Published in final edited form as:
Mol Biochem Parasitol. 2004 July ; 136(1): 11–23. doi:10.1016/j.molbiopara.2004.02.012.

The *LPG1* gene family of *Leishmania major*

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Abstract

In *Leishmania major*, the core of the abundant surface lipophosphoglycan (LPG) is structurally related to that of the smaller glycosylinositolphospholipids (GIPLs) in containing galactosylfuranose (Gal_f) residues in a Gal_f(β1, 3)Man motif. However, deletion of the putative Gal_ftransferase (Gal_fT) *LPG1* affected Gal_fincorporation in LPG but not GIPLs. We hypothesized that the presumptive GIPL Gal_ftransferases could be homologous to *LPG1*, and identified three related genes in the *L. major* genome. These were termed *LPG1L*, *LPG1R*, and *LPG1G*, the latter of which was found in three identical copies located at the telomeres of chromosomes 5, 19, and 32 based on *Leishmania* genome project data. Neither *LPG1* nor its homologues *LPG1L* and *LPG1R* were involved in the biosynthesis of GIPLs, as an *lpg1*$^-$/*lpg1l*$^-$/*lpg1r*$^-$ triple knockout (the first such in *Leishmania*) grew normally and made wild-type levels of Gal_f-containing GIPLs. In contrast, overexpression of these three led to elevated galactose incorporation in glycoproteins. Gal_f-containing glycoproteins had not been described in *Leishmania* but occur at high levels in other closely related trypanosomatids including *Trypanosoma cruzi*, *Crithidia*, *Leptomonas*, and *Endotrypanum*, and *LPG1L* and *LPG1R* homologs were detected in these species. These data suggest that the glyco-synthetic capabilities of *Leishmania* and perhaps other trypanosomatids may be larger than previously thought, with some activities being 'cryptic' in different lineages and potentially serving as reservoirs for glycoconjugate variation during evolution. Future tests will address whether the *LPG1G* family encodes the hypothesized GIPL-specific Gal_fT.

Keywords

Glycosylinositolphospholipids; Lipophosphoglycan; Galactosylfuranose; Galactosylfuranose transferase

1. Introduction

Protozoan parasites of the genus *Leishmania* are the causative agent of the disease leishmaniasis, which infects more than 10 million people worldwide [1]. *Leishmania* parasites are transmitted through sand fly bites, where the flagellated promastigote cells are introduced into the mammalian host. Parasites are then taken up by macrophages and differentiate into the non-flagellated amastigotes. Depending on parasite species, *Leishmania* infections in humans cause manifestations from self-containing cutaneous lesions to lethal visceral infections.

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The surface of the promastisgote stages of *Leishmania* is coated with a variety of interrelated glycoconjugates including lipophosphoglycan (LPG), glycosylinositolphospholipids (GIPLs), proteophosphoglycan (PPG), and GPI-anchored proteins [2–4]. In the sand fly, stage-specific modifications of LPG are responsible for the attachment and release of promastigotes from the midgut [5–7]. In the mammalian host, LPG confers resistance to complement-mediated lysis, oxidative stress, and inhibits phagolysosomal fusion [8,9]. However, LPG is down-regulated in amastigotes, whose surface is dominated instead by the abundant GIPLs, which suggested that these play critical roles in amastigote survival and virulence [10,11]. This view was recently called into question by studies of a *Leishmania major* mutant lacking the enzyme alkyldihydroxyacetone phosphate synthase (ADS1), which is required for the synthesis of ether phospholipids. The *ads1*$^-$ null mutant lacked LPG and GIPLs but maintain the presence of GPI-anchored proteins, probably bearing a modified GPI anchor [12]. Remarkably, *ads1*$^-$ mutants showed little phenotype beyond that attributable to loss of LPG (e.g. reduced ability to establish macrophage infections), and *ads1*$^-$ amastigotes can replicate in macrophages and cause disease in susceptible mice [12]. In *L. mexicana*, there are conflicting data about the role of GIPLs in parasite viability and/or virulence [13–15]. A potential drawback is that the various *Leishmania* mutants studied were pleiotropic and affected a number of glycoconjugates, making inferences about GIPL function indirect. Thus, the identification of genes that specifically affect GIPL synthesis would simplify and strengthen genetic studies of their function.

Leishmania GIPLs have different structures in different species, consisting of type I GIPLs whose structure resembles that of protein GPI anchors, type II GIPLs whose structure resembles that of the LPG core, and/or “hybrid” GIPLs [3,10,11]. *L. major* synthesize primarily type II GIPLs, which contain galactosylfuranose (Gal_f) in the $Gal_{0–2}Gal_f(\beta_3)$Man sequence, which is also found in the LPG core ([16,17], Fig. 1). We showed previously that *LPG1*, encoding a putative galactofuranosyl transferase (Gal_fT), was responsible for adding Gal_f to the LPG core structure, but that *lpg1*$^-$ mutants continued to synthesize the Gal_f-containing GIPLs [18]. Thus, there must be additional Gal_fTs that are responsible for the synthesis of GIPLs. Given the similarity of the Gal_fT acceptors for LPG and GIPLs, it seemed reasonable to postulate that these GIPL-specific Gal_fTs could be related to *LPG1*. In this report we describe the characterization of a family of *LPG1* homologs in the *L. major* genome, and studied the effect of genetic inactivation of three of these singly or in combinations (*LPG1*, *LPG1L*, and *LPG1R*).

2. Materials and methods

2.1. Leishmania cultures

L. major LV39 clone 5 (Rho/SU/59/P) cells were grown in M199 medium supplemented with 10% heat inactivated fetal calf serum at 26 °C [19]. Selective drugs used in this study included G418 (10 μg/ml), hygromycin (50 μg/ml), puromycin (10 μg/ml), nourseothricin (80 μg/ml), blasticidin (10 μg/ml), and phleomycin (10 μg/ml).

2.2. Isolation of LPG1L and LPG1R open reading frames

At the time these studies were initiated the *L. major* genome was largely incomplete. Sequence data was obtained from the Sanger Institute website at http://www.sanger.ac.uk/Projects/Lmajor/. Searches with *LPG1* first identified a 537-bp region from the *Leishmania* Genome Database; this region was PCR amplified and used to identify a cosmid (B4308) containing the full length gene from a cLHYG genomic cosmid library made from *L. major Friedlin* strain V1 (MHOM/IL/80/Friedlin). This gene was termed *LPG1L* (for *LPG1-like*) and its open reading frame and flanking regions were sequenced (GenBank accession

number AY235572). A second gene termed *LPG1R* (*LPG1-related*) was similarly identified in the *Leishmania* Genome Database; this ORF and flanking sequences were recovered by PCR and their sequences were determined (AY235573). Preliminary sequence data from *T. cruzi* was obtained from The Institute of Genomic Research through their website at http://www.tigr.org.

2.3. Molecular constructs

To generate the C-terminal *GFP* tagged proteins, full length open reading frames lacking stop codons were PCR amplified from *L. major* FV1 genomic DNA using primers SMB1307 (5′-TTATCAggatccACCATGAAGGGCAGACTAC-3′, lower case letters indicate added restriction sites) and SMB1310 (5′-ATATAAgcggccgcCGGGCTGACAGCCTGCAG) for *LPG1L* and SMB1354 (5′-TATAGCggatccACCATGAAGCGCGGGCAGAGG) and SMB1405 (5′-ACAGCTgatatcCTTTCGCCAATCCGGCTCTG) for *LPG1R*. The resulting DNA fragments were digested with appropriate restriction enzymes (*Bam*HI and *Not*I for *LPG1L*, *Bam*HI and *Eco*RV for *LPG1R*) and cloned into the expression site of pXG−/*GFP*+ (a vector for making C-terminal GFP fusion proteins; strain B2863 [20]) to make pXG-*LPG1L-GFP* (B4400) and pXG-*LPG1R-GFP* (B4455).

To make deletion constructs for *LPG1L*, a 700-bp region immediately upstream of the start codon and a 900-bp region immediately downstream of the stop codon of *LPG1L* were PCR amplified from *L. major* genomic DNA and cloned into vector pX63PAC (B1129) [21] so that the end of the upstream region is linked to the beginning of the downstream region. Next a 909-bp *NEO* marker gene (confers resistance to G418) was excised from vector pXG (B4090) [20] and inserted in between the upstream and downstream regions of *LPG1L* to make pX63PAC-KO-*LPG1L:NEO* (B4369). Similarly, a 1052-bp *HYG* marker gene (conferring resistance to hygromycin) was excised from vector pX63HYG (B617) [22] using *Spe*I and *Bam*HI and inserted in between the upstream and downstream regions of *LPG1L* to make pX63PAC-KO-*LPG1L:HYG* (B4370). Linear deletion cassettes containing *NEO* and *HYG* markers flanked by the upstream and downstream regions of *LPG1L* were generated by *Sma*I digestion.

