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Genetics

Isolation of virulence genes directing surface glycosyl-phosphatidylinositol synthesis by functional complementation of *Leishmania*

(transfection/Trypanosomatidae/glycosyltransferases/ARD1/galactofuranose)

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ABSTRACT **Trypanosomatid parasites of the genus *Leishmania* cause a spectrum of widespread tropical diseases. In the vertebrate host they reside within the macrophage phagolysosome; however, the mechanisms employed in this remarkable survival strategy are not well understood. Recent advances in the molecular genetics of these parasites prompted us to develop methods of functional genetic complementation in *Leishmania* and apply them to the isolation of genes involved in the biosynthesis of the virulence determinant lipophosphoglycan, an abundant glycosyl-phosphatidylinositol-anchored polysaccharide. LPG1, the gene product identified by complementation of the R2D2 mutant, appears to be a glycosyltransferase responsible for the addition of galactofuranosyl residues to the nascent lipophosphoglycan chain. As galactofuranose is not found in mammalian cells, inhibition of the addition of this sugar could be exploited for chemotherapy. Overall, the success of the functional complementation approach opens the way to the identification of a variety of genes involved in pathogenesis and parasitism.**

Leishmaniasis can appear as a simple cutaneous lesion, a disfiguring mucocutaneous disease, or a fatal visceral infection, depending upon the host and the species of parasite (1). Although typically considered an endemic tropical disease, infecting more than 10 million people, leishmaniasis is found throughout the Mediterranean basin and is an opportunistic pathogen of the immunocompromised and of foreign visitors (1–5).

Leishmania is transmitted by biting sand flies into the mammalian host. There the parasites enter the macrophage phagolysosome and differentiate from a flagellated promastigote form into the nonmotile amastigote stage. Here they resist the action of resident hydrolytic enzymes, inhibit activation of the oxidative burst, and mitigate the immunological attack of the host. How the parasite accomplishes this is not well understood, although it is known that at least two developmental transitions are required. Knowledge of the molecular basis of these phenomena may potentially lead to new strategies for control of *Leishmania* and provide fresh insights into basic mechanisms of intracellular pathogenesis.

Genetic approaches have been fruitful in studies of many pathogens. For example, mutants affected in interesting properties such as virulence can be obtained and the defective gene can be identified by complementation. A major advantage of this approach is that it presupposes nothing about the nature of the virulence genes. Recent advances in *Leishmania* genetics suggested that this parasite was well-poised for the introduction of similar functional complementation approaches. It is now possible to introduce DNA into a wide variety of *Leishmania* species with good plating efficiencies, using both episomal and integrating vectors. General expression vectors are available for directing the synthesis of both parasite and foreign genes *in situ*, and homologous gene replacement can be readily accomplished (6–11). These parasites do pose some unique problems in generating suitable mutants, however. Many trypanosomatid genes occur in tandem repeats, and *Leishmania* is generally diploid and lacks a manipulable sexual cycle (12–14). Consequently, at least two independent events are required to inactivate a gene, and mutations are recovered at very low frequencies (10^{-7}) even after heavy mutagenesis (15–17). This precludes simple replica plating approaches to identify mutations, and alternative strategies are required.

We have developed a selective protocol for isolating mutants defective in the biosynthesis of an abundant cell-surface lipophosphoglycan (LPG) common to all *Leishmania* species (reviewed in refs. 18 and 19). LPG is a multifunctional virulence determinant, playing key roles in the growth of *Leishmania* within the sand fly vector, its resistance to lysis by complement after infection, the recognition of the parasite by the macrophage, and the establishment of the amastigote stage therein (18). The structure of LPG from diverse *Leishmania* species has been determined and the organization of its four domains is summarized in Fig. 1. A key feature of LPG is that it is the only major surface molecule on *Leishmania donovani* that terminates in β-linked galactose, which is recognized by the lectin ricin agglutinin (15). Following heavy mutagenesis and selection against ricin binding, parasites defective in LPG expression (LPG^-) can be recovered (15, 20). Since mutations in any LPG biosynthetic step lead to the absence of terminal β-linked galactosyl residues attached to the parasite, selection against ricin-binding should lead to the recovery of a wide spectrum of mutations. Moreover, LPG components such as the disaccharide-phosphate repeats, portions of the glycan core, and the phosphatidylinositol lipid anchor appear in other parasite surface molecules, including the major surface protease gp63 (18). This extends the range of the genetic screen to a variety of other surface molecules, many of which are considered essential for virulence but not for growth *in vitro*. Thus, the LPG system provides an excellent setting for developing methods for functional complementation in *Leishmania* as well as probing the basis of virulence.§

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Abbreviations: LPG, lipophosphoglycan; Gal_f, galactofuranose; ORF, open reading frame.

