View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Digital Commons@Becker

Washington University School of Medicine
Digital Commons@Becker

Open Access Publications

2007

Comparisons of mutants lacking the golgi UDP-galactose or GDP-mannose transporters establish that phosphoglycans are important for promastigote but not amastigote virulence in Leishmania major

Althea A. Capul
Washington University School of Medicine in St. Louis

Suzanne Hickerson
Washington University School of Medicine in St. Louis

Tamara Barron
University of Kentucky Medical Center

Salvatore J. Turco
University of Kentucky Medical Center

Stephen M. Beverley
Washington University School of Medicine in St. Louis

Follow this and additional works at: http://digitalcommons.wustl.edu/open_access_pubs

Recommended Citation

Capul, Althea A.; Hickerson, Suzanne; Barron, Tamara; Turco, Salvatore J.; and Beverley, Stephen M., ,"Comparisons of mutants lacking the golgi UDP-galactose or GDP-mannose transporters establish that phosphoglycans are important for promastigote but not amastigote virulence in Leishmania major." Infection and Immunity.75,9. 4629-4637. (2007).
http://digitalcommons.wustl.edu/open_access_pubs/2382

This Open Access Publication is brought to you for free and open access by Digital Commons@Becker. It has been accepted for inclusion in Open Access Publications by an authorized administrator of Digital Commons@Becker. For more information, please contact engeszer@wustl.edu.

Infection and Immunity

Comparisons of Mutants Lacking the Golgi UDP-Galactose or GDP-Mannose Transporters Establish that Phosphoglycans Are Important for Promastigote but Not Amastigote Virulence in *Leishmania major*

Althea A. Capul, Suzanne Hickerson, Tamara Barron, Salvatore J. Turco and Stephen M. Beverley
***Infect. Immun.* 2007, 75(9):4629. DOI: 10.1128/IAI.00735-07.**
Published Ahead of Print 2 July 2007.

Updated information and services can be found at:
http://iai.asm.org/content/75/9/4629

These include:

REFERENCES

This article cites 66 articles, 28 of which can be accessed free at: http://iai.asm.org/content/75/9/4629#ref-list-1

CONTENT ALERTS

Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), more»

Information about commercial reprint orders: **http://journals.asm.org/site/misc/reprints.xhtml**
To subscribe to to another ASM Journal go to: **http://journals.asm.org/site/subscriptions/**

Journals.ASM.org

Downloaded from http://iai.asm.org/ on March 10, 2014 by Washington University in St. Louis

INFECTION AND IMMUNITY, Sept. 2007, p. 4629–4637 Vol. 75, No. 9
0019-9567/07/$08.00+0 doi:10.1128/IAI.00735-07
Copyright © 2007, American Society for Microbiology. All Rights Reserved.

Comparisons of Mutants Lacking the Golgi UDP-Galactose or GDP-Mannose Transporters Establish that Phosphoglycans Are Important for Promastigote but Not Amastigote Virulence in *Leishmania major*[▽]

Althea A. Capul,[1] Suzanne Hickerson,[1] Tamara Barron,[2] Salvatore J. Turco,[2] and Stephen M. Beverley[1]*

Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110,[1] and Department of Biochemistry, University of Kentucky Medical Center, Lexington, Kentucky 40536[2]

Received 31 May 2007/Accepted 12 June 2007

Abundant surface *Leishmania* phosphoglycans (PGs) containing [Gal(β1,4)Man(α1-PO$_4$)]-derived repeating units are important at several points in the infectious cycle of this protozoan parasite. PG synthesis requires transport of activated nucleotide-sugar precursors from the cytoplasm to the Golgi apparatus. Correspondingly, null mutants of the *L. major* GDP-mannose transporter *LPG2* lack PGs and are severely compromised in macrophage survival and induction of acute pathology in susceptible mice, yet they are able to persist indefinitely and induce protective immunity. However, *lpg2*$^-$ *L. mexicana* amastigotes similarly lacking PGs but otherwise normal in known glycoconjugates remain able to induce acute pathology. To explore this further, we tested the infectivity of a new PG-null *L. major* mutant, which is inactivated in the two UDP-galactose transporter genes *LPG5A* and *LPG5B*. Surprisingly this mutant did not recapitulate the phenotype of *L. major lpg2*$^-$, instead resembling the *L. major* lipophosphoglycan-deficient *lpg1*$^-$ mutant. Metacyclic *lpg5A*$^-$/*lpg5B*$^-$ promastigotes showed strong defects in the initial steps of macrophage infection and survival. However, after a modest delay, the *lpg5A*$^-$/*lpg5B*$^-$ mutant induced lesion pathology in infected mice, which thereafter progressed normally. Amastigotes recovered from these lesions were fully infective in mice and in macrophages despite the continued absence of PGs. This suggests that another *LPG2*-dependent metabolite is responsible for the *L. major* amastigote virulence defect, although further studies ruled out cytoplasmic mannans. These data thus resolve the distinct phenotypes seen among *lpg2*$^-$ *Leishmania* species by emphasizing the role of glycoconjugates other than PGs in amastigote virulence, while providing further support for the role of PGs in metacyclic promastigote virulence.

Leishmaniasis is considered an emerging or uncontrolled disease in many parts of the world, with more than 12 million people infected (9, 35, 45). Depending on the particular species, *Leishmania*-induced pathology ranges from self-healing cutaneous lesions to fatal, visceral disease. Several treatment regimens are available, until recently most commonly based on the metal antimony, to which resistance is widespread in some regions. As yet there are no safe vaccines available, leaving drug treatments or insect vector control measures as the major strategies for control. However, the introduction of miltefosine, a safe, orally acting compound that is effective against visceral species (35), and the ability to vaccinate effectively under some circumstances suggests the potential for progress on both chemo- and immunotherapeutic fronts in the future (33). The *Leishmania* infectious cycle comprises two phases, one extracellular within the digestive tract of the phlebotomine sand fly and one intracellular within the phagolysosome of vertebrate macrophages, both compartments where *Leishmania* must overcome a variety of host defenses. Defining the processes by which *Leishmania* survives these two hostile compartments could lead to new vaccine and drug targets, which will prevent transmission and more effectively treat new or recurrent infections arising from persistent asymptomatic parasites.

Leishmania synthesizes a variety of abundant glycoconjugates implicated in various steps of the infectious cycle (17, 39). These include lipophosphoglycan (LPG); glycosylphosphatidylinositol (GPI)-anchored proteins, including proteophosphoglycan (PPG) and glycoprotein 63 (gp63) (leishmanolysin); glycosylinositolphospholipids (GIPLs); and inositolphosphoceramide (21, 56, 63, 66). Notably these glycoconjugates share many structural motifs or domains (Fig. 1A). For example, the GPI anchors are common to LPG, proteins, and small surface glycolipids (Fig. 1A) (12). Additionally, the phosphoglycan [Gal(β1,4)Man(α1-PO$_4$)] disaccharide-phosphate repeating units (PG repeats) modify a variety of surface and secreted proteins and comprise the major portion of LPG, which contains 15 to 30 PG repeats (21, 56, 60). Thus, while studies carried out on purified PGs point to important roles such as modulating host signal transduction, inhibiting phagolysosomal fusion, and mediating oxidant resistance (reviewed by in reference 8), the structural similarity among these complex molecules often leads to imprecision in our understanding of their unique and/or overlapping roles in vivo.

To overcome this problem, we and others have pursued a strategy of generating null mutants leading to deficiencies in

* Corresponding author. Mailing address: Department of Molecular Microbiology, Washington University School of Medicine, Campus Box 8230, 660 S. Euclid Avenue, St. Louis, MO 63110. Phone: (314) 747-2631. Fax: (314) 747-2634. E-mail: beverley@borcim.wustl.edu.
▽ Published ahead of print on 2 July 2007.

