Lipophosphoglycan is a virulence factor distinct from related glycoconjugates in the protozoan parasite *Leishmania major*

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Protozoan parasites of the genus *Leishmania* undergo a complex life cycle involving transmission by biting sand flies and replication within mammalian macrophage phagolysosomes. A major component of the *Leishmania* surface coat is the glycosylphosphatidylinositol (GPI)-anchored polysaccharide called lipophosphoglycan (LPG). LPG has been proposed to play many roles in the infectious cycle, including protection against complement and oxidants, serving as the major ligand for macrophage adhesion, and as a key factor mitigating host responses by deactivation of macrophage signaling pathways. However, all structural domains of LPG are shared by other major surface or secretory products, providing a biochemical redundancy that compromises the ability of *in vitro* tests to establish whether LPG itself is a virulence factor. To study truly *lpg*$^-$ parasites, we generated *Leishmania major* lacking the gene *LPG1* [encoding a putative galactofuranosyl (Gal_f) transferase] by targeted gene disruption. The *lpg1*$^-$ parasites lacked LPG but contained normal levels of related glycoconjugates and GPI-anchored proteins. Infections of susceptible mice and macrophages *in vitro* showed that these *lpg*$^-$ *Leishmania* were highly attenuated. Significantly and in contrast to previous LPG mutants, reintroduction of *LPG1* into the *lpg*$^-$ parasites restored virulence. Thus, genetic approaches allow dissection of the roles of this complex family of interrelated parasite virulence factors, and definitively establish the role of LPG itself as a parasite virulence factor. Because the *lpg1*$^-$ mutant continue to synthesize bulk GPI-anchored Gal_f-containing glycolipids other than LPG, a second pathway distinct from the Golgi-associated LPG synthetic compartment must exist.

trypanosomatid parasites | macrophages | glycoconjugates | galactofuranose

Protozoan parasites of the genus *Leishmania* are the causative agent of leishmaniasis, a disease whose manifestations in humans range from mild cutaneous lesions to fatal visceral infections. More than 10 million people worldwide are infected, with hundreds of millions at risk (1). At present, there are no vaccines and chemotherapy relies on antiquated antimonial derivatives. *Leishmania* are transmitted by biting sand flies, where they reside in the digestive tract. After deposition in the skin, parasites are taken up by macrophages into a phagolysosome, where they differentiate to the nonflagellated amastigote stage. There, *Leishmania* resist the cytotoxic environment, and interfere with signaling pathways normally responsible for the destruction of intracellular pathogens (2–6).

The parasite surface is the primary interface of *Leishmania* with the host, and is comprised largely of three abundant classes of glycosylphosphatidylinositol (GPI)-anchored molecules: lipophosphoglycan (LPG), a smaller heterogeneous group of glycoinositolphospholipids (GIPLs), and proteins such as gp63, gp46/PSA-2, and proteophosphoglycans (PPGs) (2, 4, 7). LPG contains several domains: the GPI anchor, with an 1-*O*-alkyl-2-*lyso*-phosphatidylinositol lipid joined to a heptasaccharide glycan core; a large phosphoglycan (PG) domain containing 15–30 Gal-Man-P repeating units (which, depending on the species, bear additional substitutions); and a neutral capping oligosaccharide (2). Because of both its abundance and extension from the cell surface, attention has been focused on the role of LPG in both the sand fly and macrophage infections. LPG has been implicated in survival of *Leishmania* in the sand fly gut and stage-regulated binding to the parasite midgut (8). After inoculation into the host, LPG may participate in resistance to complement, macrophage adhesion and uptake, protection from toxic macrophage products, and down-regulation of host cell pathways critical for survival (2–6). Not surprisingly, LPG-deficient (*lpg*$^-$) mutants recovered after chemical mutagenesis are unable to establish successful infections in mice or sand flies (9–11).

Despite these findings, the conclusion that LPG itself is essential for parasite virulence is compromised by several factors. As our knowledge of the parasite surface biochemistry has grown, it has become apparent that many of the component domains of LPG are shared by other parasite molecules. The dominant PG repeating units of LPG are also borne by secreted proteins [acid phosphatase (SAP) and PPG (7, 12)] and a secreted PG (13). Likewise, the glycan core of LPG closely resembles that of GIPLs (especially in *L. major*), and the GPI anchors of LPG, GIPLs, and GPI-anchored proteins show similarities (4). Because studies investigating the role of LPG involve application of purified LPG (or components thereof), they cannot establish whether the properties arise from LPG itself or crossactivity with related molecules. Moreover, LPG mutants often show alterations in many components (14), presumably because of sharing of biosynthetic steps. The interpretation of mutant data can also be compromised by nonspecific effects associated with secondary mutations generated during heavy mutagenesis, or a general loss of virulence associated with long-term culture *in vitro*. These problems have surfaced in studies of previously complemented *lpg*$^-$ mutants, where LPG synthesis is fully restored yet virulence is not (refs. 14 and 15; unpublished data). Thus, whereas a role for LPG-related glycoconjugates in virulence seems certain, the specific role of LPG

Abbreviations: GPI, glycosylphosphatidylinositol; GIPL, glycosylinositolphospholipid; LPG, lipophosphoglycan; PG, phosphoglycan; PPG, proteophosphoglycan; SAP, secreted acid phosphatase; RT, room temperature; Gal_f, galactofuranose; WT, wild type.