To make deletion constructs for *LPG1R*, first a 2.4-kb DNA fragment containing the *LPG1R* ORF (plus sequences 550 bp from upstream and 440 bp from downstream) was PCR amplified from *L. major* genomic DNA and cloned into vector pUC18 to give pUC-*LPG1R* (B4413). Then a *Sac*II/*Sph*I fragment containing the first 500 bp of *LPG1R* ORF was replaced by drug markers (*PAC* or *SAT*, conferring resistance to puromycin or nourseothricin, respectively) amplified by PCR. The resulting constructs, pUC-KO-*LPG1R:PAC* (B4451) and pUC-KO-*LPG1R:SAT* (B4513) were digested with *Eco*RI and *Hin*dIII to generate linear deletion cassettes for *LPG1R*.

Previously, a 4.2-kb fragment containing the *LPG1* ORF and flanking sequences was cloned into the *Bam*HI site of vector pUC18 (pUC-*LPG1*-Bam, B2880). To make the deletion construct for *LPG1*, the first 700 bp from *LPG1* ORF was replaced by the *BSD* (confers resistance to blasticidin) marker gene. The resulting construct, pUC-KO-*LPG1:BSD* (B4573), was digested with *Bam*HI to generate a linear knockout cassette for *LPG1*.

An overexpression construct for *LPG1*, *LPG1L*, and *LPG1R* was made in three steps. First, the full-length *LPG1L* gene was cloned in the *Bgl*II site of vector pIR1PHLEO (B4054) derived from pIR1SAT (B3541; [23]) to make pIR1PHLEO-*LPG1L* (B4587). Second, the full-length *LPG1R* gene was cloned in the *Bam*HI site of pIR1PHLEO-*LPG1L* (B4589). The resulting construct, pIR1PHLEO-*LPG1L-LPG1R*, was linearized with *Nsi*I and blunt

ended, followed by ligation with the 4.2-kb *LPG1* fragment (*Bam*HI digestion of pUC-*LPG1*-Bam then blunt ended) to generate pIR1PHLEO-*LPG1-LPG1L-LPG1R* (B4789).

2.4. Genetic manipulations

Leishmania were transfected by electroporation as described [24]. Typically, 5–10 μg of DNA was used for each transfection. Transfected cells were plated on medium containing 1% noble agar and appropriate concentrations of selective drugs. To study the localization of LPG1L and LPG1R, *L. major* cells containing pX63HYG-LPG2-HA (a marker of the parasite Golgi apparatus; strain B1878 [22]) were transfected with pXG-*LPG1L-GFP* (B4400) or pXG-*LPG1R-GFP* (B4455) and selected with hygromycin and G418. The *lpg1*$^{-}$ *lpg1l*$^{-}$/*lpg1r*$^{-}$ triple knockout parasites were created using a loss-of-heterozygosity (LOH) approach [25]. Briefly, the first allele of *LPG1* in the *lpg1l*$^{-}$/*lpg1r*$^{-}$ double knockout was replaced by the *BSD* marker. After two passages in the presence of 50 μg/ml of blasticidin, cells were incubated in media containing 10 μg/ml of galactose-binding lectin ricin RCA120 (Sigma) at 10^7 cells/ml. Cells that failed to agglutinate were plated, and ~80% of the resulting colonies had lost the second allele of *LPG1*. All knockout strains were confirmed by Southern-blot analysis.

2.5. Molecular biology techniques

Southern-blot and Northern-blot analyses were performed as previously described [26]. The Southern blot shown in Fig. 9 was subsequently washed first in 2× SSPE/0.5% SDS for 15 min at room temperature, and then three times in 0.2× SSPE/0.5% SDS at 60 °C for 15 min each. RT-PCR analysis was used to map the mRNA splicing acceptor sites of *LPG1L* and *LPG1R*. Briefly, first strand synthesis was performed using gene-specific antisense primers SMB1228 (5′-GCGTGTCGCCATTTTGGGCGAG) for *LPG1L* and SMB1369 (5′-CCGCAGCGCGCAGCTTCGTC) for *LPG1R*. Reverse transcription was performed at 42 ′C with AMV reverse transcriptase (Sigma) and 2 μg of total RNA, followed by PCR amplification with the first strand primers plus the universal miniexon primer SMB936 (5′-AACGCTATATAAGTATCAGTTCTGTACTTTA) for *L. major* [27]. The resulting fragments were sequenced to map the mRNA splice acceptor sites.

2.6. Western blots and fluorescence microscopy

Log phase (1–5 × 10^6 cells/ml) cell extracts and culture supernatants were resolved by SDS/PAGE and electroblotted onto Hybond ECL nitrocellulose membranes (Amersham Biosciences). Mouse monoclonal antibody WIC79.3 [28] was used to detect LPG and PPG (1:1000 dilution). Mouse anti-*L. major* GP63 monoclonal antibody [29] was used to detect GP63 (1:1000 dilution). Mouse monoclonal anti-α-tubulin antibody (T5168, Sigma) was used to detect α-tubulin (1:10000 dilution). An enhanced chemiluminescence (ECL) detection system (Amersham) was used to detect signals. For fluorescence microscopy, log phase cells were immobilized on polylysine coated cover slips, followed by fixation with 3.5% formaldehyde in PBS. After being permeated by 100% ethanol at 4 °C, cells were incubated with dilutions of primary and secondary antibodies as described [30].

2.7. Mouse footpad infections

Parasite virulence was evaluated by mouse footpad infections. Briefly, all parasite lines were first inoculated at high levels (2 × 10^7) into BALB/c mice in order to guard against effects arising from transfection/culture passage associated loss of virulence. They were recovered after 1 month prior to emergence of pathology, and converted into promastigotes in vitro. After three passages in culture, stationary phase parasites (day 3) were resuspended in DMEM media at 2 × 10^7 cells/ml and 10^6 were injected into the footpads of 8-week-old female BABL/c mice (5–6 mice per group). Lesion sizes were measured weekly with a

Vernier caliper and parasite numbers in the infected foot pads were determined by limiting dilution assay [31].

2.8. GIPL analysis

Exponentially growing cells were metabolically labeled with [^{3}H]galactose (50 μCi for 8 × 10^8 cells) for 16 h, and [^{3}H]GIPLs were purified as described [32]. The [^{3}H]GIPLs were treated with 0.04 M trifluoroacetic acid for 1 h at 100 °C to cleave the labile galactofuranosidic bonds, and the [^{3}H-Gal]fragments were chromatographed on paper as described [33]. Other aliquots of unlabeled GIPLs were subjected to nitrous acid deamination [33] to remove the lipid anchors, tagged at the reducing end with the fluorophore 9-aminonaphthalene 1,3,6-trisulfate, and analyzed by GLYKO-FACE electrophoresis according to the manufacturer's specifications (GLYKO, Novato, CA).

2.9. LPG and glycoprotein analysis for the LPG1-LPG1L-LPG1R overexpressors

Parasites (5 × 10^6 cells/ml) were metabolically labeled for 16 h with [6-^{3}H]galactose (50 μCi) in 5 ml of M199 culture medium supplemented with 10% fetal calf serum and 1 μg/ml of biopterin. [^{3}H]LPG was solubilized by differential organic solvent extraction and removed by chromatography on phenyl-Sepharose as described [38]. The insoluble residue containing [^{3}H]glycoproteins was converted to [^{3}H]glycopeptides by digestion with Pronase as described [34] and desalted by chromatography on Sephadex G25. Maltohexose (1 μmole) was added to the protein residue prior to Pronase digestion as an internal standard and was quantitated by capillary electrophoresis using conditions: 20 °C, 20 psi, and 20 min.