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§The sequence reported in this paper has been deposited in the GenBank database (accession no. L11348).

FIG. 1. Structure and features of *Leishmania* LPG. A schematic of the four major domains of *Leishmania donovani* LPG is shown. LPGs from other species have identical glycan cores and phosphatidylinositol anchors but can bear additional minor substitutions on the repeat units and cap. Ricin agglutinin binds to the galactose-containing cap and CA7AE antibody binds the disaccharide-phosphate repeats. *Lyso*-PI, lyso-1-*O*-alkyl phosphatidylinositol.

MATERIALS AND METHODS

Cell Lines and Culture. *Leishmania* cells were grown in M199 medium (6, 21). The wild-type LPG$^+$ line was the 1S2D strain of *L. donovani*, and the LPG$^-$ mutant studied was R2D2 (15). R2D2 was maintained in medium with ricin agglutinin (10 μg/ml) prior to transfections.

Cosmid Libraries and Transfections. R2D2 was transfected with 20 μg of wild-type *L. donovani* cosmid library DNA per plate (6, 22). DNA was prepared from a pool of 80,000 independent recombinants from both a *Sau*3A1 partial and a shear cosmid library (22). Thirty-two thousand independently transfected colonies were obtained from 27 plates.

Ricin and Anti-LPG Monoclonal Antibody Panning. Plastic Petri dishes (100 mm; Falcon 1029) were coated with ricin (10 μg/ml) or goat anti-mouse IgM (Calbiochem) in 10 ml of 50 mM Tris (pH 9.5). Plates were swirled on a gyrotatory shaker (Belly-dancer model; Stovall Life Sciences, Greensboro, NC) for 2 hr at room temperature, the coating solution was removed, and the plates were rinsed four times with phosphate-buffered saline or M199 medium (6) lacking serum. For panning with the anti-LPG antibody CA7AE (23), cells (5×10^7 per ml) were incubated for 30 min in serum-free M199 medium containing 0.3% bovine serum albumin (M199/BSA) and CA7AE (1:1000 dilution of mouse ascites fluid), washed, resuspended in M199/BSA, and placed on anti-IgM-coated plates for 1–2 hr at 4°C. The plates were washed three times with phosphate-buffered saline, 10 ml of complete M199 medium was added, and the plates were incubated 18–48 hr at 27°C. During this period cells detached and were collected by centrifugation. For ricin panning, 1 ml of cells (1×10^7 per ml in M199/BSA) were placed on ricin-coated plates and gently swirled for 10–20 min at room temperature. Plates were washed as described above, and bound cells were eluted with 3 ml of 0.2% galactose in M199/BSA (two washes, 30 min each). Three experiments employing either three rounds of ricin panning, two rounds of antibody plus one round of ricin, or two rounds of antibody were performed; cosmids cR2D2-A and -B were recovered independently from all experiments.

Reconstruction Tests of LPG Panning. Wild-type *L. donovani* cells were transfected with the plasmid pX-βGAL (10) and an LPG$^+$ colony producing substantial β-galactosidase was identified. These cells were mixed with R2D2 at a ratio of 1:1000 and subjected to one round of panning on control, ricin-coated, or antibody-coated plates. Bound parasites were recovered and β-galactosidase was assayed. With ricin there was 0.02 fluorescence unit (FU) per cell prior to panning and 0.32 FU per cell after panning (16-fold enrichment); for CA7AE there was 0.004 FU per cell prior to panning and 0.15 FU per cell after panning (37-fold enrichment).

Agglutination Tests. *Leishmania* cells were grown to mid-logarithmic phase (about 5×10^6 per ml) and 100-μl aliquots of the culture were placed in wells of 96-well dishes. Ricin (10 μl, 20 μg/ml in serum-free M199 medium) or CA7AE (10 μl of a 1:100 dilution of mouse ascites fluid) was added, gently mixed, and incubated for 30 min at room temperature. Wild-type *L. donovani* showed clear visible agglutination, whereas R2D2 showed none.