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separate from that of other abundant related molecules is not yet established.

Here, by using gene targeting methodology, we generated null mutants of the *L. major* gene *LPG1*, which encodes a putative galactofuranosyl (Gal_f) transferase involved in biosynthesis of the LPG glycan core (15, 16). *L. major* was chosen as this species maintains virulence during *in vitro* culture. Notably, this mutant was defective only in LPG, but otherwise synthesized normal levels of related glycoconjugates. The *lpg1*$^-$ mutant was highly attenuated in virulence tests in mice and macrophages.

Materials and Methods

Molecular Constructs. *L. major LPG1* was obtained by probing a cLHYG genomic cosmid library of *L. major* Friedlin strain V1 (MHOM/IL/80/Friedlin) with the ^{32}P-labeled *Leishmania donovani LPG1* gene (15). From cosmid B2251, a 4.2-kb *Bam*HI fragment was inserted into the *Bam*HI site of pUC19, yielding pUC-LPG1BamA (strain B2880); its sequence was determined by standard methods with an Applied Biosystems ABI-373 automated DNA sequencer (GenBank accession no. AF234766). An *LPG1*::*HYG* targeting construct was made by inserting the 2.8-kb *Sma*I–*Xho*I fragment from pX63HYG (17) containing the *DHFR-TS* splice acceptor sequence and *HYG* marker, by blunt end cloning into the unique *Xho*I site located at base 598 of the *LPG1* ORF within pUC-LPG1BamA (strain B2947). The *LPG1*::*PAC* targeting construct was made with the 2.5-kb *Xho*I–*Bam*HI fragment of pX63PAC (strain B2949). pSNBR-*LPG1* (strain B3340) contains the 4.2-kb *LPG1* fragment from pUC-LPG1BamA, inserted into the *Bam*HI site of pSNBR (18). For expression of *Leishmania mexicana* SAP in *L. major*, the coding regions of *lmSAP1* and *lmSAP2* (19) were isolated by *Nco*I digestion, blunt ended, and inserted into the *Sma*I site of pXG (20), yielding pXG-LMSAP1 (strain 3093) and pXG-LMSAP2 (strain 3235).

***Leishmania* Culture and Transfection.** Wild-type (WT) *L. major* LV39 clone 5 (Rho/SU/59/P) (21) was grown in M199 medium at 26°C and transfected by electroporation (22). The first targeting round was performed with 2 μg of the 7-kb *Hin*dIII–*Sma*I *LPG1*::*HYG* fragment whose ends had been made blunt with T4 DNA polymerase, and plating on 50 μg/ml hygromycin B. A clonal line showing successful replacement of one *LPG1* allele was obtained (line H2; *LPG1*/*LPG1*::*HYG)* and taken for a second round of gene disruption with the 6.7-kb blunt-ended *Bam*HI fragment *LPG1*::*PAC*. Transfected cells were plated on media containing 50 μg/ml hygromycin B and 50 μM puromycin, and clonal lines showing inactivation of both *LPG1* alleles identified. Two colonies (A3 and B12) were transfected with 10 μg pSNBR-*LPG1* and plated on media containing 20 μg/ml G418, 50 μg/ml hygromycin B, and 50 μM puromycin.

Mouse Infection Tests. Parasite virulence was assessed following inoculation of the footpads of mice as described (23). Before quantitative tests, parasites were passed twice through the mouse at high inoculating doses (5×10^7), and were thereafter maintained for less than three passages *in vitro*. Parasites were grown to stationary phase in NNN biphasic medium (23), and groups of BALB/cJ mice (The Jackson Laboratory) were injected s.c. (10^6 parasites per mouse) in the footpad (24). Lesions were monitored by measuring the thickness of the footpad with a Vernier caliper. Lesion parasites were enumerated by limiting dilution assay (25).

Macrophage Infections. Infection of peritoneal macrophages with C3-opsonized parasites was performed as described (26), except that parasites were applied to macrophages under serum free conditions in DMEM 0.7% BSA to minimize cell aggregates. The medium was changed daily, and, at days 1, 2, and 5 postinfection, intracellular parasites were detected in formaldehyde-fixed macrophages by nuclear staining with Hoechst 33342 (0.5 μg/ml).