Downloaded from http://iai.asm.org/ on March 10, 2014 by Washington University in St. Louis

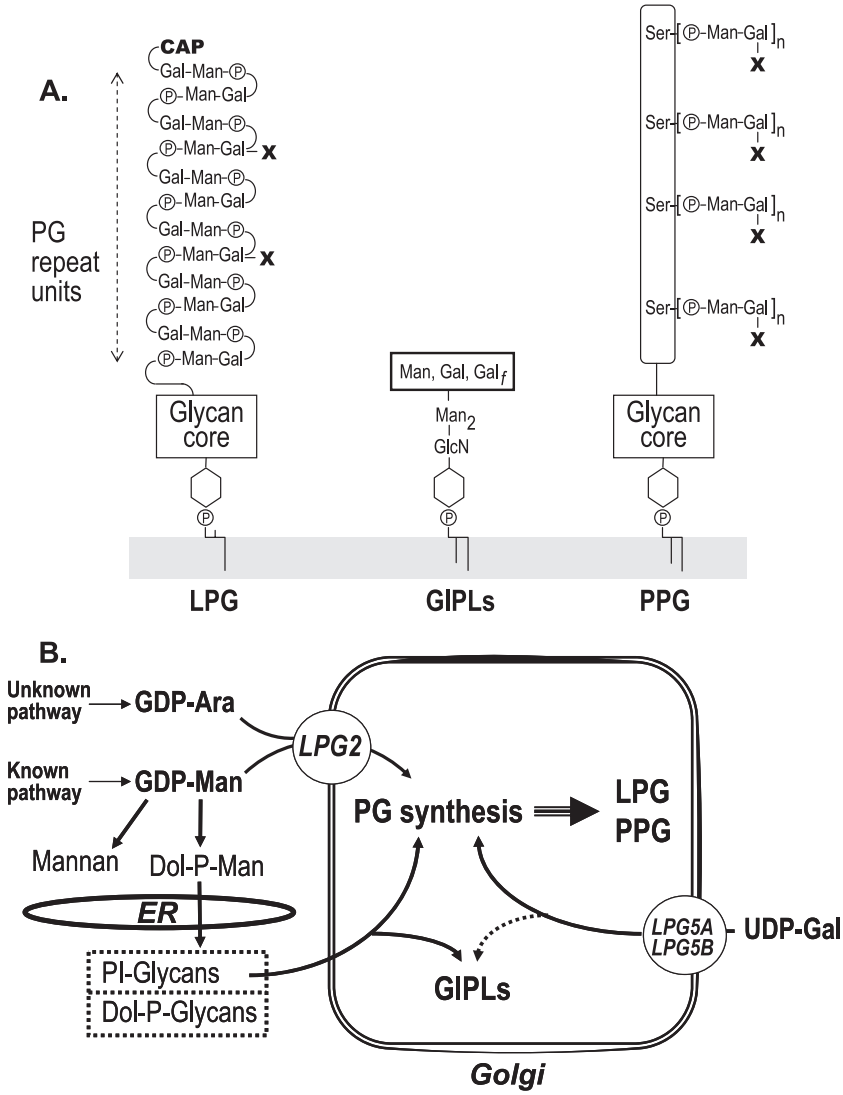

FIG. 1. *Leishmania* glycoconjugates and relevant biosynthetic pathway steps. (A) Glycoconjugates. The detailed structures of *L. major* LPG, GIPLs, and PPGs, including the glycan core, cap, linkages, and anomeric configurations, are reviewed elsewhere (21, 56). In *L. major*, "x" refers to linear β-1,3-linked galactose residues that branch off the Gal-Man-P repeating units; these can be terminated in α-1,2-Ara residues in both LPG and PPG, at greatly elevated levels in metacyclic promastigotes. In *L. mexicana*, the PG side chains consist of mono-β-1,3-glucose residues in LPG and additional sugars in PPGs. (B) Condensed cellular biosynthetic pathway. The steps and/or enzymes responsible for the synthesis of GDP-D-Ara$_p$ and its presumptive precursor D-Ara are unknown in *Leishmania* or other organisms. In contrast, the metabolic sequence leading to GDP-Man biosynthesis (Glc→Glc-6-P→Fru-6-P→Man-6-P→Man-1-P→GDP-Man) has been well characterized in *Leishmania* and other organisms (14, 15). Both PI-anchored glycans and dolichol phosphate (Dol-P) glycans can participate in the synthesis of GPI-anchored and N-linked glycoproteins (not depicted). In the Golgi apparatus, the PI-glycans can be further elaborated with additional sugars (requiring the provision of their respective nucleotide-sugar donors) forming GIPLs or forming and anchoring PG chains in the assembly of LPG and PPG (reviewed in reference 32). The *LPG5A*/*LPG5B* dependency of GIPL Gal$_p$ addition (shown by a dashed line) is considered to be likely given that the functionally similar LPG core Gal$_f$ transferase LPG1 is localized in the Golgi apparatus (2, 18).

specific single glycoconjugate or a defined group of glycoconjugates. For example, the *lpg1*$^-$ mutant lacking the LPG core galactofuranosyl transferase LPG1 is specifically deficient in LPG synthesis (50). This mutant has enabled a rigorous definition of the role of intact LPG in parasite biology, where it is important in the fly and initial mammalian stages but not thereafter in amastigotes, where its expression is developmentally extinguished (27, 50, 51). Similar gene ablation studies have addressed the role(s) of the ether lipids within GPI anchors and sphingolipids (7, 64–67). Here we focus on the role of PGs in *Leishmania* virulence, which are expressed across the *Leishmania* life cycle in the form of LPG, PPG, and other related molecules. The role of PGs was approached previously through the study of the *lpg2*$^-$ mutant, which is deficient in the Golgi nucleotide-sugar transporter (NST) responsible for GDP-mannose (GDP-Man) uptake into the lumen, where PG biosynthesis occurs (Fig. 1B) (1, 28, 29). Previous studies of known glycoconjugates showed that *L. major lpg2*$^-$ was deficient in PGs but otherwise wild type (WT) in GPI-anchored protein and GIPL levels (52). Remarkably, this mutant lost the

Downloaded from http://iai.asm.org/ on March 10, 2014 by Washington University in St. Louis

ability to induce acute pathology in susceptible mice, although it retained the ability to persist indefinitely and induce protective immunity (52, 59). Given these phenotypes and the presumption that all significant *Leishmania* glycoconjugates had been cataloged, most investigators reasoned that the lack of PGs was likely to be responsible for the *LPG2*-dependent amastigote virulence defect of *L. major*.

However, studies of the *lpg2*$^-$ mutant of *L. mexicana*, which is similarly deficient in PG synthesis but otherwise unaltered, showed that it retained amastigote virulence and the ability to induce acute pathology (22). The *lpg2*$^-$ *L. mexicana* phenotype raised the possibility that *Leishmania* species differed in their reliance upon PGs for virulence or that an *LPG2*-dependent molecule other than PGs played a critical role in *L. major* but not *L. mexicana* virulence (57). Studies of Man biosynthetic enzymes pointed to a role of Man-containing glycoconjugates in *L. mexicana* virulence, although the broad effects of these mutants through *LPG2*-independent routes, including dolichol-mediated protein N glycosylation (Fig. 1B), made it problematic to attribute the effects to any specific "virulence" glycoconjugate (14, 54). However, recent data have more directly implicated abundant cytoplasmic mannans, synthesized via gluconeogenesis, in *Leishmania* amastigote survival (38, 44). Due to their cytoplasmic localization, mannans are unlikely to be affected by the loss of *L. major LPG2*, a supposition confirmed in this work.

Given the complexity and involvement of the Man synthetic pathway in general glycoconjugate synthesis, we turned our focus to galactose, the second monosaccharide within the basic PG repeating unit (Fig. 1A). While in *Leishmania* galactose (Gal) can be obtained by salvage or through the epimerization of UDP-Glc in the glycosome (46, 58), our interest in the PG assembly and secretion via the Golgi apparatus prompted a strategy centered on this compartment. We recently described the characterization of the family of 12 *Leishmania* NSTs and functional studies of the *LPG5A* and *LPG5B* genes, which encode UDP-Gal transporters whose functions partially overlap (3). Notably, an *lpg5A*$^-$*/lpg5B*$^-$ double gene mutant completely abrogated UDP-Gal uptake into the Golgi apparatus, as this mutant lacked LPG and protein-linked PGs. Thus, the *lpg5A*$^-$*/lpg5B*$^-$ mutant provides an independent perspective from which to study the role of PGs in *L. major* (Fig. 1B). The data presented here confirm a role for PGs in the initial establishment phase of infection of vertebrate macrophages, probably reflecting the loss of LPG (50), but in neither amastigote-mediated virulence nor acute pathology. These data lend support to the existence of a vital *LPG2*-dependent molecule, unrelated to PGs, required for amastigote virulence in *L. major*.

