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Golgi GDP-mannose Uptake Requires *Leishmania LPG2*

A MEMBER OF A EUKARYOTIC FAMILY OF PUTATIVE NUCLEOTIDE-SUGAR TRANSPORTERS*

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The synthesis of glycoconjugates within the secretory pathway of eukaryotes requires the provision of lumenal nucleotide-sugar substrates. This is particularly important for eukaryotic microbes such as *Leishmania* because they must synthesize considerable amounts of extracellular and cell surface glycoconjugates that play significant roles in the infectious cycle. Here we used properly oriented sealed microsomes to characterize lumenal uptake of GDP-Man in *Leishmania donovani*. In this system, GDP-Man uptake was saturable with an apparent K_m for GDP-Man of 0.3 μM and facilitated its use as a donor substrate for lipophosphoglycan (LPG) synthesis. A *lpg2*$^-$ deletion mutant showed loss of GDP-Man but not UDP-Gal uptake, which was restored by introduction of the gene *LPG2*. Immunoelectron microscopy localized an active, epitope-tagged *LPG2* protein to the Golgi apparatus. Thus, *LPG2* is required for nucleotide-sugar transport activity and probably encodes this Golgi transporter. *LPG2* belongs to a large family of eukaryotic genes that potentially encode transporters with different substrate specificities and/or cellular locations. In the future, the amenability of the *Leishmania* system to biochemical and genetic manipulation will assist in functional characterization of nucleotide-sugar transports from this and other eukaryotes. Furthermore, since *LPG2* plays an important role in the *Leishmania* infectious cycle and mammalian cells lack a Golgi GDP-Man transporter, this activity may offer a new target for chemotherapy.

Eukaryotic glycosylation mechanisms often require the translocation of dolichol-linked saccharides and/or transport of nucleotide-sugars synthesized in the cytoplasm into the lumen of the appropriate microsomal organelle. A broad array of nucleotide-sugar transporter (NST)[1] activities with differing substrate specificities in the Golgi apparatus or endoplasmic reticulum have been reported (1). Known NST activities include those for UDP-Gal, UDP-Glc, UDP-GlcNAc, UDP-glucuronic acid, UDP-xylose, UDP-GalNAc, GDP-fucose, GDP-Man, and CMP-sialic acid (1). Three candidate NST genes have been reported recently: one for the UDP-GlcNAc transporter from *Kluyveromyces lactis* (2), and the others for the CMP-sialic acid transporter (3) and the UDP-Gal translocator (4) from mammalian cells. These genes are required for NST activity, although it is unknown whether they encode the NST itself. In different organisms and cell types, NSTs are involved in both housekeeping and specialized cellular functions (1). In this work we describe another role for NSTs, in the synthesis of glycoconjugates implicated in pathogenicity.

The protozoan parasite *Leishmania* synthesizes a variety of abundant complex glycoconjugates, whose expression permits the parasite to persevere in hostile environments throughout its life cycle in the sand fly and mammalian phagolysosome (5, 6). One of these is lipophosphoglycan (LPG), the major cell surface glycoconjugate of the promastigote (insect stage) form of the parasite. In *L. donovani*, LPG contains four domains: a lipid anchor (1-*O*-alkyl-2-*lyso*-phosphatidylinositol), a glycan core with the structure Gal(α1,6)Gal(α1,3)Gal$_4$(β1,3)[Glc(α1-PO$_4$)→6]Man(α1,3)Man(α1, 4)GlcN(α1,6), a series disaccharide-phosphate repeating units, and a cap containing one of several small oligosaccharide-phosphates containing β-galactose and α-mannose (5, 6). The repeats are attached to the C-6 hydroxyl of the distal Gal of the glycan core, and each of the 15–30 disaccharide-phosphate units has the structure [Gal(β1,4)Man(α1-PO$_4$)→6] in *L. donovani*. The repeating unit domain is the most prominent feature of LPG, and repeating units also occur on secreted proteins, most prominently an abundant secreted acid phosphatase, a molecule also implicated in parasite virulence (7–9).

