essential membrane components.

Keywords: dolichol–phosphate–mannose/glycosylphosphatidylinositol/membranes/protozoa

Introduction

Many glycoproteins on the surface of eukaryote cells are modified at their C-terminus with a glycosylphosphatidylinositol (GPI) glycolipid (Englund, 1993; reviewed in McConville and Ferguson, 1993; Kinoshita and Takeda, 1994; Stevens, 1995). In addition to acting as membrane anchors for these proteins, the GPI moiety may also regulate such processes as intracellular trafficking and the spatial organization of these proteins in the membrane bilayer (Brown and London, 1998). Nonprotein-linked, or 'free' GPIs, which are either structurally distinct from the protein anchors or present in vast excess over the requirements for protein anchoring can also be abundant membrane components in many eukaryotes (Sevlever *et al*., 1995; van't Hof *et al*., 1995; Singh *et al*., 1996). Although these free GPIs have been proposed to play a role in intracellular signaling pathways, very little is known about their function.

Free GPIs are particularly abundant in many parasitic protozoa, including the Kinetoplastid parasites (such as *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania* spp.), which cause a number of important diseases in humans. In some parasites, such as *T.brucei*, these GPIs are structurally identical to the mature GPI anchor precursors that are added to protein (McConville and Ferguson, 1993; Ferguson, 1998). However, in most other Kinetoplastid parasites free GPIs are structurally distinct from the corresponding protein anchors and may be expressed in developmental stages that lack GPI-anchored glycoproteins. This is the case in sandfly-transmitted *Leishmania* parasites, which alternate between a promastigote stage in the digestive tract of the insect vector and an amastigote stage that proliferates within the phagolysosome compartment of macrophages in the mammalian host. These parasites synthesize two distinct classes of free GPIs; a hyperglycosylated GPI, termed lipophosphoglycan (LPG) and a family of unmodified GPIs, termed glycoinositol phospholipids (GIPLs) (Figure 1; Turco and Descoteaux, 1992; McConville and Ferguson, 1993). The protein anchors, the LPG anchors and GIPLs of *Leishmania* share a common core (i.e. Manα1-4GlcN-PI) but contain distinct glycan headgroups and lipid moieties (Figure 1) and may be assembled on distinct pools of phospholipid precursors (Ralton and McConville, 1998). LPG is the dominant macromolecule on the promastigote cell surface and is essential for establishment of infection in both the insect and mammalian hosts (Turco and Descoteaux, 1992; McConville and Ferguson, 1993). However, it is not required for parasite growth in culture (Ryan *et al*., 1993; Ma *et al*., 1997) and levels of expression of this molecule are massively down-regulated (along with the expression of most GPI-anchored glycoproteins) in the intracellular amastigote stage (McConville and Blackwell, 1991; Bahr *et al*., 1993). The function of the GIPLs remains an enigma. These glycolipids form a glycocalyx over the plasma membrane, but are also present in intracellular organelles (Winter *et al*., 1994) and are abundantly expressed by all developmental stages (McConville and Blackwell, 1991). The GIPLs (and LPG) can modulate the activity of membrane-associated protein tyrosine kinases and protein kinase C in animal cells, both *in vitro* and *in vivo* (Turco and Descoteaux, 1992; Tachado *et al*., 1997), although this is unlikely to be their primary function, as structurally similar GIPLs are expressed in monogenetic Kinetoplastid parasites that do not infect a mammalian host (McConville and Ferguson, 1993).

In order to investigate the function of parasite GIPLs, we have attempted to create strains of *L.mexicana* that

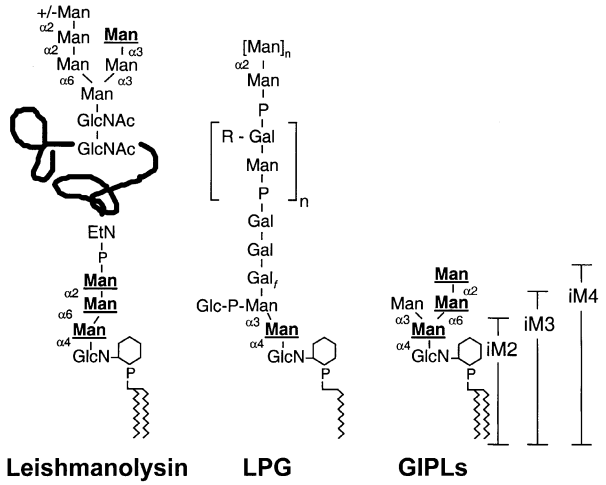

Fig. 1. Structures of anchor and free GPIs in *L.mexicana* promastigotes. Mannose residues added from DPM are underlined (Menon *et al*., 1990; Parodi 1993; this study).

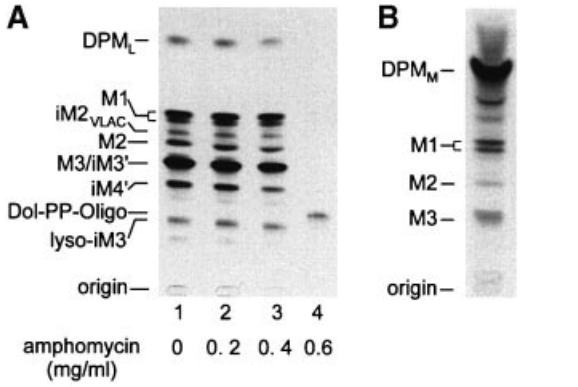

Fig. 2. DPM is utilized by three GPI-specific mannosyltransferases. Promastigotes were osmotically permeabilized and pulse (10 min) labeled with GDP-[^{3}H]Man in the presence of increasing concentrations of amphomycin (0–0.6 mg/ml) then chased with 1 mM unlabeled GDP-Man (60 min; lanes 1–4). Labeled GPIs were extracted and analyzed by HPTLC. Alternatively, the permeabilized cells were incubated with [^{3}H]DPM made from bovine dolichol–phosphate (DPM_M) for 30 min at 30°C. The elution position of DPM and intermediates in protein anchor, LPG anchor and free GPI biosynthesis are indicated (structures are given in Table I and Figure 3).

are unable to synthesize mannosylated GPIs by targeted deletion of the gene for dolichol–phosphate–mannose synthase (DPMS). DPMS catalyzes the transfer of mannose from GDP-Man to dolichol–phosphate to form DPM, the obligate mannose donor for three mannose residues that make up the trimannose backbone in the GPI protein anchor precursors (Figure 1; Menon *et al*., 1990; Orlean, 1990). Whereas DPM is required for the synthesis of several classes of glycoconjugates in other eukaryotes, i.e. the addition of outer chain mannose residues to dolichol-linked oligosaccharide *N*-glycan precursors and the core O-linked mannose in yeast glycoproteins (Hirshberg and Schneider, 1987; Orlean, 1990; Gentzsch and Tanner, 1996), most of these biosynthetic steps are absent in *L.mexicana*. Targeted disruption of both alleles of the gene that encodes DPMS in *L.mexicana*, namely *lmdpms*, resulted in amplification of the chromosomal *lmdpms* locus suggesting that this enzyme is essential for growth. As neither the GPI protein anchors nor the LPG anchors are required for growth (this study; Ryan *et al*., 1993; Mensa-Wilmot *et al*., 1994; Ma *et al*., 1997), these data provide direct evidence that the GIPLs are essential membrane components.