Antisera and Lectins. Two monoclonal antibodies specific for PG repeats were used: CA7AE, recognizing the Gal-Man-P repeating disaccharide common to all PGs (27), and WIC79.3, specific for the Gal-substituted Gal-Man-P units found in *L. major* (28). Monoclonal antibody 235 recognizes the major GPI anchored surface protease gp63 (29). Rabbit antisera against native or deglycosylated *L. mexicana amazonensis* gp46 were provided by D. McMahon-Pratt (30). Monoclonal antibody LT8.2 is specific for the *L. mexicana* SAP polypeptide (31). Fluorescein-conjugated ricin agglutinin was from Sigma.

Flow Cytometry and Immunofluorescence Microscopy. For flow cytometry on live *Leishmania*, all steps were performed at 4°C. Log-phase parasites (10^5/ml) were washed twice in PBS and incubated with 5 μg/ml fluorescein-conjugated ricin agglutinin in PBS for 10 min. Fixed cells were prepared by washing in PBS, fixation in 3.5% paraformaldehyde for 5 min at room temperature (RT), permeabilization with 100% ethanol at 4°C for 15 min, and rehydrated for 10 min at RT in PBS. For immunofluorescence labeling, cells were immobilized on poly(L-lysine)-coated glass coverslips and sequentially incubated for 20 min at 37°C with dilutions of primary and secondary antibodies as described (32). Flow cytometry was performed with a Becton Dickinson FACSCalibur system. WIC79.3 was used as a 1:500 dilution; anti-gp46 was used at a dilution of 1:1000; antibody 235 was used as 1:10 dilution of a hybridoma culture supernatant, and fluorescein-conjugated goat anti-mouse IgG from Jackson Immunochemicals was used at a dilution of 1:100. Fluorescent ricin agglutinin was used at 5 μg/ml.

Western Blot Analysis. Cell culture supernatants and cellular extracts were resolved by SDS/PAGE and electroblotted onto nitrocellulose membranes (Amersham Pharmacia). An enhanced chemiluminescence (ECL) detection system was used (Amersham Pharmacia). Antibodies WIC 79.3 and gp63–235 were used at dilutions of 1:1000 and 1:100, respectively.

Purification and Analysis of LPG and GIPLs. For LPG, late log phase cells ($\approx 1 \times 10^7$ cells/ml) were metabolically labeled with [^{3}H]mannose (50 μCi for 8×10^8 cells) for 6 h in M199 medium supplemented with 10% FBS at 26°C. LPG was extracted and solubilized as described (16). Exponentially growing cells were metabolically labeled with [^{3}H]galactose (50 μCi for 8×10^8 cells) for 16 h, and GIPLs were purified as described (33). Acid hydrolysis of GIPLs with trifluoroacetic acid and paper chromatography were performed as described (16). GIPLs were subjected to nitrous acid deamination (16), labeled with 9-aminonaphthalene 1,3,6-trisulfate, and analyzed by GLYKO-FACE electrophoresis according to the manufacturer's specifications (GLYKO, Novato, CA).

PPG Purification. PPG was partially purified from stationary phase cell extracts and culture supernatants by Triton X-114 partitioning as described (34). PPG was resolved by SDS/PAGE and analyzed by Western blotting with a 1/1000 dilution of the monoclonal anti-phosphodisaccharide antibody WIC 79.3.

Results

Targeted Disruption of *L. major LPG1*. *Leishmania* are asexual diploids and thus require two rounds of gene disruption (17). We generated targeting constructs for inactivation of *LPG1* by inserting within its coding region either *HYG* or *PAC* selectable markers. After two rounds of transfection and selection, lines in which both *LPG1* alleles had been disrupted were identified

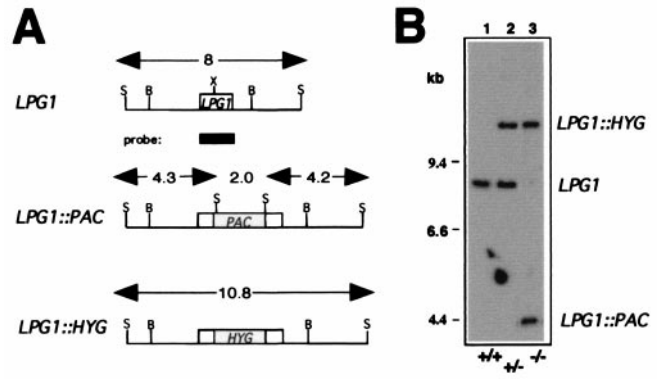

Fig. 1. Disruption of the *LPG1* locus. (*A*) Genomic organization of the *L. major LPG1* locus and the planned replacements *LPG1*::*PAC* and *LPG1*::*HYG*. S, X, and B indicate *Sma*I, *Xho*I, and *Bam*HI restriction sites, respectively; the targeting fragments contained the regions marked by the *Bam*HI sites. The sizes of fragments (in kb) expected following digestion with *Sma*I and hybridization with the probe denoted by the black bar are shown above each map. (*B*) Southern blot analysis of WT (+/+, lane 1), heterozygous (+/−, lane 2, clone H2), and homozygous mutants (−/−, lane 3, clone A3) DNAs.