RNA Isolation, Northern Blotting, and Trans-Splice-Acceptor Mapping. Promastigotes were harvested in logarithmic phase (5×10^6 per ml) and stationary phase (4×10^7 per ml). Amastigotes were recovered from the spleen of infected hamsters (24). Total RNA was extracted, poly(A)$^+$ RNA was prepared, and samples (4 μg per lane) were denatured in the presence of glyoxal, electrophoresed in 1% agarose gels, transferred to membranes, and hybridized with randomly ^{32}P-labeled probe (1.1-kb *Bgl* II–*Hin*dIII fragment; see Fig. 3*B* and ref. 25). To map the miniexon splice acceptor site, cDNA was prepared from 3.5 μg of poly(A)$^+$ RNA isolated from logarithmic-phase *L. donovani* promastigotes, in a total reaction volume of 42 μl (6). PCR (6) used 3 μl of the above reaction mixture as template in conjunction with a miniexon-specific primer (5′-ctcgggatccCAACGCTATATAAGTATCAGTTTCTGTACTTTATTG) and the LPG1-specific primer (5′-CTCCTGATAATCCATCGTTA-3′, positions 2316–2335) for 40 cycles of 94°C, 1 min; 55°C, 2 min; 72°C, 3 min.

Expression of Predicted Proteins in *Leishmania*. The LPG1 open reading frame (ORF) was amplified by using the 5′ and 3′ primers (5′-gcgggatccaccATGGCGCCGCCTCGCTGG-3′) and (5′-gcgggatccTTAGCTGGGGTCAACAGG-3′) in a reaction mixture containing 0.2 μM primer, 20 ng of *Hin*dIII-digested cR2D2-B (boiled previously for 10 min), 20 mM Tris·HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTPs, and 2.5 units of *Taq* polymerase (Boehringer). The mixture was incubated for 30 cycles of 94°C, 2 min; 55°C, 2 min; 72°C, 3 min. ARD1 was amplified by using the 5′ and 3′ primers (5′-gcgggatccaccATGACTACATATCGCCGC-3′) and (5′-gcgggatccTCACACCCACTCCAGTTC-3′) as above (lowercase letters are added sequences containing restriction sites). Amplification products were digested with *Bam*HI, purified, and inserted into the *Bgl* II site of pX63HYG (ref. 10; LPG1) or the *Bam*HI site of pX63NEO (ref. 11; ARD1). DNAs were stably transfected into R2D2 for agglutination tests.

LPG Quantitation. LPG levels were quantitated by a standard indirect ELISA. Serial dilutions of promastigotes (starting with 10^7 per ml) in 100 μl were coated onto ELISA plate wells and air-dried. CA7AE antibody (1:2000 dilution of ascites) was used as the first antibody and alkaline phosphatase-conjugated goat anti-mouse IgM/IgG (Tago) as the second antibody.

RESULTS

Mass Transfection of *L. donovani*. For functional complementation to be successful, transfection methodology must permit sampling the entire *Leishmania* genome. After electroporation in the presence of stable transfection vectors and plating on semisolid medium, 2000 *Leishmania* colonies per plate can be readily obtained (6, 22, 27). This number exceeds the number of cosmids, 1250, that would be required to cover the 50,000-kb *Leishmania* genome once. Since transfected circular DNAs are maintained extrachromosomally in *Leishmania* (1–5), we developed an episomal shuttle cosmid vector (cLHYG) which has a passenger DNA capacity of about 40 kb and transfects efficiently (22). We next developed methods for mass transfection of a wild-type *L. donovani* cosmid library into the LPG$^-$ mutant R2D2 (15), which is defective in the synthesis of the LPG glycan core (Fig. 1). Thirty-two thousand independent transfectants were obtained, constituting a 25-hit library (22).

Recovery of LPG$^+$ Transfectants. To recover the few LPG$^+$ parasites expected in the cosmid-transfected R2D2 pool, we reversed the original negative selection protocol and used ricin and/or indirect anti-LPG antibody panning as a positive selection. Parasites were passed directly over plates coated