MATERIALS AND METHODS

Cell culture, reagents, and transfection. *L. major* strain LV39c5 (Rho/SU/59/P) was grown at 26°C in M199 medium (U.S. Biologicals) containing 10% fetal calf serum (24). The Δ*lpg5A*::*HYG*/Δ*lpg5A*::*PAC* (designated *lpg5A*$^-$), *ĵlpg5B*::*BSD*/*ĵlpg5B*::*NEO* (designated *lpg5B*$^-$), Δ*lpg5A*::*HYG*/Δ*lpg5A*::*PAC*/*ĵlpg5B*::*BSD*/*ĵlpg5B*::*NEO* (designated *lpg5A*$^-$*/lpg5B*$^-$), *lpg5A*$^-$*/lpg5B*$^-$ [*pIR1SAT-LPG5B-LPG5A*] (designated *lpg5A*$^-$*/lpg5B*$^-$*/+LPG5B+LPG5A*), *ĵlpg1*::*HYG*/*ĵlpg1*/::*PAC* (designated *lpg1*$^-$), Δ*lpg2*::*HYG*/Δ*lpg2*::*HYG* (designated (*lpg2*$^-$), and *lpg2*$^-$*/Rev* mutants were described previously (3, 50, 52, 53). A second "add-back" line was generated by transformation of the *lpg5A*$^-$*/lpg5B*$^-$ line with SwaI-digested pIR1-SAT-LPG5B-LPG5A (strain B5081); this results in integration of the construct within the gene encoding the rRNA small subunit, which confers stable, strong, and uniform expression. The formal name of this line is *lpg5A*$^-$*/lpg5B*$^-$*/SSU*::*IR1SAT-LPG5B-LPG5A.* Prior to study, all lines were passed through mice once by injecting hind footpads of BALB/c mice (Charles River Laboratories, Wilmington, MA) with a large inoculum (1×10^7 to 5×10^7) of stationary-phase parasites and recovering parasites by needle aspiration of the footpad regardless of pathology 4 to 6 weeks afterwards. Cultures were identified by the number of times that they had been inoculated into mice (M1, M2, etc.) and the number of times passed in vitro after recovery from infected animals (P1, P2, etc.). As *L. major* may lose virulence during in vitro culture, parasites were passed no more than six times prior to use. (e.g., M1P6).

Dulbecco modified Eagle medium (DMEM) was purchased from Gibco BRL (under Invitrogen, Carlsbad, CA). Anti-mouse immunoglobulin G (IgG):fluorescein isothiocyanate (FITC) and anti-mouse IgM:Texas red antibodies were from Jackson Immunoresearch (West Grove, PA). Hygromycin B was from Calbiochem (San Diego, CA), puromycin was from Sigma (St. Louis, MO), G418 powder was from BioWhittaker (now under Cambrex Bio Science, Walkersville, MD), phleomycin was from InvivoGen (San Diego, CA), and nourseothricin was from Werner BioAgents (Jena, Germany). Hoechst 33342 nucleic acid dye was purchased from Molecular Probes (now under Invitrogen, Carlsbad, CA). Other reagents were purchased from Sigma or Fisher.

Mouse infections. Female BALB/c mice (6 to 10 weeks old) were purchased from Charles River Laboratories (Wilmington, MA). In a typical experiment, five mice per group were inoculated subcutaneously in the left hind footpad with 10^6 metacyclic or 10^5 amastigote stage parasites. Lesion thickness was measured using a Vernier caliper (Mitutoyo) and defined as the average difference in thickness between infected and uninfected hind footpads for each group of mice (55). Metacyclic promastigotes were prepared using the Ficoll gradient enrichment method (49), and lesion-derived amastigotes were recovered from infected lesions (≥2-mm thickness) as described previously (53). Limiting-dilution assays were performed as described previously (26).

Macrophage infections. Starch-elicited peritoneal macrophages were recovered from BALB/c mice and then plated on glass coverslips (43, 50). Metacyclic parasites were opsonized with C5-deficient serum, resuspended in DMEM containing 10% fetal calf serum, and infected at multiplicity of infection of 10. Lesion-derived amastigotes were infected at a multiplicity of infection of 3 in DMEM containing 10% fetal calf serum (53). After 2 hours, the cells were washed extensively and overlaid with fresh medium, and thereafter medium was changed daily. At 2 hours, 1 day, 2 days, and 5 days postinfection, cells were fixed in 3.7% (vol/vol) formaldehyde in phosphate-buffered saline (PBS) and stained in 2 to 2.5 μg/ml Hoechst 33342 (in PBS) prior to scoring for intracellular parasites.

Western analysis. Western analysis was done as described previously (50). Briefly, 1×10^6 cells (or serial twofold dilutions thereof) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4% polyacrylamide stacking gel and 12.5% resolving gel) and transferred to nitrocellulose membranes. WIC79.3 antibody, which recognizes Gal-modified PG repeating units, was used at a dilution of 1:500 (6). The anti-gp63 antibody 235 was used at a 1:1,000 dilution (5). A polyclonal gp46 antibody (a kind gift from D. McMahon-Pratt) was used at a 1:20,000 dilution (31). Where necessary, blots were treated with Western Re-Probe (Genotech, St. Louis, MO) and probed with a monoclonal antitubulin antibody (Sigma) at a dilution of 1:50,000. Anti-mouse IgG:horseradish peroxidase and anti-rabbit IgG:horseradish peroxidase antibodies were from Amersham (now part of GE Healthcare, Piscataway, NJ), and chemiluminescence reagents were from Perkin-Elmer (Wellesley, MA).

Indirect immunofluorescence assay. TAT-1 antibody, which recognizes trypanosome tubulin, was a kind gift from K. Gull (61). Macrophages infected for 2 days with lesion-derived amastigotes were fixed for 1 minute in 3.7% formaldehyde in PBS, followed by permeabilization with ice-cold ethanol for 15 min on ice. The cells were rehydrated for 10 min in PBS, followed by sequential incubations with TAT-1 at a 1:2 dilution, anti-mouse IgG:TR at a 1:100 dilution, WIC79.3 at a 1:500 dilution, and anti-mouse IgG:FITC at a 1:100 dilution. Cells were washed with PBS between incubations. Cells were mounted in 50% (vol/vol) glycerol in PBS, sealed, and visualized on an Olympus AX70 fluorescence microscope.

Mannan extraction and analysis. Parasites were harvested at densities of 0.7×10^7 to 2.0×10^7 cells/ml and extracted for mannans as described elsewhere (44). Briefly, cells (2×10^9 to 5×10^9) were extracted in chloroform-methanol-water (1:2:0.8) for 2 h with sonication. The samples were centrifuged at $15{,}000 \times g$ for 5 min, and the supernatant was collected, dried under a stream of nitrogen, and partitioned with water-saturated 1-butanol. The water phase (containing the mannans) was desalted by passage through a 1-ml column of AG50-X12 (H^+) layered

Downloaded from http://iai.asm.org/ on March 10, 2014 by Washington University in St. Louis

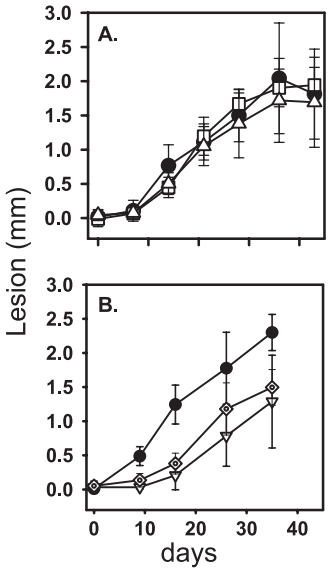

FIG. 2. The *lpg5A*$^-$/*lpg5B*$^-$ mutant shows a delayed emergence of lesion pathology, while the single-gene mutants behave as the WT. The left hind footpads of BALB/c mice ($n = 5$) were injected with 10^6 metacyclic parasites and monitored for lesion growth, defined as the mean difference in thickness between the injected footpad and the uninjected hind footpad. (A) Lesion assay comparing WT (●), *lpg5A*$^-$ (■), and *lpg5B*$^-$ (△) promastigotes. (B) Promastigotes that had been differentiated from *lpg5A*$^-$/*lpg5B*$^-$ lesion amastigotes (M2P4) (◇) were used to infect naïve mice and compared to the WT (●) and the *lpg5A*$^-$/*lpg5B*$^-$ mutant (M1P3) (▽). Error bars indicate standard deviations.

over AG1-X8 (OH^-). The desalted mannans were dried with a Speedvac drier and then fluorophore labeled at the reducing ends with 8-aminonaphthalene-1,3,6-trisulfate and analyzed by fluorophore-assisted carbohydrate electrophoresis (FACE) according to manufacturer's specifications (Glyko Inc., Novato, CA).