3. Results

3.1. Identification of a family of six LPG1-related genes in L. major

Search for sequences with similarity to *L. major LPG1* revealed the presence of fragments from related sequences in the *L. major* (Friedlin V1 strain) genome project data, which was then incomplete. The sequence for two of these genes, designated *LPG1L* (*LPG1-like*) and *LPG1R* (*LPG1-related*), was determined. Southern-blot analysis and gene deletion studies showed that these genes occurred in a single copy (data not shown or below). As these studies were being finished, the emerging *L. major* genome sequence revealed three additional related homologous genes that were named *LPG1G*, whose predicted amino acid sequences were identical and located on chromosomes 5, 19, and 32 (LmjF05.1230, LmjF19.650, and LmjF32.3900; sequence information is available at http://www.genedb.org/genedb/leish/index.jsp). Southern-blot analysis confirmed the presence of multiple *LPG1G* genes in *L. major* strain LV39 clone 5 as well (data not shown). Interestingly, all three of the *L. major* Friedlin strain *LPG1G* genes appear to be located adjacent to telomeres. *LPG1* has been described in several *Leishmania* species previously [35,36]; preliminary analysis of data arising from shotgun sequence of *L. infantum* suggest that this species also has homologs of *LPG1L*, *LPG1R*, and at least one copy of *LPG1G*, all of which show >90% identity to the homologous gene of *L. major* (data not shown).

3.2. Properties of predicted LPG1-family proteins

The predicted open reading frames for *LPG1*, *LPG1L*, *LPG1R*, and *LPG1G* contained 434, 592, 460, and 599 amino acids, respectively. All four genes (*LPG1*, *LPG1L*, *LPG1R*, and *LPG1G*) were predicted to encode type-II transmembrane proteins with short, basic cytoplasmic tails (~20 amino acids) at the N-termini, followed by single transmembrane domains (Fig. 2A; predictions were made using the TMHMM Server at http://www.cbs.dtu.dk/services/TMHMM/). Relative to *LPG1*, there were insertions in *LPG1L* (encoding amino acid 43–161) and *LPG1G* (encoding amino acid 43–149) immediately after the transmembrane domain, that were not seen in *LPG1* or *LPG1R* (Fig. 2A and B). The

overall similarity among these four genes was modest (~20% amino acid identity; Fig. 2B). Nonetheless, the predicted lumenal domains of all four genes contained several highly conserved regions as shown in the alignment in Fig. 2B. One of these regions (G-F/Y-F/L-D-E-N-F/Y-Y/F-P-A/I-Y/L-G/Y/F/M-E/D-**D**-**H**/**T**/**Y**/**I**-**D**-Y/W/L) is likely to represent the catalytic site, since it contained the metal-binding DXD motif conserved amongst glycosyltransferases (Fig. 2A and B).

In all following studies we focused exclusively on the characterization of *LPG1L* and *LPG1R*.

3.3. Detection of LPG1L and LPG1R mRNA in L. major

To test whether *LPG1L* and *LPG1R* were expressed in *L. major*, total RNA was prepared from early log phase, late log phase, stationary phase, metacyclic phase, and amastigote stage cells and subjected to Northern-blot analysis using the coding regions of *LPG1L* and *LPG1R* as probes. The 2.8-kb *LPG1L* and 2.0-kb *LPG1R* mRNAs were expressed at similar levels throughout parasite life cycle (Fig. 3). RT-PCR analysis mapped the site of *trans*-splicing sites for *LPG1L* and *LPG1R* to positions 129 and 160 nt upstream of the ATG start codon for *LPG1L* and *LPG1R*, respectively (data not shown).

3.4. Cellular localization of LPG1L and LPG1R

Previous data showed that an *LPG1::GFP* fusion protein was localized in the Golgi apparatus in *Leishmania* [20]. To examine the cellular localization of *LPG1L* and *LPG1R* gene products, we created similar fusion constructs joining the green fluorescent protein (GFP) to the C-termini of *LPG1L* and *LPG1R*. When expressed in *Leishmania* from episomal pXG vectors, both *LPG1L::GFP* and *LPG1R::GFP* fusion proteins showed strong green fluorescence in a region close to the kinetoplast, the location of the Golgi apparatus. To confirm Golgi localization, we introduced a construct expressing a hemagglutinin (HA)-tagged *LPG2* gene, which encodes a Golgi GDP-mannose transporter [22,37]. Immunofluorescence analysis of the *LPG2::HA* showed co-localization with the *LPG1L::GFP* and *LPG1R::GFP* signals (Fig. 4). This result was further confirmed by immuno-EM study using anti-GFP antibody (data not shown). Therefore, similar to *LPG1*, the gene products of *LPG1L* and *LPG1R* were also localized at the Golgi apparatus, consistent with the cellular location of most glycosyltransferases.

3.5. Generation of double and triple LPG1, LPG1L and LPG1R knockouts

Because *Leishmania* has a diploid genome, two rounds of replacement are required to inactivate any given gene. This presents an obstacle in genetically assessing the function of all six members of the *LPG1* gene family, as currently just six suitable markers exist. Here we studied the effects of inactivation of *LPG1L* and *LPG1R*, singly, together, or in combination with *LPG1*. An outline of the gene inactivation protocol is shown in Fig. 5A. Briefly, *LPG1L* alleles were replaced by *HYG* and *NEO* markers (as *lpg1l*$^-$), while *LPG1R* alleles were replaced by *PAC* and *SAT* markers (as *lpg1r*$^-$), and all four alleles were replaced in the double *lpg1r*$^-$/*lpg1l*$^-$ mutant. The triple knockout parasites (*lpg1*$^-$/*lpg1l*$^-$/*lpg1r*$^-$) were generated through a LOH approach [25], by first replacing one *LPG1* allele of the *lpg1l*$^-$/*lpg1r*$^-$ mutant with the *BSD* marker, then selecting for the loss of LPG. In all lines, Southern-blot analysis confirmed that the planned replacements had occurred; data for the *lpg1*$^-$/*lpg1l*$^-$/*lpg1r*$^-$ triple knockout parasite is shown in Fig. 5B.

The single, double and triple knockout parasites grew at similar rates as *L. major* wild-type cells in culture (data not shown). Western-blot analysis showed that all lines tested synthesized wild-type levels of PPG and GP63, and that the *lpg1l*$^-$, *lpg1r*$^-$, and *lpg1l*$^-$/*lpg1r*$^-$ mutants synthesized WT levels of LPG (Fig. 6 and data not shown). These data

indicated that *LPG1L* and *LPG1R* were not involved in the synthesis of large surface glycoconjugates such as LPG, PPG, or the GPI-anchored protein GP63. Additionally, expression of *LPG1L* and *LPG1R* from episomal vectors did not restore the synthesis of LPG in the *lpg1*$^-$ mutant, arguing that *LPG1L* and *LPG1R* could not substitute for the function of *LPG1* even when overexpressed (data not shown).

3.6. Deletion of LPG1L and/or LPG1R did not affect the biosynthesis of GIPLs

The effect of *LPG1*-family mutants on GIPL synthesis was evaluated in two ways. First, total GIPL fractions from wild type and mutant cells were extracted, dilipidated, fluorophore-labeled, and analyzed by FACE (fluorophore-assisted carbohydrate electrophoresis). GIPLs from single (*lpg1l*$^-$, *lpg1r*$^-$) or double (*lpg1l*$^-$/*lpg1r*$^-$) knockouts showed a pattern similar to the GIPL pattern seen for wild type (Fig. 7A and data not shown). In contrast, the GIPL pattern for the triple knockout (*lpg1*$^-$/*lpg1l*$^-$/*lpg1r*$^-$) containing the deletion of *LPG1* showed a pattern similar to the *lpg1*$^-$ knockout alone (Fig. 7A). Accumulation of the expected LPG biosynthetic intermediate (Glc-P-Man_2-GlcN) was observed in all lines bearing the homozygous *lpg1*$^-$ deletions. Second, cells were metabolically labeled with [^{3}H]galactose and total GIPLs were extracted and treated with trifluoroacetic acid to cleave the Gal_f-Man linkage in GIPLs with the structure $Gal_{0–2}$-Gal_f-Man-Man-GlcN. After separation by paper chromatography, similar profiles were seen in WT and all mutant lines (Fig. 7B and data not shown). Therefore, it is unlikely that any of genes (*LPG1*, *LPG1L*, and *LPG1R*) were involved in the biosynthesis of the Gal_f-containing type-II GIPLs in *L. major*.

