Two Functionally Divergent UDP-Gal Nucleotide Sugar Transporters Participate in Phosphoglycan Synthesis in Leishmania major

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In the protozoan parasite Leishmania, abundant surface and secreted molecules, such as lipophosphoglycan (LPG) and proteophosphoglycans (PPGs), contain extensive galactose in the form of phosphoglycans (PGs) based on (Gal-Man-PO4) repeating units. PGs are synthesized in the parasite Golgi apparatus and require transport of cytoplasmic nucleotide sugar precursors to the Golgi lumen by nucleotide sugar transporters (NSTs). GDP-Man transport is mediated by the LPG2 gene product, and here we focused on transporters for UDP-Gal. Data base mining revealed 12 candidate NST genes in the L. major genome, including LPG2 as well as a candidate endoplasmic reticulum UDP-glucose transporter (HUT1L) and several pseudogenes. Gene knock-out studies established that two genes (LPG5A and LPG5B) encoded UDP-Gal NSTs. Although the single lpg5A− and lpg5B− mutants produced PGs, an lpg5A+/B− double mutant was completely deficient. PG synthesis was restored in the lpg5A−/B− mutant by heterologous expression of the human UDP-Gal transporter, and heterologous expression of LPG5A and LPG5B rescued the glycosylation defects of the mammalian Lec5 mutant, which is deficient in UDP-Gal uptake. Interestingly, the LPG5A and LPG5B functions overlap but are not equivalent, since the lpg5A− mutant showed a partial defect in LPG but not PPG phosphoglycosylation, whereas the lpg5B− mutant showed a partial defect in PPG but not LPG phosphoglycosylation. Identification of these key NSTs in Leishmania will facilitate the dissection of glycoconjugate synthesis and its role(s) in the parasite life cycle and further our understanding of NSTs generally.

Protozoan parasites of the genus Leishmania must survive in two separate and harsh environments to complete their life cycle: extracellularly within the midgut of the sand fly and intracellularly within the phagolysosome of vertebrate macrophages. A variety of abundant secreted and surface glycoconjugates have been implicated in key steps of the infectious cycle (1). Several of the most abundant of these contain repeating phosphoglycan (PG) polymers, such as lipophosphoglycan (LPG) and proteophosphoglycans (PPGs) (2). Secreted acid phosphatases also contain PGs, and other less abundant PG-containing molecules exist (3, 4). Leishmania PG repeating units contain the disaccharide-phosphate Gal(β1,4)-Man(α1)PO4 (referred to hereafter as the PG repeat unit) (5). PG repeats frequently contain additional sugar substitutions in different species and strains, most commonly on the Gal residue, although modifications of the Man also occur (Fig. 1) (6).

Leishmania PGs can be linked to the cell surface via glycosylphosphatidylinositol anchor attachment, directly as in LPG or through attachment to the protein backbone in PPGs (Fig. 1). The basic LPG structure has four domains: a 1-alkyl-2-lyso-P1 anchor, a heptasaccharide core consisting of Gal(α1,6)-Gal(α1,3)-Gal(β1,3)-Glc(α1-P04)6-Man(α1,3)-Man(α1,4)-GlcN(α1,6), the poly-PG “backbone” consisting of (Gal(β1,4)-Man(α1))P PG repeat units (average n ~ 15–30), and a terminating oligosaccharide cap (Fig. 1). In Leishmania major, LPG plays several roles in parasite survival, including control of parasite binding to the sand fly midgut wall, resistance to lysis by complement, protection from oxidative damage, and delaying phagolysosomal fusion (6–8). PPGs arise from a large gene family that encodes large proteins (>200 kDa) containing Ser/Thr-rich regions to which PG repeating units are attached (9, 10). PPGs occur in different forms, including membrane-bound PPG, filamentous PPG, and secreted PPG (9). These PG-containing molecules play roles in parasite transmission and virulence following sand fly biting, such as modulating macrophage immune functions (11–13). The role(s) of secreted acid phosphatases in parasite virulence are not well understood, since their levels are relatively low in L. major (14), and secreted acid phosphatase null mutants in Leishmania mexicana are avirulent (15).

The assembly of Leishmania PG repeating units to form LPG and PPGs occurs in the Golgi apparatus (16–18). Nucleotide sugar transporters (NSTs) transport cytoplasmically synthesized nucleotide sugars into the endoplasmic reticulum (ER) or Golgi lumen, where they are consumed in glycosylation reactions (19–22). A large number of NSTs have been identified,

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S3 and Figs. S1–S4.

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The abbreviations used are: PG, phosphoglycan; PPG, proteophosphoglycans; LPG, lipophosphoglycan; NST, nucleotide sugar transporter; Galβ, galactofuranose; ER, endoplasmic reticulum; WT, wild type; ORF, open reading frame; RT, reverse transcription; CHO, Chinese hamster ovary.
containing poly-\(N\)-acetyl-lactosamine (27), although this modification is not known to occur in *Leishmania*. The phenotype of galactose-deficient trypanosomes has been investigated previously through inactivation of UDP-glucose epimerase (28), a gene also found in *Leishmania*.

Since our interests concerned the role of galactosylation reactions occurring within secretory compartments, our strategy focused on inactivation of UDP-Gal transport. Thus, it was necessary first to identify the parasite’s UDP-Gal transporters. Twelve candidate NST sequences were identified in the *L. major* genome, with five showing varying degrees of similarity to known UDP-Gal transporters, including two pseudogenes. Our data indicate that two candidates, *LPG5A* and *LPG5B*, both encode UDP-Gal transporters and are required for phosphoglycan synthesis in *L. major*, although their functions differ significantly. More limited studies of another candidate, *HUUT1L*, suggest that it may be the functional homolog of the ER UDP-Glc NST, shown recently to be important to the protein folding glucosylation/reglucosylation process (29, 30).

### EXPERIMENTAL PROCEDURES

**Cell Culture, Reagents, and Transfection—*Leishmania*** were grown at 26 °C in M199 medium (U. S. Biological, Swampscott, MA) containing 10% fetal calf serum and other supplements as described (31). Unless otherwise indicated, the wild type (WT) *L. major* strain LV39 clone 5 (RHO/SU/59/P) was used (LV39). More limited studies were performed on *L. major* strains SD (MHOM/SN/74/SD) and Friedlin V1 (WHOM/IL/80/FN), whose genome was recently completed (32). *L. major* strains are closely related, showing on average of less than 1% sequence divergence (33). *L. mexicana* (MNYC/BZ/1962/M379) and *Leishmania donovani* (MHOM/SD/00/1S-2D) were also used.