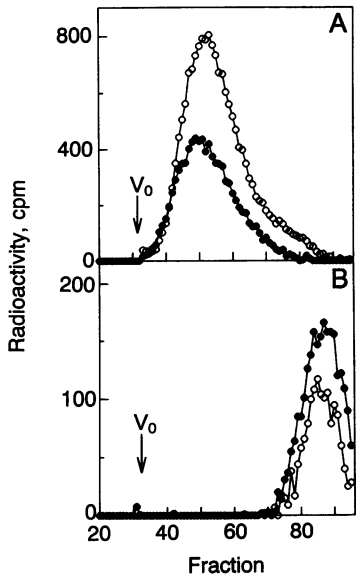

FIG. 2. Metabolic labeling of LPG in wild-type and transfected *L. donovani*. Late-logarithmic-phase promastigotes (10^9) were metabolically labeled with [^{3}H]mannose and extracted, the extract was treated with phosphatidylinositol-specific phospholipase C, and the released carbohydrate was characterized by Sephadex G-150 chromatography (29). Similar total cpm were analyzed. ○, Wild-type *L. donovani*; ●, R2D2 transfected with cosmid R2D2-B; no significant incorporation was found with control R2D2. (*A*) Intact LPG. (*B*) Samples were pretreated with mild acid (0.02 M HCl, 5 min, 100°C) to release the disaccharide-phosphate repeats. V_0, void volume.

with ricin or, alternatively, were incubated with an anti-LPG monoclonal antibody (CA7AE, directed to the subterminal disaccharide-phosphate repeats; Fig. 1; ref. 23) and passed over plates coated with an anti-IgM antiserum; bound parasites were then recovered. Reconstruction experiments showed that the enrichment obtained in one panning step ranged from 16- to 37-fold. This suggested that two or three successive steps would be sufficient for the required 1000-fold enrichment. Accordingly, the cosmid-transfected R2D2 pool was independently subjected three times to three rounds of ricin and/or antibody panning.

These three populations all agglutinated with both ricin and anti-LPG monoclonal antibody. The cosmids within these cells were shuttled into *E. coli*, and mapping of 85 cosmids revealed 9 different ones, all sharing a subset of fragments totaling about 20 kb. Two cosmids were recovered from all three populations, 3 were obtained from two populations, and 4 were unique to a single population. The recovery of 9 independent but related cosmids argues that we have reached saturation and obtained the only locus capable of complementing R2D2. To confirm that the complementing activity did not arise from spontaneous reversions, two of the cosmids (cR2D2-A and cR2D2-B) were retransfected into R2D2; both conferred agglutination with ricin and monoclonal antibody. In contrast, transfection of these cosmids into two different, biochemically distinct LPG$^-$ mutants (28) yielded cells that failed to agglutinate with either ricin or CA7AE. It appears that LPG biosynthetic genes are not clustered within the region defined by the these two complementing cosmids.

Biochemical characterizations showed that the transfectants bearing cR2D2-B synthesized authentic LPG. Its size was identical to that of wild-type *L. donovani* (Fig. 2*A*), and mild acid hydrolysis showed the presence of disaccharide-phosphate repeats as in wild-type LPG (Fig. 2*B*). Indirect immunofluorescence with the CA7AE antibody showed LPG on the parasite surface (data not shown). LPG in the transfectants was at 5–10% wild-type levels, which we attribute to the possibility that genes carried on episomal cosmids may not be properly regulated (30).

Identification of the LPG1 Gene. Molecular karyotype and Southern blot analysis mapped both cosmids cR2D2-A and -B to a single 850-kb chromosome (data not shown). The two cosmids shared a 19.5-kb region, and Southern blot analysis showed that the cR2D2-B map represented the genomic structure (Fig. 3*A*; cosmid cR2D2-A contained additional sequences of unknown origin). A series of deletions were tested and ultimately localized the LPG$^+$-complementing region to a 3.5-kb *Hin*dIII fragment (Fig. 3*B*). Deletions within the *Hin*dIII fragment mapped the LPG$^+$-complementing activity to a 2.4-kb *Sst* I–*Hin*dIII fragment, while the overlapping 2.4-kb *Hin*dIII–*Bgl* II fragment failed to complement (Fig. 3*B*). The sequence of the 3.5-kb *Hin*dIII fragment was obtained and two ORFs were identified, of 187 and 434 amino acids (Fig. 3*B*). The regions encoding ORFs were transcribed into stable mRNAs (described below). The 434-residue ORF was located entirely within the LPG$^+$ *Sst* I–*Hin*dIII fragment and was truncated in the LPG$^-$ *Hin*dIII–*Bgl* II fragment; the 187-residue ORF lay entirely within the LPG$^-$ *Hin*dIII–*Bgl* II fragment. This implicated the 434-residue ORF in LPG complementation, and this ORF is hereafter termed LPG1.