The [Gal(β1,4)Man(α1-PO$_4$)→6] repeating units are synthesized by the sequential transfer of mannose 1-phosphate and galactose from their respective nucleotide donors (10, 11). The cellular compartment in which repeating unit synthesis likely occurs is the Golgi apparatus, as monensin treatment inhibits repeating unit attachment to both LPG and the secreted acid phosphatase (12). Since no evidence was obtained for the participation of lipid intermediate carriers for the two sugars in this process (10), the nucleotide-sugar substrates for these reactions need to be transported from their cytosolic site of synthesis to the site of utilization in the Golgi lumen. Thus, due to the large amounts of LPG and related glycoconjugates synthesized by the parasite, considerable demands are placed upon NST activity during the infectious cycle.

One of the attractive features of the LPG system is the ability to identify LPG-deficient mutants (13–15) and then by functional genetic rescue isolate genes involved in LPG biosynthesis (16, 17). Previously we described the C3PO mutant, which shows a complete lack of repeating units on LPG and the secreted acid phosphatase (17). Despite its *lpg*$^-$ phenotype,

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[1] The abbreviations used are: NST, nucleotide-sugar transporter; LPG, lipophosphoglycan; EST, expressed sequence tag; DIDS, 4,4-diisothiocyanatostilbene-2,2′-disulfonic acid; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; HA, hemagglutinin.

crude microsomal preparations of C3PO were able to conduct LPG synthesis, suggesting that the defect in this mutant lay in the assembly or compartmentalization of LPG biosynthesis, rather than in a specific glycosyltransferase activity. The gene *LPG2* was shown to be deleted in C3PO, and the predicted *LPG2* protein encoded a hydrophobic membrane protein. From these data, we proposed that *LPG2* was involved in the transport of a key LPG biosynthetic precursor or activity into a new cellular compartment, presumably the lumen of the Golgi apparatus.

In this paper we describe the development of an *in vitro* assay to demonstrate intravesicular GDP-Man transport in *Leishmania* membrane vesicles, and use this system to establish that the *Leishmania* gene *LPG2* is essential for GDP-Man uptake. Sequence data base searches indicate that *LPG2* is a member of a large eukaryotic gene family, which is proposed to include members affecting NST activity with different substrates and cellular compartments in distinct species and cell types.

EXPERIMENTAL PROCEDURES

Materials—Materials were obtained as follows. GDP-[1-^{14}C]Man (50 mCi/mmol), UDP-[6-^{3}H]Gal (10.2 Ci/mmol), GDP-[1-^{14}C]fucose (10 Ci/mmol), and CMP-[^{3}H]sialic acid (20 Ci/mmol) were from American Radiolabeled Chemicals; Dulbecco's modified Eagle's medium and M199 medium were from Life Technologies, Inc.; thin layer chromatography cellulose plates were from Eastman Kodak Corp.; and AG 1-X8 anion exchange resin were from Bio-Rad. All other chemicals were from Sigma.

Cell Cultures—Promastigotes of *L. donovani* (1S2D strain) were grown at 25 °C in Dulbecco's modified Eagle's medium (13). The LPG-defective, *L. donovani* mutant C3PO (14) was grown in the above medium supplemented with 10% fetal bovine serum. C3PO transfected with either cLHYG cosmids containing *Leishmania* genomic DNA bearing *LPG2* or the expression vector pX63HYG-LPG2(HA) (17) were maintained in M199 medium supplemented with 10% fetal bovine serum and hygromycin B (50 μg/ml).

Isolation of Membrane Vesicles—Stationary phase *L. donovani* were harvested, pelleted at 3,000 $\times$ *g* for 15 min, washed with phosphate-buffered saline (13), and resuspended in the reaction buffer (10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 1 mM TLCK, 1 μg/ml leupeptin, 1.0 μg/ml pepstatin A, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM 2,3-dimercaptopropanol). The cells were disrupted by nitrogen cavitation at 1,500 p.s.i. for 25 min at 4 °C. Microsomal vesicles were isolated according to the procedures of Goud *et al.* (18). Vesicle integrity was determined by measuring the latency of galactosyltransferase-catalyzed transfer of [^{3}H]galactose from UDP-[^{3}H]Gal to GlcNAc (19).