*Leishmania* cells were transfected by electroporation using either a low voltage (31) or high voltage protocol (34). Following transfection, cells were allowed to grow 16–24 h in M199 medium with 10% fetal calf serum and then plated on semisolid media containing 1% Noble agar (Fisher) and the appropriate selective drugs. Individual colonies were picked and grown in liquid medium. Clones were maintained in selective medium and then removed from selection for one passage prior to experiments. Hygromycin B was from Calbiochem (now under EMD Biosciences (San Diego, CA)), G418 was from BioWhittaker (now under Cambrex Bio Science (Walkersville, MD)), puromycin powder was from Sigma, blasticidin was from Werner BioAgents (Jena, Germany), and phleomycin was from InvivoGen (San Diego, CA).

\[\text{[\alpha-32P]dCTP} \text{ was from PerkinElmer Life Sciences. UDP-[6-3H]Gal and GDP-[6-3H]Man were from American Radiolabeled Chemicals (St. Louis, MO). The protease inhibitors leupeptin, 4-(2-aminoethyl)-benzenesulfonyl fluoride, and E-64 were from Sigma. Other reagents not mentioned were from either Sigma or Fisher. PCR was done using Taq polymerase (Roche Applied Science) or Platinum Taq Hi Fidelity (Invitrogen). Oligonucleotide primers were purchased from IDT (Corvallis, IA), and their sequences are described in the supplemental materials. Restriction enzymes were from New England Biolabs Inc. (Ipswich, MA). Standard methods for general

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**FIGURE 1. PG and LPG and PPG structures.** Schematic diagrams of the basic PG repeating unit (A) and LPG and PPG structures (B) are shown. The detailed structures of LPG and PPG, including the cap, linkages, and anomeric configurations, are described elsewhere (2, 9). The GPI-anchored form of PPG is depicted, but other forms of PPG are similar regarding the PG structures. X, linear β1,3-linked galactose residue(s) that branch off the Gal-Man-PO4 repeating unit backbone (A). EN, ethanolamine.

and they now constitute a well characterized family of membrane proteins (20, 23). Previously, we showed that the *Leishmania* LPG2 gene encoded the Golgi GDP-Man transporter, one of the earliest NSTs identified, and the first multispecific NST, since it was additionally able to transport GDP-\(\alpha\)-Ara and GDP-\(\gamma\)-Fuc (17). *lpg2\(^{-}\)* mutants in three species of *Leishmania* were completely devoid of PGs (24–26); however, the consequences to parasite virulence differed greatly. Mutant *lpg2\(^{-}\)* *L. major* were unable to survive within macrophages or to cause pathology (8), whereas *lpg2\(^{-}\)* *L. mexicana* were able to survive and induce disease (26).

Because Golgi GDP-Man transport is required for PG synthesis, we hypothesized there would be a matching requirement for UDP-Gal transport as well. Loss of UDP-Gal transport in *L. major* should therefore render parasites PG-deficient and provide an additional tool to study PG function. Galactose is found in other surface glycoconjugates of trypanosomatids (18), such as the glycan head groups of Type II glycosylphosphatidylinositol anchor side chains and the glycan core structures of Type II glycosylphosphatidylinositol anchor side chains.
molecular biological techniques including Southern blotting were used as described (35).

**L. major Constructs**—Molecular manipulations are summarized in Table S1. Briefly, ORFs were amplified from *L. major* DNA and cloned into the unique BamHI site of the *Leishmania* expression vectors pXG(NEO) (B1288) or pXG(PHLEO) (B3324). For expression, oligonucleotide primers (Table S2) added an optimal translation initiation sequence (CCACC) upstream of the ORF start codon. PCR products were directly cloned into the TA vector pGEM-TEasy™ (Promega, Madison, WI), sequenced, and then liberated with BamHI and inserted into *Leishmania* expression vectors. Where indicated, ORFs were liberated from pXG vectors with BamHI and cloned into the unique BamHI site of pCDNA3 (B1974; Table S1). pXG(NEO)-hUGT1-cHA (B5541) and pMKIT-hUGT1-cHA (B5034) were gifts from H. Segawa.

**LPG5A** apleic replacement constructs were made by inserting ORFs encoding hygromycin B (HYG) or puromycin (PAC) resistance between 0.95-kb 5′ and 3′ *LPG* flanking regions present in pBSK-LPG5Aflanks (B4748; Table S1), making pBSK-LPG5A-HYGO (B5019) and pBSK-LPG5A-PACKO (B5020). Disruption constructs for *LPGSB* were made by inserting a 0.92-kb Xhol fragment from pXG(BSD) (B4908), which contains the DHFR-TS splice acceptor site and the BSD resistance gene, into the unique BsrGI site of pBSK-LPG5B-BSDisrupt (B5025; Table S1). A 1.9-kb Accl/Xhol fragment from pXG(NEO) (B1288), which contains the DHFR-TS splice acceptor site and the NEO resistance gene, was cloned into the BsrGI site of pBSK-LPG5Bsubclone to make pBSK-LPG5B-NEOdisrupt (B5026; Table S1).

**Mapping of the LPG5A Splice Acceptor Site**—Mapping of splice acceptor sites was accomplished using RT-PCR (36). FV1 RNA was prepared from exponentially growing parasites using Trizol (Roche Applied Science) and used as a template to generate randomly primed cDNAs using Superscript II (Stratagene, La Jolla, CA). A universal miniexon primer (SMB936) was used in conjunction with oligonucleotide SMB1581 (Table S2) to amplify portions of gene-specific cDNAs from the pool of cDNAs generated ORFs encoding hygromycin B (HYG) or puromycin (PAC) resistance between 0.95-kb 5′ and 3′ *LPG* flanking regions present in pBSK-LPG5Aflanks (B4748; Table S1), making pBSK-LPG5A-HYGO (B5019) and pBSK-LPG5A-PACKO (B5020). Disruption constructs for *LPGSB* were made by inserting a 0.92-kb Xhol fragment from pXG(BSD) (B4908), which contains the DHFR-TS splice acceptor site and the BSD resistance gene, into the unique BsrGI site of pBSK-LPG5B-BSDisrupt (B5025; Table S1). A 1.9-kb Accl/Xhol fragment from pXG(NEO) (B1288), which contains the DHFR-TS splice acceptor site and the NEO resistance gene, was cloned into the BsrGI site of pBSK-LPG5Bsubclone to make pBSK-LPG5B-NEOdisrupt (B5026; Table S1).

