A Specialized Pathway Affecting Virulence Glycoconjugates of Leishmania

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For virulence and transmission, the protozoan parasite Leishmania must assemble a complex glycolipid on the cell surface, the lipophosphoglycan (LPG). Functional complementation identified the gene LPG2, which encodes an integral Golgi membrane protein implicated in intracellular compartmentalization of LPG biosynthesis. Lpg2 mutants lack only characteristic disaccharide-phosphate repeats, normally present on both LPG and other surface or secreted molecules considered critical for infectivity. In contrast, a related yeast gene, VAN2/VRG4, is essential and required for general Golgi function. These results suggest that LPG2 participates in a specialized virulence pathway, which may offer an attractive target for chemotherapy.

In Leishmania, developmentally regulated glycoconjugates are essential during the infectious cycle. LPG, the major cell surface glycoconjugate of promastigotes, is a multifunctional virulence determinant required for development within the fly, resistance to complement in the bloodstream, and establishment of the infection within the macrophage (1, 2). In L. donovani, LPG consists of a phosphoglycan polymer of repeating disaccharide-PO4 units [Gal(β1-4)Man(α1-PO4)]6 (where Gal is galactose and Man is mannose) attached by means of a glycan core to an unusual phosphatidylinositol (PI)-lipid anchor (2). These components occur on other parasite molecules (sometimes with further modifications), and their expression can be constitutive or specific to a particular stage (2–5).

LPG is experimentally attractive because its biosynthesis can be studied in vitro and lpg− mutants can be readily generated (6–8). We recently demonstrated the feasibility of functional genetic complementation in Leishmania by identification of LPG1, which mediates addition of the Gal moiety (where "F" indicates furanose) within the LPG glycan core (9, 10). LPG1 thus represents a class of genes (class I) encoding LPG biosynthetic enzymes. Here, we report a second class of LPG mutants that affect compartmentalization and LPG assembly. These genes are functionally distinct from those affecting protein secretion and glycosylation.

The mutant C3PO synthesizes a truncated LPG containing only the glycan core and lipid anchor and lacking repeating units (8, 11). A wild-type L. donovani cosm! library in the shuttle vector cLYG was introduced into C3PO, and LPG+ transfectants were recovered by antibody screening (9, 10, 12). From these, four cosmids

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Fig. 1. Functional and physical mapping of LPG2. (A) Restriction map of the LPG2 locus. C3PO contained a deletion (indicated by parentheses) of 6.0 kb containing LPG2 (black box). Repeating sequences are indicated by shaded boxes. WT, wild type. (B) Position of the four overlapping cosmids recovered from LPG2 C3PO transfectants. (C) Localization of LPG2 by deletional analyses. The indicated restriction fragments were tested for LPG expression after cloning into appropriate Leishmania vectors (13). N, Not I; R, Eco RV; H, Hind III; S, Spe I.

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with overlapping inserts were recovered, and the functionally active gene was mapped to a single 3-kb Not I–Eco RV fragment (Fig. 1) (13). The sequence revealed a single open reading frame (ORF; termed LPG2) encoding a hypothetical protein of 341 amino acids (Figs. 1 and 2A). LPG2 was predicted to be hydroporphic and to possess up to 10 transmembrane domains (Fig. 2B). Sequence database searches showed that LPG2 was related to three yeast proteins (14). Of these, Vrg4/Van2 showed 28% identity (55% similarity) (Fig. 2) and has recently been found to be essential and required for correct protein N-glycosylation and maintenance of Golgi function and structure (15). Consistent with this, an active LPG2 protein tagged with an influenza hemagglutinin epitope at its COOH-terminus was localized to the Leishmania Golgi apparatus (Fig. 2C) (16).

Several lines of evidence implicated LPG2 in the lpg- defect of the C3P0 mutant. First, transfection of an LPG2 expression construct fully restored LPG levels to those present in the wild type (17). Second, molecular karyotype analysis showed that the LPG2 locus was deleted in C3P0 (Fig. 3A). Southern (DNA) blot analysis revealed that C3P0 contained a homzygous deletion of about 6 kb encompassing LPG2 (Fig. 1) (18). These studies showed that LPG2 is flanked by repeated DNA (Fig. 1) that may have served as the site of rearrangement (18). Although C3P0 was obtained after nitrosoquinidine mutagenesis, classic point mutants can also induce DNA rearrangements. Leishmania are thought to be diploid, which was confirmed for LPG2 specifically (19). The homozygous LPG2 deletion in C3P0 may be the result of a two-step process, whereby an initial heterozygous deletion was rendered homozygous. Finally, lpg- null mutants obtained by homologous gene replacement (19) lacked LPG and exhibited properties identical to those of the C3P0 mutant. LPG2 is thus not required for viability.