3.7. LPG1L and LPG1R were not required for metacyclogenesis nor mouse infectivity

The percent metacyclic promastigotes (infectious form) present in stationary phase cultures showed very little difference amongst the wild type and various knockout cells (data not shown). To test if *LPG1L* and/or *LPG1R* were implicated in virulence, stationary phase parasites were inoculated in susceptible BABL/c mice. There was no significant difference in lesion formation between wild type and various knockout cells (*lpg1l*$^-$, *lpg1r*$^-$, and *lpg1l*$^-$/*lpg1r*$^-$), arguing that *LPG1L* and *LPG1R* were not required for virulence in the mammalian host (Fig. 8).

3.8. Potential role of LPG1L and LPG1R in adding Gal_f to glycoproteins

Since neither *LPG1L* nor *LPG1R* appeared to function in GIPL biosynthesis, we asked whether these genes might participate in other glycoconjugate synthetic pathways. Gal_f residues are not synthesized by the mammalian hosts, but are found in glycoconjugates synthesized by a number of pathogens including *Mycobacterium*, *T. cruzi*, and *Aspergillus* [38–40]. In *Leishmania*, Gal_f residues have only been found in glycolipids [41]. In contrast, other Kinetoplastid species such as *T. cruzi*, *Crithidia fasculata*, *Leptomonas samueli*, and *Endotrypanum schaudinni* contain abundant amounts of Gal_f residues in their glycoproteins, often β1, 3-linked to mannose as in the LPG and GIPLs in *L. major* [38,42–44]. Southern-blot analysis was performed on the genomic DNA from the species mentioned above using the coding regions of *LPG1*, *LPG1L*, and *LPG1R* from *L. major* as probes. After several washes at moderate stringy (Section 2), strong signals were seen for several of these *LPG1* family members in all species (Fig. 9). Additionally, *LPG1-related* genes have been identified in the emerging genome sequence of *T. cruzi* (Fig. 2).

Since Gal_f had never been observed previously in *Leishmania* proteins, we undertook an overexpression approach to determine whether *LPG1* and its homologues (*LPG1L* and *LPG1R*) had any effect on protein galactosylation. In these studies, we generated a construct which simultaneously expressed *LPG1*, *LPG1L*, and *LPG1R* and integrated it into the ribosomal RNA locus of *L. major*, a position which gives high levels of expression [23]. WT

and transfectants were metabolically labeled with [^{3}H]galactose, and incorporation of the radiolabel into glycoproteins was measured. The results showed a small but reproducible increase in protein galactosylation (Fig. 10A). Efforts to assign this activity to either *LPG1*, *LPG1R*, or *LPG1L* in single gene overexpressors were inconclusive, possibly due to the low level of incorporation.

Unlike several other protozoans, the presence of Gal$_f$ in N-linked carbohydrate chains in *L. mexicana* has been investigated and was not detected [45,46], possibly due to the low abundance of the monosaccharide. To examine whether *L. major* might possess Gal$_f$-containing proteins, [^{3}H-Gal]glycopeptides were prepared by Pronase digestion of glycoproteins from wild-type cells and aliquots were incubated in the presence or absence of exo-β-galactofuranosidase [47], desalted by anion exchange that removes most of the glycopeptides, and analyzed by paper chromatography. No radioactive material that co-migrated with standard galactose was present in the chromatogram from the untreated [^{3}H]glycopeptides (Fig. 10B, upper panel) whereas galactofuranosidase treatment to the release of low but measurable levels of [^{3}H]galactose (Fig. 10B, lower panel), indicating the presence of terminal Gal$_f$ in *Leishmania* glycopeptides. The large majority of radiolabeled glycopeptides was not susceptible to exo-β-galactofuranosidase digestion, which could be due to carbohydrate chains with internal Gal$_f$ residues and/or could be glycopeptides containing galactopyranose. Efforts to further characterize [^{3}H]glycopeptides using treatment with dilute trifluoracetic acid to release Gal$_f$-containing fragments were inconclusive, possibly because of the low level of incorporation.

4. Discussion

In this study we described the *LPG1* gene family of *L. major*, and reported detailed characterization of two of the genes, *LPG1L* and *LPG1R*. The five new genes predict proteins showing strong similarity to the active LPG-specific Gal$_f$T encoded by *LPG1*, in being type II transmembrane glycoproteins with a short N-terminal cytoplasmic domain and a lumenal C-terminal domain bearing a canonical glycosyltransferases DXD motif (Fig. 2A). Both *LPG1R* and *LPG1L* are constitutively expressed in stable mRNA throughout the parasite life cycle (Fig. 3). Furthermore, episomally expressed LPG1L and LPG1R-GFP fusion proteins localized to the parasite Golgi apparatus (Fig. 4). Thus, these genes were reasonable candidates to be the GIPL-specific Gal$_f$ transferase postulated previously [9], and their modest degree of overall sequence conservation (~20% identity) provides the requisite opportunity for divergence in Gal$_f$T acceptor specificity.

Due to the late emergence of the three *LPG1G* gene sequences and the availability of only six selectable genetic markers, we focused functional studies on the *LPG1*, *LPG1L*, and *LPG1R* genes. Single and double mutants of *LPG1L* and *LPG1R* were made, and a mutant lacking all three was also constructed, the first such triple knockout mutant reported in *Leishmania* (Fig. 5). These studies showed collectively that *LPG1L* and *LPG1R* had no effect on parasite growth, differentiation or the ability to induce disease in animals (Fig. 8). Analysis of glycoconjugate synthesis showed similarly that loss of *LPG1L* and *LPG1R* had no effect on the synthesis of the abundant glycoconjugates GP63, PPG, or LPG (Fig. 6). Lastly, while the triple mutant showed the expected adverse effect on LPG synthesis due to deletion of the *LPG1* gene, there was no effect on GIPL biosynthesis (Fig. 7). These data suggest that none of these three genes are essential for the GIPL-specific Gal$_f$T. Obviously, the proposal that the *LPG1G* genes encode this activity is attractive, but the possibility that a completely unrelated gene encodes this activity cannot be excluded. It is interesting that *L. infantum* appears to contain an *LPG1G* homolog; while the GIPL composition of this species isolate has not been investigated, its close relative *L. donovani* lacks Gal$_f$-containing GIPLs [11]. While we were unable to test the effect of *LPG1G* knockouts in these studies,

our repertoire of six selectable markers will be sufficient to permit the eventual inactivation of all three *LPG1G* genes simultaneously in the future.

In *L. major* Gal_f residues are present almost exclusively in glycolipids such as LPG and GIPLs, whereas in other trypanosomatid species other glycoconjugates contain Gal_f similarly linked to Man [38,42–44]. This suggested that perhaps one or more of the *LPG1* family members participate in Gal_fT reactions involving other acceptors including the carbohydrate chains of proteins. Although Parodi and coworkers reported that *L. mexicana* lacks Gal_f-containing glycoproteins [45,46], a low abundance of this substituent was not excluded. Consistent with the possibility of Gal_f in *Leishmania* glycoproteins, several species known to express Gal_f-containing proteins possess genes homologous to *LPG1*, *LPG1L*, and *LPG1R* (Fig. 9), and the *T. cruzi* genome project also has revealed a family of *LPG1*-homologs (http://www.tigr.org/tdb/e2k1/tca1/). Significantly, simultaneous overexpression of *LPG1*, *LPG1R*, and *LPG1L* resulted in increased incorporation of galactose into *L. major* proteins (Fig. 10), at least some of which was in the form of terminal Gal_f. Although we were unable to assign this function to a single gene, it is likely that one or more of the *LPG1*-family genes encode proteins with protein-glycoconjugate Gal_fT activity. This would be especially relevant to studies of *T. cruzi*, where the abundant mucins are highly modified by Gal_f and are thought to contribute to parasite virulence [48,49].

One scenario consistent with our data is that *Leishmania LPG1L* and *LPG1R* may be cryptic, relatively “silent counterparts” of genes highly active in other trypanosomatids. This further suggests that the potential genomic glyco-synthetic repertoire of these parasites may be larger than revealed by biochemical assays, which typically focus on the most abundant molecules or subsets thereof. A similar finding was reached in studies of the *SCG* family of LPG side chain galactosyltransferases, as only a subset of the seven genes of this family have shown significant GalT activity [6]. The presence of a number of silent but potentially active glycosyltransferases genes is intriguing and raises a number of speculative possibilities. Potentially, these genes represent a source for the emergence of novel or modified glycoconjugate synthesis during evolution, which may contribute in any number of ways to parasite virulence and host range. In protozoa such as *Plasmodium* or *Trypanosoma* variant surface proteins represent an interesting analogy, as gene families in these organisms comprise a group of cryptic genes that are expressed in various ways amongst strains and species and contribute to pathology [50–52]. In these species many such genes are located at telomeres and it is interesting to note that the three *LPG1G* genes appear to be located at telomeres in *L. major* and *L. infantum* (unpublished data; *Leishmania* Genome Project). Presumably during the evolutionary divergence of trypanosomatids, different lineages have chosen to emphasize the synthesis of particular glycoconjugates relevant to their biological niche within the mammalian and insect hosts. This raises the possibility that this cryptic glyco-synthetic capability may be reactivated and expressed at higher levels in the future, with consequences best understood at present by the parasite.