Results

DPM is required for the synthesis of all classes of GPIs in L.mexicana

In this study we used a genetic approach to investigate the function of GPIs in *Leishmania*. As described below, we cloned the *L.mexicana* gene for DPMS (*lmdpms*). This enzyme catalyzes the formation of DPM, the sugar donor for the three mannose residues in the GPI protein anchor precursors (Figure 1; Menon *et al*., 1990; Orlean, 1990), so that deletion of *lmdpms* should lead to a block in protein anchor biosynthesis beyond the early precursor GlcN-PI. To test whether deletion of *lmdpms* would also inhibit the biosynthesis of the LPG anchor and GIPLs, the requirement for DPM in the biosynthesis of these GPI intermediates was tested using an *in vitro* assay system. *Leishmania mexicana* promastigotes were permeabilized in hypotonic buffer and pulse–chase labeled with GDP-[^{3}H]Man (Ralton and McConville, 1998). During the pulse, [^{3}H]Man was initially incorporated into endogenous DPM and subsequently chased into a complex family of GPIs (Figure 2, lane 1). These labeled GPIs have the structure, $Man_{1–4}$GlcN-PI, where the PI moiety contains alkylacylglycerol with either C18:0 or very long [C24:0, C26:0 (VLAC)] *sn*-1 alkyl chains (Ralton and McConville, 1998; summarized in Table I). GPI species containing C18:0 alkyl chains possibly correspond to GIPL precursors, whereas those with C24:0 or C26:0 alkyl chains (i.e. $M1_{VLAC}$ and $iM2_{VLAC}$ in Figure 2A) probably correspond to protein and LPG anchor precursors (Table I; Ralton and McConville, 1998). Inclusion of increasing concentrations of the lipopeptide antibiotic, amphomycin, an inhibitor of DPM synthesis *in vitro*, inhibited the synthesis of both endogenous DPM and all GPIs in these permeabilized cells (Figure 2A, lanes 2–4). The effect of amphomycin was reversed when exogenous dolichol–phosphate was included in the reactions (data not shown), as expected for the mode of action of this inhibitor which forms a complex with dolichol–phosphate (Banerjee, 1989). The novel lipid species labeled in the presence of 0.6 mg/ml amphomycin (Figure 2A, lane 4) has been partially characterized as $Man_4GlcNAc_2$-P-P-dolichol (data not shown). This putative *N*-glycan precursor is synthesized by GDP-Man-dependent mannosyltransferases and reflects the presence of a small pool of dolichol–oligosaccharide intermediates that are not depleted by tunicamycin treatment. These data confirm that DPM is minimally required for the addition of the first mannose residue to GlcN-PI to form Man_1GlcN-PI, a common precursor in all three biosynthetic pathways. To determine whether DPM is required for the addition of other mannose residues, the permeabilized cells were labeled with [^{3}H]DPM rather than GDP-[^{3}H]Man. Label from this donor was incorporated into both molecular species of Man_1GlcN-PI (M1), a single species of Man_2GlcN-PI (M2; Manα1-6Manα1-4GlcN-PI) and Man_3GlcN-PI (M3 Manα1-2Manα1-6Manα1-4GlcN-PI) (Figure 2B), suggesting that both the α1-6- and α1-2-linked mannose

Table I. Structures of GPI lipids synthesized in permeabilized *L.mexicana* promastigotes

GPI[a]	Class of precursor[b]	Structure
M1	GIPL	$Man_1GlcN\text{-}PI_{18:0'-18:0}$
$M1_{VLAC}$	protein/LPG anchor	$Man_1GlcN\text{-}PI_{C24:0/C26:0-18:0}$
$iM2_{VLAC}$	LPG anchor	$Man\alpha1\text{-}3Man\text{-}GlcN\text{-}PI_{24:0/C26:0-18:0}$
iM2′	GIPL	$Man\alpha1\text{-}3Man\text{-}GlcN\text{-}PI_{18:0'-18:0}$
M2	GIPL	$Man\alpha1\text{-}6Man\text{-}GlcN\text{-}PI_{18:0'-18:0}$
M3	GIPL	$Man\alpha1\text{-}2Man\alpha1\text{-}6Man\text{-}GlcN\text{-}PI_{18:0'-18:0}$
iM3′	GIPL	$Man\alpha1\text{-}6[Man\alpha1\text{-}3]Man\text{-}GlcN\text{-}PI_{18:0'-18:0}$
iM4′	GIPL	$Man\alpha1\text{-}2Man\alpha1\text{-}6[Man\alpha1\text{-}3]Man\text{-}GlcN\text{-}PI_{18:0'-18:0}$
EPM3	protein anchor	EtN-P- Manα1-2Manα1-6Man-GlcN-PI

[a]The nomenclature used is from Ralton and McConville (1998). GPIs may contain one (M1), two (M2) or three (M3) mannose residues. The prefix 'i' refers to species containing the α1-3-linked mannose residue. Species denoted by a prime (′) have the same glycan headgroup as mature GIPLs but contain an unremodelled lipid moiety. VLAC denotes GPI species which contain very long alkyl chains (i.e. C24:0 or C26:0).
[b]The involvement of these precursors in protein anchor, LPG anchor and GIPL biosynthesis is based on the characterization of their structures and *in vivo* pulse–chase labeling experiments (Ralton and McConville, 1998). EtN, ethanolamine; P, phosphate; Man, mannose; GlcN, glucosamine; PI, phosphatidylinositol.

residues are transferred from DPM. Significantly, incorporation of label into the LPG anchor precursor, $iM2_{VLAC}$, or into GIPL precursors containing the α1-3-linked mannose branch was not observed, providing the first evidence that the putative GPI-specific α1-3mannosyltransferase may utilize GDP-Man rather than DPM.