**Generation of LPG5 Mutants and Add-back Lines**—Targeting fragments for LPG5A and LPG5B were liberated from their respective vectors and purified prior to transfection into *L. major* LV39. For single-gene mutants, two rounds of transfection were done to recover homozygous mutants, and correct targeting was confirmed using Southern analysis. The names below follow the formal nomenclature for *Leishmania* (37). The Δlps5A/Δlps5A clonal strain A1 was chosen for biochemical analysis and is referred to as *lps5A*−. The Δlps5B/Δlps5B clonal strain A3 was chosen for biochemical analysis and is referred to as *lps5B*−. To produce a strain targeted in both *LPG5A* and *LPG5B*, *LPG5B* was disrupted in the *lps5A*− mutant. Clonal strain 2A-2, whose genotype Δlps5A/Δlps5A/Δlps5B/Δlps5B was confirmed by Southern analysis, is referred to as *lps5A*−/ *lps5B*−. Further details are discussed in supplemental materials.

Parasite strains were passed in vitro no more than six times (M1P6) before use.

**Immunoblotting**—Whole cell lysates from *L. major* promastigotes were prepared from exponential growth or stationary phase parasites in SD5 sample buffer; 5 × 106 cell equivalents/lane were separated by discontinuous SDS-PAGE on 4% stacking and 12.5% resolving gels and then electrotransferred to nitrocellulose membranes (Hybond-ECL; Amersham Biosciences). Protein loading was assessed by staining with 5% Ponceau S. 1% glacial acetic acid (w/v) prior to Western analysis. Monoclonal antibody WIC79.3 (38) was used at a 1:1000 dilution; CA7AE (39) was used at a 1:1000 dilution. Secondary antibodies conjugated to peroxidase were from Amersham Biosciences, and ECL reactions were conducted using Pierce or PerkinElmer Life Sciences chemiluminescence kits.

**Heterologous Expression**—WT Chinese hamster ovary (CHO) and Lec8 cells (40, 41) were from ATCC (Manassas, VA) and maintained in culture following ATCC recommendations. Lec8 cells were transformed using Lipofectamine2000 (Invitrogen) per manufacturer’s instructions. Transfectants were selected in medium containing 640 μg/ml G418; 5,000–10,000 stable transfectants were pooled and maintained in 300 μg/ml G418. Lec8 cells were transfected with constructs pCDNA3, pCDNA-LPG5A, pCDNA-LPG5B, pCDNA-HUT1L, and pMKIT-hUGT1-cHA to make the strains Lec8/pCDNA3 (hereafter referred to as Lec8/empty), Lec8/LPG5A, Lec8/LPG5B, Lec8/HUT1L, and Lec8/hUGT1, respectively.

**Fluorescent Lectin Binding**—The binding of fluorescein isothiocyanate-labeled *Maackia amurensis* agglutinin (EY Laboratories, San Mateo, CA) was assessed by fluorescence microscopy and fluorescence-activated cell sorting. Transfectants were removed from antibiotic selection for one passage prior to experiments. Cells grown on coverslips for IFA or scraped from flasks for fluorescence-activated cell sorting were washed once with phosphate-buffered saline and fixed for 1 min in 3.7% formaldehyde (v/v) in phosphate-buffered saline and incubated for 1 h at 25°C in 20 or 40 μg/ml *M. amurensis* fluorescein isothiocyanate in phosphate-buffered saline. Cells were washed three times with phosphate-buffered saline and visualized using an Olympus AX-70 microscope (Melville, NY) or a BD Biosciences FACSCalibur

**Purification and Analysis of LPG**—LPG was prepared from exponential and stationary phase parasites using previously described methods (42). To assess side chain modification, phosphoglycan repeating units were obtained by depolymerization of LPG using mild acid hydrolysis, dephosphorylated, and covalently labeled with either 8-aminonaphthalene-1,3,6-trisulfate or 1-aminopyrene-3,6,8-trisulfonate and analyzed using fluorescence-assisted carbohydrate electrophoresis and capillary electrophoresis (43).

The number of PG repeating units per LPG molecule was determined using two methods. The first method used gas chromatography-mass spectrometry (44). Briefly, the lipid anchor was cleaved by nitrous acid deamination, and the anhydromannose residue at the reducing end was further reduced with NaB3H4 to produce a single anhydromannitol residue for each LPG molecule. The LPG was hydrolyzed with 2 N trifluoroacetic acid to release neutral monosaccharides and phos-
phosphorylated monosaccharides (mostly galactose-6-phosphate). The phosphorylated monosaccharides were removed by anion exchange chromatography, and then the neutral monosaccharides were acetylated before gas chromatography-mass spectrometry. The ratio of mannose to anhydromannitol was determined using more sensitive PSI-BLAST comparisons (Table 1). Two additional potential \textit{L. major} NSTs were only detected using more sensitive PSI-BLAST comparisons (Table 1). 

**RESULTS**

\textit{Data Base Mining for NST Candidates}—We used the BLAST algorithm to identify candidate NSTs in the \textit{Leishmania} Genome Project data bases (45), focusing primarily on queries with proteins with experimentally confirmed NST activity. In the completed \textit{Leishmania} major genome, 12 sequences showing similarity to one or more NSTs were found (Table 1). To prioritize those mostly likely to encode UDP-Gal transporters, we compared the candidate \textit{Leishmania} NSTs with UDP-Gal transporters from humans, yeast, and plants as well as the GDP-Man transporter \textit{LPG2} and human YEA4 (Table 1).

Focusing first on those showing a relationship to known UDP-Gal transporters, \textit{LmjF24.0360} and \textit{LmjF18.0400} showed similarity to human UDP-Gal transporter hUGT1 and other UDP-Gal transporters (29–38\% identity, Table S3), but no significant similarity to \textit{Schizosaccharomyces pombe HUT1}, \textit{Arabidopsis thaliana AtlUTr1}, or \textit{LPG2} (Table 1 and S3). These two \textit{Leishmania} proteins were nearly as divergent from each other as they were from other UDP-Gal NSTs (26\% versus 29–38\% amino acid identity; Table S3). \textit{LmjF22.1010} (\textit{HUT1L}) showed similarity to the \textit{A. thaliana} AtUTr1 (30\% identity; Table S3), shown recently to be a UDP-Glc/Gal/NST located within the ER (29), and to \textit{S. pombe HUT1} (27\%), which has been associated with Golgi UDP-Gal uptake (46). \textit{LmjF15.0840} bore two internal stop codons (confirmed here), arguing that it was a pseudogene; otherwise, it showed a relationship to other UDP-Gal NSTs, including \textit{LmjF24.0360} and \textit{LmjF18.0400}.*\textit{LmjF15.055*} showed more than 95\% nucleotide identity with \textit{LmjF24.0360} (LPG5A) and mapped between the unrelated genes \textit{LmjF18.0400} and \textit{LmjF24.0360}.