Northern (RNA) blot analysis revealed a single, 2.8-kb LPG2 mRNA in wild-type but not C3P0 promastigotes (Fig. 3B). This mRNA was abundant in log phase promastigotes and down-regulated in stationary phase promastigotes and lesion amastigotes. Amastigotes exhibited reduced amounts of the 2.8-kb mRNA, as well as larger forms ranging up to 9 kb, which probably represent unprocessed precursors. LPG2 mRNA amounts were correlated with the developmental expression of molecules such as LPG and the secretory acid phosphatase (sAP), which bear the repeating units (2–5).

The accumulation of a truncated LPG in C3P0 suggested a defect in the addition of the first mannose-phosphate residue (11). However, C3P0 microsomal membranes catalyzed the addition of repeating units onto endogenous LPG glycan core acceptors. C3P0 LPG synthesis was 65% of that in the wild type in the presence of detergent and was 11% of that of the wild type in its absence (20). In contrast, microsomes from the R2D2 mutant, which is defective in the synthesis of the LPG core, synthesized only background levels of LPG (6, 9, 21). Most importantly, the labeled product made by C3P0 microsomes was the same size as LPG synthesized in vitro from wild-type extracts, and mild acid hydrolysis released repeating units labeled with 14C-labeled Man (21). Thus, the enzymatic machinery for LPG synthesis is present in C3P0, which indicates that LPG2 must affect the localization or compartmentalization of a key LPG biosynthetic precursor or enzyme.

Previous studies have shown that C3P0 completely lacks repeating units (8). For sAP, this is shown by altered electrophoretic mobility in non-denaturing gels (Fig. 4A) and its failure to react with an antibody to the repeating unit, CAT7AE (Fig. 4B). Both electrophoretic mobility and repeating unit modification returned to levels found in the wild type when LPG2 expression was re-added in C3P0 (Fig. 4). Addition of repeating units to sAP is thought to occur in the Golgi apparatus, which is consistent with the localization of LPG2 (Fig. 2C) (3). The normal activity of the C3P0 sAP implies that it does not require repeating unit modifications for either secretion or catalysis (3, 8, 22).

Otherwise, the rate of growth, and the
expression or modifications of other membrane proteins and glycolipids, were unaffected in the lpg-2 mutants. The levels and pattern of total membrane proteins labeled with 3H-Man were similar in C30P and wild-type cells (8). Protein immunoblot analysis of the glycosylated abundant protein gp63 from the surface membrane revealed only fully modified gp63 in both C30P and lpg-2 knockout mutants (23). Similarly, sAP activity in the lpg-2 mutants implied that sAP N-glycosylation was unaffected, as this is known to be required for activity (23). C30P contained normal amounts of a heterogeneous group of smaller glycosinothiophospholipids (GPIs), which include the biosynthetic precursors of the GPI (glycosylphosphatidylinositol) lipid anchors of LPG and proteins (2, 11). Staining with the membrane dye DiOC6 (15) showed only normal morphology.

Although Leishmania LPG2 and yeast Van2p/Vrg4p are clearly related, their role or roles must be different. VAN2/VRG4 is essential and required for general Golgi function and structure, whereas lpg-2 cells lack only repeating units and are otherwise normal. This suggests that an ancestral gene has functionally diverged and specialized for LPG-related modifications in Leishmania. Consistent with this proposal, attempts to cross-complement Leishmania lpg-2 mutants with Vrg4p and yeast vrg4p mutants with LPG2 have been unsuccessful (24). Alternatively, the different mutant phenotypes may reflect more stringent demands placed on a common, shared pathway in yeast than in Leishmania, which differ in numerous aspects of protein secretion and glycolipid metabolism.