The properties of the GPI-specific α1-3mannosyltransferase were investigated further. We have found that the α1-3-linked mannose residues are only added to GPI precursors during the chase in the presence of unlabeled GDP-Man (Figure 3B, lanes 1 and 4). In the absence of the GDP-Man chase, only unbranched GPIs (i.e M1–3 species and their *lyso* derivatives) were synthesized (Figure 3B, lanes 1 and 2). This observation was exploited to investigate the nature of the sugar donor in this step. Permeabilized cells were pulse-labeled with GDP-[^{3}H]Man for 10 min (to label unbranched GPI precursors), then incubated in the presence or absence of amphomycin (10 min, 0°C) before the GDP-Man chase. If addition of the α1-3-linked mannose residue is dependent on enhanced synthesis of DPM during the chase, then these additions should be inhibited following amphomycin treatment (Figure 3A). Alternatively, if the α1-3mannosyltransferases utilizes GDP-Man, inclusion of amphomycin should have no effect on the addition of this residue. As shown in Figure 3B, the addition of amphomycin before the chase did not inhibit the formation of branched GIPLs (i.e. iM2′, iM3′ and iM4′) or LPG anchor intermediate, $iM2_{VLAC}$, suggesting that the α1-3-linked mannose is transferred directly from GDP-Man. The stimulatory effect of the GDP-Man chase on the addition of this residue was not affected by the length of the GDP-[^{3}H]Man pulse (indicating that addition of this residue is not dependent on a residual pool of dolichol–phosphate) and was maximal at ~0.25 mM GDP-Man (data not shown).

In addition to GPI biosynthesis, DPM is required for the addition of a single terminal mannose residue to dolichol-linked oligosaccharides (*L.mexicana* lacks other DPM-dependent mannosyltransferases from this pathway), which are subsequently transferred to protein as *N*-glycans (reviewed in Parodi, 1993; Figure 1). However, it is not required for the addition of mannose residues in the phosphoglycan chains of LPG or the heavily phosphoglycosylated glycoproteins and proteoglycans of these parasites (Carver and Turco, 1992; Moss *et al*., 1999). Finally we have not been able to obtain evidence for any *O*-mannosylation of leishmanial glycoproteins (data not shown), which is initiated by a DPM-dependent step in yeast (Gentzsch and Tanner, 1996). Collectively, these data indicate that DPM is primarily used for GPI biosynthesis in *Leishmania*.

Cloning and characterization of lmdpms

The *L.mexicana* gene encoding DPMS was isolated by screening a genomic library using a PCR amplicon of the *T.brucei dpms* gene (Mazhari-Tabrizi *et al*., 1997). The coding region of *lmdpms* spanned 789 nucleotides (Figure 4B) and encoded a catalytically active DPMS when expressed in *Escherichia coli* (data not shown). The predicted amino acid sequence of *L.mexicana* DPMS (~29 kDa) lacked an N-terminal signal sequence, but contained a hydrophobic domain at the C-terminus (average hydropathy value >1.6 over 19 amino acid residues), which probably constitutes a transmembrane domain (Kyte and Doolittle, 1982) (Figure 4C). An alignment of the predicted amino acid sequences of DPMS from different species showed that *L.mexicana* DPMS was most closely related to the synthases from *T.brucei*, *Ustilago maydis* and *Saccharomyces cerevisiae* (Figure 4B). The sequences from these organisms are related to, but more divergent than those of the *Schizosaccharomyces pombe*, *Caenorhabditis briggsiae* and human DPMS, which lack a C-terminal hydrophobic sequence (Colussi *et al*., 1997; Figure 4B). Other features of the predicted sequence were the presence of a putative cAMP kinase phosphorylation site and the absence of a putative dolichol recognition sequence, which is proposed to occur in other enzymes that utilize a dolichol-containing substrate (Albright *et al*., 1989). A combination of Southern analysis of *L.mexicana* genomic DNA using the *lmdpms* probe and DNA sequencing of the *lmdpms* clone (data not shown) was used to map restriction sites within and adjacent to the predicted coding region of *lmdpms* (Figure 4A). These analyses were consistent with *L.mexicana* having a single DPMS gene per haploid genome.

Creation of lmdpms null mutants

Targeted deletion of *lmdpms* was performed by sequential replacement of the two alleles with genes conferring antibiotic resistance to G418 (*neo*), puromycin (*pac*) and

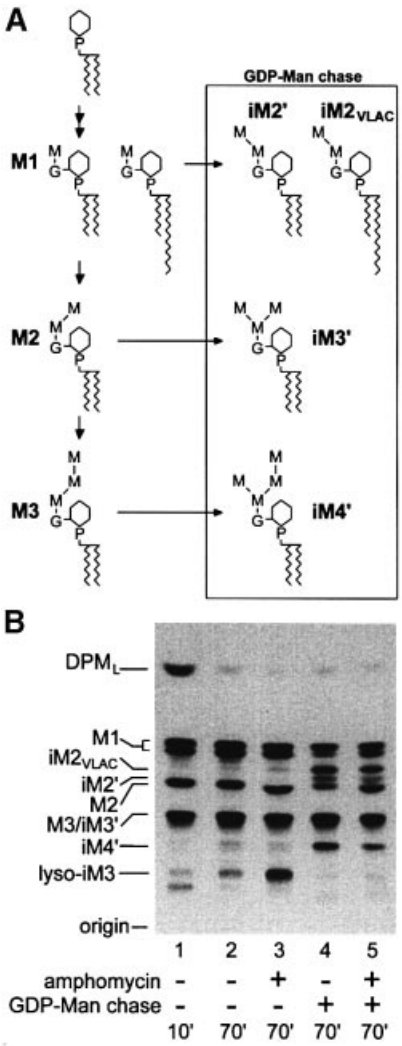

Fig. 3. Addition of the α1-3-linked mannose does not require DPM synthesis. (**A**) Structures of mannosylated GPIs synthesized in permeabilized *L.mexicana* promastigotes, pulse–chase labeled with GDP-[^{3}H]Man. Structures outside the box are the predominant species synthesized during the pulse. Boxed structures are only synthesized during the chase with unlabeled GDP-Man (see B). (**B**) Permeabilized promastigotes were incubated with GDP-[^{3}H]Man for 10 or 70 min with no chase (lanes 1–3), or chased after 10 min with 1 mM unlabeled GDP-Man (lanes 4 and 5). Where indicated, cells were incubated with amphomycin (10 min, 0°C) after the pulse and then chased with unlabeled GDP-Man (lanes 3 and 5). Labeled GPIs were extracted and analyzed by HPTLC.