Sequence database searches revealed that the 187-residue ORF (termed ARD1) was highly related to the yeast ARD1

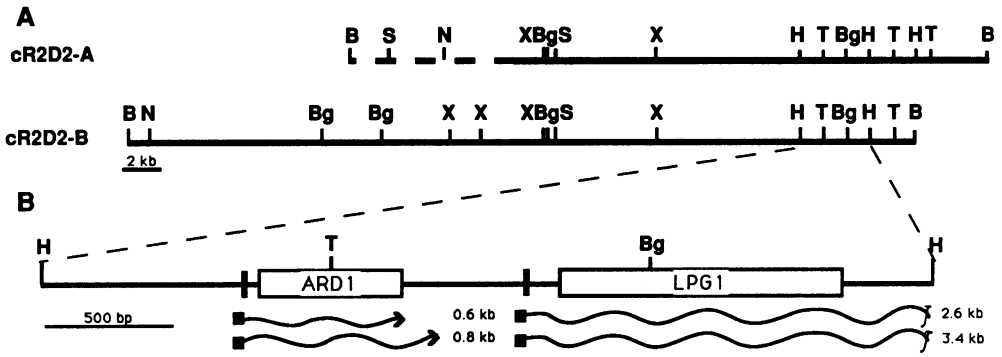

FIG. 3. Restriction maps, features, and DNA sequence of the LPG1 region. (*A*) Cosmid map. Aligned maps of the inserts within cosmids cR2D2-A and -B are shown; the dashed lines delimit the sequences of unknown origin in cR2D2-A. B, *Bam*HI; Bg, *Bgl* II; H, *Hin*dIII; N, *Not* I; S, *Spe* I; T, *Sst* I; X, *Xba* I. All sites for *Xba* I and *Bgl* II and only relevant sites for the other enzymes are shown. (*B*) LPG1 region. An expanded map of the 3.5-kb *Hin*dIII fragment is shown. The ORFs encoding ARD1 and LPG1 are shown as open boxes, splice acceptor sites by small vertical lines, and mRNAs as wavy lines with small filled boxes and arrowheads representing the trans-spliced miniexon and poly(A) tails, respectively (data for LPG1 are discussed in the text; data for ARD1 are not shown). Restriction sites are as in *A*. The sequence of this fragment has been determined on both strands in its entirety and has been assigned GenBank database accession no. L11348.

protein (24% amino acid identity; $P = 3.2 \times 10^{-6}$), encoding the regulatory subunit of protein *N*-acetyltransferase (31–33); LPG1 showed no significant matches. Both reading frames were excised by PCR, inserted into *Leishmania* expression vectors, and transfected into R2D2. Neither LPG complementation nor other phenotypes were detectable in the *Leishmania* ARD1 expressors; yeast ARD1 overexpressors also exhibit no phenotype (31–33). In contrast, agglutination and biosynthetic labeling studies revealed that only the LPG1 expressors synthesized authentic LPG, as observed in the cR2D2-B transfectants (data not shown). Thus, *LPG1* is the gene within the cosmids responsible for LPG complementation.

Potential Function of LPG1. Preliminary *in vitro* studies of LPG biosynthesis in the R2D2 mutant suggest that it is deficient in the addition of the single galactofuranose (Gal_f) within the glycan core (Fig. 4*C*; ref. 40). Several defects could bestow such a phenotype, such as loss of Gal_f transferase, the biosynthesis of a Gal_f donor, or alterations in some regulatory element. The first possibility was favored by the observation that LPG1 contains a short tract exhibiting similarity to mannose-binding proteins (Fig. 4*A*, overlined region; ref. 34), as an LPG-specific glycosyltransferase would be required to bind the nascent LPG precursor bearing terminal mannosyl residues. Hydrophilicity analysis of LPG1 revealed the presence of a putative signal-anchor sequence at amino acids 19–38 (Fig. 4*B*), predicting a topology consisting of a short, 18-amino acid N-terminal cytoplasmic tail, a 20-residue membrane anchor sequence, and a 396-residue luminal domain containing four N-linked glycosylation sites (Fig. 4 *A* and *C*). This arrangement resembles that of a large class of glycosyltransferases which possess short N-terminal cytoplasmic tails and large C-terminal luminal domains; comparisons amongst these also fail to reveal amino acid similarities (reviewed in ref. 35). Thus we propose that LPG1 may be a glycosyltransferase responsible for Gal_f addition in the endoplasmic reticulum of the parasite (Fig. 4*C*). This can be explored by using a cell-free system for LPG biosynthesis (36, 37).