(*LPG1*::*HYG*/*LPG1*::*PAC*; Fig. 1*B*, lane 3). To restore *LPG1* expression, several lines were transfected with an *LPG1* expression construct (pSNBR-*LPG1*), yielding transfectants that were now *LPG1*::*HYG*/*LPG1*::*PAC* [pSNBR-*LPG1*]. Because sibling clonal lines behaved similarly, only results with a single doubly disrupted line (A3, referred to as "*lpg1*$^-$") and its *LPG1*-restored "add-back" derivative (A3ab, termed "*lpg1*$^-$/+*LPG1*") are presented below.

Loss of LPG Expression in the *lpg1*$^-$ Mutant. LPG was quantitated after metabolic labeling and purification (Fig. 2*A*). Only background levels were detected in the *lpg1*$^-$ mutant (<5% WT), whereas the *lpg1*$^-$/+*LPG1* line showed normal levels (Fig. 2*A*). Western blot analysis with anti-PG antibody confirmed the absence of LPG in the *lpg1*$^-$ line, and the presence of full-length LPG in the *lpg1*$^-$/+*LPG1* line (Fig. 2*B*). LPG was localized to the cell surface, as shown by flow cytometry and immunofluorescence with fluorescein-ricin agglutinin against live cells (Fig. 2*C*, and data not shown).

Phosphoglycosylation Is Unaffected in the *lpg1*$^-$ Mutant. *L. major* expresses high levels of a mucin-like PG-containing protein, PPG (35). Secreted and cell-associated PPGs were purified from WT, *lpg1*$^-$ and *lpg1*$^-$/+*LPG1* lines, and detected by Western blotting with anti-PG antibody (Fig. 3*A*). Comparable levels of similarly sized PPGs were detected in culture supernatants and in the aqueous phase of Triton X-114-treated cell extracts, suggesting that *LPG1* does not affect PG synthesis (Fig. 3*A*). Although it has been suggested that PPG may be localized to the parasite surface through a putative GPI anchor (36), PPG was not detected in the Triton X-114 detergent phase (Fig. 3*A*).

Unlike other species of *Leishmania*, *L. major* does not express another PG-containing protein, SAP (37). This allowed heterologous expression of *L. mexicana* SAP in WT and *lpg1*$^-$ *L. major* to be used as a reporter of phosphoglycosylation activity (38). In both lines, *SAP1* and *SAP2* were expressed at comparably high levels, reacted comparably with anti-PG and anti-SAP protein antibodies, and showed comparable electrophoretic mobilities characteristic of heavily PG-modified proteins (data not shown).

Localization and Quantitation of *Leishmania* PGs. The cellular localization of PGs was determined by indirect immunofluorescence

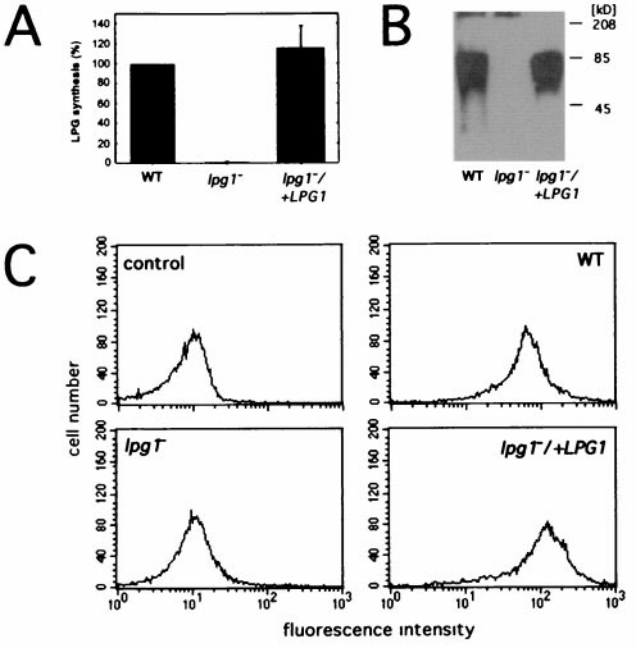

Fig. 2. *lpg1*$^-$ *L. major* lack LPG. (*A*) LPG was metabolically labeled with [^{3}H]mannose, purified, incorporation determined by liquid scintillation counting, and the levels expressed relative to WT. Only background levels were detected in *lpg1*$^-$ cells. (*B*) Purified LPGs were subjected to Western blot analysis with anti-PG antibody WIC 79.3. No reactive material was detected in the *lpg1*$^-$ cells. (C) Flow cytometry of live *Leishmania* labeled with fluorescein-conjugated ricin agglutinin. The control shows unlabeled WT parasites.