bleomycin (*ble*). A scheme for the restriction fragments predicted to hybridize to various DNA probes and diagnostic for chromosomal integration of the antibiotic resistance genes into the *lmdpms* locus is shown in Figure 3A. Following one round of transfection with a knockout construct containing *neo* linked to 5′ and 3′ sequence found flanking the *lmdpms* locus, G418-resistant mutants (Δ*lmdpms-neo*) in which one allele of *lmdpms* had been deleted were generated (Figure 5B, lane 2). Null/+ *lmdpms* mutants were also obtained using a knockout construct containing *pac* (Figure 5B, lane 3). Southern blot analyses of *Pst*I-digested genomic DNA demonstrated that one allele of the *lmdpms* gene had been replaced with the antibiotic resistance marker in each mutant cell line (Figure 5C, lanes 2 and 3). The Δ*lmdpms-neo* and Δ*lmdpms-pac* mutants were used for a second round of transfection with constructs containing *pac* or *ble*, respectively, and doubly resistant cell lines were isolated. Genomic integration of the second antibiotic resistance marker in both cell lines was confirmed by Southern analysis (Figure 5B, lanes 4 and 5). Significantly, hybridization of the same blot with the *lmdpms* probe revealed that these clonal double-knockout cell lines still retained at least one copy of *lmdpms* (Figure 5C, lanes 4 and 5). Retention of *lmdpms* was consistently seen using other combinations of selectable markers to generate double knockouts (data not shown). These results suggested that one or more chromosomal copies of *lmdpms* were amplified during homologous replacement, a hallmark of an essential gene (Cruz *et al*., 1993; Dumas *et al*., 1997). To confirm that this amplification was specific for the deletion of *lmdpms*, the Δ*lmdpms-pac* cell line was transfected with the episomal vector pX containing a copy of *lmdpms*, and doubly resistant colonies were subjected to a third round of transfection with the *lmdpms-ble* construct to obtain null/null mutants expressing an episomal copy of *lmdpms*. Southern analysis confirmed that both the *pac* and *ble* constructs had been incorporated into the *lmdpms* locus (Figure 5B, lane 6). Significantly, the *lmdpms* probe bound only to a 6.3 kb restriction fragment derived from the episome containing *lmdpms* and not to a 2.5 kb chromosomal fragment (Figure 5C, lane 6), demonstrating that a *lmdpms* double-knockout can be obtained when an episomal copy of the gene is present. These data confirm that there is only one copy of *lmdpms* in the genome and that this gene is essential for growth.

Reduced levels of GPI-anchored protein expression do not affect promastigote growth

The apparent lethality of the DPMS knockout may be due to lack of surface expression of one or more GPI-anchored glycoproteins. To investigate this possibility, *L.mexicana* promastigotes were transfected with the *T.brucei* gene for GPI-specific phospholipase C (GPI-PLC), a cytosolically orientated membrane protein that has previously been shown to cause the selective down-regulation of protein anchor precursors, but not LPG anchor or type-2 (LPG-like) GIPL intermediates when ectopically expressed in *Leishmania major* (Mensa-Wilmot *et al*., 1994). As shown in Figure 6, expression of GPI-PLC in *L.mexicana* resulted in a 50-fold decrease in levels of the major surface glycoprotein, leishmanolysin (Figure 6A). Analysis of total cellular proteins after Triton X-114 phase partitioning indicated that levels of other GPI-anchored proteins had similarly decreased (data not shown). In contrast, cellular levels of GIPLs and LPG were essentially the same in cells expressing either the GPI-PLC or an irrelevant cytosolic protein (green fluorescent protein, GFP), as determined by steady state [^{3}H]inositol labeling (Figure 6B, lanes 1 and 3) and compositional analysis of purified LPG and GIPL pools (data not shown). When [^{3}H]inositol-labeled lipids were lysed in Triton X-100 (37°C, 120 min), all the GIPLs were hydrolyzed in the GPI-PLC-expressing cells but not in control transfected cells (Figure 6B, lanes 2 and 4), demonstrating that levels of expression of this enzyme are sufficient to hydrolyze the cellular pool of GPIs. Significantly, the growth characteristics of the transfected cell lines were very similar (Figure 6C), indicating that the requirement for DPM is unlikely to

