

# Targeted Gene Deletion of *Leishmania major* UDP-galactopyranose Mutase Leads to Attenuated Virulence\*

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Considering the high incidence of galactofuranose (Gal<sub>f</sub>) in pathogens and its absence from higher eukaryotes, the enzymes involved in the biosynthesis of this unusual monosaccharide appear as attractive drug targets. However, although the importance of Gal<sub>f</sub> in bacterial survival or pathogenesis is established, its role in eukaryotic pathogens is still undefined. Recently, we reported the identification and characterization of the first eukaryotic UDP-galactopyranose mutases. This enzyme holds a central role in Gal<sub>f</sub> metabolism by providing UDP-Gal<sub>f</sub> to all galactofuranosyltransferases. In this work, the therapeutic potential of Gal<sub>f</sub> metabolism in *Leishmania major* was hence evaluated by targeted replacement of the *GLF* gene encoding UDP-galactopyranose mutase. In *L. major*, Gal<sub>f</sub> is present in the membrane anchor of the lipophosphoglycan (LPG) and in glycoinositolphospholipids. Accordingly, the generated *glf*<sup>-</sup> mutant is deficient in LPG backbone and expresses truncated glycoinositolphospholipids. These structural changes do not influence the *in vitro* growth of the parasite but lead to an attenuation of virulence comparable with that observed with a mutant exclusively deficient in LPG.

Protozoan parasites of the genus *Leishmania* are the etiologic agents of leishmaniasis, a widespread group of diseases that affect millions of people. These diseases encountered in tropical and subtropical areas of the world range from self-healing cutaneous leishmaniasis to lethal visceral leishmaniasis. Throughout the world, pentavalent antimonials have been the treatment of choice for more than 50 years. However, increasing drug resistance as well as the high cost and toxicity of these drugs considerably limit their use. Nowadays, other medications such as amphotericin B, liposomal amphotericin B, pentamidine, or miltefosine offer an alternative for treatment, but like antimonials they are toxic and/or expensive. In addition, the emergence of resistant strains, which already seriously compromise the efficacy of pentamidine, is also expected with the latest antileishmanial drug miltefosine because of its long half-life (1). Efforts to develop new effective treatments have thus to be pursued.

The promastigote form of *Leishmania* parasites transmitted to human and other mammalian reservoirs by a sandfly bite is coated by molecules of the glycosylphosphatidylinositol (GPI)<sup>2</sup> family comprising lipophosphoglycan (LPG), GPI-anchored proteins such as membrane proteophosphoglycans (mPPG) or the metalloprotease gp63 and a heterogeneous group of glycoinositolphospholipids (GIPLs) (2). Once in the mammalian host, the promastigotes enter hematopoietic cells of the monocyte/macrophage lineage by phagocytosis (3) and differentiate into amastigotes responsible for disease propagation. This latter form is covered by a thinner glycocalyx mainly composed of GIPLs and glycosphingolipids acquired from the host (4, 5) because both LPG and GPI-anchored proteins are strongly down-regulated at this stage.

As the major macromolecule present on the promastigote, LPG has received much attention and its contribution to *Leishmania* pathogenesis has been critically defined. LPG is essential for the binding and detachment of parasites to the midgut of the insect vector and therefore for the transmission of the parasites to the mammalian host (6). It also protects the parasite from hydrolytic enzymes, oxidants, and human complement (7). LPG is hence crucial for *Leishmania major* virulence as demonstrated using a mutant exclusively deficient in LPG obtained by targeted gene replacement of the putative galactofuranosyltransferase *LPGI* involved in the anchor synthesis (8). Interestingly, LPG and some other related glycoconjugates are dispensable for *L. mexicana* virulence that seems to have evolved different mechanisms of host cell manipulation (9–13).

In contrast, the role of GIPLs is still controversial. GIPLs are the predominant glycoconjugates of the intracellular amastigote stage and might thus play important roles in macrophage invasion and parasite survival within phagocytes. Although several studies support such roles (14–18), the decisive contribution of GIPLs in these processes was recently called into question by a *L. major* mutant deficient in all ether lipids including LPG and GIPLs (19).

Interestingly, LPG membrane anchor and GIPLs of *L. major* are structurally related molecules that contain notably a galactofuranose residue (Gal<sub>f</sub>) (2, 20). This uncommon monosaccharide is highly immunogenic and present in the sur-

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<sup>2</sup> The abbreviations used are: GPI, glycosylphosphatidylinositol; PI, phosphatidylinositol; Gal<sub>f</sub>, galactofuranose; Gal<sub>p</sub>, galactopyranose; GIPL, glycoinositolphospholipid; LPG, lipophosphoglycan; PPG, proteophosphoglycan; UGM, UDP-galactopyranose mutase; SAP, secreted acid phosphatase; GFP, green fluorescent protein; PBS, phosphate-buffered saline.

face glycoconjugates of many pathogenic bacteria, fungi, and protozoan parasites (21). It originates from the action of the UDP-galactopyranose mutase (UGM) that catalyzes the interconversion of UDP-galactopyranose (UDP-Gal<sub>p</sub>) into UDP-galactofuranose (UDP-Gal<sub>f</sub>) (22–26). Because Gal<sub>f</sub> is essential for the survival or virulence of various pathogenic bacteria (27) but is absent from higher eukaryotes, UGM is a recognized drug target and has been extensively studied in prokaryotes (28–33). Recently, we and others identified and characterized the first eukaryotic UGMs from the human pathogens *L. major*, *Trypanosoma cruzi*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* (22, 23). Interestingly, the phylogenetic distribution of the *GLF* gene (encoding UGM) suggests that Gal<sub>f</sub> is more widespread in lower eukaryotes than previously thought. Nevertheless its role in lower eukaryotes is still undefined.