The specific molecular step or steps carried out by LPG2 are under investigation. Most probably, LPG2 mediates transport of an essential LPG precursor or biosynthetic enzyme into the secretory network. There are several proteins closely associated with LPG, such as the B protein and the strongly immunogenic protein KMP-11, both of which lack typical N-terminal signal peptides (25). Because putatively LPG-deficient lines show alterations in the surface localization of the B protein (26), there could be an LPG-coupled pathway for translocation of proteins into the secretory network involving LPG2.

The pleiotropic roles of LPG2 have implications for genetic studies of the role of LPG in the Leishmania infectious cycle. In both L. donovani and L. major, LPG mutants show alterations in virulence, as assessed by survival in host macrophages and modulation of the immune response (27). Because genes such as LPG2 affect multiple cellular components simultaneously, some caution must be exercised when attributing the phenotype of LPG mutants to specific molecules.

The properties of LPG2 point to the existence of a previously unknown class (II) of Leishmania biosynthetic genes, a class that is specifically devoted to the correct targeting and assembly of LPG and related molecules implicated in parasite virulence. At least some portion of this pathway is distinct from that mediating the assembly, modification, and targeting of secreted proteins and smaller glycolipids. Preliminary studies of other L. donovani mutants suggest the existence of other class II genes that primarily affect LPG assembly and targeting (28). Leishmania thus resembles prokaryotes in possessing pathways focused on the targeting of complex surface polysaccharides involved in virulence (29), whose dissection promises to provide a rich ground for basic cellular research. As in other pathogens, this Leishmania-specific pathway may well offer an attractive target for chemotherapeutic intervention.

REFERENCES AND NOTES

8. T. B. McNeely et al., Glycobiology 1, 63 (1990).
11. Preliminary studies show that C3PO synthesizes a truncated LPG that contains only the glycan core Gal-Gal-Gal-Glc-P-D-Man-Man-GlcNAc-P (where Glc is glucose and Ac is acetate) (A. Descoteaux, Y. Luo, S. J. Turco, S. M. Beverley, unpublished results) (2). This truncated LPG domain, when LPG2 expression is restored by transfection.
12. Independent transfectants (12,000) were obtained, providing about 10-fold coverage of the Leishmania genome. LPG2 transfection was recovered by two rounds of LPG screening with monoclonal antibody CA7AE (10).
13. Deletions shown in Fig. 1C were prepared either by partial digestion of cosmids C3PO-14 and C3PO-18 with HindIII or by transfection of fragments into the Leishmania shuttle vector pSNER (30). Each construct was transfected into C3PO and tested for LPG expression by agglutination (10).
16. LPG bearing the influenza hemagglutinin (HA) epitope at its COOH-terminus was prepared by polymerase chain reaction (PCR) with the use of the oligonucleotides SM25-5' (5'-gggatccgggctacgggtgcgggggctacgggtgcggg-3') and SM26-3' (5'-cgccgccgatgctggccggc-3'). The PCR product was digested with BamHI, inserted into the Bgl II site of pX3HfYG (37), and transfected into C3PO. LPG synthesis was shown by agglutination with CA7AE (10). Transfected cells were then fixed on a glass slide and fixed in 4% paraformaldehyde for 30 min at 4°C. After permeabilization with acetone (–20°C), the HA epitope was immunolocalized with the mouse monoclonal antibody 12CA5 (Boehringer Mannheim) and Texas red–conjugated antibody to mouse immunoglobulin. The slide was also stained with DAPI to visualize the kinetoplast and nuclear DNA. Golgi localization of C3PO is shown in Figs. 6 and 7.
17. The C3PO OFF was amplified by PCR with the oligonucleotides SM25-16 (5'-gggatccctactcaggttggagtgtc-3') and SM26-16 (5'-gggatccctactcaggttggagtgtc-3'). This C3PO transfectants were assayed for LPG expression as described (10). Surface localization determined by immunofluorescence with the CA7AE monoclonal antibody (32).
18. Repeating elements (Fig. 1A) were mapped by Southern blot analysis of wild-type and C3PO genomic DNAs with probes surrounding LPG2 (not shown in the figure). The gap indicated for C3PO marks only the absence of LPG2 sequences; the rearrangement is more complex than a simple deletion and possibly involves duplication of adjacent sequences or insertion of non-adjacent DNA.
19. The 5.5-kb Eco RIII fragment containing the LPG2 ORF was cloned into a pUC vector. The 0.6-kb Sph l-Spe I fragment from the LPG2 ORF was replaced by a 2.0-kb Sal–Bam HI fragment from pX3HfYG containing resistance to hygromycin B gene with the resistance gene pHYG and an upstream splice acceptor site. This construct was linearized with Not I and Xba I and transfected into the wide-type L. donovani 1506 strain. Southern blot analysis of seven colonies showed that all possessed heterozygous LPG2 replacements. For homologous replacements, we used a loss-of-heterozygosity protocol (33) (F. Guerres-Filho and S. M. Beverley, unpublish-
Interaction of Tyrosine-Based Sorting Signals with Clathrin-Associated Proteins