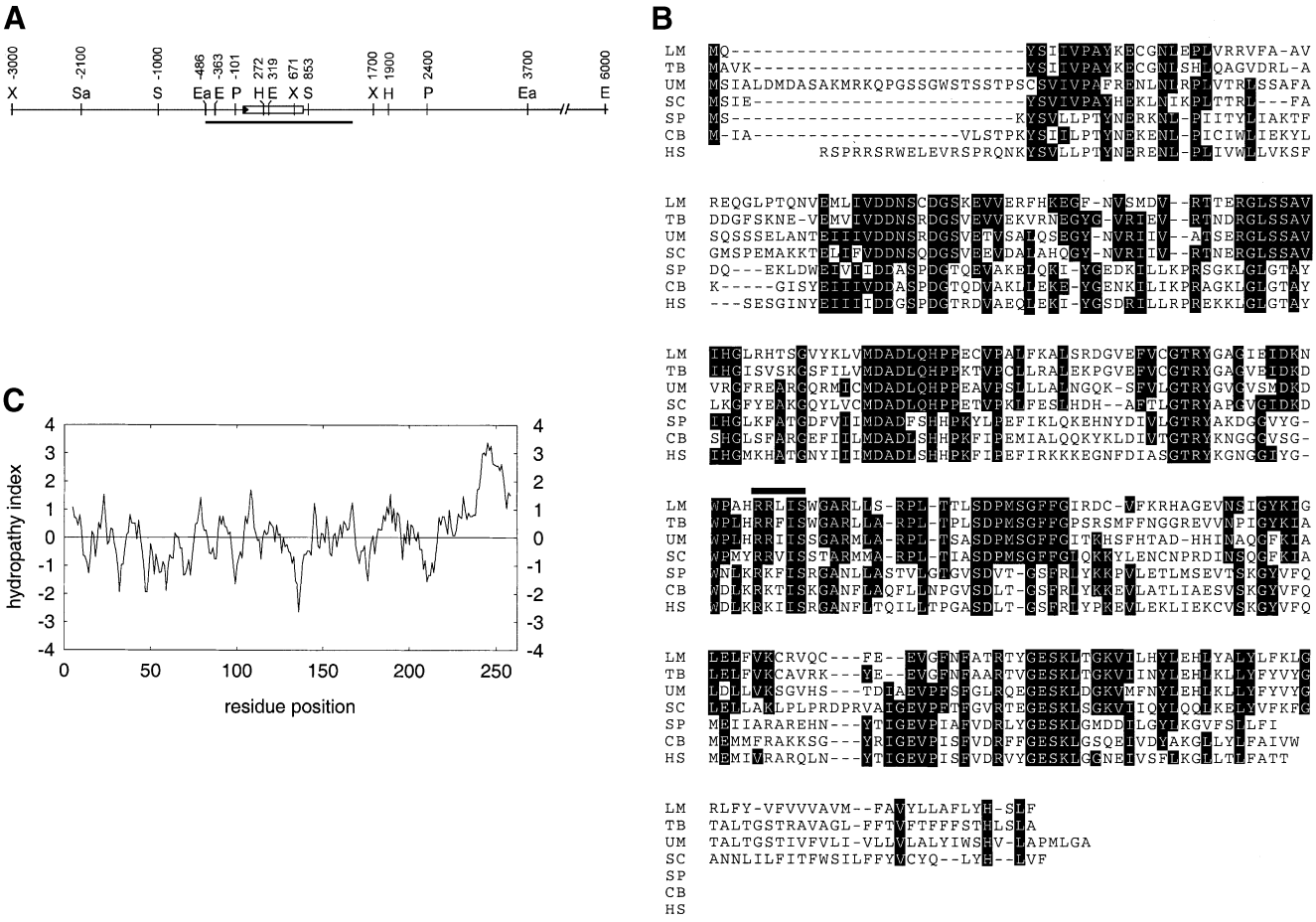

Fig. 4. *Leishmania mexicana* DPMS. (**A**) Map of *lmdpms*. Restriction sites immediately flanking the coding region of *lmdpms* were determined by Southern analysis. The restriction sites are labeled *Eag*I (Ea), *Eco*47III (E), *Hin*dIII (H), *Pst*I (P), *Sal*I (S), *Sau*3A (Sa), *Xho*I (X). The sites for each restriction endonuclease further 5′ and 3′ were not mapped. The coding region of *lmdpms* is indicated by the boxed region. Numbering is relative to the translation start site. The region underlined spans the sequenced portion of the genomic clone. (**B**) Alignment of predicted amino acid sequences of DPM-synthases from *L.mexicana* (LM), *T.brucei* (TB), *U.maydis* (UM), *S.pombe* (SP), *Caenorhabditis briggsiae* (CB) and humans (HS) (A). Amino acid sequences identical in at least four of the six sequences are highlighted in black. A putative cAMP-dependent kinase phosphorylation site is indicated by heavy line. (**C**) Hydropathy plot of *L.mexicana* DPMS hydropathy values were calculated according to Kyte and Doolittle (1982) using a window of nine residues.

be due to a requirement for a specific GPI-anchored glycoprotein.

Discussion

In this study we provide evidence that DPMS is essential for growth of *L.mexicana* promastigotes. This is the first demonstration that some glycosylation biosynthetic pathways are essential for the viability of any parasitic protozoan and validates the development of anti-parasite drugs that target enzymes in these pathways (Smith *et al*., 1997; Sutterlin *et al*., 1997). It is possible that DPM is required for the growth of *L.mexicana* because of its role in GPI biosynthesis. Specifically, DPM is the mannose donor for the common core mannose residue in all leishmanial GPIs, as well as for two other mannose residues that are found in both the protein anchors and the polar GIPLs. Although DPM also acts as a sugar donor for a single terminal mannose residue in the major *N*-glycan precursor, $Man_{5-6}GlcNAc_2$-P-P-dolichol (Parodi, 1993; Figure 1), intermediates in this pathway are at least 100-fold less abundant than GPI intermediates (J.E.Ralton and M.J.McConville, unpublished data). Moreover, this step is not required for oligosaccharide transfer to protein or for the viability of either yeast (Orlean *et al*., 1988) or mammalian cells (Chapman *et al*., 1980) and is unlikely to be essential in *Leishmania*. Indeed, *N*-glycosylation does not appear to be required for correct folding or intracellular transport of specific leishmanial glycoproteins (Funk *et al*., 1994; McGwire and Chang, 1996). Finally, there is no evidence for other endoplasmic reticulum (ER)-glycosylation pathways in *L.mexicana* that require DPM (i.e. *O*-mannosylation of glycoproteins; data not shown) or that DPM is required for the assembly of abundant phosphoglycan chains on either the LPG anchors or the secreted glycoproteins and proteoglycans in the Golgi apparatus (Carver and Turco, 1992; Mengeling *et al*., 1997; Moss *et al*., 1999).

Several lines of evidence suggest that it is the GIPLs, rather than the GPI-anchored glycoproteins and LPG, that are required for growth of cultured leishmanial promastigotes. First, we show that the surface expression of GPI-anchored glycoproteins in *L.mexicana* can be downregulated (>95%) by ectopic expression of the *T.brucei* GPI-PLC without affecting growth. These results are similar to those obtained by Mensa-Wilmot *et al*.