Transcripts of LPG1. LPG1 was encoded by 2.6- and 3.4-kb mRNAs (Figs. 3*B* and 5). All trypanosomatid mRNAs bear a common 5′ 39-base leader sequence (the miniexon or spliced leader; reviewed in ref. 38) added by trans-splicing, and PCR with miniexon and gene-specific primers was used to map the splice acceptor sites for the LPG1 mRNA (32). A single product was obtained, mapping the splice site 133 bases upstream of the LPG1 initiation codon (data not shown). Because only a single site was identified, the size heterogeneity of the LPG1 mRNAs must reflect 3′ length heterogeneity as seen in numerous other trypanosomatid genes.

LPG biosynthesis is regulated during *Leishmania* development, with large amounts synthesized in logarithmic-phase promastigotes, somewhat increased amounts in stationary phase (containing metacyclic promastigotes), and 1000-fold less in amastigotes (18, 26). Surprisingly, *LPG1* was expressed throughout the life cycle, with only a 2-fold decrease in the 3.4-kb mRNA in amastigotes, which additionally lacked the minor 2.6-kb mRNA (Fig. 5). Translation of LPG1 mRNA may be highly regulated, as seen for another *Leishmania* gene (39). We consider it more likely that LPG1 participates in the biosynthesis of other cellular constituents present throughout the *Leishmania* life cycle. For example, a related class of glycosyl-phosphatidylinositol phospholipids is comparably abundant in both promastigote and amastigote stages, and several of these share the Gal_f-Man linkage in LPG whose synthesis is dependent upon LPG1 (Fig. 4*C*; ref. 19).

DISCUSSION

We have demonstrated the feasibility of functional complementation in the protozoan parasite *Leishmania* and recovered a gene relevant to the biosynthesis of a key virulence determinant. Recently we have been able to complement and