Standard GDP-Man Transport Assay—The transport assays were started by mixing 100 μl of vesicle protein (1 mg) with 100 μl of reaction buffer (above) containing GDP-[^{14}C]Man (final concentration of 3 μM). After incubation at 28 °C for 6 min, the samples were placed on ice and diluted with 1.5 ml of reaction buffer and applied onto a filtration apparatus (Millipore 1225 Sampling Manifold) containing HA filters (24 mm diameter; 0.45 μm pore size). The filters were washed with 25–30 ml of buffer and radioactivity on the filters was measured by scintillation counting. The amount of GDP-[^{14}C]Man that was nonspecifically bound to the outside of the vesicles was determined by measuring the radioactivity associated with the vesicles at zero time of incubation of vesicles with solute.

Identification of GDP-Man within Vesicles—Two methods were used to demonstrate the presence of GDP-[^{14}C]Man within the vesicles following transport. In one method, after a 6-min transport assay, the washed filters were placed into 1 ml of water, sonicated for 15 min at 4 °C, and extracted twice with 5 ml of chloroform/methanol (3:2) in the final proportion of chloroform:methanol:water of 3:2:1. The aqueous phases were combined and desalted by gel filtration on a column of Bio-Gel P2. An aliquot of the desalted sample was applied to a thin layer cellulose plate using methanol/formic acid/water (80:15:5) as the solvent system and GDP-mannose as the chemical standard. GDP-Man was detected with silver nitrate and ethanolic NaOH (20). Radioactive samples were scanned by a Bioscan detector.

In a second method, identification of GDP-[^{14}C]Man by chromatography on concanavalin A-Sepharose was performed based on the protocol described by Rush and Waechter (21). Briefly, another aliquot of the desalted sample was dried under a steam of nitrogen, redissolved in 0.1 ml of water, and applied to a column (4 ml) of concanavalin A-Sepharose equilibrated in 15 mM ammonium phosphate, pH 3.5, containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$. GDP-[^{14}C]Man was eluted with 50 mM α-methylmannoside in the equilibration buffer.

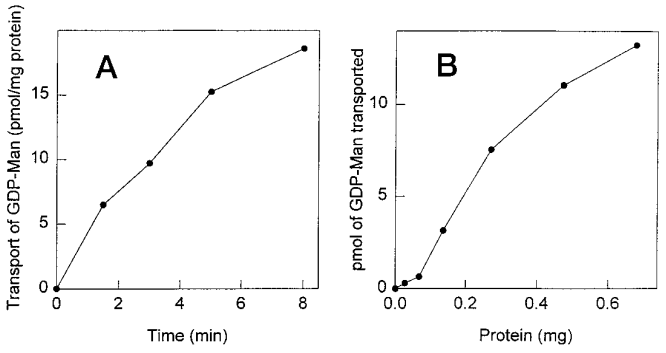

FIG. 1. **Time and protein dependence of GDP-Man transport by microsomal vesicles.** Vesicles were incubated with 0.16 μCi of GDP-[^{14}C]Man (3 μM) at 28 °C in a final volume of 200 μl. After incubation the amount of GDP-Man transported into the vesicles was determined as described under "Experimental Procedures." *Panel A*, time course for which the final vesicle protein concentration was 5 mg/ml. *Panel B*, protein concentration curve for which the time of incubation was 6 min.

Cell-free Biosynthesis Assay of LPG—In vitro LPG biosynthesis was performed as described by Carver and Turco (10). The microsomal vesicles (1 mg of protein) were incubated with 2 μM GDP-[^{14}C]Man (0.4 μCi), 10 μM UDP-galactose in HEPES buffer (50 mM HEPES, pH 7.4, 25 mM KCl, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 0.1 mM TLCK, 1 μg/ml leupeptin, 0.8 mM ATP, 0.5 mM dithiothreitol) at 28 °C for 1 h in a final volume of 250 μl. The reaction was terminated by addition of chloroform/methanol to the incubation mixture to give a final ratio of chloroform/methanol/water (4:8:3) and sonicated. By protocols described elsewhere (10), the membranes were sequentially extracted and the radioactive LPG product was purified and quantitated.