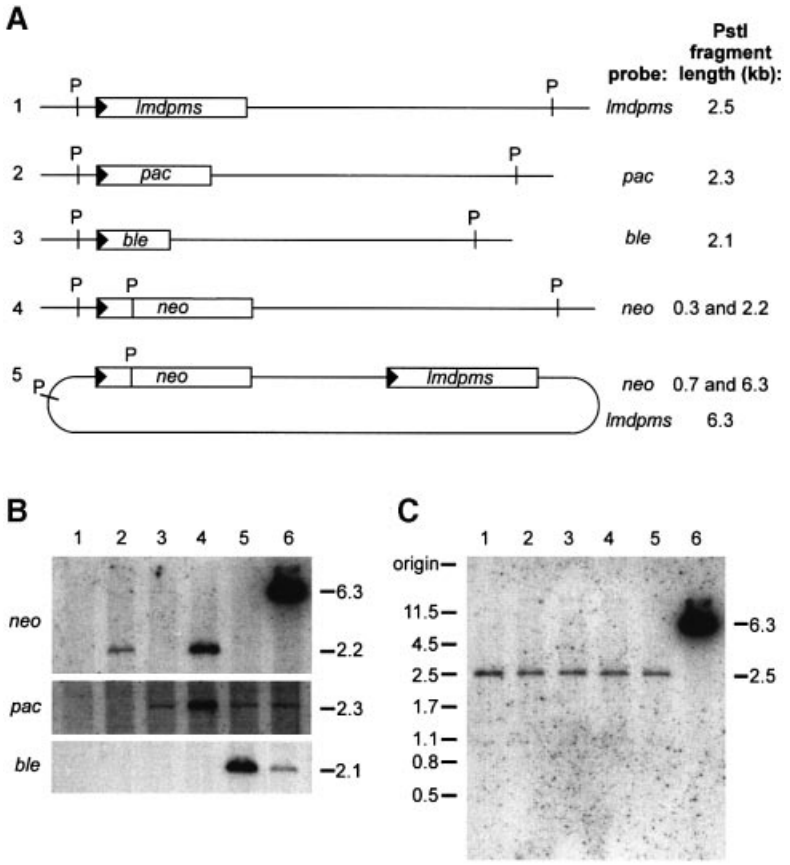

Fig. 5. Targeted replacement and episomal expression of *lmdpms*. (**A**) Schematic representation of the genomic organization of wild-type *lmdpms* (1), the *lmdpms* replacement events with *pac* (2), *ble* (3) and *neo* (4), and the episomal expression vector pDPMS23 (5). *Pst*I restriction sites are indicated (P). The sizes of *Pst*I restriction fragments predicted to hybridize with corresponding DNA probes in transfected cell lines are listed. (**B**) Southern analysis of total DNA from wild-type and transfected cell lines. *Pst*I-digested DNAs were sequentially probed with *neo*, *pac* and *ble* DNA probes. Lane 1, wild-type *L.mexicana*; lane 2, single-knockout *neo* replacement clone, Δ*lmdpms-neo*; lane 3, single-knockout *pac* replacement clone, Δ*lmdpms-pac*; lane 4, double-knockout *pac*/*neo* replacement clone; lane 5, double-knockout *pac*/*ble*-replacement clone; lane 6, double-knockout *pac*/*ble* replacement clone containing episomal pDPM23 plasmid. (**C**) Same Southern blot probed with a *lmdpms* probe. Molecular sizes (kb) are indicated.

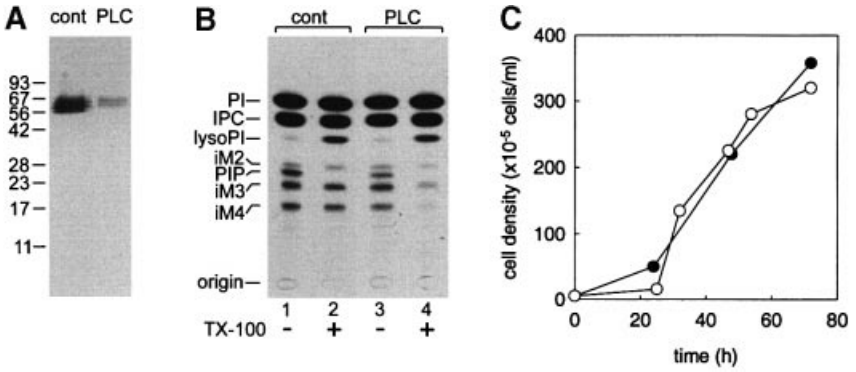

Fig. 6. Decreased levels of expression of GPI-anchored proteins are not associated with a growth phenotype. *Leishmania mexicana* promastigotes were stably transfected with a gene encoding either *T.brucei* GPI-PLC or cytosolic GFP (cont) and cellular levels of GPI-anchored leishmanolysin, GIPLs and LPG in each cell line analyzed. (**A**) Leishmanolysin was recovered after Triton X-114 solubilization and phase partitioning and levels quantitated by immunoblotting after SDS–PAGE and transfer to nitrocellulose. Molecular sizes (kDa) are shown on the left-hand side. (**B**) Inositol lipids were labeled to steady state (24 h) with [^{3}H]inositol and extracted directly from the cell pellet (lanes 1 and 3) or from a 0.1% Triton X-100 cell lysate (lanes 2 and 4) after incubation at 37°C for 120 min to confirm the capacity of ectopically expressed GPI-PLC to degrade endogenous GIPLs. IPC, inositolphospho-ceramide. (**C**) Growth curve of cell lines expressing GFP (●) or GPI-PLC (○).

(1994) in *L.major*. This cytosolically orientated lipase can degrade all classes of GPI when cells are lysed in detergent (Figure 6), but only hydrolyzes protein anchor precursors *in vivo*, presumably reflecting differences in the topology and/or subcellular localization of intermediates in these different pathways (Mensa-Wilmot *et al*., 1994). Secondly, targeted disruption of a gene encoding one of the components in the transamidase complex that attaches mature GPI anchor precursors to protein is not lethal in *L.mexicana* promastigotes (Hilley *et al*., 1998). Thirdly, a number

of viable LPG-deficient leishmanial mutants have been identified (Ryan *et al*., 1993; Ma *et al*., 1997). These mutants have defects in either the assembly of the phosphoglycan chains or in post-mannosylation steps in GPI anchor assembly (McConville and Homans, 1992; Ryan *et al*., 1993; Ma *et al*., 1997). Strikingly, none of the defects in LPG biosynthesis occur in enzymes common to both the LPG anchor and free GPI biosynthetic pathways. Finally, surface expression of both LPG and GPI-anchored proteins, but not of the GIPLs, is downregulated following differentiation of promastigotes to amastigotes in the phagolysosome of mammalian macrophages (McConville and Blackwell, 1991; Bahr *et al*., 1993), suggesting that the lethality of the *lmdpms* knockout is probably not due to the cumulative effect of deleting both classes of GPI anchor.

The primary function of the GIPLs in *Leishmania* and other Kinetoplastid parasites remains unclear, although, as the major class of glycolipids in these cells and abundant components in both the plasma and intracellular membranes, they could fulfill one or more essential functions. Intracellular compartments containing GIPLs include the megasomes (large lysosomes) in *L.mexicana* amastigotes (Winter *et al*., 1994) and a distinct subdomain of the ER in rapidly dividing promastigotes (S.Ilgoutz, K.Mullin, B.Southwell and M.McConville, manuscript in preparation). By analogy with the role of glycosphingolipids in animal cells, these cell surface and intracellular GIPLs may be involved in forming a protective surface glycocalyx, in protecting lysosomal membranes from lumenal hydrolases or in maintaining specific membrane microdomains within the secretory pathway (Brown and London, 1998). Alternatively, the GIPLs could act as mediators in endogenous signal transduction pathways, as they have been proposed to do in mammalian host cells (Tachado *et al*., 1997). Interestingly, free GPIs are relatively abundant in other eukaryotes such as *S.cerevisiae* (Sipos *et al*., 1994) and in some cultured animal cells (Sevlever *et al*., 1995; van't Hof *et al*., 1995; Singh *et al*., 1996). Studies on the function of these glycolipids are complicated by the fact that protein-linked GPIs per se can be essential for growth. For example, GPI biosynthesis is probably essential for growth in yeast (Leidich *et al*., 1994), in part because a defect in GPI biosynthesis leads to the accumulation and eventual degradation of non-anchored yeast glycoproteins in the ER (Doering and Schekman, 1996) and prevents some cell-surface glycoproteins from being covalently attached to cell wall glucans via their GPI moiety (Nobel and Lipke, 1994). Thus further studies on the function of free GPIs in *Leishmania* may provide insights into a possible role(s) of this class of glycolipid in other eukaryotes.