microscopy of permeabilized parasites with anti-PG antibody (Fig. 3*B*). The LPG surface coat of WT parasites was strongly labeled and, additionally, labeling of intracellular vesicular structures was observed. In the *lpg1*$^-$ mutant, the surface labeling was lost, and only intracellular vesicular structures were identified

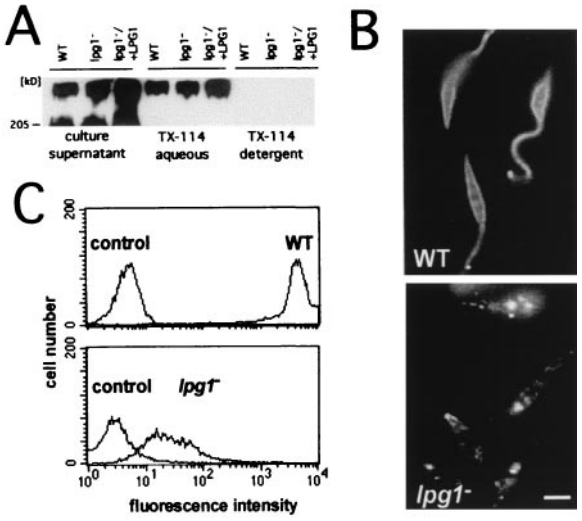

Fig. 3. *lpg1*$^-$ cells express normal levels of PPG. (*A*) PPG was purified from culture supernatants or parasites fractionated by Triton X-114 extraction and subjected to Western blot analysis with anti-PG antibody WIC 79.3. (*B*) Indirect immunofluorescence microscopy is shown with anti-PG antibody WIC79.3. The exposure time for WT was 1/15 s, whereas for *lpg1*$^-$ it was 1/4 s. The bar corresponds to 5 μm. (*C*) Flow cytometry of fixed permeablized parasites labeled with anti-phosphoglycan antibody WIC 79.3. Control represents cells incubated only with secondary antibody.

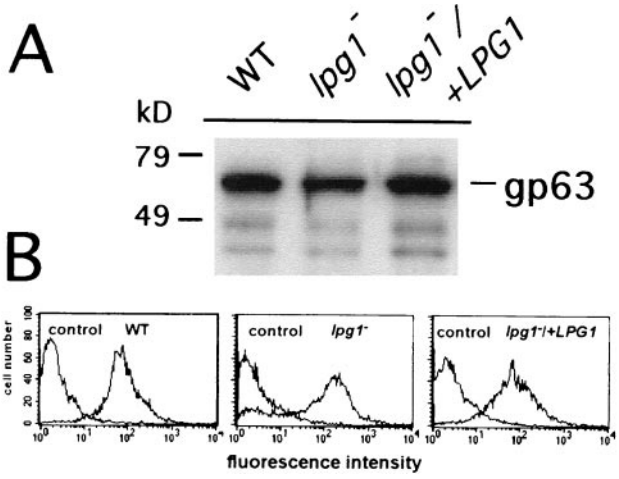

Fig. 4. *LPG1* does not affect gp63 expression. (*A*) Western blot analysis. Crude cell extracts (2.5×10^6 cells) from logarithmic cultures were subjected to SDS/PAGE and Western blotting with anti-gp63 antibody. (*B*) Flow cytometry. Fixed permeabilized parasites (log phase) were labeled with anti-gp63 antibody. Control parasites were treated identically except that anti-gp63 antibodies were omitted.

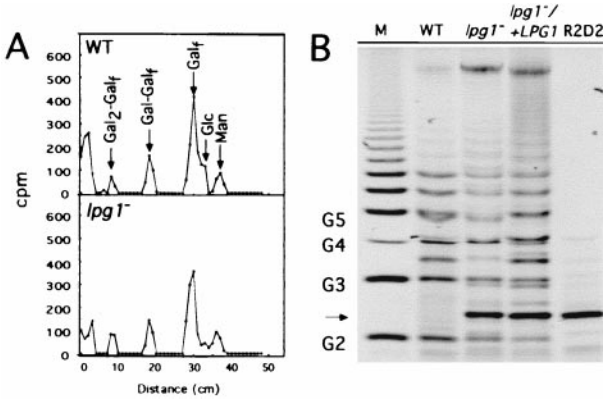

Fig. 5. *lpg1*$^-$ cells maintain Gal$_f$-containing GIPLs. (*A*) WT and *lpg1*$^-$ GIPLs, metabolically labeled with [^{3}H]galactose were separated by paper chromatographic procedures. The mobility of unlabeled standards are indicated; Gal, galactose; Glc, glucose; Gal$_f$, galactofuranose; Man, mannose. (*B*) Total GIPL analysis. Purified GIPLs were delipidated, fluorophore-labeled, separated by the GLYKO-FACE system (GLYKO), and visualized by UV fluorescence. The arrow indicates the mobility of the derivative corresponding to LPG precursor Glc-P-Man$_2$-GlcN-PI. G2–G5 represent oligomeric derivatives of glucose.