In this study we address the role of Gal<sub>f</sub> in *L. major* pathogenesis by targeted replacement of *GLF*. Absence of UGM is expected to affect not only the synthesis of the virulence factor LPG but also that of GPIs. Moreover, the *L. major* genome encodes at least six putative galactofuranosyltransferases homologous to LPG1 whose functions are still undefined (34). Therefore, targeting a central enzyme of Gal<sub>f</sub> metabolism such as UGM rather than individual galactofuranosyltransferases could have a more pronounced effect on *Leishmania* pathogenesis.

## EXPERIMENTAL PROCEDURES

**Materials**—Vectors pXG(NEO) (B1288), pXG-GFP+2 (B2952), pXG-GFP+ (B2799), and pXG-LmexSAP1 (B3092) were kind gifts from Stephen M. Beverley (St. Louis, MO). Antibody gp63–235 (35) was generously provided by W. Robert McMaster (Vancouver, Canada).

**Parasite Culture**—Promastigotes of *L. major* MHOM/SU/73/5ASKH were grown at 27 °C in M199 medium supplemented with 10% fetal bovine serum, 40 mM Hepes, pH 7.5, 0.1 mM adenine, 0.0005% hemin, 0.0002% biotin, and 50 units/ml penicillin. As needed, G418, hygromycin B, and phleomycin were added at 30, 50, and 5 μg/ml, respectively. Selection of individual clones after transfection was done on semi-solid M199 medium containing 1% agar.

**Targeted Gene Replacement of *L. major* *GLF***—A 1.5-kb region directly upstream of the start codon of *GLF* and a 1.5-kb region directly downstream of the stop codon were amplified by PCR from *L. major* genomic DNA using the primer pairs CAAACTTCTTCGAGGCTATCAAG, GTCTGGATCCATGATGGATTTGCTGCGTGTG and CAAGTCTAGAGGCCAAGGTCGGCAGCCA, GCCTGGAGCTCAAACCTCCGCCAAAAC, respectively. An existing XhoI site and the underlined BamHI, XbaI, and SacI restriction sites were used for successive cloning into pBluescript SK(+) (Stratagene), resulting in 5'3'pBSK+. The *GLF::HYG* targeting construct was then made by amplifying the hygromycin resistance gene (*HYG*) from plasmid pCR2.1hyg<sup>3</sup> with primers GACGCAACGCAGGCACACGCAGCAAATCCATCATGAAAAAGCCTGAACTCA and TCCTCCTCGTCCATGGCTGCCGACCTTGGCCTCTATTCCTTTGCCCTCGGA and subsequently introducing *HYG*

in 5'3'pBSK+ by homologous recombination in *Escherichia coli* YZ2000 as instructed by the manufacturer (GeneBridges). The same strategy was used to obtain the *GLF::PHLEO*-targeting construct from plasmid pCR2.1phleo<sup>3</sup> using the primers CATCAACTGACGCAACGCAGGCACACGCAGCAAATCCATCATGGCCAAGTTGACCAGTGCC and CTCCCTCGTCCCTCCTCGTCCATGGCTGCCGACCTTGGCCTTCAGTCTGCTCCTCGGCCAC. Parts homologous to 5'3'pBSK+ are underlined. The start and stop codons of the antibiotic resistance genes replace those of *GLF* in the final constructs. *GLF::HYG* and *GLF::PHLEO* were digested with XhoI and SacI and the desired fragments purified from agarose gel and precipitated by ethanol. The heterozygous mutant  $\Delta glf::PHLEO/GLF$  was first obtained by electroporation of 2 μg of *GLF::PHLEO* fragment in *L. major* as described previously (36). A second targeting round with the *GLF::HYG* fragment resulted in  $\Delta glf::HYG/\Delta glf::PHLEO$ , referred to as *glf*<sup>-</sup> mutant.

*GLF* expression was restored in several lines of the *glf*<sup>-</sup> mutant by transfection with 10 μg of pXG-*GLF* construct. The latter was made by amplification of *GLF* with the primers ATATCCCGGGATGAGCGCTGACAAGGTGGTC and GCCTGGGATCCTACGAGGCCGTCGACGAC and insertion in the SmaI and BamHI sites of pXG(NEO) (37). For reason of simplicity, the transfectants  $\Delta glf::HYG/\Delta glf::PHLEO$  [pXG *GLF*] are designated *glf*<sup>-</sup>/+*GLF*.

**Subcellular Localization of *L. major* UGM**—To generate an N-terminal GFP-tagged UGM, the full-length open reading frame lacking the start codon was amplified using the primers GTCTGGATCCAGCGCTGACAAGGTGGTCATA and CCTGGGATCCTACGAGGCCGTCGACGAC and inserted in the sense orientation into the BamHI site of pXG-GFP+2 (37). 10 μg of the resulting plasmid were transfected in wild-type *L. major* and in the *glf*<sup>-</sup> mutant. Cells were immobilized to poly-L-lysine-coated coverslips and fixed in 4% paraformaldehyde in PBS for 15 min at room temperature. 4',6-Diamidino-2-phenylindole staining (8 μg/ml in PBS) was performed to visualize the nucleus and kinetoplast. GFP encoded by pXG-GFP+ was used as control.

**Western Blot Analysis**—Total cell lysates of Log phase promastigotes were separated by SDS-PAGE and transferred to nitrocellulose membranes (Whatman Schleicher & Schuell). The monoclonal antibody WIC79.3 (38) and a secondary anti-mouse antibody coupled to horseradish peroxidase (Dianova) were used at a dilution of 1:4,000 and 1:50,000, respectively. Monoclonal antibody gp63–235 was used at a dilution of 1:50 of a hybridoma culture supernatant. Detection was performed with an enhanced chemiluminescence system (Pierce) followed by autoradiography.