### Table 1

**NST candidates identified in the \textit{L. major} strain Friedlin genome**

| Gene      | Systematic ID | Accession number | Homo sapiens | Saccharomyces pombe HUT1 | Arabidopsis thaliana UDP-Gal (AtUTr1) | H. sapiens YEA4 UDP-GA | L. major LPG2 GDP-Man | ORF (nucleotides) | Number of amino acids | TM domains |
|-----------|---------------|------------------|--------------|--------------------------|---------------------------------------|------------------------|-----------------------|---------------------|----------------------|-------------|
| LPG5A     | \textit{LmjF24.0360} | AY949108        | 10<sup>-20</sup> | NS<sup>a</sup> | NS          | NS          | NS          | 1,350<sup>h</sup> | 451                  | 10          |
| LPG5B     | \textit{LmjF18.0400} | AY949109        | 10<sup>-20</sup> | NS<sup>a</sup> | NS          | NS          | 1,686                  | 561                  | 10          |
| \textit{(LPG5-like)} | \textit{LmjF15.0840} | AY949106        | 10<sup>-13</sup> | NS          | 10<sup>-15</sup> | 1,050<sup>i</sup> | Pseudogene           | NA<sup>i</sup>       | 10          |
| HUT1L     | \textit{LmjF22.1010} | AY9491010       | NS          | 10<sup>-13</sup> | 10<sup>-15</sup> | NS          | 1,623                  | 580                  | 5–13       |
| LPG5A-like | \textit{LmjF15.055*} | AY949107       | N/A         | NS          | NS          | NS          | ~1,304<sup>c</sup> | Pseudogene           | NA          |
| LPG2      | \textit{LmjF34.3120} | CAJ08033        | NS          | NS          | NS          | NS          | 1,032                  | 341                  | 8–9        |
| LmjF19.1490 | CAJ07245       | NS          | NS          | NS          | NS          | 10<sup>-4</sup>  | 966                   | 322                  | 8–10       |
| LmjF19.1510 | CAJ07248       | NS          | NS          | NS          | NS          | 10<sup>-4</sup>  | 966                   | 322                  | 8–10       |
| LmjF30.2680 | CAJ06589       | NS          | NS          | NS          | NS          | 10<sup>-6</sup>  | 978                   | 325                  | 9–10       |
| LmjF18.1540 | CAJ04305       | NS          | NS          | 10<sup>-25</sup> | NS          | 1,089                  | 363                  | 7          |
| LmjF36.0670 | CAJ09020       | NS<sup>i</sup> | NS          | NS          | NS          | 1,389                  | 463                  | 7–10       |
| LmjF70.0400 | CAJ07000       | NS<sup>i</sup> | NS          | NS          | NS          | 1,833                  | 611                  | 8–10       |

<sup>a</sup> Sequence comparisons were done using the BLAST or PSI-BLAST algorithms (available on the World Wide Web at www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi).

<sup>b</sup> Assigned by the \textit{Leishmania} Genome Project.

<sup>c</sup> TM domains determined by submitting amino acid sequence to three different topology programs (see supplemental materials).

<sup>d</sup> NS, no significant similarity.

<sup>e</sup> \textit{LmjF24.0360} was assigned a start codon by the \textit{Leishmania} Genome Project leading to a predicted 600-amino acid ORF; however, our results show that the correct ORF is 451 amino acids.

<sup>f</sup> \textit{LmjF15.0840} is a pseudogene containing two internal stop codons, identified by both our and the \textit{Leishmania} Genome Project sequence. In \textit{L. infantum} this gene appears intact (\textit{LmjF15.0890}), but it is absent in \textit{L. braziliensis}. The BLASTP comparison was performed with the conceptual protein sequence.

<sup>g</sup> NA<sup>i</sup>, not applicable to pseudogenes.

<sup>h</sup> The \textit{Leishmania} Genome Project release 5.2 annotates this as “\textit{LmjF15.055*},” although it is located on chromosome 20. Although clearly a pseudogene with multiple gaps and substitutions, it shows strong homology to LPG5A (27\%), which has been associated with other functionally characterized UDP-Gal NSTs (26\% versus 29–38\% amino acid identity; Table S3). \textit{LmjF22.1010} (\textit{HUT1L}) showed similarity to the \textit{A. thaliana} AtUTr1 (30\% identity; Table S3), shown recently to be a UDP-Glc/Gal/NST located within the ER (29), and to \textit{S. pombe HUT1} (27\%), which has been associated with Golgi UDP-Gal uptake (46).

<sup>i</sup> \textit{LmjF36.0670} showed a relationship to members of the solute carrier family A3 in PSI-BLAST comparisons (\textit{p} < 10<sup>-10</sup>), but it is absent in \textit{L. braziliensis}.

<sup>j</sup> \textit{LmjF07.0400} showed a relationship to members of the solute carrier family F5 in PSI-BLAST comparisons (\textit{p} < 10<sup>-10</sup>).
Leishmania UDP-Gal Transporters

We identified orthologs for LPG5A, LPG5B, and HUT1L using reverse transcription-PCR and the fact that all Leishmania mRNAs bear a common 39-nucleotide 5’ leader sequence added by trans-splicing. This placed the splice acceptor at a position located 384 nucleotides 3’ of the annotated AUG for the 600-amino-acid LPG5A ORF (Fig. 3A).

The next AUG following this mapped splice acceptor occurred 63 nucleotides downstream (Fig. 3B), leading to a predicted LPG5A protein of 451 amino acids (Table 1). As shown below, the 451-amino-acid LPG5A ORF was functional. Although efforts to similarly map the LPG5B 5’ end were unsuccessful, current data suggest that the 561-amino-acid ORF for LPG5B is correct (discussed below).

Several transmembrane (TM) prediction algorithms suggested the occurrence of 8–10 TM domains for both LPG5A and LPG5B, consistent with predictions for other NSTs (Table 1, Figs. 4 and S1). We incorporated previous studies of the murine CMP-sialic acid transporter, whose topology has been experimentally determined and whose sequence is closely related to UDP-Gal transporters (50). From this and sequence alignment criteria, we developed models predicting 10 transmembrane domains in the LPG5A and LPG5B proteins (Figs. 4 and S1; Fig. S2 presents an amino acid alignment with shaded predicted TM domains).

One striking feature in both LPG5A and LPG5B was the prediction of a large cytoplasmic loop (134 and 199 amino acids, respectively) between TM domains 2 and 3 (Figs. 4 and S1). For comparison, this loop is 25–26 amino acids in the mammalian UDP-Gal and CMP-Sia NSTs, and no other characterized NSTs to date bear a loop comparable with those of LPG5A or LPG5B.