(Fig. 3*B*). In contrast to the results obtained with the V121 *L. major* strain (36), the absence of surface PG labeling in these cells, as well as the absence of PPG in the Triton membrane fraction, suggests that PPG is not expressed on the cell surface of *L. major* strain LV39.

The specific absence of LPG in the *lpg1*$^-$ cells allowed us to quantify the relative contribution of LPG to the total cellular PG content, by flow cytometry of permeabilized cells labeled with anti-PG antibodies (Fig. 3*C*). WT cells showed strong fluorescence, which was reduced 200-fold in the *lpg1*$^-$ line (which showed fluorescence markedly over background). Assuming that intracellular PGs stain as efficiently as surface PG, these data suggest LPG accounts for >99% of the total cellular PG content.

Analysis of GPI-Anchored Proteins. The synthesis of two surface GPI-anchored proteins, gp63 and gp46/PSA-2, was assessed by Western Blots and flow cytometry. Loss of *LPG1* and LPG had little effect on production of gp63 (Fig. 4 *A* and *B*) and gp46 (not shown). Similarly, gp63 expression was not affected in mutagenized LPG-deficient strains of *L. donovani* and *L. major* (14, 39).

***lpg1*$^-$ Shows Normal Expression of Bulk GIPLs.** In *L. major*, the predominant ("bulk") GIPLs consist of Gal$_{(0-2)}$-Gal$_f$-[Glc-P]-Man$_2$-GlcN-PI, a structure also found in the LPG glycan core (40). This suggested that the GIPL Gal$_f$-Man linkage might also arise through the action of LPG1 (15, 16). GIPLs in WT and *lpg1*$^-$ parasites were metabolically labeled with [^{3}H]galactose, purified, and treated with trifluoroacetic acid to cleave the Gal$_f$-Man bonds. Separation of the [^{3}H]Gal fragments by paper chromatography (Fig. 5*A*) showed that the patterns were very similar in both lines; notably, the mobility and amounts of several GIPLs containing Gal$_f$ were unaffected. Thus, the Gal$_f$ transferase responsible for GIPL biosynthesis is not affected by loss of *LPG1*.

Previous studies of the R2D2 mutant of *L. donovani*, which is also defective in *LPG1* (ref. 15; R. Zufferey and S.M.B., unpublished data), showed the accumulation of the expected Glc-P-Man$_2$-GlcN-PI intermediate. To visualize this, total GIPLs were delipidated by treatment with nitrous acid, fluorophore-labeled, separated by electrophoresis, and visualized under UV light (Fig. 5*B*). Again, the GIPL pattern was very similar, except for the accumulation of a new species migrating between the G2 and G3 markers in the *lpg1*$^-$ parasites (marked by an arrow in Fig. 5*B*). This species is derived from and corresponds to the expected Glc-P-Man$_2$-GlcN-PI, as revealed by its comigration with the same GIPL derivative present in R2D2 (Fig. 5*B*; ref. 41). The identity of this precursor was confirmed by treatment with mild acid followed by alkaline phosphatase digestion (data not shown). Surprisingly, high levels of the Glc-P-Man$_2$ GIPL remained in the *lpg1*$^-$/+*LPG1* line, despite complete restoration of LPG expression (Fig. 2). This was reproducibly seen, in multiple independent *LPG1* restoration lines, and in similar studies of the R2D2 mutant (data not shown). The basis of this is not yet understood, especially as we know that the LPG1 protein is correctly targeted to the parasite Golgi apparatus even when overexpressed (20).

***lpg1*$^-$ *Leishmania* Are Attenuated for Virulence in Mice.** Virulence was assessed by infection of susceptible BALB/c mice. When inoculated into footpads, 10^6 stationary-phase WT parasites rapidly gave a lesion by day 15, which progressed steadily and ultimately resulted in death (Fig. 6*A*). In contrast, 10^6 stationary phase *lpg1*$^-$ parasites showed greatly delayed lesion formation, appearing only after 60 days. In these studies, lesion size correlated with parasite burden, as assessed by limiting dilution assay (not shown).

The delayed appearance of parasites in the *lpg1*$^-$ infections could reflect the emergence of LPG+ revertants. To test this, amastigotes [which normally express very little LPG (42–45)] were recovered and allowed to differentiate back to the promastigote stage. These *lpg1*$^-$ parasites lacked LPG and thus were not revertants. This finding was confirmed by subsequent mouse infections, which showed the expected attenuation of lesion progression (data not shown).