**Immunofluorescence Microscopy**—*Leishmania* wild-type parasites, *glf*<sup>-</sup> and *glf*<sup>-</sup>/+*GLF* promastigotes were washed in PBS, immobilized onto poly-L-lysine-coated coverslips, and fixed in 2% paraformaldehyde for 15 min at room temperature. Cells were washed and incubated with 50 mM NH<sub>4</sub>Cl for 15 min and eventually permeabilized with 0.1% saponin. Preincubation, antibody incubation, and washes were conducted in PBS buffer containing 2% bovine serum albumin. The monoclonal antibody WIC79.3 and the secondary antibody goat anti-mouse

<sup>3</sup> M. Wiese, unpublished data.

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Cy3 (Sigma) were used at a dilution of 1:1000 and 1:500, respectively. Monoclonal antibody gp63–235 was used at a dilution of 1:10 of a hybridoma culture supernatant. Cells were stained with 8  $\mu\text{g}/\text{ml}$  4',6-diamidino-2-phenylindole for 30 min in the dark before embedding in Mowiol and inspection by fluorescence microscopy.

**Purification and Analysis of PPGs**—PPGs were partially purified from cell lysates of stationary phase promastigotes by Triton-X114 partitioning and analyzed by Western blotting with WIC79.3 at a 1:4000 dilution. *L. mexicana* secreted acid phosphatase (SAP) was heterologously expressed in the different cell lines by transfection with pXG-LmexSAP1 and immunoprecipitated with the anti-SAP monoclonal antibody LT8.2. Phosphoglycosylation was analyzed by Western blot analysis with WIC79.3 and LT8.2 using the Super-Signal West Femto ECL substrate (Pierce).

**Purification and Analysis of GIPLs**—A pellet of  $4 \times 10^8$  parasites washed with PBS was disrupted by  $5 \times 30$  s of sonication in 500  $\mu\text{l}$  of chloroform/methanol/water (1:2:0.8) and centrifuged at  $11,000 \times g$  for 10 min. The supernatants obtained from two successive extractions were dried under a nitrogen stream, resuspended in 1.5 ml of  $\text{H}_2\text{O}$ , and applied to a  $\text{C}_{18}/\text{SepPak}^{\text{®}}$  Plus column (Waters) pre-equilibrated with 5 ml of methanol and 20 ml of  $\text{H}_2\text{O}$ . After washing the column with 10 ml of  $\text{H}_2\text{O}$ , GIPLs were eluted in 1.5 ml of methanol, dried under a nitrogen stream, and dissolved in 200  $\mu\text{l}$  of chloroform/methanol/water (3:6:0.8). For mass spectrometry measurement, 1  $\mu\text{l}$  of 6-aza-2-thiothymine matrix (5  $\mu\text{g}/\mu\text{l}$ ), 5  $\mu\text{l}$  of sample, and 1  $\mu\text{l}$  of matrix were successively deposited on a metal target plate under a hot air stream. Mass spectra were recorded on a Bruker ULTRAFLEX<sup>TM</sup> matrix-assisted laser desorption ionization time-of-flight/time-of-flight spectrometer in the negative mode. The instrument was used at a maximum accelerating potential of 20 kV.

**Mouse Infection Studies**—Parasites were first passed through BALB/c mice (Charles River) and maintained for less than three passages *in vitro*. Then  $2 \times 10^6$  stationary phase promastigotes were injected subcutaneously into the footpad of BALB/c mice (5 mice/group), and lesions were monitored by measuring the thickness of the infected footpad with a Vernier caliper and comparing it to the non-infected footpad. Mice were sacrificed when necrosis appeared in the group. Lesion-derived parasites were enumerated by limiting dilution assay (39). Amastigotes recovered from infected animals were differentiated into promastigotes, and LPG expression was checked by immunofluorescence as described above.

## RESULTS

***L. major* UGM Is Localized in the Cytoplasm**—The protein UGM encoded by *GLF* was described previously (22, 23). There is no signal in the polypeptide sequence of UGM typical of membrane association or anchorage suggesting that this protein is soluble. However, there is precedent in trypanosomatids and other organisms for the compartmentalization of proteins involved in nucleotide sugar interconversion (40–42). The cellular localization of UGM was thus examined using episomal expression of N-terminal GFP-tagged protein (*GFP::GLF*) in wild type *L. major* and *glf*<sup>−</sup> mutant (see below). Fluorescence microscopy showed that the GFP-tagged UGM is distributed