FIGURE 2. Phylogenetic tree of Leishmania NSTs. This evolutionary tree depicts relationships among L. major NST candidates and key NSTs of known function. These include ScVRG4, Saccharomyces cerevisiae GDP-Man transporter (P40107), S. pombe GMS1, S. pombe UDP-Gal transporter (BAA24703), mouse CMP-Sia transporter (CAAs5855), human UGT1 UDP-Gal transporter (BAA12673), A. thaliana AtrUTR1 ER UDP-Glc/Gal transporter (AAM48281), and the S. cerevisiae HUT1 transporter (Q12250). The scale represents amino acid substitutions. Amino acid sequences were aligned using the M-Coffee algorithm (71), which incorporates input from a number of pairwise and multiple alignment procedures and generates a consensus. Following the removal of gap positions, a bootstrap consensus evolutionary tree was constructed using the neighbor-joining algorithm (1000 replicas) implemented in the MEGA 3.1 evolutionary analysis package (72). The smaller numbers at the nodes represent the bootstrap consensus percentages and are a measure of confidence. Tests of a variety of different alignment and tree algorithms did not reveal any significant differences within the major lineages depicted here (data not shown). A table of pairwise percentage identities can be found in Table S3. Due to its different alignment and tree algorithms did not reveal any significant differences within the major lineages depicted here, this cluster contained transporters with specificities for a variety of UDP-sugars as well as CMP-sialic acid transporters (23), reinforcing prior observations that phylogeny is of only limited usefulness in predicting nucleotide sugar specificity in the NST family (20, 21, 49). Nonetheless, we used evolutionary relationship as a guide to focus on the two candidates showing the best homology to hUGT1, which we named LPG5A and LPG5B (Table 1), and carried out limited studies on the HUT1-related gene LmjF22.1010, which we named HUT1L (HUT1-like).

We identified orthologs for LPG5A, LPG5B, and HUT1L in the provisional genome sequences of Leishmania infantum and Leishmania braziliensis. Interestingly, seemingly intact orthologs for pseudogenes LmjF15.0840 and LmjF15.1055 were found in L. infantum (LinJ15.0890 and LinJ15.1080, respectively) but not L. braziliensis. We did not identify additional NSTs in these species beyond orthologs of those of L. major, although this conclusion is tentative, since their genomes were not complete.

Properties of the LPG5A and LPG5B Open Reading Frames—Typical eukaryotic NSTs are about 320–400 amino acids in length; however, the predicted LPG5A and LPG5B proteins comprised 600 and 561 amino acids, respectively (Table 1). We mapped the 5’ end of the LPG5A mRNA using reverse transcription-PCR and the fact that all Leishmania mRNAs bear a common 39-nucleotide 5’ leader sequence added by trans-splicing. This placed the splice acceptor at a position located 384 nucleotides 3’ of the annotated AUG for the 600-amino-acid LPG5A ORF (Fig. 3A). The next AUG following this mapped splice acceptor occurred 63 nucleotides downstream (Fig. 3B), leading to a predicted LPG5A protein of 451 amino acids (Table 1). As shown below, the 451-amino-acid LPG5A ORF was functional. Although efforts to similarly map the LPG5B 5’ end were unsuccessful, current data suggest that the 561-amino-acid ORF for LPG5B is correct (discussed below).

Several transmembrane (TM) prediction algorithms suggested the occurrence of 8–10 TM domains for both LPG5A and LPG5B, consistent with predictions for other NSTs (Table 1, Figs. 4 and S1). We incorporated previous studies of the murine CMP-sialic acid transporter, whose topology has been experimentally determined and whose sequence is closely related to UDP-Gal transporters (50). From this and sequence alignment criteria, we developed models predicting 10 transmembrane domains in the LPG5A and LPG5B proteins (Figs. 4 and S1; Fig. S2 presents an amino acid alignment with shaded predicted TM domains).

One striking feature in both LPG5A and LPG5B was the prediction of a large cytoplasmic loop (134 and 199 amino acids, respectively) between TM domains 2 and 3 (Figs. 4 and S1). For comparison, this loop is 25–26 amino acids in the mammalian UDP-Gal and CMP-Sia NSTs, and no other characterized NSTs to date bear a loop comparable with those of LPG5A or LPG5B.
Other conserved features included several buried charged residues, including conserved Glu and Lys residues in the second predicted TM2 domains of LPG5A, LPG5B, murine CMP-Sia, and human UDP-Gal NSTs (Figs. 4, S1, and S2). Residues shown to be functionally important in the mammalian UDP-Gal and CMP-Sia (21) were conserved in LPG5A and LPG5B as well (Figs. 4, S1, and S2).

LPG5A and LPG5B Rescue the Glycosylation Defect of the UDP-Gal NST-deficient Lec8 Mutant—To test the function(s) of the three UDP-Gal transporter candidates LPG5A, LPG5B, and HUT1L, we asked whether heterologous expression of these ORFs could rescue the CHO cell mutant Lec8. This mutant is deficient in UDP-Gal transport, leading to decreased glycoprotein galactosylation, deficiency in sialylation, and a failure to react with the sialic acid (α2,3)-galactose-specific M. amurensis agglutinin (41, 51). When a functional UDP-Gal transporter is expressed in these cells, M. amurensis agglutinin binding is restored (52), a widely used assay to functionally identify and characterize UDP-Gal transporters from a variety of species (19, 21, 22).

M. amurensis agglutinin binding was examined in stably transfected Lec8 cells bearing constructs expressing LPG5A, LPG5B, or HUT1L. By fluorescence microscopy (Fig. 5A) and flow cytometry (Fig. 5B), LPG5A and LPG5B transfectants showed strong M. amurensis agglutinin binding, similar to that of WT CHO cells or to Lec8 cells expressing the human UDP-Gal transporter HUGT1. A caveat to the negative HUT1L result is that a specific antiserum to HUT1L was not available, precluding verification of its correct expression and/or localization in the transfectants. These data suggested that LPG5A and LPG5B probably encoded proteins with UDP-Gal transporter activity.

Generation of Null LPG5A, LPG5B, and Double Mutants—To further study the candidate UDP-Gal transporters, we created null mutants. Leishmania are asexual and predominantly disomic, necessitating the use of two successive rounds of transfection to generate null mutants (53, 54). For LPG5A, we designed constructs that would precisely replace the 451-amino acid LPG5A ORF with drug resistance marker ORFs encoding hygromycin B (HYG) or puromycin (PAC) resistance (Fig. 6A). Starting with the WT LV39 strain of L. major, correct replacement of both alleles was confirmed by Southern analysis (Fig. 6C), and this mutant is termed lpg5A−/−.