RESULTS

The *lpg5A*$^-$/*lpg5B*$^-$ mutant shows a delayed lesion emergence phenotype. Parasites lacking *LPG5A*, *LPG5B*, or both genes, were tested for their ability to mount an infection in susceptible mice. Cultured in vitro as promastigotes, these mutant strains grow at similar rates and to similar densities as the WT (3). BALB/c mice infected with purified metacyclic promastigotes from either the *lpg5A*$^-$ or *lpg5B*$^-$ mutant developed lesions similarly to those infected with the WT (Fig. 2A). In contrast, the *lpg5A*$^-$/*lpg5B*$^-$ mutant showed a delay in the emergence of lesions, of about 10 days when an inoculum of 10^6 metacyclic promastigotes was used (Fig. 2B and 3A) and of about 30 days with an inoculum of 10^5 metacyclic promastigotes (not shown). Delays in lesion appearance were seen in all five independent experiments (data not shown). Following the appearance of overt pathology, the lesions grew in size at rates similar to those caused by the WT (Fig. 2B).

Several essential controls behaved as anticipated. First, to test if the *lpg5A*$^-$/*lpg5B*$^-$ mutant parasites that emerged in growing lesions differed from the inoculated parasites by adaptation or reversion, cells were recovered from the infected animal footpads and allowed to differentiate back to promastigotes, and purified metacyclics were retested in naïve mice. As before, lesion emergence was delayed, arguing against adaptation or reversion among the *lpg5A*$^-$/*lpg5B*$^-$ parasites in lesions (Fig. 2B). Second, to confirm that the growth delay arose specifically from loss of *LPG5A* and *LPG5B*, expression of both genes was restored simultaneously by transfection of *lpg5A*$^-$/*lpg5B*$^-$ parasites with plasmid pIR1SAT-LPG5B-LPG5A, which was shown previously to confer complete restoration of PG synthesis (3). As expected, with the *lpg5A*$^-$/*lpg5B*$^-$/+*LPG5B*+*LPG5A* parasites the delay in lesion emergence in mice was alleviated, with lesions appearing and progressing thereafter like those caused by the WT (Fig. 3A).

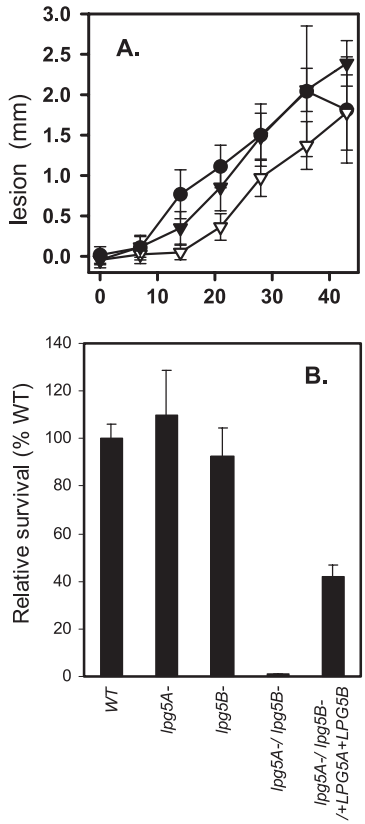

FIG. 3. Defects in promastigote infections with the *lpg5A*$^-$/*lpg5B*$^-$ mutant. (A) Lesion assay comparing WT (●), *lpg5A*$^-$/*lpg5B*$^-$ (▽), and *lpg5A*$^-$/*lpg5B*$^-$/*pIR1-LPG5B-LPG5A* strains. BALB/c mice were injected in one hind footpad and monitored for lesion growth as described in Materials and Methods. (B) Macrophage infection. Peritoneal macrophages were infected with Ficoll-enriched metacyclic parasites opsonized in C5-deficient serum and scored for intracellular parasites after 5 days. Data are plotted as percent survival based on the average number of parasites/macrophage relative to WT parasites (1.3/macrophage). Error bars indicate standard deviations.

Limiting-dilution assays were performed to assess whether the initial delay in lesion pathology reflected decreased parasite numbers (Fig. 4). These experiments showed that indeed parasite numbers were much less than WT parasite numbers for the *lpg5A*$^-$/*lpg5B*$^-$ mutant, from 18- to 270-fold less when measured after 10 or 28 days, respectively (Fig. 4).

***lpg5A*$^-$/*lpg5B*$^-$ metacyclic promastigotes are defective in establishment of macrophage infections.** The delayed-lesion phenotype observed in the *lpg5A*$^-$/*lpg5B*$^-$ mutant was reminiscent of that for the *lpg1*$^-$ mutant (when inoculated at comparable

Downloaded from http://iai.asm.org/ on March 10, 2014 by Washington University in St. Louis

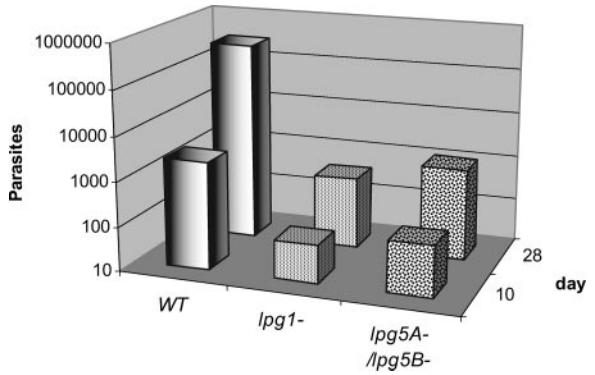

FIG. 4. Limiting-dilution assay of *L. major* mutant survival following infection of BALB/c mice. Metacyclic WT, *lpg1*$^-$, and *lpg5A*$^-$/*lpg5B*$^-$ parasites (10^5) were purified and inoculated into the hind footpad of BALB/c mice as described in Materials and Methods (three mice/group). With this number of parasites, WT infections showed only small lesions by day 28 (0.04 ± 0.04 mm). At the indicated times the animals were sacrificed and the number of parasites per footpad was assessed by limiting dilution.

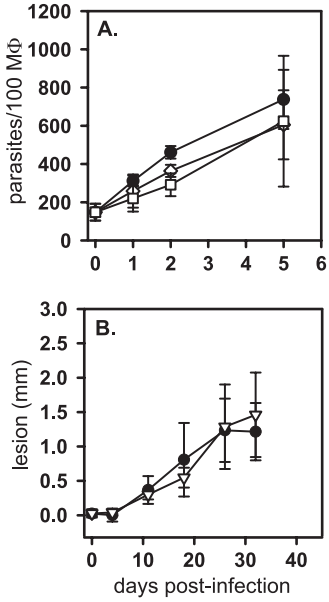

FIG. 5. Amastigote survival, replication, and lesion induction are not affected by loss of *LPG5A* and *LPG5B*. (A) Macrophage (MΦ) infection showing amastigote survival. Peritoneal macrophages were infected with three amastigotes/macrophage, and survival was monitored over time as described in Materials and Methods. Symbols correspond to WT (●), *lpg1*$^-$ (□), and *lpg5A*$^-$/*lpg5B*$^-$ (◇) strains. (B) Footpad lesion assay. BALB/c mice were infected with 10^5 lesion amastigotes per footpad and monitored for lesion growth as outlined in Materials and Methods. Symbols correspond to WT (●) and *lpg5A*$^-$/*lpg5B*$^-$ (▽) strains. Error bars indicate standard deviations.

numbers of infectious metacyclic promastigotes), which lacked only LPG and was thus defective in the initial establishment steps of intracellular infection (50, 51). In fact, the decreased initial parasite levels seen in vivo for the *lpg5A*$^-$/*lpg5B*$^-$ mutant were very similar to those seen with the *lpg1*$^-$ mutant tested in parallel in this experiment (Fig. 4). To pursue this, mouse peritoneal macrophages were infected with *lpg5A*$^-$/*lpg5B*$^-$ metacyclic promastigotes and parasite survival scored (Fig. 3B). Following an initial decrease in number, the WT and the single *lpg5A*$^-$ or *lpg5B*$^-$ mutant *L. major* survived and replicated thereafter (Fig. 3B and data not shown). In contrast, the *lpg5A*$^-$/*lpg5B*$^-$ mutant experienced a more drastic decline within the first 1 to 2 days and did not increase thereafter (Fig. 3B and data not shown). Restoration of *LPG5A* and *LPG5B* expression in the *lpg5A*$^-$/*lpg5B*$^-$ mutant (*lpg5A*$^-$/*lpg5B*$^-$/*+LPG5A+LPG5B*) restored macrophage survival, albeit to levels somewhat less than seen with the WT (Fig. 3B). In one experiment we found that integration of the LPG5A and LPG5B expression vector into the rRNA locus, which typically increases transgene expression, restored *lpg5A*$^-$/*lpg5B*$^-$ macrophage survival fully (data not shown).