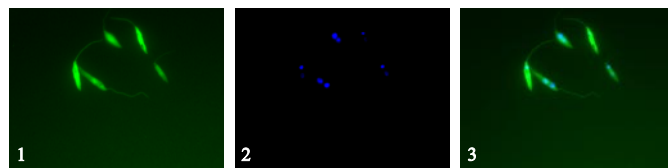


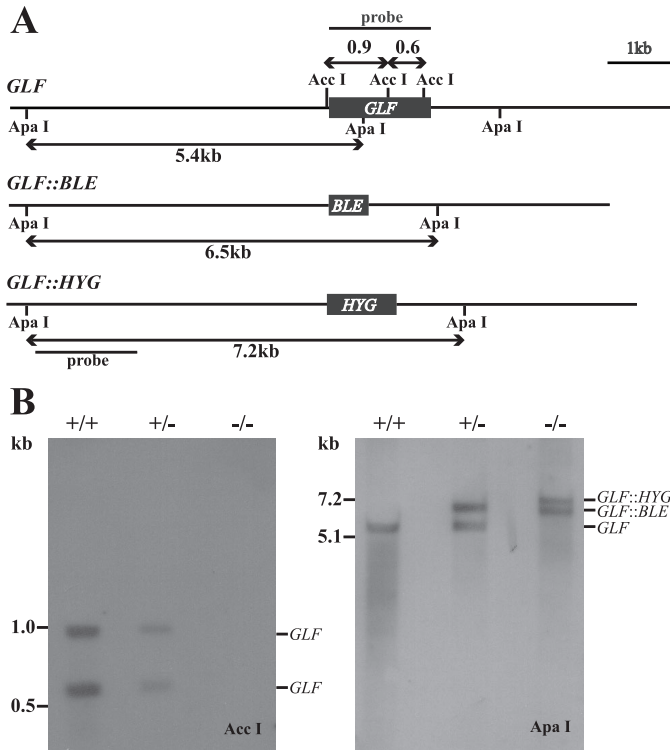
FIGURE 1. *L. major* UGM is localized in the cytoplasm. Wild-type *L. major* expressing N-terminal GFP-tagged UGM after transfection with pXG-GFP + 2-GLF. Panel 1, GFP fluorescence; panel 2, 4',6-diamidino-2-phenylindole staining; panel 3, overlay of panels 1 and 2.

throughout the whole cell within *L. major* wild-type (Fig. 1), supporting the cytoplasmic location of UGM. The cytoplasmic localization is consistent with the absence of peroxisomal targeting sequence type 1 or type 2 in UGM amino acid sequence. As a control, LPG that is absent from the *glf*<sup>−</sup> mutant was re-expressed after transfection, demonstrating that the GFP-tagged protein is enzymatically active (data not shown).

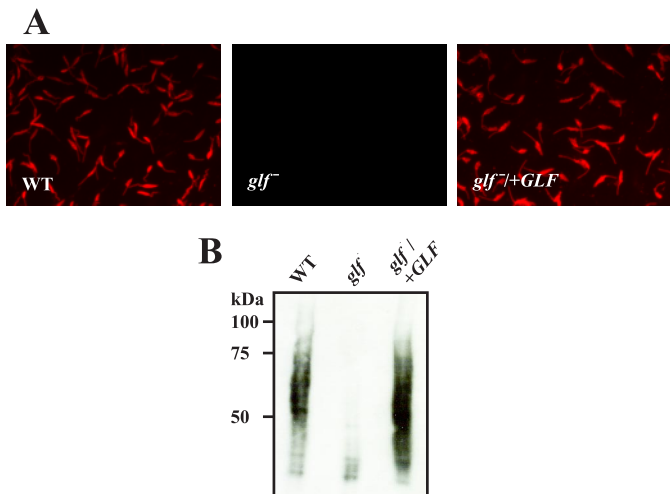
**Generation of *L. major glf* Null Mutant**—Southern blot analysis indicated that *GLF* is a single copy gene (data not shown), which was confirmed by targeted gene replacement. Moreover, *L. major* genome sequencing was recently completed (43) and revealed a single homologue of *GLF* present on chromosome 18. Because there is evidence that gene expression in *Leishmania* is directed by sequences present in the 3'- and 5'-flanking regions, the targeting constructs were prepared by homologous recombination to allow a precise replacement of *GLF* by the antibiotic resistance genes and thus ensure correct expression of these markers. Two successive rounds of gene replacement were required to obtain a *glf* null mutant ( $\Delta glf::HYG/\Delta glf::BLE$ ) because *Leishmania* has a diploid genome. Southern blots of *AccI*-digested genomic DNA from wild-type, single, and double targeted parasites were hybridized with a digoxigenin-labeled *GLF* probe (Fig. 2). Bands corresponding to the predicted size of *AccI* fragments were observed in wild-type and *GLF/\Delta glf::BLE* heterozygous mutant. In contrast, no hybridization was observed in the *glf*<sup>−</sup> mutant, confirming successful deletion of both *GLF* alleles. Integration of the *BLE* and *HYG* markers at the correct locus was confirmed by an additional Southern blot hybridized with a digoxigenin-labeled probe situated in the *GLF* 5'-flanking region outside the region used for homologous recombination (Fig. 2). Further Southern blots using digoxigenin-labeled probes specific for *BLE* and *HYG* excluded random integration of the markers (data not shown).

All *glf*<sup>−</sup> clones appeared morphologically normal and grew in culture as well as the parental wild-type strain. As a control for subsequent analysis, several lines of *glf*<sup>−</sup> mutant were transfected with an *GLF* expression plasmid, yielding transfectants designated *glf*<sup>−</sup> + *GLF* ( $\Delta glf::HYG/\Delta glf::BLE$  [pXG-*GLF*]). In all analyses, sibling clonal lines behaved similarly, and thus results from a single *glf*<sup>−</sup> and *glf*<sup>−</sup> + *GLF* mutant will be shown.