Acknowledgments

We thank A. Capul, D. Dobson, and K. Robinson for comments on the manuscript, M. Cunningham for pIR1PHLEO, W. Beatty for immuno-EM studies, everyone in our laboratories for helpful discussions, the *Leishmania* and *T. cruzi* Genome Projects for generously making available preliminary partial sequence of *LPG1L*, *LPG1R*, and the sequences *LPG1G*, and Maria Julia Manso Alves for the gift of exo-β-galactofuranosidase. This work was supported by NIH grant NIH AI 31078. Sequencing of the *L. major* genome was accomplished as part of the *Leishmania* Genome Network with support by The Wellcome Trust. Sequencing of *T. cruzi* was accomplished by TIGR with support from the NIAID.

Abbreviations

LPG	lipophosphoglycan
GIPL	glycosylinositolphospholipids
PPG	proteophosphoglycan
Gal_f	galactosylfuranose
Gal_f *T*	galactofurranosyl transferase
GFP	green fluorescent protein
NEO	G418 resistance marker
SAT	nourseothricin resistance marker
PAC	puromycin resistance marker
BSD	blasticidin resistance marker
HYG	hygromycin resistance marker
LOH	loss of heterozygosity

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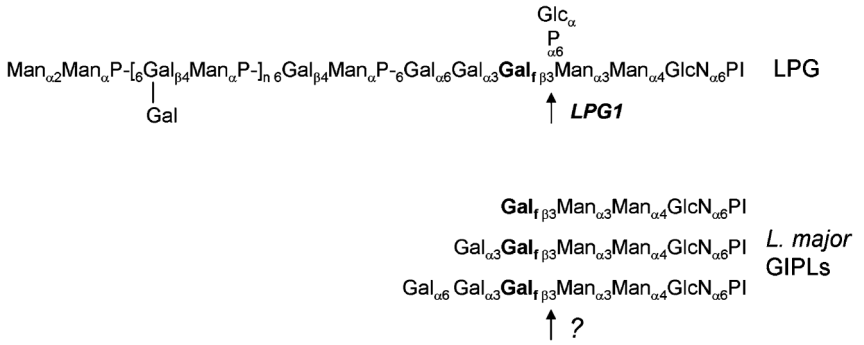

Fig. 1.

Structures of LPG and GIPLs in *L. major*. Abbreviations: Gal, galactose; Gal$_f$, galactosylfuranose; Man, mannose; Glc, glucose; GlcN, glucosamine; P, phosphate; PI, phosphatidylinositol anchor.

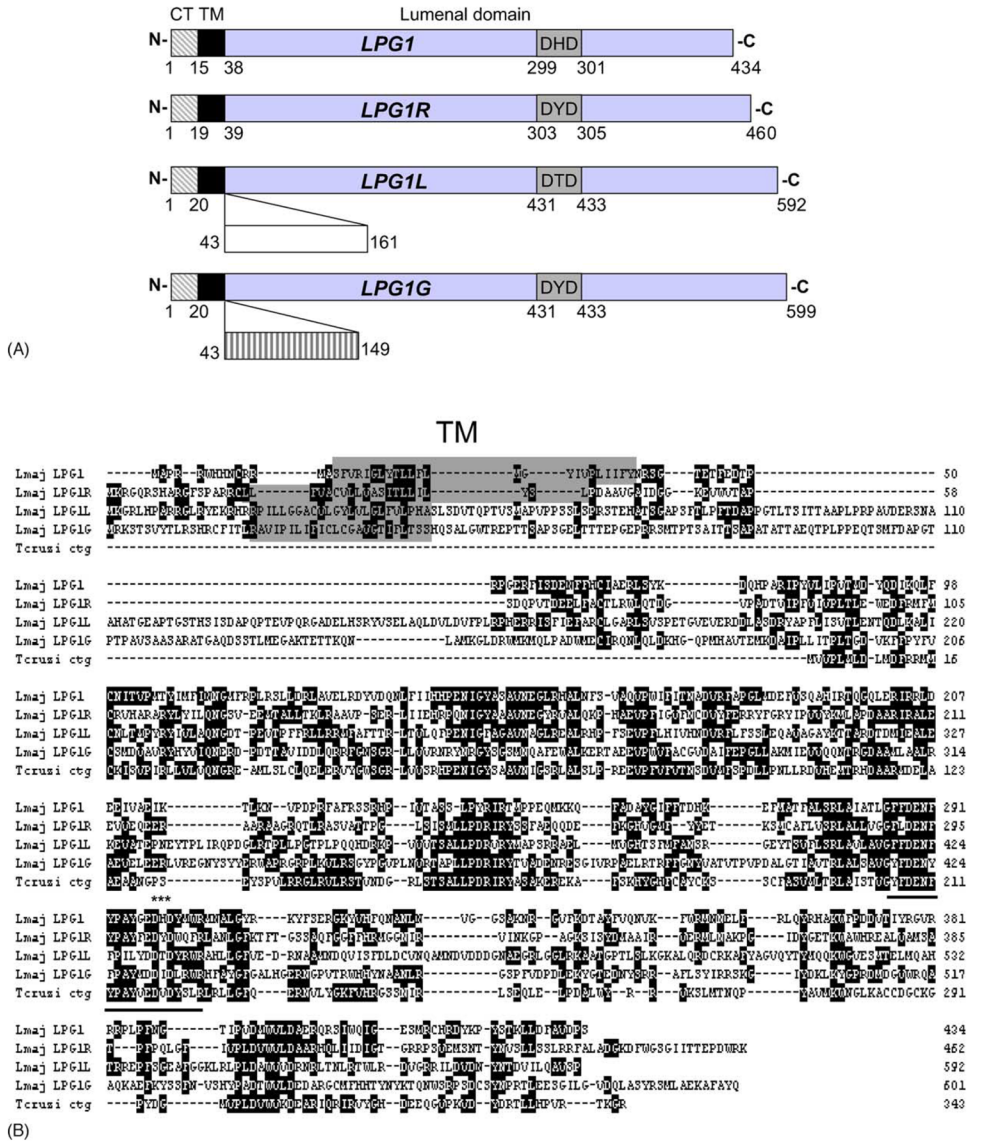

Fig. 2.

LPG1 gene family. (A) Schematic representations of *LPG1*, *LPG1L*, *LPG1R*, and *LPG1G*. Numbers indicate amino acids. Positions of the conserved DXD catalytic motifs are indicated. CT, cytoplasmic tail; TM, transmembrane domain. Compared to *LPG1* and *LPG1R*, *LPG1L* and *LPG1G* contain additional "inserts" (from amino acid 43–161 for *LPG1L* and 43–149 for *LPG1G*) after the transmembrane domain, as indicated. Our analysis indicates that the three copies of LPG1G on chromosomes 5, 19, and 32 are identical. (B) Alignment of *LPG1*, *LPG1L*, *LPG1R*, and *LPG1G* from *L. major* (~20% identity among these genes at amino acid level) and a preliminary partial open reading frame (contig) from *T. cruzi* (*T. cruzi* ctg). Alignments were performed using the ClustaIW algorithm as implemented in the program Megalign (DNA Star). Transmembrane domains were highlighted. The underlined region contains the DXD catalytic motif (marked by asterisks).