Immunolocalization of an Epitope-tagged LPG2 Protein—Immunoelectron microscopy was performed on frozen sections from transfected promastigotes fixed in 1% glutaraldehyde in Hepes saline and infiltrated in polyvinylpyrrolidine/sucrose as described previously (22, 23). Sections were probed with rabbit antibody raised against a synthetic peptide YPYDVPDYASL, corresponding to the influenza hemagglutinin tag, coupled to ovalbumin. The anti-HA antibody was affinity-purified on peptide-Sepharose and used at 1 μg/ml diluted in Hepes saline with 5% fetal bovine serum and 5% goat serum. Binding of primary antibody was visualized by incubation with goat anti-rabbit IgG conjugated to 18 nm gold (Jackson Immunoresearch Laboratories). Controls were conducted with non-transfected promastigotes and by omission of the primary antibody. Under the conditions used background labeling remained below 4% of the specific label.

RESULTS

Development of a GDP-Man Transport Assay in L. donovani

Properties of the GDP-Man Transport System—L. donovani parasites were disrupted by nitrogen cavitation, centrifuged at 10,000 $\times$ *g* to remove large organelles, and sealed microsomal vesicles were obtained by centrifugation at 100,000 $\times$ *g*. The integrity of the microsomal vesicles was estimated by measuring the latency of galactosyltransferase to proteolytic digestion with trypsin (19). The results from this intactness assay indicated that at least 90% of the vesicles were sealed in the proper orientation (data not shown).

To optimize conditions for transport of GDP-Man into *Leishmania* microsomal vesicles, it was important to establish that transport had characteristics of a protein carrier-mediated system. GDP-Man transport was: (i) time-dependent with linearity up to 6 min (Fig. 1*A*), (ii) temperature-dependent with approximately 15% of the activity at 4 °C compared to that obtained at 28 °C, and (iii) protein-dependent with linearity

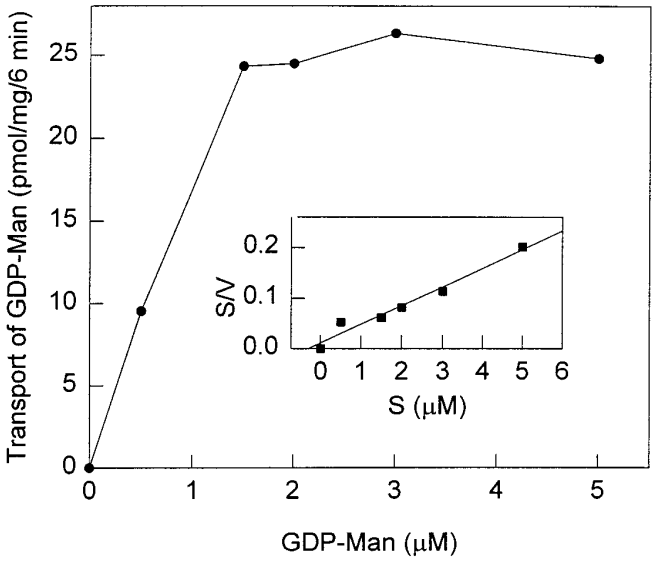

FIG. 2. **GDP-Man uptake by *Leishmania* microsomes is saturable.** GDP-Man transport assays contained reaction buffer (see "Experimental Procedures"), microsomes (1 mg), and the indicated concentration of GDP-[^{14}C]Man (540 cpm/pmol) in a total volume of 200 μl. After incubation for 6 min at 28 °C, the amount of GDP-Man transported into the vesicles was determined as described under "Experimental Procedures." *Inset*, Eadie plot. *S*, substrate; *V*, velocity.

from 0.05–0.3 mg (Fig. 1*B*). As shown in Fig. 2, transport was saturable with an apparent K_m of 0.3 μM for GDP-Man and a V_{max} of 25 pmol/mg/6 min. Under identical assay conditions, no transport of CMP-NANA was observed whereas the transport of GDP-fucose was approximately 5% the rate compared to GDP-Man. On the other hand, translocation of UDP-Gal into the microsomal vesicles was substantial (detailed below) as would be expected due to its utilization for lumenal synthesis of LPG and related glycoconjugates.