**The *glf* Mutant Lacks LPG**—Presence of phosphoglycans in *L. major* wild-type, *glf*<sup>−</sup>, and *glf*<sup>−</sup> + *GLF* mutants was first analyzed by immunofluorescence microscopy using the monoclonal antibody WIC79.3. This antibody specifically recognizes the galactose-substituted repeat units present in *L. major* LPG and PPGs and strongly labels the cell surface of wild-type and *glf*<sup>−</sup> + *GLF* parasites. In contrast, no fluorescent signal was discernable at the cell surface of the *glf*<sup>−</sup> mutant, indicating the

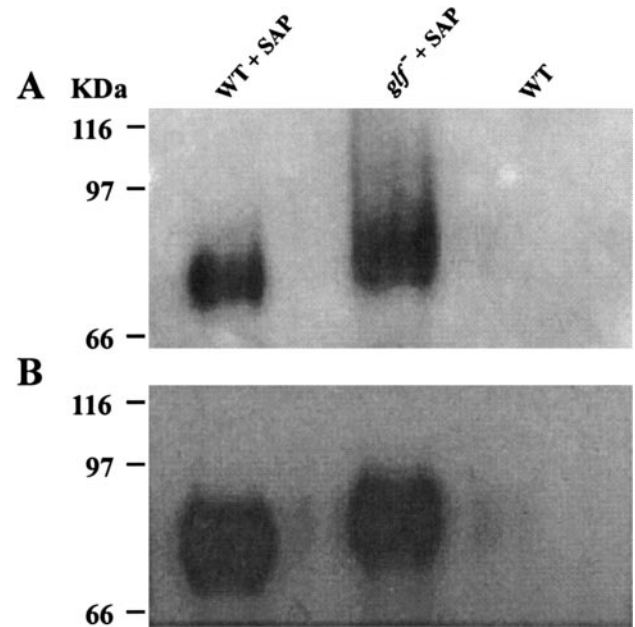


**FIGURE 2. Targeted gene replacement of *GLF* alleles.** *A*, schematic representation of *L. major* *GLF* locus in wild-type and *glf*<sup>-</sup> replacement lines indicating *AccI* and *ApaI* fragments expected after hybridization with a *GLF* probe (upper lines) or a 5'-flanking probe (lower lines), respectively. *B*, Southern blot analysis of genomic DNA from wild-type (+/+), heterozygous *GLF*/ $\Delta$ *glf*:*BLE* mutant (+/-), and homozygous *glf*<sup>-</sup> mutant (-/-). DNA was digested with either *AccI* (left panel) or *ApaI* (right panel), separated on agarose gel, blotted onto nylon membrane, and hybridized with digoxigenin-labeled *GLF* (left panel) or a digoxigenin-labeled 5'-flanking probe (right panel).



**FIGURE 3. The *glf*<sup>-</sup> mutant lacks LPG.** *A*, indirect immunofluorescence microscopy of fixed permeabilized promastigotes with the monoclonal antibody WIC79.3. *B*, Western blot of wild-type, *glf*<sup>-</sup>, and *glf*<sup>-</sup>/*+GLF* cell extracts detected with WIC79.3 and ECL.

absence of phosphoglycans (Fig. 3*A*). Lack of LPG was confirmed by Western blotting of total cell extracts labeled with WIC79.3. A characteristic smear of LPG migrating around 30–80 kDa is observed with wild-type *L. major* but not with the *glf*<sup>-</sup> mutant (Fig. 3*B*). As expected, re-expression of UGM restores LPG synthesis (Fig. 3).



**FIGURE 4. Phosphoglycosylation of reporter secreted acid phosphatase is more extensive in the *glf*<sup>-</sup> mutant.** *A*, SAP expressed in wild-type (lane 1) and *glf*<sup>-</sup> (lane 2) parasites was immunoprecipitated with the monoclonal antibody LT8.2 and analyzed by Western blotting using the antibody WIC79.3. Untransfected wild-type parasites were used as control (lane 3). *B*, equal loading of samples was confirmed by staining with LT8.2.

*Phosphoglycosylation of Reporter Secreted Acid Phosphatase Is More Extensive in glf*<sup>-</sup>—Secreted and cell-associated PPGs were partially purified from culture supernatants and cell extracts by Triton X114 partitioning and analyzed by Western blotting with WIC79.3. Surprisingly, only very low levels of PPGs were detectable in the fractions obtained from *L. major* MHOM/SU/73/5ASKH and derivative cell lines. In contrast, PPGs were clearly visible in the stacking gel in control experiments with *L. major* strain MHOM/IL/80/Friedlin (data not shown). These results suggest the low abundance of PPGs in *L. major* MHOM/SU/73/5ASKH and are consistent with the absence of immunolabeling of permeabilized *glf*<sup>-</sup> parasites with WIC79.3 (Fig. 3).

To study phosphoglycosylation, *L. mexicana* secreted acid phosphatase (SAP) was heterologously expressed in wild-type *L. major* and the *glf*<sup>-</sup> mutant, immunoprecipitated with the anti-SAP monoclonal antibody LT8.2, and analyzed by Western blotting with WIC79.3 and LT8.2 (Fig. 4). As expected, SAP was only detected in cell lines transfected with pXG-LmexSAP and not in untransfected cell lines. Moreover, SAP expressed by the *glf*<sup>-</sup> mutant migrated more slowly when compared with molecules expressed by wild type, suggesting that the degree of phosphoglycosylation is elevated in this cell line.

*L. major glf*<sup>-</sup> Mutant Synthesizes Truncated GIPLs and Accumulates Residual LPG Anchors—GIPLs were purified from wild-type and *glf*<sup>-</sup> mutants and analyzed by matrix-assisted laser desorption ionization mass spectrometry in the negative mode. *L. major* synthesizes different type 2 GIPLs consisting of Gal<sub>1</sub>( $\beta$ 1-3)Man( $\alpha$ 1-3)Man( $\alpha$ 1-4)GlcN-PI, Gal( $\alpha$ 1-3)Gal<sub>1</sub>( $\beta$ 1-3)Man( $\alpha$ 1-3)Man( $\alpha$ 1-4)GlcN-PI, and Gal( $\alpha$ 1-6)Gal( $\alpha$ 1-3)Gal<sub>1</sub>( $\beta$ 1-3)Man( $\alpha$ 1-3)Man( $\alpha$ 1-4)GlcN-PI, known as GIPL-1, GIPL-2, and GIPL-3 according to the number of Gal