***lpg5A*$^-$/*lpg5B*$^-$ amastigotes are fully virulent.** The ability of the *lpg5A*$^-$/*lpg5B*$^-$ parasites to induce pathology following an initial delay could be explained by the need for these gene products during the initial establishment of macrophages, such as PGs (including LPG and/or PPGs), but not for survival as amastigotes thereafter, as seen previously for LPG with the *lpg1*$^-$ mutant (50). To test this idea, we purified *lpg5A*$^-$/*lpg5B*$^-$ amastigotes from visibly progressing lesions (such as those shown in Fig. 2B or 3A) and used them directly in macrophage or mouse infections (Fig. 4).

In macrophage infections, WT amastigotes entered and replicated rapidly thereafter without any delay (Fig. 5A). Similar results were obtained with *lpg5A*$^-$/*lpg5B*$^-$ amastigotes and with *lpg1*$^-$ amastigotes, used for comparison (Fig. 5A). In mouse infections, the *lpg5A*$^-$/*lpg5B*$^-$ amastigotes induced lesions which appeared and progressed at the same rate as those induced by the WT (Fig. 5B). These data argued that like *lpg1*$^-$ and unlike *lpg2*$^-$ *L. major* (50, 51), *lpg5A*$^-$/*lpg5B*$^-$ amastigotes are as virulent as WT amastigotes.

***lpg5A*$^-$/*lpg5B*$^-$ amastigotes lack PGs.** In the previous studies leading to the identification of the *LPG5A* and *LPG5B* UDP-Gal NSTs, 10 additional NSTs were identified, including several showing relationship to NSTs known in other species to transport UDP-sugars including UDP-Gal (3). This raised the possibility that one of these NSTs could mediate UDP-Gal uptake in amastigotes, by either normal developmental up-regulation or selection during the derivation of the *lpg5A*$^-$/*lpg5B*$^-$mutant. This could then lead to PG expression in *lpg5A*$^-$/*lpg5B*$^-$ amastigotes, and mitigation of their phenotype, if PGs were the ultimate source of the amastigote defect.

To test this, macrophages were infected with amastigotes purified from lesions as described above and, after 2 days, were examined by indirect immunofluorescence with anti-PG monoclonal antibodies. Macrophages and parasites were visualized by phase microscopy and reactivity with an α-tubulin antiserum (Fig. 6A to F). In macrophages infected by WT amastigotes, PGs were readily detected, not only on the parasite itself but also shed into the macrophage, as seen previously (Fig. 6G) (19, 50). In contrast, *lpg5A*$^-$/*lpg5B*$^-$ amastigotes were devoid of anti-PG reactivity (Fig. 6H). As a control, we performed parallel tests with amastigotes from the *lpg2*$^-$/*Rev* mutant, which similarly survives within macrophages despite the absence of PGs (Fig. 6I). Thus, the survival of the *lpg5A*$^-$/*lpg5B*$^-$ amastigotes cannot be explained by activation of an alternative NST or other pathway leading to PG synthesis.

Downloaded from http://iai.asm.org/ on March 10, 2014 by Washington University in St. Louis

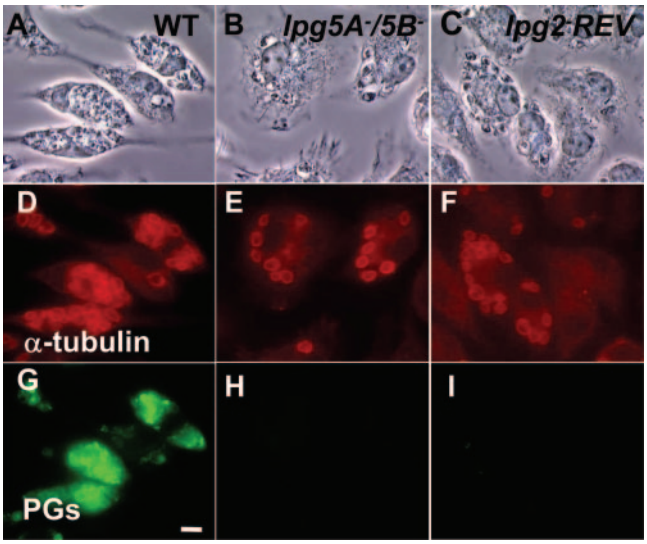

FIG. 6. *lpg5A*$^{-}$/*lpg5B*$^{-}$ amastigotes lack PGs. Macrophages infected with lesion-derived amastigotes were fixed and permeabilized before probing for PGs using WIC79.3 and tubulin using an anti-trypanosome tubulin antibody. (A to C) Phase-contrast image. (D to F) Detection of parasite α-tubulin (Texas Red-conjugated secondary antibody). (G to I) PGs detected with WIC79.3 antibody (FITC-conjugated secondary antibody). WT amastigotes (A, D, and G), *lpg5A*$^{-}$/*lpg5B*$^{-}$ amastigotes (B, E, and H), and *lpg2*$^{-}$/*Rev* amastigotes (C, F, and I) were used. Bar, 10 μm.

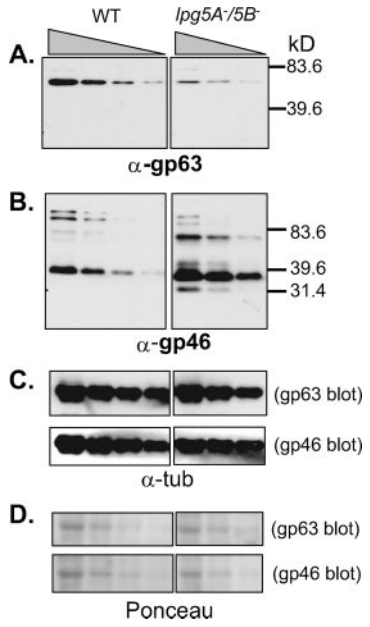

FIG. 7. Western analysis of GPI-anchored proteins in the WT versus the *lpg5A*$^{-}$/*lpg5B*$^{-}$ mutant. (A and B) Serial twofold dilutions of whole-cell extracts were subjected to Western blot analysis with antiserum to GPI-anchored protein gp63 (A) or gp46 (B). (C) The membranes used for the experiments shown in panels A and B were stripped and immunoblotted with antiserum to α-tubulin. (D) Total protein (revealed by Ponceau S staining) bound to the membranes used in panels A to C prior to immunoblotting.

The levels of two GPI-anchored proteins are minimally altered in the *lpg5A*$^{-}$/*lpg5B*$^{-}$ mutant. Another explanation for the results above was a compensatory up-regulation of other parasite membrane components. While neither *Leishmania* protein GPI anchors nor N-linked glycans have been shown to contain galactose (13, 40), current data are not sufficient to rule out the occurrence at low levels, and both modifications occur in trypanosomes, where they may be essential (47). We thus examined the pattern and levels of gp63, encoding the abundant surface protease leishmanolysin, and gp46/PSA-2. Both of these proteins are encoded by large gene families and show higher expression in the promastigote stage (34, 37). Immunoblot analysis showed a modest change in their levels, with a two- to fourfold decrease in gp63 expression (Fig. 7A), versus a two- to fourfold increase of gp46 (Fig. 7B), compared to the WT. We believe that these relatively modest differences are unlikely to be significant and may reflect clonal variation arising during the rounds of transfection and cloning on semisolid medium employed in our studies. Indeed, small variations in gp63 expression and/or gene copy number in various clonal variants have been reported by many laboratories, often without strong correlation to virulence (11, 36).

Mannan levels in *lpg2*$^{-}$ *L. major* and *L. mexicana* are normal. *Leishmania* spp. synthesize an abundant cytoplasmic β-1,2-mannan (25, 42), which is suggested to play an important role during environmental stress and as an energy reservoir in amastigote survival (44). In principle, the activity of the *LPG2* Golgi GDP-Man transporter should have little or no effect on cytoplasmic mannan synthesis (Fig. 1A), but given the results obtained with the *lpg5A*$^{-}$/*lpg5B*$^{-}$ mutant, this supposition warranted testing.