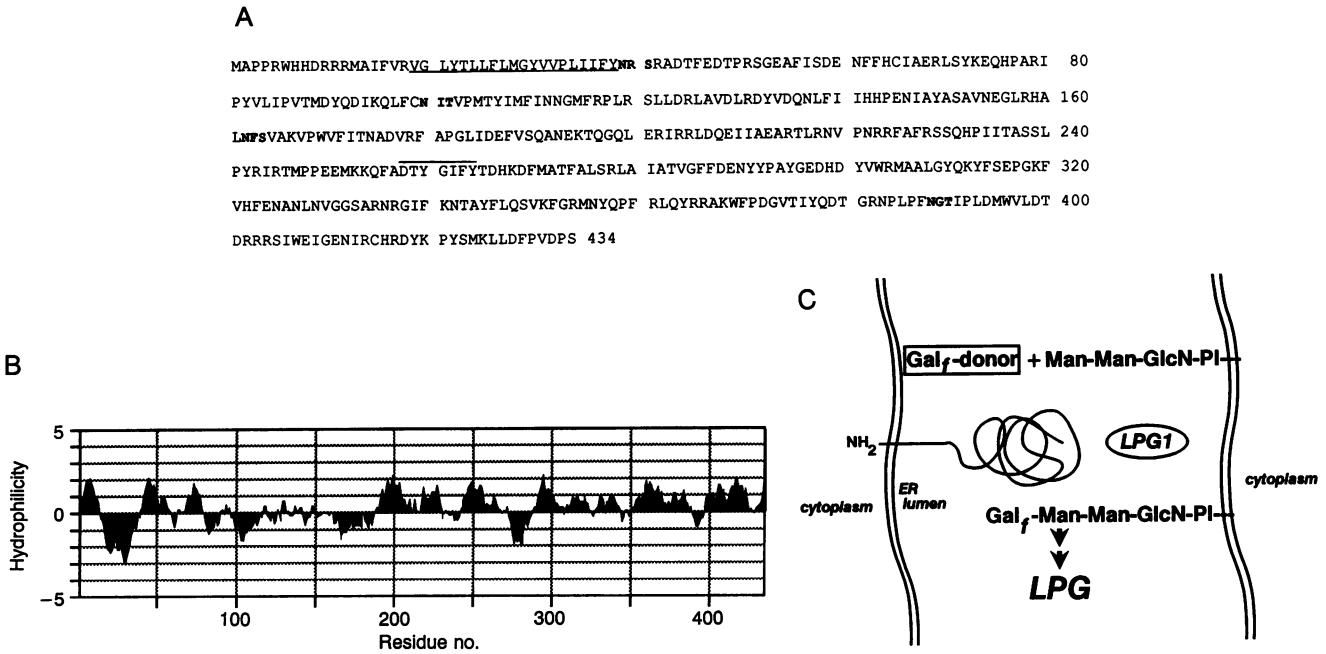

FIG. 4. Sequence homologies and properties of LPG1. (*A*) Predicted protein sequence of LPG1. The translated ORF corresponding to LPG1 is shown in the standard one-letter code. A predicted transmembrane signal-anchor sequence is underlined, and four potential N-linked glycosylation signals are shown in small boldface type. A segment with homology to mannose-binding proteins (34) is overlined. (*B*) Hydrophilicity plot of LPG1. A Kyte–Doolittle (41) analysis was performed with a window of 12 amino acids. (*C*) Potential topology and function of LPG1. LPG1 is shown with a large C-terminal endoplasmic-reticulum luminal domain and a short N-terminal cytoplasmic domain, a motif common in glycosyltransferases (35). The structure of the LPG glycan core precursor lacking Gal_f and present in R2D2 mutants is shown above the predicted LPG1 structure (Man, mannose; GlcN, glucosamine; PI, *lyso*-1-*O*-alkyl phosphatidylinositol). LPG1 is postulated to function in Gal_f addition, probably as the Gal_f glycosyltransferase but potentially in the synthesis of the Gal_f donor.

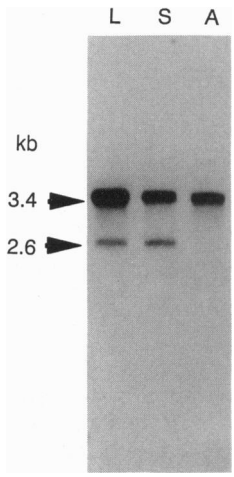

FIG. 5. Developmental expression of LPG1 mRNA. Northern blot analysis of logarithmic (L) and stationary (S)-phase promastigote and amastigote (A) mRNA with an LPG1-specific probe (1.1-kb *Bgl* II–*Hin*dIII; Fig. 3*B*) is shown. Only the results with poly(A)$^+$ RNA are shown, as the results were identical with total RNA preparations.

recover cosmids for two additional independent LPG$^-$ mutants defective in other LPG biosynthetic steps (unpublished data). Complementation of the R2D2 mutant was successful even though only partial expression was restored. These findings suggest that the complementation approach is robust and likely to be generally applicable in *Leishmania*.

LPG is required for parasite infectivity, and proteins involved in LPG biosynthesis are interesting since some LPG linkages are parasite-specific (18). LPG1 itself is implicated in the addition of Gal_f to the glycan core, and Gal_f is a unique linkage not present in host molecules. *In vitro* biochemical characterizations (36, 37) of the role of LPG1 and other LPG biosynthetic proteins may result in the development of selective inhibitors superior to the available pentavalent antimonials.

LPG has superb potential to be a rich grounds for exploring the parasite surface and genome. We have generated a large panel of LPG$^-$ mutants, and preliminary biochemical and genetic characterizations suggest that many are independent (unpublished data). LPG biosynthesis requires at least 25 enzymatic steps, which must be accompanied by accessory proteins responsible for proper regulation and cellular localization. Many will be required to synthesize LPG components occurring in other parasite molecules whose developmental expression differs from that of LPG. Thus, the developmental regulation of these genes is likely to prove interestingly complex and provide new insights into basic regulatory mechanisms in trypanosomatids. In a sense, we have initiated a study of genetic function and organization along the classic lines established in the analysis of operons in other microorganisms.

The success and potential of the LPG system have prompted us to consider the incorporation of other types of genetic screens into the functional complementation methodology. For example, transfection of cosmid library DNA prepared from virulent *Leishmania* into avirulent mutants, followed by screening for restoration of infectivity, could identify previously unrecognized virulence factors. More directed screens could focus on specific steps involved in metacyclogenesis, macrophage survival, and immunoregulation. We are confident that these methods may be extended to a variety of interesting phenotypes spanning every aspect of *Leishmania* biology.

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