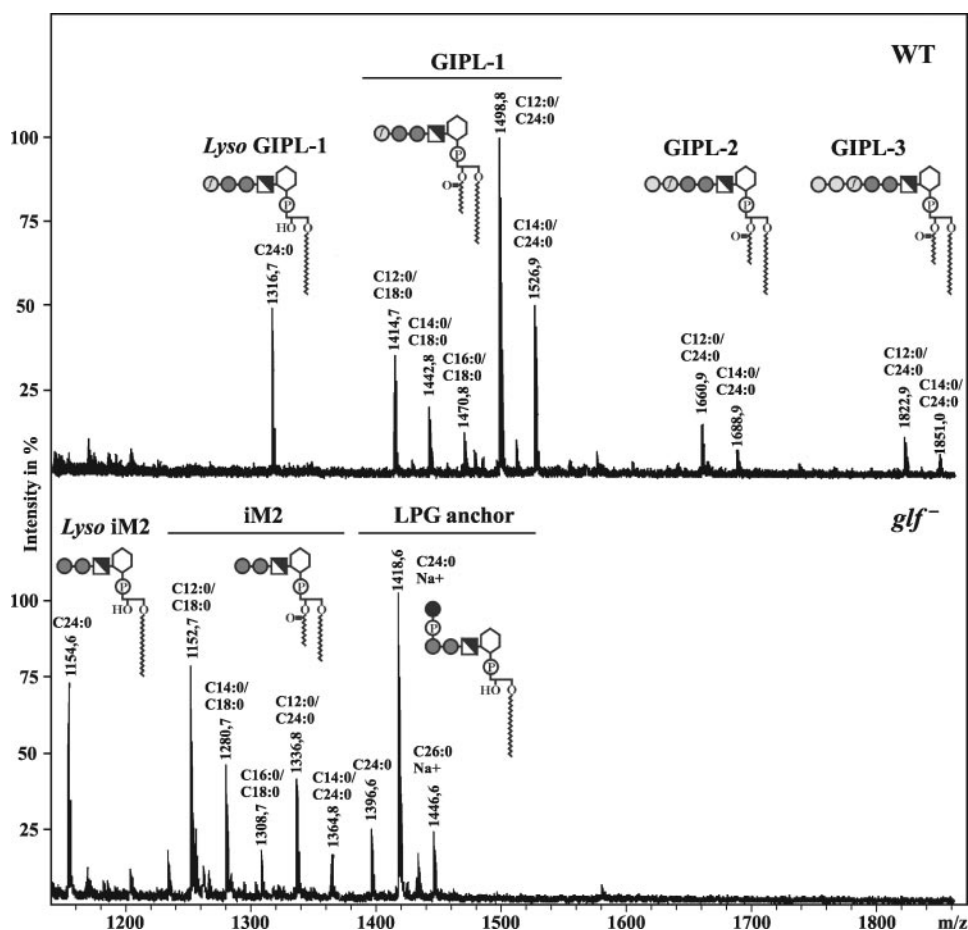


FIGURE 5. The *glf*<sup>-</sup> mutant synthesized truncated GPIs. Negative ion matrix-assisted laser desorption/ionization spectrum of GPIs isolated from wild-type (WT) and *glf*<sup>-</sup> mutant. The identities of the ions are indicated by the schematics and can be inferred from the structure of GIPL-3, which is Gal $\alpha$ 1-6Gal $\alpha$ 1-3Gal $\beta$ 1-3Man $\alpha$ 1-3Man $\alpha$ 1-4GlcN $\alpha$ 1-6myo-inositol-1-HPO4-3(*sn*-1-alkyl-2-acylglycerol). The length and saturation of the acyl and alkyl chain, respectively, is indicated above each peak. Light shaded circles, Gal $\beta$ ; light shaded circles with f, Gal $\alpha$ ; dark shaded circles, Man; solid circle, Glc; half shaded squares, GlcN; and P, phosphate.

residues that they contain (20). Because of additional heterogeneity in the lipid part, 10 distinct glycolipids ions can be observed in the negative ion spectrum of *L. major* MHOM/SU/73/5ASKH GPIs (Fig. 5). The ions at *m/z* 1498.8, 1526.9, 1660.9, 1688.9, 1822.9, and 1851.0 represent GIPL-1, GIPL-2, and GIPL-3 with C24:0 alkyl and C12:0 or C14:0 acyl chains. Additionally, the spectrum presents ions at *m/z* 1414.7, 1442.8, and 1470.8 corresponding to GIPL-1 with C18:0 alkyl and C12:0, C14:0, or C16:0 acyl chains, respectively. These assignments were confirmed by tandem mass spectrometry and are in agreement with the structures previously described in *L. major* V121 (20, 44). Interestingly GIPL-1 containing a *sn*1-alkyl-2-lyso-PI with C24:0 alkyl chain (*m/z* 1316.7) is also present in the wild-type spectrum. The latter was not reported in *L. major* V121 but seems to be present in *L. major* Friedlin V1 (19). The negative ion spectrum of GPIs extracted from *GLF*/ $\Delta$ *glf*::*BLE* heterozygous mutant was identical to the wild-type spectrum (data not shown). In contrast, analysis of *glf*<sup>-</sup> mutant revealed the presence of truncated GPIs (Fig. 5). As expected the biosynthesis of GPIs seems to stop after the addition of the second mannose, giving rise to GPIs having the basic structure Man( $\alpha$ 1-3)Man( $\alpha$ 1-4)GlcN-PI known as iM2. The same lipid