Inoculation of fewer parasites is known to result in the delayed appearance of lesions, a finding replicated here with WT parasites (Fig. 6*B*). Lesion progression following inoculation of 10^4 WT parasites closely resembled that obtained with 10^6 *lpg1*$^-$ parasites (Fig. 6*B*). This suggested that loss of LPG and *LPG1* attenuates parasite survival by a factor of approximately 100, and that lesion progression observed in *lpg1*$^-$ parasites arose from survival of a small number of inoculated parasites. Consistent with this, inoculation of higher "saturating" doses of parasites minimized and even eliminated the difference between the WT

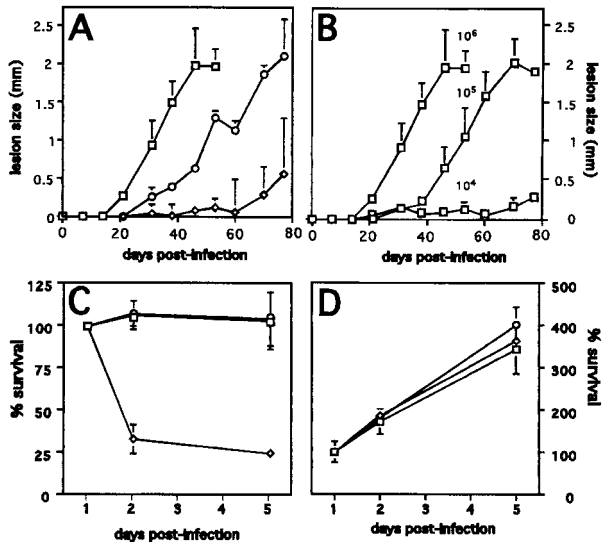

Fig. 6. Virulence of *lpg1*$^-$ promastigotes is attenuated. Mouse infections: (*A*) 10^6 stationary-phase WT (□), *lpg1*$^-$ (◇), and *lpg1*$^-$/+*LPG1* (○) parasites were inoculated into the footpad, and lesion formation as measured by the increase in size is shown. (*B*) Quantitation of lesion formation. 10^6, 10^5, and 10^4 stationary phase WT parasites were inoculated and lesion formation monitored. Macrophage survival (symbols are as in *A*): (*C*) C3-opsonized stationary phase WT, *lpg1*$^-$ and *lpg1*$^-$/+*LPG1* promastigote-stage parasites were inoculated into peritoneal macrophages and parasite survival monitored. (*D*) Amastigote stage parasites were recovered from infected mice (*A*) and used to inoculate peritoneal macrophages following C3 opsonization at a ratio of 10 parasites/macrophage. Parasite survival was determined as the number of parasites/100 macrophages and is expressed relative to the WT, and was determined in at least two independent triplicate experiments. In all panels, bars show the standard deviations.

and *lpg1*$^-$ lines, whereas inoculation of lower doses of parasite accentuated the difference in virulence (data not shown).

Significantly, the virulence of the "add-back" *lpg1*$^-$/+*LPG1* parasites was restored to levels approaching that of WT parasites: only a minimal delay in lesion appearance occurred, and lesion size reached 70% of that attained by the parental line at day 46 (Fig. 6*A*). This suggests that the phenotype of the *lpg1*$^-$ mutants arose from loss of *LPG1* alone. Although complete restoration of virulence was anticipated, it is a common finding that complemented lines frequently do not fully recover virulence (46, 47).

***lpg1*$^-$ Parasites Are Defective in Macrophage Survival.** *Leishmania* survival in macrophages was assesed following infection of peritoneal macrophages (Fig. 6 *C* and *D*), with stationary phase parasites that had previously been opsonized with complement (48, 49). The absence of *LPG1* and LPG expression had no influence on invasion of the host cell by either promastigote or amastigote cells, as the efficiency of infection ranged between 84% for WT to 97% for *lpg1*$^-$ parasites. However, more than 75% of the *lpg1*$^-$ parasites were rapidly eliminated within the first 2 days. Restoration of LPG expression in the *lpg1*$^-$/+*LPG1* parasites led to full restoration of macrophage survival (Fig. 6*C*).

We purified amastigotes from the lesions of mice infected with the three strains and evaluated their ability to survive in macrophages. In contrast to the results obtained with promastigotes, loss of *LPG1* expression had no effect on survival of amastigotes (Fig. 6*D*). This was expected as *Leishmania* amastigotes express very little LPG (42–45).

Discussion

We generated null mutants of *L. major* lacking the *LPG1* gene and showed they lack the surface virulence glycoconjugate LPG but otherwise maintain WT levels of other related glycoconjugates including PPG, GIPLs, and GPI-anchored proteins. We used this finding to dissect the pathways involved in LPG and related glycoconjugate biosynthesis, and establish the importance of LPG as a virulence factor in the mammalian phase of the infectious cycle.