The requirement for an intact permeability barrier for GDP-Man transport was demonstrated by showing that uptake of the nucleotide-sugar was abolished when the vesicles were incubated with 0.1% (w/v) Triton X-100 (Table I). Furthermore, in the absence of detergent, GDP-Man transport was completely abrogated by DIDS, a stilbene derivative that has been utilized as an inhibitor of nucleotide-sugar transport into mammalian Golgi vesicles (24, 25). Transport was also inhibited by pretreatment of the microsomal vesicles with protease XIV (Table I), suggesting the presence of an important cytoplasmically oriented domain of the transporter. GDP-Man transport was not affected significantly by the addition of 10 μM mannose, 10 μM mannose 1-phosphate, or 1 mM EDTA (data not shown).

Identification of GDP-Man Transported into Vesicles—To provide direct evidence that GDP-[^{14}C]Man was transported into the lumen of the membrane vesicles, the nature of [^{14}C]mannose-labeled, vesicle-associated material was studied. Following a 6-min incubation of the vesicles with GDP-[^{14}C]Man, the vesicles were washed by filtration and then extracted with organic solvents to isolate LPG and water-soluble solutes. [^{14}C]LPG accounted for the majority (75%) of the vesicular radioactivity. This was as expected since the glycosyltransferases involved in the assembly of repeat units of LPG and cap structure are lumenally oriented and would have been able to utilize the transported nucleotide-sugar as a donor substrate. The remaining radioactivity (25%) was water-soluble, and an aliquot was subjected to TLC (Fig. 3). The main peak of radioactivity co-migrated with standard GDP-Man. The water-soluble ^{14}C-labeled material was confirmed further as GDP-

TABLE I
Transport of GDP-[^{14}C]Man after a 2-min incubation

Microsomal vesicles (5 mg/ml) were incubated with 0.25 μCi of GDP-[^{14}C]Man (3 μM) at 28 °C for 2 min in a final volume of 200 μl. After incubation the amount of GDP-Man transported into the vesicles was determined as described under "Experimental Procedures."

Incubation mixture	Amount
	pmol/mg
Complete	31
+ Triton X-100 (0.1%)	<1
+ DIDS (4 mM)	<1
+ Protease XIV (6 units)	<1

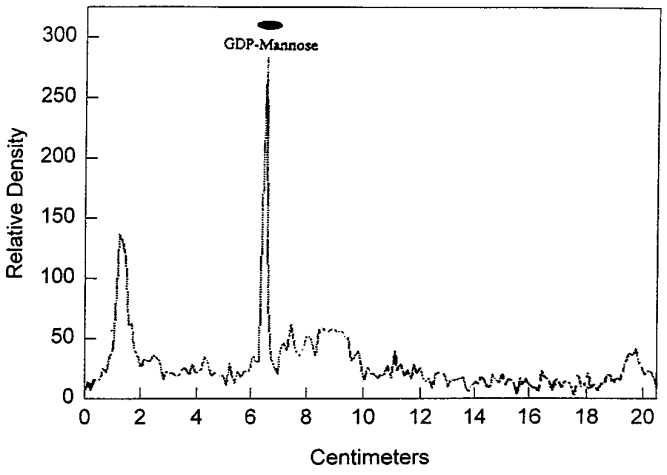

FIG. 3. **TLC of aqueous soluble material in microsomal vesicles.** Following a 6-min incubation of vesicles with 0.16 μCi of GDP-[^{14}C]Man (3 μM) at 28 °C, an aliquot of the desalted, aqueous-soluble sample was applied to a thin layer cellulose plate using methanol/formic acid/water (80:15:5) as the solvent system and GDP-mannose as the chemical standard. The radioactive sample was scanned by a Bioscan detector.