Total cellular mannans were extracted from WT and *lpg2*$^{-}$ *L. major* and *L. mexicana*, fluorophore derivatized, and analyzed by FACE analysis (Fig. 8). WT *L. mexicana* mannans were highly polymerized, ranging upwards of 10 mannose residues, and were absent in a null mutant (*gdmp*$^{-}$) lacking cytoplasmic GDP-Man pyrophosphorylase (Fig. 8A) (44). Notably, mannan levels were unaltered in the *L. mexicana* or *L. major* *lpg2*$^{-}$ mutants relative to the WT (Fig. 8A and B). Similar results were obtained with WT *L. donovani* and *lpg2*$^{-}$ mutants (data not shown). Interestingly, the degree of mannan polymerization in *L. major* (<10) was less than that in *L. mexicana*

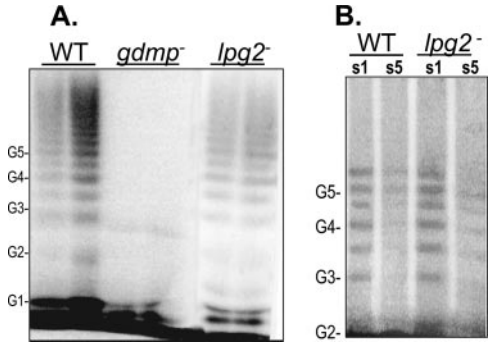

FIG. 8. Mannan levels are unchanged in *Leishmania* *lpg2*$^{-}$ mutants. Total cellular mannans were purified, labeled, and subjected to FACE analysis as described in Materials and Methods. (A) Stationary-phase WT, *gdmp*$^{-}$, and *lpg2*$^{-}$ *L. mexicana*. (B) Stationary-phase WT and *lpg2*$^{-}$ *L. major*. s1, 1 day in stationary phase; s5, 5 days in stationary phase.

Downloaded from http://iai.asm.org/ on March 10, 2014 by Washington University in St. Louis

upon entry into stationary phase, and it declined further after 5 days in stationary phase (Fig. 7B). This would be consistent with a role for mannan in energy metabolism in the metacyclic as well as amastigote stage of *Leishmania* parasites (44). Regardless, the equivalence of mannan levels in WT and *lpg2*$^-$ comparisons within both *Leishmania* species makes it unlikely that mannans contribute to *LPG2*-dependent virulence effects.

DISCUSSION

The results presented here show that metacyclic promastigotes of the *L. major* PG-deficient *lpg5A*$^-$/*lpg5B*$^-$ mutant are attenuated in the early steps of infection of mammalian macrophages but thereafter are able to survive, replicate, and induce disease normally as amastigotes. In this regard the phenotype of this mutant most closely resembles that of the *lpg1*$^-$ mutant of *L. major*, which lacks LPG alone (50, 51). However, the macrophage survival defect of the *lpg5A*$^-$/*lpg5B*$^-$ metacyclic promastigotes was more pronounced (<1%) (Fig. 3) and was nearly as severe as that of the completely PG-deficient *lpg2*$^-$ *L. major* mutant (52). As expected, restoration of *LPG5A* and *LPG5B* expression in the *lpg5A*$^-$/*lpg5B*$^-$ mutant restored promastigote virulence to levels identical or close to that of the WT (Fig. 3 and 5). In total, these studies further support the importance of PGs, including LPG, in the establishment of successful infections in host macrophages by *L. major*.

We ruled out the possibility that the amastigote virulence phenotype arose through second-site alterations not involving the *LPG5A* and *LPG5B* genes (Fig. 2B) or that the *lpg5A*$^-$/*lpg5B*$^-$ parasites possessed amastigote-specific UDP-Gal NSTs or other alternate pathways leading to PG synthesis that could potentially bypass the *lpg5A*$^-$/*lpg5B*$^-$ mutant (Fig. 6) (3). Thus, in contrast to the situation for promastigote virulence, we conclude that PGs are not essential for amastigote virulence in *L. major*, as suggested previously for *L. mexicana* (22).

Our findings were also consistent with previous work on a second-site revertant of *L. major lpg2*$^-$, *lpg2*$^-$/*Rev*, which, like the *lpg5A*$^-$/*lpg5B*$^-$ mutant, lacked PGs and showed initial macrophage establishment phase defects as promastigotes but otherwise retained amastigote virulence in mouse infections (53). As yet the nature of the second-site mutation in the *lpg2*$^-$/*Rev* mutant and its consequences for glycoconjugate synthesis (if any) have not been determined.

These data suggest that there is a PG-independent, *LPG2*-dependent pathway required for amastigote virulence specifically in *L. major*. Given the known role of LPG2 in Golgi nucleotide sugar transport, it seems most likely that a deficiency of some LPG2-dependent glycoconjugate underlies the virulence phenotype. Since we have now ruled out all of the known major glycoconjugate candidates of *L. major*, it seems likely that an uncharacterized, and most likely nonabundant, glycoconjugate is responsible. The intense study that the Man-containing glycoconjugate pathway has received experimentally could be viewed as arguing against the involvement of a Man-containing glycoconjugate (14, 44). Nonetheless, when new methods and approaches are applied, new glycoconjugates may emerge, as exemplified by the recent discovery of the abundant cytoplasmic mannan that may play an important role in amastigote virulence (44). However, we have shown here that mannan levels exhibit no *LPG2*-dependent changes in either *L. major* or *L. mexicana* (Fig. 7), as anticipated, eliminating this cytoplasmic molecule as a candidate (44). While GDP-Man synthetic mutants of *L. mexicana* are avirulent, their lack of general dolichol-mediated protein N glycosylation (Fig. 1B) and structural abnormalities make it difficult to attribute virulence defects to any specific glycoconjugate (14, 54). Thus, the possibility that undiscovered, less-abundant LPG2-dependent Man-containing glycoconjugates essential for *L. major* amastigote virulence exist cannot be excluded.

LPG2 is a multispecific GDP-sugar transporter with specificities for both GDP-D-Ara$_p$ and GDP-L-Fuc in addition to GDP-Man (20, 28). Potentially the critical role of *LPG2* involves these sugars, most likely arabinose (Ara), since Fuc has not been reported in *L. major* (48). In *L. major*, two D-Ara$_p$ transferases mediating terminal arabinosylation of Gal-modified PG repeats of metacyclic promastigotes have been identified, SCA1 and SCA2 (10). The terminal D-Ara$_p$ substitutions disrupt binding of LPG to the sand fly midgut lectin, allowing detachment of *L. major* promastigotes and subsequent transmission (23, 30, 41). We believe that the general lack of PG dependency suggests that it is unlikely that Ara-containing or other modifications of the PG repeating unit account for *LPG2*-dependent amastigote virulence, although arabinosylation of other, as-yet-unknown glycoconjugates remains a possibility. Interestingly, Ara-containing glycolipids have been reported previously in *L. donovani* (62); however, their role in virulence has not been investigated.

In recent work we have shown that *lpg2*$^-$ *L. donovani* (16) also shows decreased virulence in both mouse and hamster infections (M. Wilson and S. M. Beverley, unpublished data). These findings raise the important question as to why *L. mexicana* does not show the same *LPG2* dependency for amastigote virulence as in *L. major* and *L. donovani*, despite the extensive similarities in the structures of known *LPG2*-dependent glycoconjugates. Potentially *L. mexicana* may synthesize novel glycoconjugates and/or other molecules that fulfill this role in the absence of *LPG2*. Alternatively, the molecules required for amastigote virulence may differ between the species due to differences in the parasite biology, with one example being the natures of the parasitophorous vacuoles formed by *L. mexicana* versus *L. donovani* and *L. major* (4). The availability of well-characterized mutants of all three species should greatly facilitate the resolution of this important question in the future.

ACKNOWLEDGMENTS

We thank T. Ilg and J. Mottram for the *L. mexicana lpg2*$^-$ and *gdmp*$^-$ mutants; K. Gull, R. McMaster, and D. McMahon Pratt for antisera; M. Wilson for discussions and preliminary results for the *L. donovani lpg2*$^-$ mutant; N. Novozhilova for discussions; and M. Mandell and K. Zhang for comments.

This work was supported by NIH grant AI 31078 to S.M.B. and S.J.T.