heterogeneity as seen in wild-type GPIs leads to the observation of six different iM2 peaks at *m/z* 1154.6, 1252.7, 1280.7, 1308.7, 1336.8, and 1364.8. As a result of UDP-Gal $\alpha$  absence, LPG biosynthesis is also prematurely stopped, leading to the accumulation of a residual LPG anchor with the structure Glc $\alpha$ 1-HPO $\alpha$ -6-Man( $\alpha$ 1-3)Man( $\alpha$ 1-4)GlcN-*sn*1-alkyl-2-lyso-PI. The spectrum is therefore dominated by an ion at *m/z* 1418.6 corresponding to a sodium adduct ([M-2H+Na]<sup>-</sup>) of this residual LPG anchor with a C24:0 alkyl chain. The formation of a sodium adduct is enabled by the presence of two phosphate groups in this glycolipid. The molecular ion [M-H]<sup>-</sup> at *m/z* 1396.6 corresponds to the same molecule. Additionally, a small amount of truncated LPG anchor with a C26:0 alkyl chain can be observed (sodium adduct at *m/z* 1446.6). All assignments were confirmed by tandem mass spectrometry.

Interestingly, iM2 molecules with a C24:0 alkyl and a C12:0 or C14:0 acyl chain (ions at *m/z* 1336.8 and 1364.8) are not abundant in the *glf*<sup>-</sup> mutant, although these two PIs are the main lipids found in wild-type GIPL-1 (ions at *m/z* 1498.8 and 1526.9), GIPL-2 (ions at *m/z* 1660.9 and 1688.9), and GIPL-3 (ions at *m/z* 1822.9 and 1851.0). As remodeling of the acyl chain, but not of the alkyl chain, has previously been described (45), this suggests that these two iM2 molecules can be directed toward LPG biosynthesis by deacylation and Glc-1-P addition.

*The glf*<sup>-</sup> Mutant Synthesizes Normal Amount of GPI-anchored Proteins—The synthesis of the abundant GPI-anchored metalloprotease gp63 was examined by immunofluorescence and Western blot analysis with the *L. major*-specific monoclonal antibody gp63-235. Coomassie blue staining was used to confirm equal loading of samples. As expected, loss of *GLF* did not affect the expression or localization of gp63 (data not shown).

*Galactofuranose Biosynthesis Is Important for Establishment of Infections in Mice*—The virulence of the *glf*<sup>-</sup> mutant was assessed by infection of susceptible BALB/c mice. The results presented in Fig. 6 are the average of two individual experiments. When inoculated with wild-type or *glf*<sup>-</sup> + *GLF* stationary phase promastigotes, mice presented lesions at the site of infection after ~3 to 4 weeks. In contrast, disease appearance was delayed to 9 weeks in mice infected with the *glf*<sup>-</sup> mutant. In both cases, lesions developed progressively thereafter and the lesion size correlated with parasite burden. Importantly *glf*<sup>-</sup>

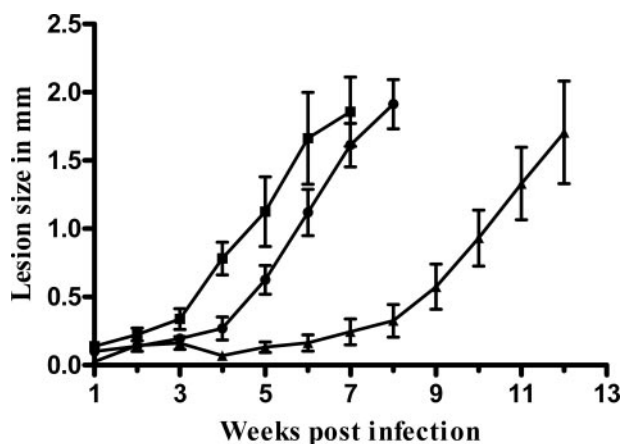


FIGURE 6. Virulence of the *glf*<sup>-</sup> mutant is attenuated in a murine infection model. Mice were inoculated in the footpad with  $2 \times 10^6$  wild type (■), *glf*<sup>-</sup> (▲), and *glf*<sup>-</sup> + GLF (●), and lesion formation was monitored over a period of 12 weeks. Average of two individual experiments.

parasites recovered from infected animals did not express any LPG, thus excluding the presence of contaminants.

## DISCUSSION

In recent years, different enzymes involved in Gal<sub>f</sub> metabolism have been characterized in bacteria and revealed the crucial role of Gal<sub>f</sub> for survival or virulence of several pathogenic bacteria (e.g. Mycobacteria) (21). In contrast, the role of Gal<sub>f</sub> in eukaryotes is still undefined. In this work, the role of Gal<sub>f</sub> for *L. major* virulence was investigated by targeted deletion of the *GLF* gene encoding UGM. We and others recently identified and partially characterized this central enzyme in Gal<sub>f</sub> biosynthesis that converts UDP-Gal<sub>p</sub> into UDP-Gal<sub>f</sub> (22, 23). *L. major* UGM was localized in the cytoplasm as predicted from the lack of polypeptide sequences typical of peroxisomal targeting, membrane association, or anchorage. Interestingly, the UDP-glucose 4'-epimerase of *Trypanosoma brucei* has been localized in the glycosome (42) and several other trypanosomatid enzymes involved in UDP-Gal production are predicted to be in this microbody (46). The cytoplasmic localization of UGM implies thus the existence of a UDP-Gal transporter exporting UDP-Gal from the glycosome into the cytoplasm and of a Golgi-localized UDP-Gal<sub>f</sub> transporter because the galactofuranosyltransferase LPG1 is situated in this organelle (37). The latter transporter is expected to be distinct from UDP-Gal<sub>p</sub> transporters.