LPG1 was first identified by functional rescue of the *L. donovani lpg*$^-$ mutant R2D2 and is required for the synthesis of the Gal_f-Man linkage present in the LPG glycan core, probably by acting as the Gal_f-transferase (15, 16). For the most part, the specificity of the *lpg1*$^-$ phenotype toward LPG alone was anticipated, as the Gal_f-Man bond has not been found in other *Leishmania* PG-containing glycoconjugates or GPI-anchored proteins (50). One interesting result was that *lpg1*$^-$ mutants maintained normal levels of bulk GIPLs, which contain the same Gal_f-Man bond present in LPG (Fig. 5; ref. 40). Although several Gal_f-bearing GIPLs in *L. major* are identical to expected LPG intermediates, metabolic-labeling studies suggest that bulk GIPLs are not biosynthetic intermediates of LPG (51–53). These and other data (20, 54) suggest that the pathways of protein, LPG, and GIPL GPI anchor biosynthesis are segregated in different cellular compartments. Our data strengthen this hypothesis, and further suggest that there must be a second Gal_f transferase responsible for synthesis of the GIPL Gal_f-Man linkages in *L. major*. Because LPG1 and other LPG biosynthetic proteins are localized in the Golgi apparatus (14, 20), the presumptive GIPL Gal_f transferase may represent a marker of the GIPL biosynthetic compartment, whose identity is presently unknown. Our data further suggest that absence of intact LPG, a major component of the parasite surface membrane, has no effect on the trafficking of other molecules destined for secretion or the cell surface such as PPG, gp46/PSA-2, and gp63 (Figs. 3 and 4).

LPG Is a Virulence Factor for Establishment of Mammalian Infection. The *lpg1*$^-$ parasites only lacked LPG and thus provided an opportunity to assess whether LPG itself, rather than related glycoconjugates, was required for virulence. In two different assays, *lpg*$^-$ parasites showed strong defects in virulence.

In mouse infectivity tests, *lpg1*$^-$ parasites were strongly attenuated, showing a delay in lesion progression associated with a 100-fold decrease in survival. This was strongly related to inoculum size, as infections with high cell numbers (5×10^7) showed little difference between *lpg1*$^-$ and WT parasites, whereas infections with lower doses (10^6 and fewer) showed strongly decreased lesion progression in the *lpg1*$^-$ parasites (Fig. 6*A*; data not shown). Because infected sand flies typically deposit less than 100 parasites in natural transmission (55), an LPG-dependent 100-fold decrease in survival would have strong consequences in nature.

Ultimately, lesions appeared and progressed normally in the *lpg1*$^-$ parasites. The emergent parasites were recovered and shown not to be LPG$^+$ revertants. In combination with the quantitative results above (Fig. 6*B*), it is likely that delayed lesion formation arises from survival of a small number of parasites during the initial phase of *lpg1*$^-$ infections. Correspondingly, amastigotes recovered from *lpg1*$^-$ parasites were as infectious as WT parasites in macrophage infections (Fig. 6*D*), as expected because LPG is expressed at very low levels in the amastigote stage of the infectious cycle LPG (42–45). *In vitro* infections of mouse macrophages support the view that LPG is required for the establishment but not maintenance of intracellular survival (Fig. 6 *C* and *D*).

Previous studies using purified LPG were subject to concerns arising from cross-activity with related glycoconjugates and the biological relevance of the amounts and delivery route of purified LPG. The availability of specifically *lpg*$^-$ mutants now allows an assessment of the role of LPG in an appropriate

biological context. For example, whereas LPG has been suggested to be a major ligand for macrophage uptake (56), C3-opsonized *lpg1*$^-$ parasites enter macrophages as successfully as WT (see *Results*). Future studies will use the *lpg*$^-$ mutants to assess the role of LPG in carrying out other steps of the infectious cycle, such as resistance to complement and oxidants, and its ability to modulate host signaling pathways. It is possible that functional redundancy may occur among different LPG-related glyconjugates. For example, under some circumstances, PG-containing molecules such as PPG may substitute for the PG-dependent roles of LPG.

A key finding was that restoration of LPG and *LPG1* expression in *lpg1*$^-$ parasites completely restored survival in macrophages (Fig. 6*C*), and substantially restored virulence in mouse infections (Fig. 6*A*). This contrasts with previously *lpg*$^-$ mutants, which showed pleiotropic defects in other glycoconjugate synthesis and/or effects unrelated to LPG biosynthesis (refs. 14 and 15, and unpublished data). Thus, LPG and *LPG1* satisfy the requirements for virulence factors/genes as set forth by the modern "Molecular Koch's postulates" (57), establishing LPG itself as a *Leishmania* virulence factor.

In summary, we have shown through biochemical characterization of the *lpg1*$^-$ mutant that LPG itself is an important virulence determinant for *Leishmania* during the mammalian infectious cycle. Previously, we showed that the *lpg1*$^-$ parasites are unable to survive in the natural sand fly vector *Phlebotomus papatasi* (10), establishing the importance of LPG throughout the entire parasite infectious cycle. Our studies emphasize the importance of combining parasite genetics and biochemical characterizations to establish the role of LPG from other members of this complex family of glyconconjugates. We are now pursuing other LPG genes and mutants to assess the relative contribution of other LPG related molecules such as PPG and GIPLs to virulence.

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