For LPG5B, we were unable to determine the mRNA start site by RT-PCR using a variety of LPG5B-specific primers and amplification conditions (data not shown); the reasons for this are not evident to us. The assignment of the LPG5B ORF, however, was supported by functional assays (Fig. 5), sequence alignment (Fig. S2), and transfection assays showing that several shorter ORFs initiating at downstream AUGs were inactive (data not shown). Thus, for LPG5B an insertional inactivation approach was used where autonomous cassettes mediating resistance to blastocidin (BSD) and G418 (NEO) were inserted into the LPG5B ORF (Fig. 6B). Correct targeting was confirmed by Southern analysis (Fig. 6D), and this mutant contained lpg5B−. To make the lpg5A−/lpg5B− mutant, we inactivated LPG5B in the background of the lpg5A− mutant above. Successful alteration of both genes was confirmed by Southern analysis (Fig. 6, C and D). All mutants grew at normal rates and to normal densities in standard M199 culture media in vitro (data not shown).

LPG5A or LPG5B Is Sufficient for PG Synthesis, but the lpg5A−/lpg5B− Mutant Lacks PGs—L. major PG repeats are often modified by side chain Gal residues, added in consecutive β1,3-
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linkages to the Gal residue within the PG repeating unit by specific Gal-transferases (Fig. 1) (55). Gal-modified PG repeats can be recognized by the monoclonal antibody WIC79.3 (56), whereas unmodified PG repeats can be recognized by monoclonal antibody CA7AE (39). We used these in immunoblotting to probe the effects of LPGSA and LPGSB mutations on PG synthesis; similar results were obtained by immunofluorescence microscopy (data not shown).

WT extracts showed strong reactivity to WIC79.3 in the 25–100-kDa region, corresponding to LPG, whereas a much fainter reactivity was observed within the stacking gel, where the large PPGs migrate under these conditions (Fig. 7A, lane 1) (7, 10). In the lpg5A mutant, strong WIC79.3 reactivity remained, but it migrated faster with an apparent molecular mass of 20–40 kDa (Fig. 7A, lane 2). A slight increase in WIC79.3 reactivity in the stacking gel/PPG region was also observed (Fig. 7A, lane 2). In contrast, for the lpg5B mutant, strong WIC79.3 reactivity in the 25–100-kDa “LPG region” remained, whereas greatly elevated reactivity was observed within the stacking gel/“PPG region” (Fig. 7A, lane 3). These data suggested that loss of LPGSA primarily affected PGs associated with LPG, whereas loss of LPGSB primarily affected PGs associated with PPG.

Both the lpg5A and lpg5B mutant showed a small increase in CA7AE reactivity in the stacking gel/PPG region (Fig. 7B, lanes 2 and 3 versus lane 1). This was consistent with the idea that loss of UDP-Gal NST activity leads to decreased levels of galactosylated PGs. The lpg5B PPG showed slightly decreased mobility relative to that of lpg5A (Fig. 7B, lanes 2 and 3). The significance of this observation is uncertain, since structural studies of the PPG were not undertaken; potentially, the CA7AE-reactive PPG population is a minor fraction of total PPGs.

In contrast to the single mutants, no reactivity with either monoclonal antibody was seen for the double lpg5A−/5B− mutant, in either the LPG or stacking gel/PPG regions (Fig. 7, A and B, lanes 5). This phenotype was identical to that seen in the lpg2− mutant (Fig. 7, A and B, lanes 6), which is completely deficient in PGs through loss of the GDP-Man transporter (17). Thus, lpg5A−/5B− had similarly completely lost PG synthesis. PG expression was restored by reintroduction of either LPGSA or LPGSB, singly (data not shown) or in combination (Fig. 7C).

Altered LPG Structure in lpg5A− but Not lpg5B− Mutants—The altered mobility of lpg5A− LPG (Fig. 7A, lane 2) suggested that it was structurally altered, presumably arising through decreased levels of galactosylation. This could arise through decreased numbers of PG side chain Gal residues or PG repeats or both (Fig. 1A). We thus purified LPG from WT and mutant strains and determined its structure (Table 2). Side-chain galactosylation was assessed by depolymerizing LPG with mild acid to release the PG repeating units, which were then separated by capillary electrophoresis and quantitated. To examine the number of PG repeats in the LPG backbone of the lpg5A− mutant, we measured the ratio of mannose to anhydromanitol, the former arising from the PG repeats and the latter from the LPG core, following removal of the lipid anchor by nitrous acid deamination and reduction with NaBH₄ and hydrolysis to monosaccharides (see “Experimental Procedures”).

For lpg5A− LPG, a general decrease in both types of galactosylation was seen (Table 2). The fraction of PG repeats lacking Gal side chains was 3–4-fold higher (0.40 versus 0.09 WT in log phase), and 43% fewer Gal residues per PG repeat were added.
Moreover, the number of lpg5A− PG repeats was reduced to 50–74% of WT (13.5 and 10 versus 18.2 and 19.8 for WT log and stationary phase LPG, respectively). In contrast, LPG from the lpg5B− mutant closely resembled that of WT, in the number of side chain Gal residues/PG repeat, the fraction of unmodified PG repeats, and the total number of repeats (Table 2). These results were consistent with the immunoblot analysis (Fig. 7).

Thus, loss of lpg5A, but not lpg5B, resulted in a general deficit in LPG galactosylation, affecting both side chain and PG repeat galactosylation quantitatively, to about the same extent.

Loss of LPG5B Primarily Affects PPG Modification—Although LPG was unaffected by loss of lpg5B, PPGs in the lpg5B− but not lpg5A− mutant reacted much more strongly with antibody WIC79.3 (Fig. 7A, lanes 1–3). This gain seemed contrary to the expectation that loss of a UDP-Gal transporter would lead to a deficit in some aspect of PG galactosylation, which might lead to a loss (or perhaps no change) in WIC79.3 reactivity. However, if WIC79.3 were more strongly reactive with PG repeats bearing fewer side chain Gal residues, increased reactivity could arise by an increased level of monogalactosylated PG repeats, at the expense of polygalactosylated PG repeats. Thus, the immunoblotting results are explicable as follows. The much greater reactivity of PPGs with WIC79.3 in the lpg5B− mutant can be attributed to increased levels of monogalactosylated PG repeats, relative to WT PPGs that bear predominantly oligo-/polygalactosylated PG repeats. Thus, loss of lpg5B appears to result in a decrease in galactosylation, but specifically affecting PPGs and not LPG (Table 1), in contrast to loss of LPG5A, where the reverse is seen.