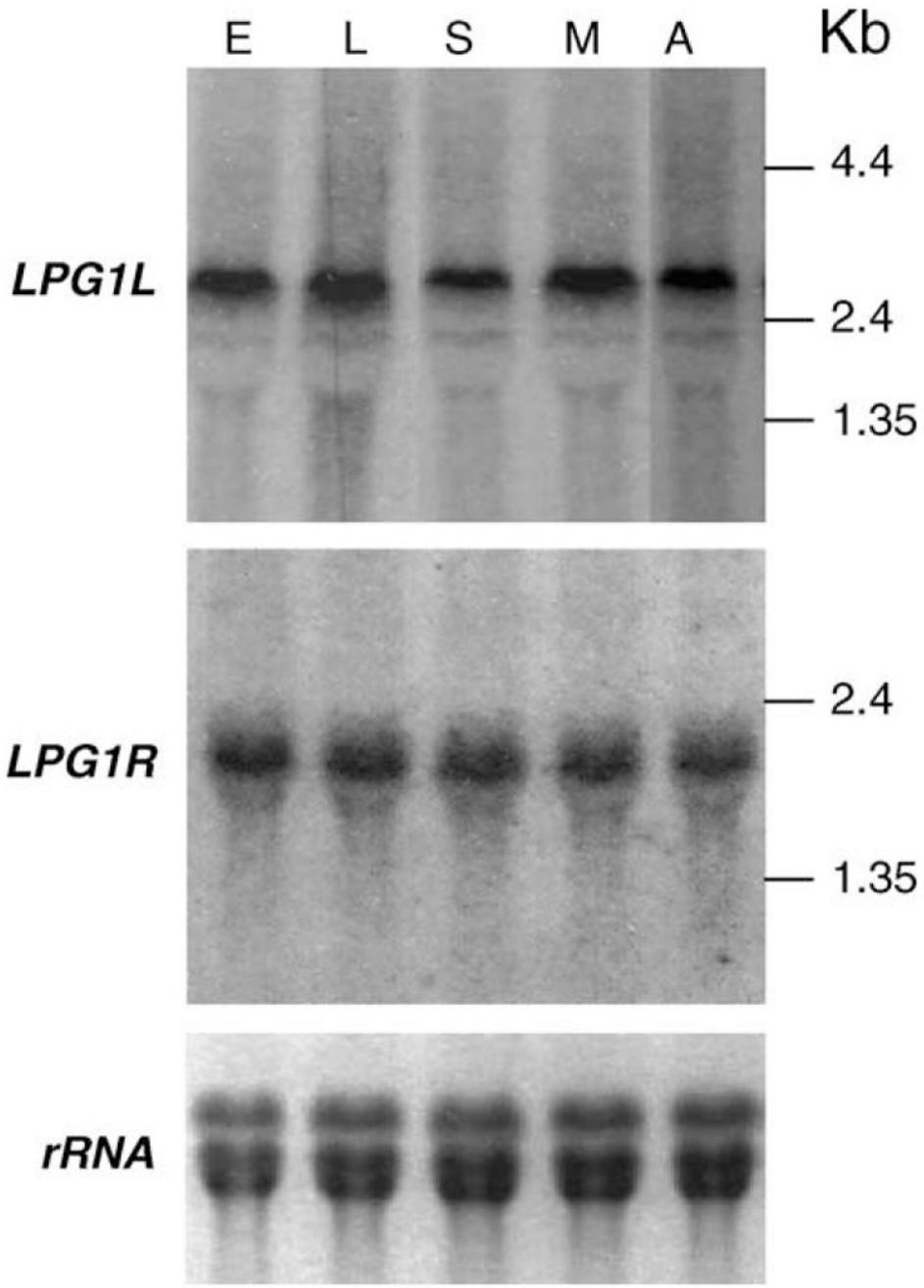

Fig. 3.

LPG1L and *LPG1R* are expressed throughout *L. major* life cycle. Northern-blot analysis was performed as described in Section 2. Probes were made from coding regions of *LPG1L* and *LPG1R*. E, early log phase; L, late log phase; S, stationary phase; M, metacyclic phase (prepared by negative selection for binding with peanut agglutinin; [53]); A, amastigotes (from infected mouse foot pads). Expression of rRNA was used as controls for loading.

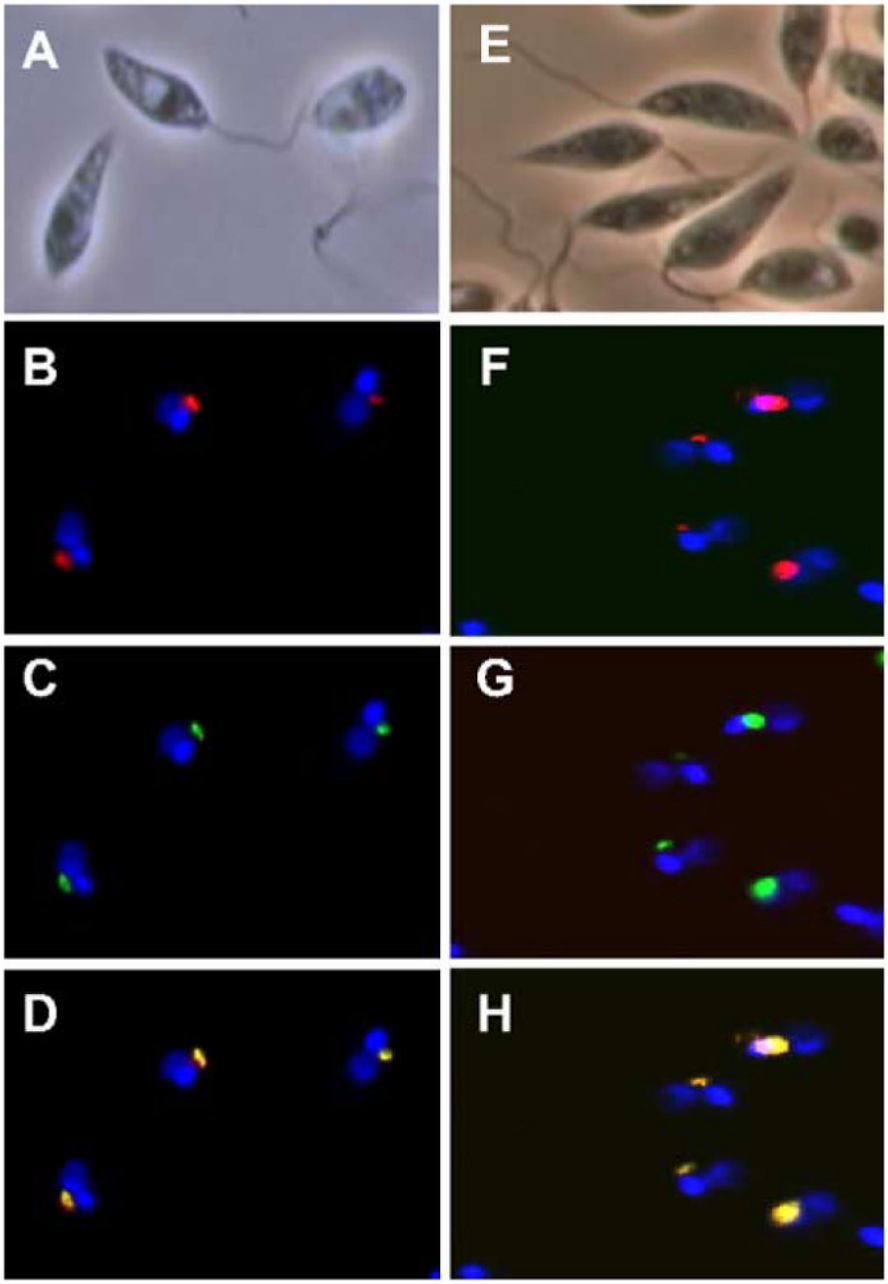

Fig. 4.

LPG1L and LPG1R are localized at the Golgi apparatus in *L. major*. LV39 WT cells were cotransfected with pX63HYG-*LPG2-HA* (Golgi marker) and pXG-*LPG1L-GFP* (A–D) or pXG-*LPG1R-GFP* (E–H). Localizations of LPG1L and LPG1R were determined by GFP fluorescence (C and G, respectively). The golgi apparatus was revealed by staining cells with rabbit anti-HA polyclonal antibody, followed by Texas Red-labeled mouse anti-rabbit monoclonal antibody (B and F). Hoechst 33258 dye was used to stain DNA (both nuclear and kinetoplast). D: merge of B and C; H: merge of F and G.