DPM is synthesized on the cytoplasmic face of the ER (Bossuyt and Blanckaert, 1993). However, all mannose additions involving this sugar donor appear to occur in the lumen of the ER. These include the last four mannose additions to dolichol-linked oligosaccharides involved in *N*-glycan biosynthesis, the *O*-linked mannose core on yeast glycoproteins and at least one mannose addition to the GPI protein anchor precursor (Hirshberg and Schneider, 1987; Gentzsch and Tanner, 1996; Takahashi *et al*., 1996). In contrast, no mannose additions in the lumen of the ER have been shown to involve GDP-Man, possibly because the ER lacks a GDP-Man transporter. Interestingly, a GDP-Man transporter has been found in *Leishmania*, although this protein is localized exclusively to the Golgi apparatus (Ma *et al*., 1997). In this context, the involvement of GDP-Man as the direct sugar donor for the α1-3-linked mannose residue in the LPG anchor and GIPL precursors was surprising. We have shown recently that the α1-3-linked mannose and the DPM-dependent mannose residues are added in a novel pre-Golgi compartment which has a subcellular distribution distinct from the Golgi apparatus (S.Ilgoutz, K.Mullin, B.Southwell and M.McConville, manuscript in preparation). Thus in order to be modified with the α1-3 mannose residue, GIPL and LPG anchor intermediates may have to be 'flipped' from the ER lumen (where DPM-dependent mannose additions occur) to the cytoplasmic face of the ER. Flipping of GPI precursors across the ER membrane has previously been proposed to explain how early intermediates (i.e. GlcN-PI and GlcNAc-PI) are made on the cytoplasmic side of the ER, whereas the mature GPI anchor precursors are added to newly synthesized proteins in the lumen of the ER (Vidugiriene and Menon, 1993, 1994; Watanabe *et al*., 1996). Our findings provide evidence that the flipping of GPIs can be a bi-directional process. Whether all mannosylated GPIs flip in both directions is still not clear as the protein anchor precursors in *Leishmania* do not appear to be modified with the the α1-3 mannose branch. Preferential flipping of different classes of GPI intermediates could be regulated by differences in their lipid moiety and/or the spatial organization of these intermediates relative to the putative transmembrane 'flippase'. These findings point to a possible mechanism by which GPI intermediates from different biosynthetic pathways could be segregated and elaborated with different glycan headgroups.

Materials and methods

Parasites

Leishmania mexicana (strain MNYC/BZ/62/M379) promastigotes were cultured at 27°C in RPMI medium supplemented with 10% heat inactivated fetal bovine serum (pH 7.5) and, when appropriate, antibiotics for selection of transfectants. For selection, G418 (Geneticin) at 20–50 μg/ml, hygromycin B at 50 μg/ml, bleomycin sulfate at 5 μg/ml and puromycin sulfate at 20 μg/ml were added singly or in combination.

Cloning of lmdpms

lmdpms was isolated from a library of *L.mexicana* genomic DNA comprised of *Sau*3A partially digested genomic DNA ligated into Lambda DASHII phage arms (Weise *et al*., 1995). The library was probed by plaque hybridization with a radiolabeled PCR amplicon spanning most of the coding region of the DPMS gene from *T.brucei* (nucleotides 213–1015 relative to the translation start site; Mahari-Tabrizi *et al*., 1997) amplified directly from *T.brucei* genomic DNA. Two subclones derived from a single positive plaque overlapped by 759 bp and spanned the putative coding region of *lmdpms*. These subclones (designated pDPMS3 and pDPMS4) extended from –488 to 3700 and from –2100 to 272, relative to the translation start site, respectively. The double-stranded nucleotide sequence obtained from both clones spanned from –488 to 1436.

DNA constructs

DPMS knockout constructs consisted of a selectable marker (*neo*, *ble* or *pac*) flanked by 5′ and 3′ genomic DNA sequence found flanking the *lmdpms* coding region (derived from the clones pDPMS3 and pDPMS4). pDPMS61 consisted of a neomycin phosphotransferase (*neo*) gene amplified by PCR using primers 28/29 and pX (LeBowitz *et al*., 1990) as template, flanked by 2.1 kb of 5′ sequence (amplified by PCR using primers 26/27 and pDPMS4 as template) and 2.9 kb of 3′ sequence

(amplified using primers 30/31 and pDPMS3 as template). The three primary PCR amplicons were purified from 1% agarose, TAE gels using Qiaquick spin column (Qiagen) and then fused together by spliced overlap extension PCR (Horton *et al*., 1990). The second DPMS knockout construct, pDPMS7, contained the puromycin *N*-acetyl transferase (*pac*) gene [amplified from a parent vector (Mottram *et al*., 1996) using primers 61/62] flanked by 503 bp of 5′ sequence and 487 bp of 3′ sequence amplified from pDPMS3 using primers combinations 52/60 and 63/64, respectively. The third DPMS knockout construct, pDPMS8, contained the bleomycin hydrolase (*ble*) gene [amplified from a parent vector (Mottram *et al*., 1996) using primers 73/74] and the same 5′ and 3′ sequence as used in pDPMS7 (amplified with primer combinations 52/72 and 75/64, respectively). pDPMS7 and pDPMS8 were also synthesized by spliced overlap extension PCR. The resulting amplicons were cloned into *Eco*RI and *Xba*I sites (for pDPMS61) or the *Bam*HII and *Hin*dIII sites (in the case of pDPMS7 and pDPMS8) in pBluescriptII SK^+ using restriction sites engineered in the PCR primers. pDPMS61, pDPMS7 and pDPMS8 were used to generate Δ*lmdpms-neo*, Δ*lmdpms-pac* and Δ*lmdpms-ble* mutant cell lines respectively. pDPMS23, containing *lmdpms* in pX was used for the episomal expression of DPMS. *lmdpms* was amplified by PCR from the genomic clone pDPMS3 using primers 20/19, then cloned into *Bam*HI and *Xba*I sites in pX. pGPI-PLC, consisting of the pX plasmid containing the gene for the *T.brucei* GPI-PLC, was kindly provided by Dr K.Mensa-Wilmot and synthesized as described previously (Mensa-Wilmot *et al*., 1994).

Oligonucleotide primers for PCR were as follows (5′ to 3′). Nucleotides in bold were not complementary to the template DNA, but were added for cloning. Lower case denotes a sequence that was added to allow fusion between amplicons by spliced overlap extension PCR.