The lpg5A−/5B− Mutant Is Rescued by Expression of a Heterologous UDP-Gal Transporter—Attempts to demonstrate decreased UDP-Gal uptake with purified lpg5A−/B− microsomes were unsuccessful (supplemental materials). This raised the formal possibility that the PG deficiency of the lpg5A−/5B− mutant might arise through mechanisms other than decreased UDP-Gal uptake. If this idea were correct, we reasoned that expression of a distantly related heterologous UDP-Gal transporter would be unable to rescue PG synthesis. Thus, we expressed the human UDP-Gal transporter hUGT1 in the lpg5A−/5B− mutant. However, in these transfectants, WIC79.3-reactive LPG was restored fully (Fig. 7D, lane 3 versus lane 1). It is highly unlikely that hUGT1 expression would rescue PG synthesis by any mechanism other than restoration of UDP-Gal uptake, and thus we conclude that the PG deficiency of the lpg5A−/B− double mutant arises from a lack of UDP-Gal transport activity.

**DISCUSSION**

LPG5A and LPG5B Encode UDP-Gal Transporters Required for L. major Phosphoglycan Synthesis—Twelve NST candidate sequences were identified in the L. major genome by data base mining. Of these, LPG5A, LPG5B, and HUT1L showed an evolutionary relationship to NSTs able to transport UDP-Gal in other species (Fig. 2). Since NST phylogeny does not always reflect substrate specificity (21, 23, 49), we tested these three candidates in functional assays. Expression of both LPG5A and LPG5B, but not HUT1L, could rescue M. amurensis agglutinin binding in UDP-Gal transport-deficient Lec8 cells (Fig. 5). Although L. major mutants inactivated in either LPG5A or LPG5B still made PGs, we found that a mutant genetically defective in both LPG5A and LPG5B lacked detectable PGs (Fig. 7) and that PG levels were restored by re-expression of either (or both) of these genes (Fig. 7) but not by overexpression of HUT1L (data not shown). We were unable to measure UDP-Gal uptake in purified *Leishmania* microsomes using methods polygalactosylated PG repeats (Fig. S3). This matches the finding that the LPG present in the immunogen for WIC79.3 was probably monoglactosylated LPG (38).

Thus, the immunoblotting results are explicable as follows. The much greater reactivity of PPGs with WIC79.3 in the lpg5B− mutant can be attributed to increased levels of monogalactosylated PG repeats, relative to WT PPGs that bear predominantly oligo-/polygalactosylated PG repeats. Thus, loss of LPG5B appears to result in a decrease in galactosylation, but specifically affecting PPGs and not LPG (Table 1), in contrast to loss of LPG5A, where the reverse is seen.

The lpg5A−/5B− Mutant Is Rescued by Expression of a Heterologous UDP-Gal Transporter—Attempts to demonstrate decreased UDP-Gal uptake with purified lpg5A−/B− microsomes were unsuccessful (supplemental materials). This raised the formal possibility that the PG deficiency of the lpg5A−/5B− mutant might arise through mechanisms other than decreased UDP-Gal uptake. If this idea were correct, we reasoned that expression of a distantly related heterologous UDP-Gal transporter would be unable to rescue PG synthesis. Thus, we expressed the human UDP-Gal transporter hUGT1 in the lpg5A−/5B− mutant. However, in these transfectants, WIC79.3-reactive LPG was restored fully (Fig. 7D, lane 3 versus lane 1). It is highly unlikely that hUGT1 expression would rescue PG synthesis by any mechanism other than restoration of UDP-Gal uptake, and thus we conclude that the PG deficiency of the lpg5A−/B− double mutant arises from a lack of UDP-Gal transport activity.
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applied successfully previously to the LPG2 Golgi GDP-Man transporter, possibly due to the presence of a strong interfering galactosylation activity, whose source was not identified (supplemental material). Thus, to confirm that the loss of PG synthesis in the lpg5A+/5B- mutant arose specifically from a lack of UDP-Gal NST activity, we introduced the human UDP-Gal NST hUGT1, which completely restored PG synthesis. Together, these data indicate that LPG5A and LPG5B both encode UDP-Gal transporters, whose combined inactivation results in loss of PGs in L. major.

From the structures of known Leishmania glycoconjugates, one could predict that in addition to GDP-Man and UDP-Gal, NSTs mediating uptake of UDP-Glc, GDP-D-arabinopyranose, and UDP-galactofuranose are required. Many NSTs are multispecific and able to transport more than one nucleotide sugar, such as LPG2, which can transport guanine diphosphoarabinose and guanine diphosphofucose in addition to GDP-Man (17, 23, 57). Since UDP-Glc epimerase is localized to the glycosome in trypanosomatids (58), potentially UDP-Gal transporters, possibly due to the presence of a strong interfering transporter, might be involved in UDP-galactofuranose transport. Immunoblotting with antibody CA7AE, which recognizes unmodified PGs. Loss of LPG5A and LPG5B leads to a small increase in unmodified PGs in the PPG fraction, whereas the lpg5A+/5B- mutant lacks unmodified PGs. Intervening lanes not related to this experiment were removed. C. PGs are rescued in the lpg5A+/5B- mutant following expression of the human UDP-Gal transporter. Immunoblotting was with WIC79.3. Lane 1, WT; lane 2, lpg5A-/lane 3, lpg5B-/lane 4, empty; lane 5, lpg5A+/5B-; lane 6, lpg5A-/5B-. Loss of LPG5A in LPG5BWT gene (data not shown), a common finding in other studies in Leishmania (available on the World Wide Web at www.genedb.org; data not shown). Of the remaining NSTs, several showed a distant relationship to the LPG2 GDP-Man transporter (LmjF19.1490/19.1510 and LmjF30.2680), and one showed a relationship to the YEA4 group of NSTs, which includes UDP-glucuronic acid/UDP-GalNAc transporters (LmjF15.0840).

Orthologous genes related to LmjF36.0670 were restricted to Leishmania species, whereas an ortholog of LmjF07.0400 was present in T. brucei but not T. cruzi, and one related to LmjF19.1490/19.1510 occurred in the T. cruzi but not T. brucei genomes. Potentially these NSTs could be redundant, have specificities for nucleotide sugars required uniquely by each species, or act in distinct cellular compartments. One attractive role for NSTs shared by L. major and T. cruzi, but not T. brucei, involves UDP-galactofuranose, since UDP-galactopyranose mutase, the enzyme responsible for UDP-Gal, synthesis, is absent in the T. brucei genome (59). Correspondingly, Leishmania and T. cruzi, but not T. brucei, synthesize a variety of Gal- containing glycoconjugates (see references cited in Ref. 59).