REFERENCES

1. **Bates, P. A., and D. M. Dwyer.** 1987. Biosynthesis and secretion of acid phosphatase by *Leishmania donovani* promastigotes. Mol. Biochem. Parasitol. **26:**289–296.
2. **Capul, A. A.** 2005. UDP-galactose transporters in *Leishmania.* Ph.D. thesis. Washington University, St. Louis, MO.
3. **Capul, A. A., T. Barron, D. E. Dobson, S. J. Turco, and S. M. Beverley.** 2007. Two functionally divergent UDP-GAL nucleotide-sugar transporters partic-

Downloaded from http://iai.asm.org/ on March 10, 2014 by Washington University in St. Louis

ipate in phosphoglycan synthesis in *Leishmania major*. J. Biol. Chem. **282:** 14006–14017.
4. **Castro, R., K. Scott, T. Jordan, B. Evans, J. Craig, E. L. Peters, and K. Swier.** 2006. The ultrastructure of the parasitophorous vacuole formed by *Leishmania major*. J. Parasitol. **92:**1162–1170.
5. **Connell, N. D., E. Medina-Acosta, W. R. McMaster, B. R. Bloom, and D. G. Russell.** 1993. Effective immunization against cutaneous leishmaniasis with recombinant bacille Calmette-Guerin expressing the *Leishmania* surface proteinase gp63. Proc. Natl. Acad. Sci. USA **90:**11473–11477.
6. **de Ibarra, A. A., J. G. Howard, and D. Snary.** 1982. Monoclonal antibodies to *Leishmania tropica major:* specificities and antigen location. Parasitology **85:**523–531.
7. **Denny, P. W., D. Goulding, M. A. Ferguson, and D. F. Smith.** 2004. Sphingolipid-free *Leishmania* are defective in membrane trafficking, differentiation and infectivity. Mol. Microbiol. **52:**313–327.
8. **Descoteaux, A., and S. J. Turco.** 2002. Functional aspects of the *Leishmania donovani* lipophosphoglycan during macrophage infection. Microbes Infect. **4:**975–981.
9. **Desjeux, P.** 2004. Leishmaniasis: current situation and new perspectives. Comp. Immunol. Microbiol. Infect. Dis. **27:**305–318.
10. **Dobson, D. E., B. J. Mengeling, S. Cilmi, S. Hickerson, S. J. Turco, and S. M. Beverley.** 2003. Identification of genes encoding arabinosyltransferases (SCA) mediating developmental modifications of lipophosphoglycan required for sand fly transmission of *Leishmania major*. J. Biol. Chem. **278:** 28840–28848.
11. **Espinoza, J. R., A. C. Skinner, C. R. Davies, A. Llanos-Cuentas, J. Arevalo, C. Dye, W. R. McMaster, J. W. Ajioka, and J. M. Blackwell.** 1995. Extensive polymorphism at the Gp63 locus in field isolates of *Leishmania peruviana*. Mol. Biochem. Parasitol. **72:**203–213.
12. **Ferguson, M. A.** 1999. The structure, biosynthesis and functions of glycosylphosphatidylinositol anchors, and the contributions of trypanosome research. J. Cell Sci. **112:**2799–2809.
13. **Ferguson, M. A.** 1997. The surface glycoconjugates of trypanosomatid parasites. Philos. Trans. R. Soc. London **352:**1295–1302.
14. **Garami, A., and T. Ilg.** 2001. Disruption of mannose activation in *Leishmania mexicana:* GDP-mannose pyrophosphorylase is required for virulence, but not for viability. EMBO J. **20:**3657–3666.
15. **Garami, A., and T. Ilg.** 2001. The role of phosphomannose isomerase in *Leishmania mexicana* glycoconjugate synthesis and virulence. J. Biol. Chem. **276:**6566–6575.
16. **Goyard, S., H. Segawa, J. Gordon, M. Showalter, R. Duncan, S. J. Turco, and S. M. Beverley.** 2003. An in vitro system for developmental and genetic studies of *Leishmania donovani* phosphoglycans. Mol. Biochem. Parasitol. **130:**31–42.
17. **Guha-Niyogi, A., D. R. Sullivan, and S. J. Turco.** 2001. Glycoconjugate structures of parasitic protozoa. Glycobiology **11:**45R–59R.
18. **Ha, D. S., J. K. Schwarz, S. J. Turco, and S. M. Beverley.** 1996. Use of the green fluorescent protein as a marker in transfected *Leishmania*. Mol. Biochem. Parasitol. **77:**57–64.
19. **Handman, E.** 1990. Study of *Leishmania major*-infected macrophages by use of lipophosphoglycan-specific monoclonal antibodies. Infect. Immun. **58:** 2297–2302.
20. **Hong, K., D. Ma, S. M. Beverley, and S. J. Turco.** 2000. The *Leishmania* GDP-mannose transporter is an autonomous, multi-specific, hexameric complex of LPG2 subunits. Biochemistry **39:**2013–2022.
21. **Ilg, T.** 2000. Proteophosphoglycans of *Leishmania*. Parasitol. Today **16:**489–497.
22. **Ilg, T., M. Demar, and D. Harbecke.** 2001. Phosphoglycan repeat-deficient *Leishmania mexicana* parasites remain infectious to macrophages and mice. J. Biol. Chem. **276:**4988–4997.
23. **Kamhawi, S., M. Ramalho-Ortigao, V. M. Pham, S. Kumar, P. G. Lawyer, S. J. Turco, C. Barillas-Mury, D. L. Sacks, and J. G. Valenzuela.** 2004. A role for insect galectins in parasite survival. Cell **119:**329–341.
24. **Kapler, G. M., C. M. Coburn, and S. M. Beverley.** 1990. Stable transfection of the human parasite *Leishmania major* delineates a 30-kilobase region sufficient for extrachromosomal replication and expression. Mol. Cell. Biol. **10:**1084–1094.
25. **Keegan, F. P., and J. J. Blum.** 1992. Utilization of a carbohydrate reserve comprised primarily of mannose by *Leishmania donovani*. Mol. Biochem. Parasitol. **53:**193–200.
26. **Lima, H. C., J. A. Bleyenberg, and R. G. Titus.** 1997. A simple method for quantifying *Leishmania* in tissues of infected animals. Parasitol. Today **13:** 80–82.
27. **Lodge, R., and A. Descoteaux.** 2005. Modulation of phagolysosome biogenesis by the lipophosphoglycan of *Leishmania*. Clin. Immunol. **114:**256–265.
28. **Ma, D., D. G. Russell, S. M. Beverley, and S. J. Turco.** 1997. Golgi GDP-mannose uptake requires *Leishmania LPG2*. A member of a eukaryotic family of putative nucleotide-sugar transporters. J. Biol. Chem. **272:**3799–3805.
29. **McConville, M. J., K. A. Mullin, S. C. Ilgoutz, and R. D. Teasdale.** 2002. Secretory pathway of trypanosomatid parasites. Microbiol. Mol. Biol. Rev. **66:**122–154.
30. **McConville, M. J., S. J. Turco, M. A. Ferguson, and D. L. Sacks.** 1992. Developmental modification of lipophosphoglycan during the differentiation of *Leishmania major* promastigotes to an infectious stage. EMBO J. **11:**3593–3600.
31. **McMahon-Pratt, D., Y. Traub-Cseko, K. L. Lohman, D. D. Rogers, and S. M. Beverley.** 1992. Loss of the GP46/M-2 surface membrane glycoprotein gene family in the *Leishmania braziliensis* complex. Mol. Biochem. Parasitol. **50:** 151–160.
32. **Mengeling, B. J., S. M. Beverley, and S. J. Turco.** 1997. Designing glycoconjugate biosynthesis for an insidious intent: phosphoglycan assembly in *Leishmania* parasites. Glycobiology **7:**873–880.
33. **Modabber, F.** 1995. Vaccines against leishmaniasis. Ann. Trop. Med. Parasitol. **89**(Suppl. 1)**:**83–88.
34. **Montgomery, J., T. Ilg, J. K. Thompson, B. Kobe, and E. Handman.** 2000. Identification and predicted structure of a leucine-rich repeat motif shared by *Leishmania major* proteophosphoglycan and parasite surface antigen 2. Mol. Biochem. Parasitol. **107:**289–295.
35. **Murray, H. W., J. D. Berman, C. R. Davies, and N. G. Saravia.** 2005. Advances in leishmaniasis. Lancet **366:**1561–1577.
36. **Murray, P. J., E. Handman, T. A. Glaser, and T. W. Spithill.** 1990. *Leishmania major*: expression and gene structure of the glycoprotein 63 molecule in virulent and avirulent clones and strains. Exp. Parasitol. **71:**294–304.
37. **Myung, K. S., J. K. Beetham, M. E. Wilson, and J. E. Donelson.** 2002. Comparison of the post-transcriptional regulation of the mRNAs for the surface proteins PSA (GP46) and MSP (GP63) of Leishmania chagasi. J. Biol. Chem. **277:**16489–16497.
38. **Naderer, T., M. A. Ellis, M. F. Sernee, D. P. De Souza, J. Curtis, E. Handman, and M. J. McConville.** 2006. Virulence of *Leishmania major* in macrophages and mice requires the gluconeogenic enzyme fructose-1,6-bisphosphatase. Proc. Natl. Acad. Sci. USA **103:**5502–5507.
39. **Naderer, T., J. E. Vince, and M. J. McConville.** 2004. Surface determinants of *Leishmania* parasites and their role in infectivity in the mammalian host. Curr. Mol. Med. **4:**649–665.
40. **Olafson, R. W., J. R. Thomas, M. A. Ferguson, R. A. Dwek, M. Chaudhuri, K. P. Chang, and T. W. Rademacher.** 1990. Structures of the N-linked oligosaccharides of Gp63, the major surface glycoprotein, from Leishmania mexicana amazonensis. J. Biol. Chem. **265:**12240–12247.
41. **Pimenta, P. F., S. J. Turco, M. J. McConville, P. G. Lawyer, P. V. Perkins, and D. L. Sacks.** 1992. Stage-specific adhesion of *Leishmania* promastigotes to the sandfly midgut. Science **256:**1812–1815.
42. **Previato, J. O., M. T. Xavier, R. P. Brazil, P. A. Gorin, and L. Mendonca-Previato.** 1984. Formation of (1–2)-linked beta-D-mannopyranan by *Leishmania mexicana amazonensis*: relationship with certain *Crithidia* and *Herpetomonas* species. J. Parasitol. **70:**449–450.
43. **Racoosin, E. L., and S. M. Beverley.** 1997. *Leishmania major*: promastigotes induce expression of a subset of chemokine genes in murine macrophages. Exp. Parasitol. **85:**283–295.
44. **Ralton, J. E., T. Naderer, H. L. Piraino, T. A. Bashtannyk, J. M. Callaghan, and M. J. McConville.** 2003. Evidence that intracellular beta1-2 mannan is a virulence factor in *Leishmania* parasites. J. Biol. Chem. **278:**40757–40763.
45. **Remme, J. H., E. Blas, L. Chitsulo, P. M. Desjeux, H. D. Engers, T. P. Kanyok, J. F. Kayondo, D. W. Kioy, V. Kumaraswami, J. K. Lazdins, P. P. Nunn, A. Oduola, R. G. Ridley, Y. T. Toure, F. Zicker, and C. M. Morel.** 2002. Strategic emphases for tropical diseases research: a TDR perspective. Trends Microbiol. **10:**435–440.
46. **Roper, J. R., M. L. Guther, J. I. Macrae, A. R. Prescott, I. Hallyburton, A. Acosta-Serrano, and M. A. Ferguson.** 2005. The suppression of galactose metabolism in procyclic form *Trypanosoma brucei* causes cessation of cell growth and alters procyclin glycoprotein structure and copy number. J. Biol. Chem. **280:**19728–19736.
47. **Roper, J. R., M. L. Guther, K. G. Milne, and M. A. Ferguson.** 2002. Galactose metabolism is essential for the African sleeping sickness parasite *Trypanosoma brucei*. Proc. Natl. Acad. Sci. USA **99:**5884–5889.
48. **Schneider, P., M. J. McConville, and M. A. Ferguson.** 1994. Characterization of GDP-alpha-D-arabinopyranose, the precursor of D-Arap in *Leishmania major* lipophosphoglycan. J. Biol. Chem. **269:**18332–18337.
49. **Spath, G. F., and S. M. Beverley.** 2001. A lipophosphoglycan-independent method for isolation of infective *Leishmania* metacyclic promastigotes by density gradient centrifugation. Exp. Parasitol. **99:**97–103.
50. **Spath, G. F., L. Epstein, B. Leader, S. M. Singer, H. A. Avila, S. J. Turco, and S. M. Beverley.** 2000. Lipophosphoglycan is a virulence factor distinct from related glycoconjugates in the protozoan parasite *Leishmania major*. Proc. Natl. Acad. Sci. USA **97:**9258–9263.
51. **Spath, G. F., L. A. Garraway, S. J. Turco, and S. M. Beverley.** 2003. The role(s) of lipophosphoglycan (LPG) in the establishment of *Leishmania major* infections in mammalian hosts. Proc. Natl. Acad. Sci. USA **100:**9536–9541.
52. **Spath, G. F., L. F. Lye, H. Segawa, D. L. Sacks, S. J. Turco, and S. M. Beverley.** 2003. Persistence without pathology in phosphoglycan-deficient *Leishmania major*. Science **301:**1241–1243.
53. **Spath, G. F., L. F. Lye, H. Segawa, S. J. Turco, and S. M. Beverley.** 2004. Identification of a compensatory mutant (*lpg2*$^{-}$*Rev*) of *Leishmania major*