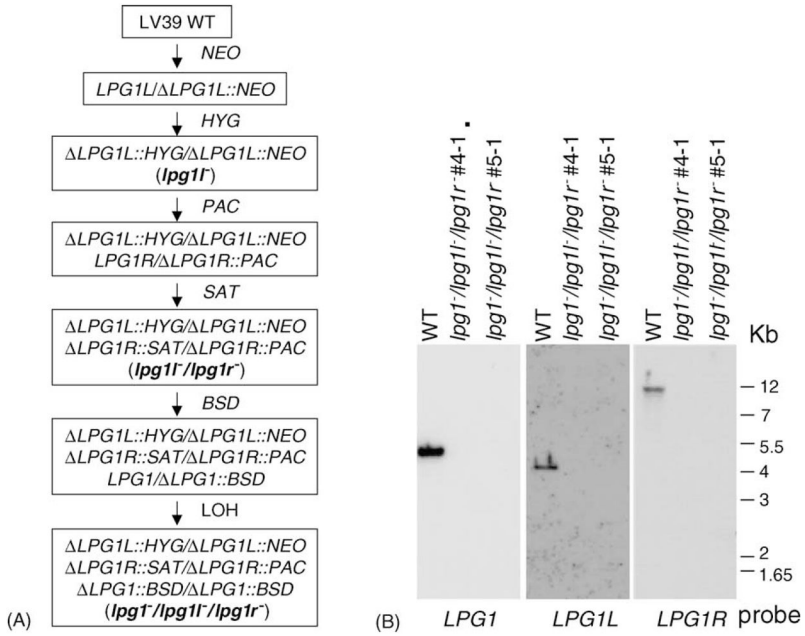

Fig. 5.

Deletions of *LPG1L*, *LPG1R*, and *LPG1* in *L. major*. (A) Procedure to generate the *lpg1*⁻/*lpg1l*⁻/*lpg1r*⁻ triple knockout parasites. (B) Southern-blot analysis to confirm the *lpg1*⁻/*lpg1l*⁻/*lpg1r*⁻ triple knockout parasites (nos. 4-1 and 5-1 are two independent clones). Genomic DNA was digested with *Hin*dIII and hybridized with probes made from the coding regions of *LPG1*, *LPG1L*, and *LPG1R*.

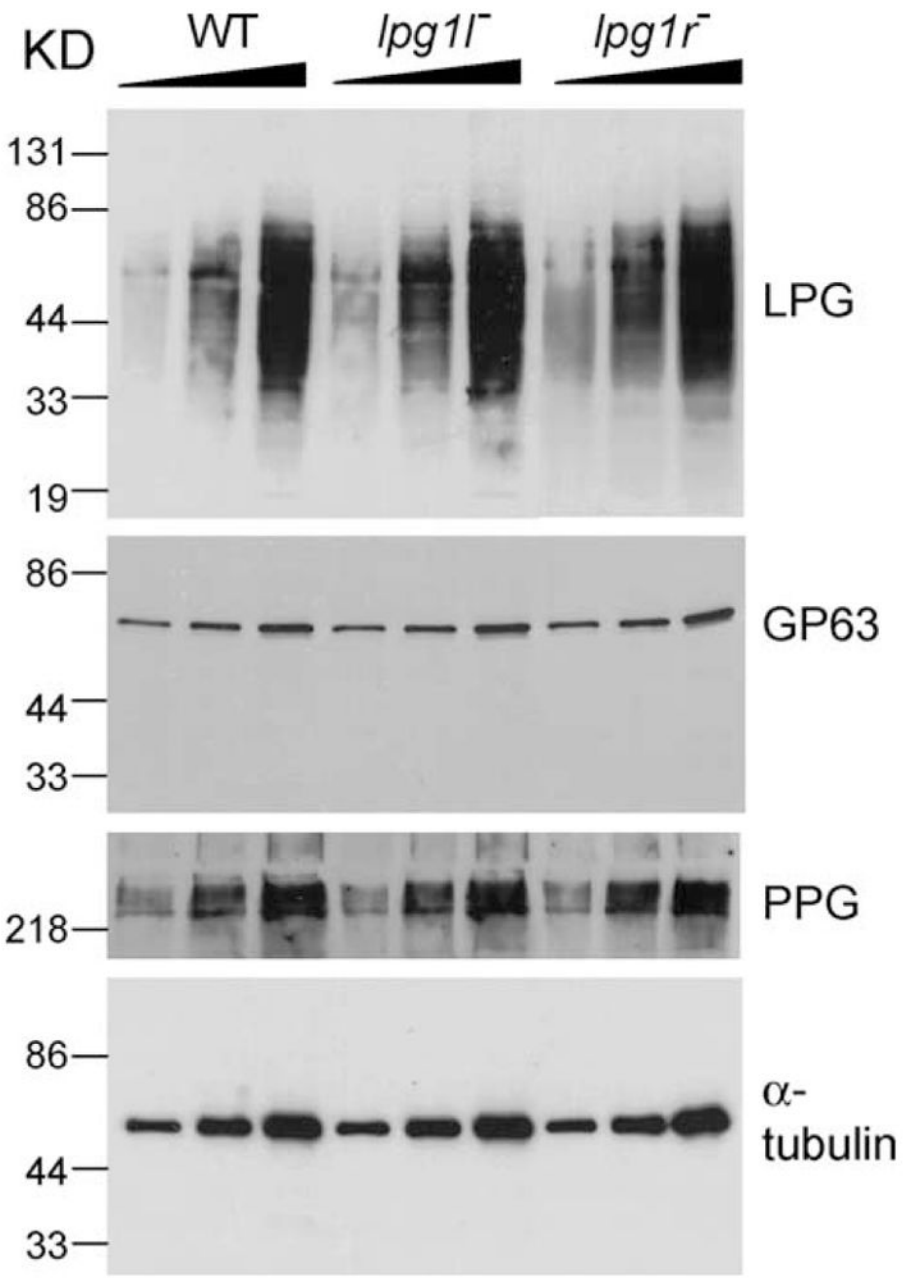

Fig. 6.

LPG1L and *LPG1R* are not required for the synthesis of LPG, PPG, or GPI-anchored proteins. Western blots of cell extracts (for LPG, GP63, and α-tubulin) and culture supernatants (for PPG) were performed as described in Section 2. For each cell type (WT, *lpg1l*⁻, and *lpg1r*⁻), materials from 2.5×10^5, 5×10^5, and 10^6 cells (from left to right) were loaded.

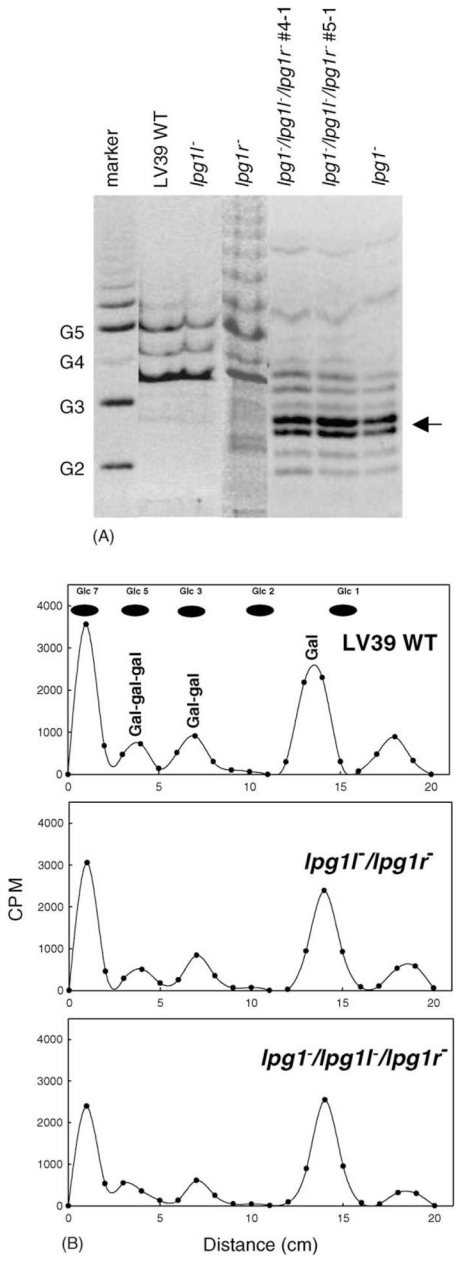

Fig. 7.