19 **GATCTGAATTCCTAGA**CCTAGAAGAGGGAATGGTAGAG
20 **GACTGGATCC**CATGCAGTACTCCATTATCG
26 **CTGCAGGAATTCG**GATCCTC
27 **ggccgatcccat**GTTTCCGAGCTAAAACAATGAAAAAG
28 **tagctcggaaa**CATGGGATCGGCCATTGAAC
29 **tgcacaaggggc**TCAGAAGAACTCGTCAAGAAGGC
30 **gagttcttctga**GCCCCTTGTGCACTCCTG
31 **TAGCTCTAGA**GCACACCGCACCTTACCAG
52 **GTCAGGATCC**GTGATTGGAGGTGCGGCT
60 **gtactcggtcat**GTTTCCGAGCTAAAACAATGAAAAAG
61 **tagctcggaaac**ATGACCGAGTACAAGCCCAC
62 **tgcacaaggggc**TCAGGCACCGGGCTTG
63 **cccggtgcctga**GCCCCTTGTGCACTCCTG
64 **CATGAAGCTT**AGAGACTGGCAGATGGCAAG
72 **caacttggccat**GTTTCCGAGCTAAAACAATGAAAAAG
73 **tagctcggaaac**ATGGCCAAGTTGACCAGTG
74 **tgcacaaggggc**TCAGTCCTGCTCCTCGGC
75 **gagcaggactga**GCCCCTTGTGCACTCCTG

Transfections

Leishmania promastigotes were transfected by electroporation, essentially as described by Kapler *et al*. (1990). For integration events, purified plasmid DNA was digested with appropriate restriction endonucleases to release the knockout constructs and the DNA recovered by chloroform/phenol extraction and ethanol precipitation, prior to being resuspended in 10 mM Tris–HCl pH 8 at 1 μg/ml. DNA (5 μg for integration or 30 μg for episomal expression) was used per transfection. Cells were suspended in RPMI containing 10% fetal bovine serum for 18 h, before being transferred to fresh medium containing appropriate antibiotics. Clonal cell lines were obtained by limit dilution.

Southern analysis

A probe for *lmdpms* was obtained by digesting the pDPMS23 plasmid with *Bam*HI and *Xba*I. Probes for the various selectable markers were obtained by digestion of pX with *Spe*I and digestion of previously described vectors (Mottram *et al*., 1996) containing *pac* and *ble* genes with *Bam*HI and *Spe*I. Genomic DNA was isolated from *L.mexicana* promastigotes (Medina-Acosta and Cross, 1993), exhaustively digested with restriction endonucleases and resolved by electrophoresis in 1% agarose, Tris–borate–EDTA gels. DNA was vacuum blotted onto Hybond N+ membranes (Amersham), which were washed in 2× SSC (1× SSC is 150 mM NaCl, 15 mM trisodium citrate, pH 7.0), air dried and baked at 80°C for 2 h. Membranes were pre-hybridized by incubation in 15 ml Rapid-hyb buffer (Amersham) for 30 min at 65°C, then hybridized in the same buffer with 50 ng of DNA probe for 2.5 h at 65°C. The membranes were washed sequentially with 2× SSC, 0.1% (w/v) SDS (20 min, 25°C) and twice with 0.1× SSC, 0.1% SDS (w/v) (15 min, 65°C). Bound probe was detected by autoradiography.

Radiolabeling GPI glycolipids in permeabilized L.mexicana promastigotes

Promastigotes (1×10^8 cells/ml) were incubated in RPMI–10% fetal bovine serum containing 1 μg/ml tunicamycin for 30 min at 27°C, then hypertonically permeabilized (at a concentration of 2×10^8 cells/ml) in water containing 2 mM EGTA, 0.1 mM TLCK, 0.1 μM leupeptin and 1 mM dithiothreitol (DTT) for 10 min at 0°C. Permeabilized cells were pelleted by centrifugation (16 000 *g*, 5 min, 4°C), washed once with hypotonic solution and resuspended (4×10^8 cell equivalents/ml) in 50 mM HEPES–NaOH buffer pH 7.4, containing 100 mM KCl, 2 mM EGTA, 1 mM ATP, 0.2 mM TLCK, 0.1 mM leupeptin, 0.5 mM DTT and 1 μg/ml tunicamycin (buffer A). In some experiments, amphomycin (10 mg/ml suspension in water) was added to permeabilized cells to give a final a concentration range of 0–0.6 mg/ml. This inhibitor was added either before radiolabeling (for 10 min at 0°C) or, in the experiments shown in Figure 5, before the chase with unlabeled GDP-Man (again for 10 min at 0°C). Labeling was initiated by adding 10 μl of GDP-[^{3}H]Man (0.5 μCi) and UDP-GlcNAc (10 mM) in buffer A to 60 μl permeabilized cells. After 10 min at 20°C, the label was chased for a further 60 min by adding 10 μl GDP-Man (10 mM in buffer A). At the end of the incubation period, lipids were extracted by adding 300 μl chloroform:methanol (1:2 v/v) to give a final chloroform/methanol/water ratio of 1:2:0.8 v/v (CMW) and sonicated intermittently over 2 h at 25°C. Insoluble material was removed by centrifugation (16 000 *g*, 5 min) and re-extracted with CMW. The pooled CMW supernatants were dried under a stream of N_2 and free GPIs recovered by partitioning in a biphasic system of 1-butanol (90 μl) and water (90 μl) (McConville and Blackwell, 1991). The lower aqueous phase was extracted twice more with water-saturated 1-butanol (2×90 μl) and the combined upper 1-butanol phases back extracted with water (100 μl), before being dried on a Speed-vac (Savant) concentrator. For analysis of radiolabeled GPIs, samples in 40% 1-propanol were applied to aluminium-backed Silica Gel 60 HPTLC plates (Merck) which were developed in chloroform/methanol, 1 M ammonium acetate, 13 M NH_4OH, water (180:140:9:9:23 v/v; Solvent A). HPTLC sheets were sprayed with En^{3}Hance (DuPont) and exposed to BioMax film (Kodak) at –70°C.

Analysis of GPI-PLC-expressing cell lines

The phenotype of the *L.mexicana* promasitgotes transfected with pXGPI-PLC was compared with cells containing the control plasmid pX-GFP, which express a cytosolic green fluorescent protein (Ha *et al*., 1996). GPI-anchored glycoproteins were isolated by Triton X-114 phase partitioning and detected by immunoblotting with the monoclonal antibody (mAb) L3.8 after SDS–PAGE under nonreducing conditions and transfer to nitrocellulose membranes (Ilg *et al*., 1993). Cellular levels of GIPLs and LPG were determined by monosaccharide analysis of semi-purified fractions isolated in CMW and 9% 1-butanol, respectively (McConville and Blackwell, 1991), or by HPTLC analysis of lipids which had been metabolically labeled to steady state with [^{3}H]inositol (Ralton and McConville, 1998).

The sequence of *lmdpms* has been submitted to the DDBJ/EMBL/GenBank databases under accession No. AJ131960.

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