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Tyrosine-based signals within the cytoplasmic domain of integral membrane proteins mediate clathrin-dependent protein sorting in the endocytic and secretory pathways. A yeast twowhybrid system was used to identify proteins that bind to tyrosine-based signals. The medium chains (μ1 and μ2) of two clathrin-associated protein complexes (AP-1 and AP-2, respectively) specifically interacted with tyrosine-based signals of several integral membrane proteins. The interaction was confirmed by in vitro binding assays. Thus, it is likely that the medium chains serve as signal-binding components of the clathrin-dependent sorting machinery.

Targeting of integral membrane proteins to endosomes, lysosomes, the basolateral plasma membrane, and the trans-Golgi network (TGN) is largely mediated by sorting signals contained within the cytoplasmic domain of the proteins [reviewed in (1)]. Many of these sorting signals consist of continuous sequences of four to six amino acids containing a critical tyrosine residue. A subset of tyrosine-based signals conforms to the canonical motif YXXΦ, where Φ is tyrosine, X is any amino acid, and Φ is an amino acid with a bulky hydrophobic side chain (1). Although much has been learned in recent years about the structure and function of tyrosine-based signals, the molecular mechanisms involved in their recognition are still poorly understood. Previous studies have provided evidence for an association of cytoplasmic domains bearing tyrosine-based signals with clathrin-associated protein complexes (2). However, the exact identity of the signal-binding proteins and the molecular details of the recognition event remain to be established.

We decided to search for proteins that interact with tyrosine-based sorting signals, using a yeast two-hybrid approach (3). As a "bait" in the two-hybrid system, we used a triple repeat of the tyrosine-containing sequence SDYQRL (4, 5) from the cytoplasmic tail of the integral membrane protein TGN38 (6). This sequence has the characteristics of a YXXΦ motif and mediates both internalization from the cell surface and localization to the TGN (7). Screening of a mouse spleen complementary DNA (cDNA) library (~2.5 × 10^8 clones) resulted in the isolation of two clones that interacted specifically with the (SDYQRL)₄ bait sequence (8). The two clones (termed 3M2 and 3M9) corresponded to the medium chain (μ2) of the plasma membrane, clathrin-associated protein complex AP-2 (9). In addition to μ2, the AP-2 complex contains two large chains (α- and β-adaptin) and one small chain (σ2) (10).

Using growth on histidine-deficient (-His) plates as an assay (11), we found that proteins encoded by both 3M2 and 3M9 interacted not only with the (SDYQRL)₄ repeat but also with a single SDYQRL sequence and with the full-length TGN38 cytoplasmic tail (Fig. 1A). Mutation of the tyrosine (Y) residues in all three contexts abolished interaction with the μ2 clones (Fig. 1A). The binding specificity of μ2 was further characterized by mutation of each residue of the SDYQRL sequence individually to alanine. Only the Y and L residues were absolutely required for interaction with 3M9, whereas mutation of the S, D, and Q residues had no detectable effect, and mutation of the R residue decreased but did not completely abolish the ability to grow on -His plates (Fig. 1B). Thus, μ2 was capable of interacting with the sequence SDYQRL in various contexts and under sequence requirements that were consistent with those defined in studies in vivo (7).

To corroborate the results obtained with the two-hybrid system, we tested whether in vitro-translated, [35S]-methionine-labeled μ2 was capable of interacting with various sequences appended to glutathione-S-transferase (GST) (Fig. 2). We observed that both the 3M2 and 3M9 forms of μ2 bound to GST-(SDYQRL)₄ but not to GST-(SDGQRL)₄ or to GST (Fig. 2A). In vitro-translated luciferase, used as a negative control, did not interact with any of the GST fusion proteins tested (Fig. 2A). Binding of