A Probable Role for Leishmania HUT1L as an ER UDP-Glc NST—Leishmania HUT1L was of interest, since its closest relatives mediate UDP-Gal uptake in other species (30). However, heterologous expression of HUT1L did not rescue the UDP-Gal transport defect of Lec8 cells (Fig. 5). In preliminary studies, we attempted to generate null mutants for Leishmania HUT1L with the same methods successful with LPG5A and LPG5B (Fig. 6; data not shown). Although heterozygous replacements were readily obtained, we were unable to obtain homozygous null mutants (data not shown). Instead, a variety of aneuploid and tetraploid cell lines were recovered bearing the second replacement as well as the WT gene (data not shown), a common finding in other studies in Leishmania attempting to knock out essential genes (60).

Recently, it was shown that the Arabidopsis HUT1L relative AtUTr1 encodes a UDP-Glc/Gal NST activity localized to the ER (29), as do fungal HUT1s (30). Interestingly, both HUT1L and AtUTr1 bear a C-terminal lysine-rich motif implicated in ER localization (61). Within the ER, AtUTr1 participates in the
unfolded protein response, through provision of ER UDP-Glc required for the glucosylation/glucohydrolase cycle occurring as part of protein folding and quality control (29, 62, 63). Were Leishmania HUT1L similarly involved in the protein folding cycle in Leishmania, its loss could prove lethal to the cell, since unlike other organisms, the core glycans added to nascent trypanosomatid glycoproteins lack Glc residues and must necessarily be acquired in the ER (64). Preliminary data show localization of a green fluorescent protein-tagged HUT1L protein to the Leishmania ER, and thus we believe that Leishmania HUT1L may likely be the parasite’s ER UDP-Glc transporter. Functional studies will be required in the future to confirm the specificity of this and other novel NSTs identified here.

_LPG5A and LPG5B Show Functional Divergence_—Although complete ablation of PG synthesis required inactivation of both LPG5A and LPG5B, both the single lpg5A− and lpg5B− mutants made abundant PGs, albeit with different phenotypes. For the lpg5A− mutant, a structurally altered LPG was made (Fig. 7A), containing less side chain galactosylation and fewer PG repeating units (Table 2), but alterations in PPG immunoreactivity were not detected. In contrast, the structure of LPG was unchanged in the lpg5B− mutant (Table 2), but alterations in PPG immunoreactivity were seen (Fig. 7A). These were attributed to decreased galactosylation, leading to an increase in WIC79.3-reactive monogalactosylated PGs at the expense of oligo- or polygalactosylated PGs linked to PPG (Figs. 7 and S3).

What underlies the functional differences between LPG5A and LPG5B? Some NSTs display specific interactions with glycosyltransferases, as in the case of the mammalian Golgi UDP-galactose transporter UGT1 with UDP-galactosylceramide galactosyltransferase (65). Building upon this model, perhaps key Gal transferases responsible for LPG versus PPG PG synthesis preferentially associate with the LPG5A and LPG5B UDP-Gal NSTs, respectively. Another possibility invokes differential localization within the secretory pathway of LPG5A and LPG5B. Current data strongly indicate that PGs incorporated into both LPG and PPG are synthesized in the parasite Golgi apparatus (16–18, 26). Although it has been generally assumed that relevant NSTs and glycosyltransferases are colocalized in the same compartment, recent data also raise the possibility that luminal nucleotide sugars may traffic between compartments as well (66, 67). Moreover, considerable data point to the existence of functionally divergent subcompartments within and after the eukaryotic Golgi apparatus (68).

Either model above could explain why, despite some degree of functional separation, both LPG5A and LPG5B function must be lost in order to completely ablate PG synthesis. For the association model, the coupling could be leaky, and for the compartmentalization, anterograde and/or retrograde vesicular trafficking of luminal UDP-Gal could result in limited redistribution.

The structure of the LPG5A and LPG5B proteins themselves provide some opportunities for functional divergence. Although most features closely resemble those of other known NSTs, LPG5A and LPG5B are unique in possessing a large, divergent 134–199-amino acid insert between the second and third TM domains (Figs. 4 and S1). Moreover, the C-terminal domain of several NSTs has been implicated in targeting NSTs to the ER and/or Golgi apparatus, through diphosphate retention and/or hydrophobic export motifs (66, 69, 70). Both LPG5A and LPG5B show potential motifs of this sort (Fig. S2). Thus, it is reasonable to propose that these or other domains contribute in some way toward divergent functions of these two NSTs. Preliminary data suggest that at least some portion of C-terminally tagged LPG5A and LPG5B proteins are found within the parasite Golgi apparatus, and efforts are under way to more finely map the cellular distribution of these two proteins and the functional roles of the unique peptide insert domains in LPG, PPG, and other secretory pathway glycoconjugates, such as the glycosylinositolphospholipids. (4)

In summary, we have presented data for the existence of multiple NST candidate sequences in _L. major_. Pursuant to our interests in UDP-Gal transport, we found two functionally divergent UDP-Gal transporters, _LPG5A_ and _LPG5B_, which both contribute to PG synthesis in _L. major_. These two NSTs show novel structural features and properties, studies of which are likely to increase our knowledge of the detailed _Leishmania_ PG synthetic and secretory pathway in the future. Given the evolutionary diversity of glycoconjugates in both _Leishmania_ and trypanosomes and their key roles during their infectious cycles, our studies further emphasize the roles of the large and diverse NST family in these important pathogens.

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**TABLE 2**

Biochemical analysis of WT and mutant LPGs

| Growth phase/cell line | Side chain Gal/PG repeat | Fraction PG repeats unmodified | PG repeat units/LPG |
|------------------------|--------------------------|-------------------------------|---------------------|
|                        | Log                      | Stationary                   | Log                 | Stationary                   | Log        | Stationary                   |
| WT                     | 2.8 ± 0.6                | 2.3 ± 1.1                    | 0.09 ± 0.04         | 0.17 ± 0.15                | 18.2      | 19.8                          |
| lpg5A−                 | 1.2 ± 0.4*               | 1.0 ± 0.3                    | 0.40 ± 0.08*        | 0.47 ± 0.11                | 13.5*     | 10*                           |
| lpg5B−                 | 3.0 ± 0.1                | 1.9 ± 0.1                    | 0.06 ± 0.01         | 0.14 ± 0.01                | 21        | 23                            |

*Significantly different from WT at the _p _< 0.05 level by a _t_ test of means.

**a** Significantly different from WT at the _p _< 0.10 level.

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3. T. Nicholson, A. Capul, and S. M. Beverley, unpublished observations.

4. A. Capul, T. Nicholson, F. Hsu, J. Turk, S. J. Turco, and S. M. Beverley, manuscript in preparation.
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