Triple knockout cells (*lpg1*¯/*lpg1l*¯/*lpg1r*¯) still synthesize Gal*f*-containing GIPLs. (A). Total GIPLs from LV39 WT, *lpg1*¯, *lpg1l*¯, *lpg1r*¯, and *lpg1*¯/*lpg1l*¯/*lpg1r*¯ cells were extracted and analyzed by a GLYCO-FACE gel electrophoresis as described in Section 2. The arrow indicates material derived from truncated form of LPG (GlcP-Man_2-GlcN-PI), which is minimal in LV39 WT cells. The marker (G2–G5) contains oligomeric derivatives of glucose. (B) WT (top panel), *lpg1l*¯/*lpg1r*¯ (middle panel), and *lpg1*¯/*lpg1l*¯/*lpg1r*¯ (lower panel) cells were metabolically labeled with [^{3}H]galactose and [^{3}H]GIPLs were purified. The [^{3}H]GIPLs were treated with TFA to cleave galactofuranosidic bonds and the fragment were separated by paper chromatography. Migration of unlabeled glucose standards is indicated.

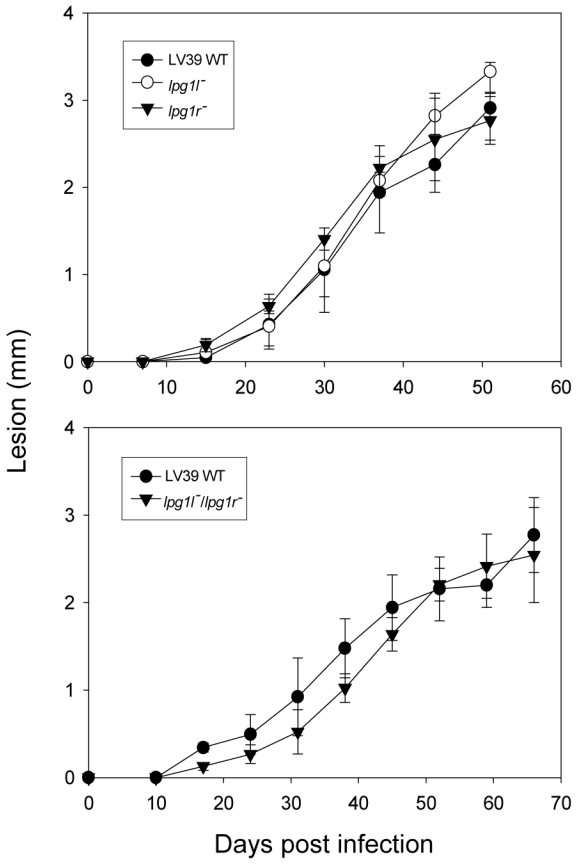

Fig. 8.

LPG1L and *LPG1R* are not required for *L. major* virulence. Mouse footpad infections were performed as described in Section 2. Stationary (WT, *lpg1*⁻, *lpg1r*⁻, and *lpg1l*⁻/*lpg1r*⁻) cells (10^6) were injected into each mouse. Five to six mice were used in each experiment. Error bars represent standard deviations.

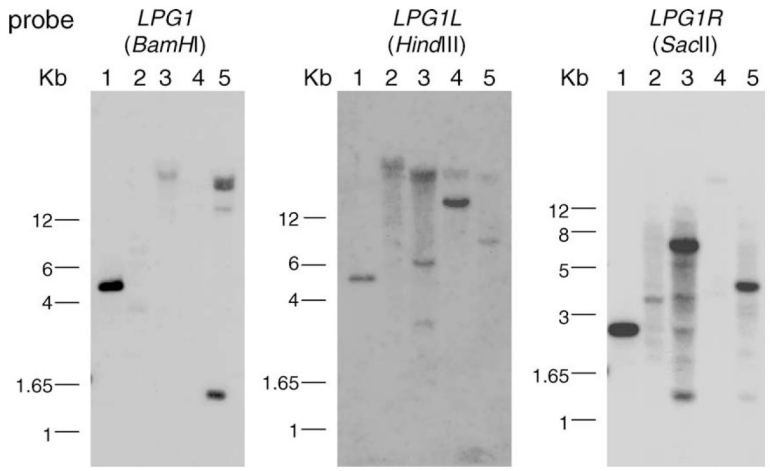

Fig. 9.

Homologues of *LPG1L* and *LPG1R* exist in other Kinetoplastida parasites. Genomic DNAs from *L. major* LV39 (lane 1), *Trypanosoma cruzi* (lane 2), *Crithidia fasculata* (lane 3), *Leptomonas samuli* (lane 4), and *Endotypanum schaudinni* (lane 5) were digested with restriction enzymes and hybridized with probes corresponding to the coding regions of *LPG1*, *LPG1L*, and *LPG1R*. The blots were washed under 'moderately' stringent conditions as described in the Section 2 (0.2× SSPE/0.5% SDS at 60 °C).

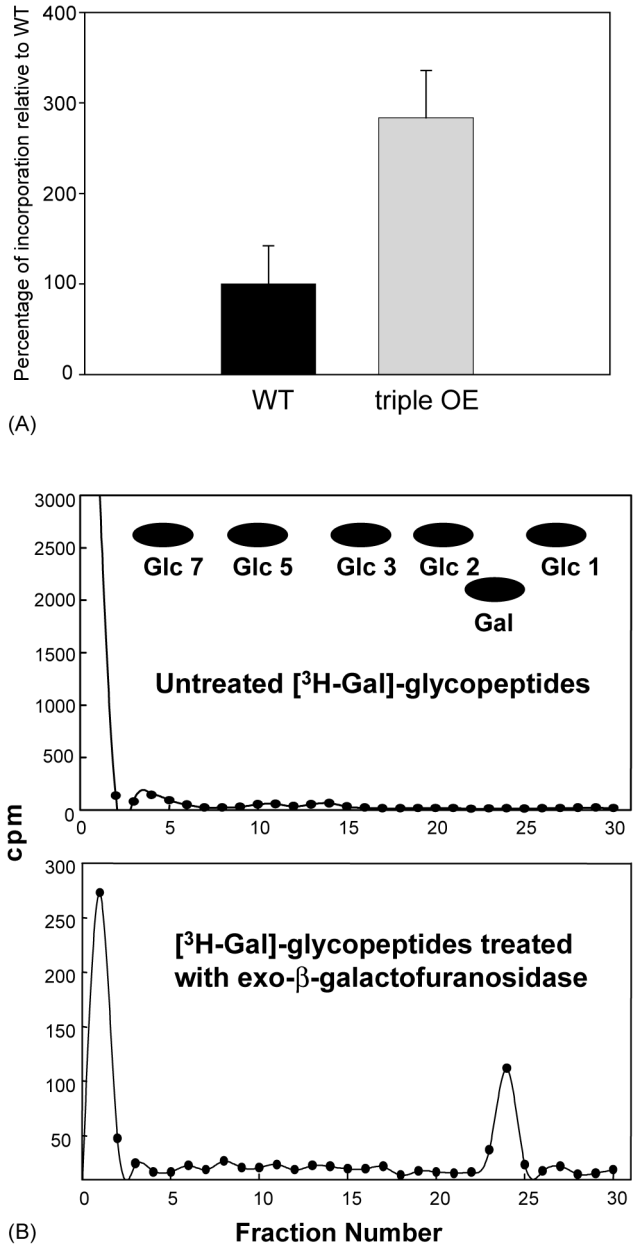

Fig. 10.

(A) Overexpression of *LPG1*, *LPG1L*, and *LPG1R* caused increased incorporation of [^{3}H]galactose in glycoproteins. LV39 WT cells and cells overexpressing *LPG1*, *LPG1L*, and *LPG1R* (triple OE: triple overexpressor) were metabolically labeled with [^{3}H]galactose and glycopeptides were extracted as described in Section 2. Radioactivity in these glycoproteins was determined using a scintillation counter; several experiments were done and the results of one with three replicas is shown; error bars represent standard deviation. (B) Demonstration of terminal Gal$_f$ residues in *L. major* glycoproteins. [^{3}H-Gal]Glycoproteins from LV39 WT cells were digested with pronase and desalted [25], and 10,000 cpm were incubated in the presence or absence of exo-β-galactofuranosidase [47]. After 24 h of incubation at 37 °C, the samples were applied to a column (2 ml) of Dowex AG1-X8 (acetate) over Dowex AG50-X8 (hydrogen) and the eluent was subjected to descending

chromatography on paper using the solvent *n*-butanol:pyridine:water (6:4:3). Radioactivity was measured and plotted for samples without (upper panel) or with galactofuroanosidase (lower panel) treatment, along with migration of glucose oligomeric standards.