Downloaded from http://iai.asm.org/ on March 10, 2014 by Washington University in St. Louis

able to survive as amastigotes within macrophages without *LPG2*-dependent glycoconjugates and its significance to virulence and immunization strategies. Infect. Immun. **72:**3622–3627.
54. **Stewart, J., J. Curtis, T. P. Spurck, T. Ilg, A. Garami, T. Baldwin, N. Courret, G. I. McFadden, A. Davis, and E. Handman.** 2005. Characterisation of a *Leishmania mexicana* knockout lacking guanosine diphosphate-mannose pyrophosphorylase. Int. J. Parasitol. **35:**861–873.
55. **Titus, R. G., I. Muller, P. Kimsey, A. Cerny, R. Behin, R. M. Zinkernagel, and J. A. Louis.** 1991. Exacerbation of experimental murine cutaneous leishmaniasis with CD4+ *Leishmania major*-specific T cell lines or clones which secrete interferon-gamma and mediate parasite-specific delayed-type hypersensitivity. Eur. J. Immunol. **21:**559–567.
56. **Turco, S. J., and A. Descoteaux.** 1992. The lipophosphoglycan of *Leishmania* parasites. Annu. Rev. Microbiol. **46:**65–94.
57. **Turco, S. J., G. F. Spath, and S. M. Beverley.** 2001. Is lipophosphoglycan a virulence factor? A surprising diversity between *Leishmania* species. Trends Parasitol. **17:**223–226.
58. **Turco, S. J., M. A. Wilkerson, and D. R. Clawson.** 1984. Expression of an unusual acidic glycoconjugate in *Leishmania donovani*. J. Biol. Chem. **259:** 3883–3889.
59. **Uzonna, J. E., G. F. Spath, S. M. Beverley, and P. Scott.** 2004. Vaccination with phosphoglycan-deficient *Leishmania major* protects highly susceptible mice from virulent challenge without inducing a strong Th1 response. J. Immunol. **172:**3793–3797.
60. **Wiese, M., T. Ilg, F. Lottspeich, and P. Overath.** 1995. Ser/Thr-rich repetitive motifs as targets for phosphoglycan modifications in *Leishmania mexicana* secreted acid phosphatase. EMBO J. **14:**1067–1074.
61. **Woods, A., T. Sherwin, R. Sasse, T. H. MacRae, A. J. Baines, and K. Gull.** 1989. Definition of individual components within the cytoskeleton of *Trypanosoma brucei* by a library of monoclonal antibodies. J. Cell Sci. **93:** 491–500.
62. **Wyder, M. A., D. Sul, and E. S. Kaneshiro.** 1999. The fatty acid and monosaccharide compositions of three neutral and three phosphorylated glycolipids isolated from *Leishmania donovani* promastigotes grown in a chemically defined medium. J. Parasitol. **85:**771–778.
63. **Yao, C., J. E. Donelson, and M. E. Wilson.** 2003. The major surface protease (MSP or GP63) of *Leishmania* sp. Biosynthesis, regulation of expression, and function. Mol. Biochem. Parasitol. **132:**1–16.
64. **Zhang, K., F. F. Hsu, D. A. Scott, R. Docampo, J. Turk, and S. M. Beverley.** 2005. *Leishmania* salvage and remodelling of host sphingolipids in amastigote survival and acidocalcisome biogenesis. Mol. Microbiol. **55:**1566–1578.
65. **Zhang, K., J. M. Pompey, F. F. Hsu, P. Key, P. Bandhuvula, J. D. Saba, J. Turk, and S. M. Beverley.** 2007. Redirection of sphingolipid metabolism toward *de novo* synthesis of ethanolamine in *Leishmania*. EMBO J. **26:**1094–1104.
66. **Zhang, K., M. Showalter, J. Revollo, F. F. Hsu, J. Turk, and S. M. Beverley.** 2003. Sphingolipids are essential for differentiation but not growth in *Leishmania*. EMBO J. **22:**6016–6026.
67. **Zufferey, R., S. Allen, T. Barron, D. R. Sullivan, P. W. Denny, I. C. Almeida, D. F. Smith, S. J. Turco, M. A. Ferguson, and S. M. Beverley.** 2003. Ether phospholipids and glycosylinositolphospholipids are not required for amastigote virulence or for inhibition of macrophage activation by *Leishmania major*. J. Biol. Chem. **278:**44708–44718.

Editor: W. A. Petri, Jr.

Downloaded from http://iai.asm.org/ on March 10, 2014 by Washington University in St. Louis