In *L. major* Gal<sub>f</sub> is present in the GPI anchor of the abundant cell surface LPG as in other *Leishmania* species and in the GIPLs (2). Accordingly, targeted replacement of *GLF* resulted in the absence of LPG backbone and the presence of truncated GIPLs similar to the iM2 glycolipids present in *L. mexicana*. Additionally, phosphoglycosylation of reporter secreted acid phosphatase was increased when expressed in the *glf*<sup>-</sup> mutant. Such influence on PPG phosphoglycosylation was also observed in the *ads1*<sup>-</sup> mutant that lacks both LPG and GIPLs (19) and could be seen as an indirect effect of LPG synthesis blockage because the carbohydrate repeating units building PPGs and LPG are identical. However, such increase of phosphoglycosylation was not reported in *L. major* and *L. mexicana*

*lpg1*<sup>-</sup> mutants that are exclusively deficient in LPG (8, 11) and its basis remains to be determined.

The structural changes resulting from UGM absence did not affect the viability or growth of *Leishmania in vitro*. This was anticipated because other mutants defective in one or more surface glycoconjugates are perfectly viable and do not display any growth phenotype *in vitro*. In contrast, deletion of *GLF* impairs the growth of the fungi *Aspergillus fumigatus* and *Aspergillus niger*.<sup>4</sup> Such variation in the influence of Gal<sub>f</sub> metabolism on growth has also been observed in bacteria (25, 27).

*L. major* synthesizes type 2 GIPLs that can be distinguished from the LPG anchor by the absence of Glc-1-P residue on the α-1-3-linked Man and by their lipid moiety. LPG is exclusively anchored via a *sn*-1-alkyl-2-lyso-PI with long C24:0 or C26:0 alkyl chains, whereas type 2 GIPLs contain principally *sn*-1-alkyl-2-acyl-PI with C18:0 (GIPLs<sub>C18:0</sub>) or C24:0 alkyl chains (GIPLs<sub>C24:0</sub>) (2). GIPLs<sub>C24:0</sub> that constitute the vast majority of mature GIPLs synthesized by wild-type *L. major* strain MHOM/SU/73/5ASKH are considerably reduced in the *glf*<sup>-</sup> mutant. In the absence of Gal<sub>f</sub> addition, these molecules seem to be deacylated and modified by the addition of a Glc-1-P residue to generate the residual LPG anchor that accumulates in the mutant. These data strongly suggest that GIPLs<sub>C24:0</sub> and LPG originate from a common iM2 precursor and thus share a common biosynthetic pathway (47). In *L. mexicana* that exclusively express hybrid type GIPLs with a C18:0 alkyl chain, GIPLs and LPG anchor are thought to be the products of independent biosynthetic pathways (45). Such separation of the biosynthetic pathways of GIPLs<sub>C18:0</sub> on one hand and LPG/GIPLs<sub>C24:0</sub> on the other hand might also occur in *L. major*. The predominance of GIPLs<sub>C18:0</sub> in *L. major* amastigotes in which the LPG pathway is strongly down-regulated (48) and the involvement of distinct galactofuranosyltransferases in LPG and GIPLs biosynthesis (8) would be consistent with this hypothesis. To date it is still unclear whether different galactofuranosyltransferases are involved in the biosynthesis of GIPLs<sub>C24:0</sub> and LPG (8) and thus whether the biosynthetic pathway of these molecules diverges after the assembly of iM2. Additionally, the accumulation of residual LPG with a Glc-1-P residue and a *lyso*-alkyl in the *glf*<sup>-</sup> mutant indicate that the enzymes involved in these modifications do not require the prior addition of Gal<sub>f</sub>. As expected, GIPL<sub>C18:0</sub> are not processed by these enzymes either because of their specificity or because of compartmentalization of the pathway.

Using genetic inactivation of the putative galactofuranosyltransferase LPG1 that results in the exclusive loss of LPG, it was clearly established that LPG is a virulence factor in *L. major* (8). Thus, the reduction of *L. major glf*<sup>-</sup> virulence observed in a mouse model was anticipated. The role of GIPLs, on the other hand, is more disputed. Unlike LPG and the major surface proteins, they are abundant in both promastigote and amastigote developmental stages. The  $4 \times 10^6$  GIPL molecules present at the surface of each amastigote are thought to cover 45–60% of the parasite plasma membrane (48, 49) and play a protective

<sup>4</sup> F. H. Routier and W. Vervecken, unpublished results.

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role. Importantly, the terminal Gal<sub>f</sub> residue present in GIPL-1 was proposed to participate in the interaction of the parasites with macrophage and their internalization (50). Gal<sub>f</sub> would indeed be exposed at the apex of GIPLs and thus easily accessible (49). More recently, however, the *ads1*<sup>-</sup> mutant that is deficient in all ether phospholipids including LPG and GIPLs oppugned the importance of GIPLs for *Leishmania* pathogenesis (19). In agreement with this last study, we found that the complete lack of Gal<sub>f</sub> reflected by the absence of LPG backbone and truncation of the GIPLs results in a delay of disease appearance comparable with that obtained when LPG alone is deficient (8). Thus, the additional truncation of GIPLs had no profound effect on the virulence of *Leishmania* toward mice. Once infection has been established, disease progression in animals infected with wild-type *Leishmania* or *glf*<sup>-</sup> mutants is comparable, suggesting that Gal<sub>f</sub> is not essential for the amastigote-macrophage interaction or the survival of amastigotes into these phagocytes. All effect observed might thus be due to the sole loss of LPG. Gal<sub>f</sub> contributes significantly nevertheless to *Leishmania major* pathogenesis.

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