[^{14}C]Man by chromatography on concanavalin A-Sepharose according to the method of Rush and Waechter (21). As shown in Fig. 4*A*, GDP-[^{14}C]Man binds to the lectin and can be eluted in the presence of α-methylmannoside. Prior to chromatography on concanavalin A-Sepharose, pretreatment of another aliquot of the sample under mild acid conditions that cleaves the labile nucleotide-sugar resulted in quantitative recovery of radioactivity in the void volume (Fig. 4*B*). In a key control experiment, only negligible radioactivity (<1% compared to wild type microsomal vesicles) was extractable using microsomal vesicles prepared from LPG-deficient C3PO parasites (discussed below).

Nucleotide-sugar Transport by Microsomal Vesicles from C3PO and LPG2-transfected C3PO Cells

Among the possible roles for *LPG2* transporter activity, we considered transport of GDP-Man since Man-P addition to the glycan core is the first step of repeating unit biosynthesis (see Introduction). In these studies we made use of mutants lacking the *LPG2* gene (C3PO) and of C3PO to which *LPG2* expression had been restored by transfection of a *LPG2* expression vector (pX63HYG-LPG2).

First, the relative ability to transport GDP-[^{14}C]Man was assayed in microsomal vesicles. As shown in Table II, vesicles from C3PO cells failed to transport significant amounts of GDP-[^{14}C]Man. This defect, in turn, was responsible for the inability of sealed vesicles to synthesize sizable amounts of LPG *in vitro* in the absence of Triton X-100 (Fig. 5, *lower panel*), since without translocation the precursor would be unavailable inside vesicles. Incorporation of radioactive man-

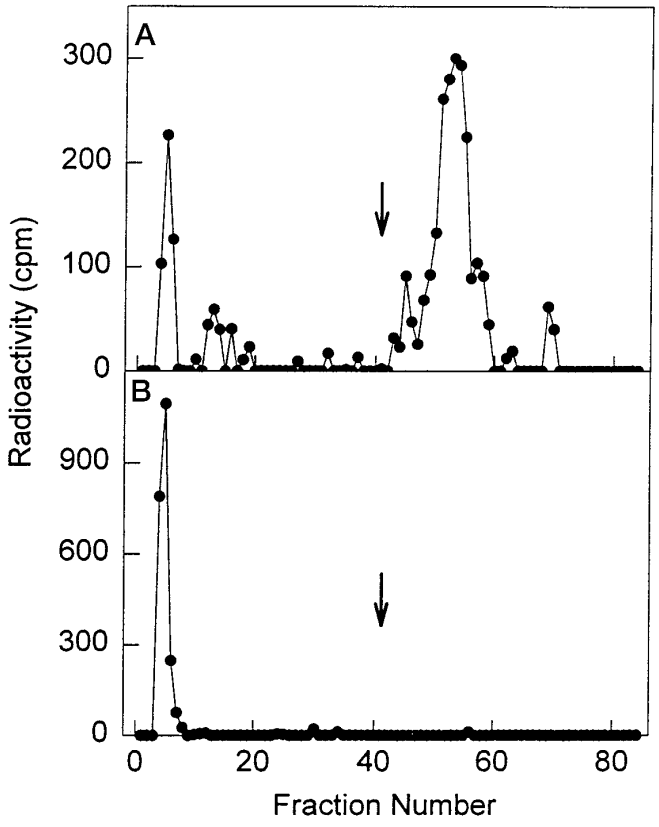

FIG. 4. **Concanavalin A-Sepharose chromatography of aqueous soluble material in microsomal vesicles.** Following a 6-min incubation of vesicles with 0.16 μCi of GDP-[^{14}C]Man (3 μM) at 28 °C, an aliquot of the desalted, aqueous-soluble sample was chromatographed on a column of concanavalin A-Sepharose (4 ml) equilibrated in 15 mM ammonium phosphate, pH 3.5, containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$. The column was washed with the equilibration buffer for 40 fractions (0.6 ml/fraction). The *arrow* designates the fraction number at which the elution was switched to an equilibration buffer containing 50 mM α-methylmannoside. *Panel A*, no mild acid pretreatment. *Panel B*, the sample was pretreated with 0.02 N HCl, 5 min, 100 °C, dried under nitrogen, and resuspended in equilibration buffer.

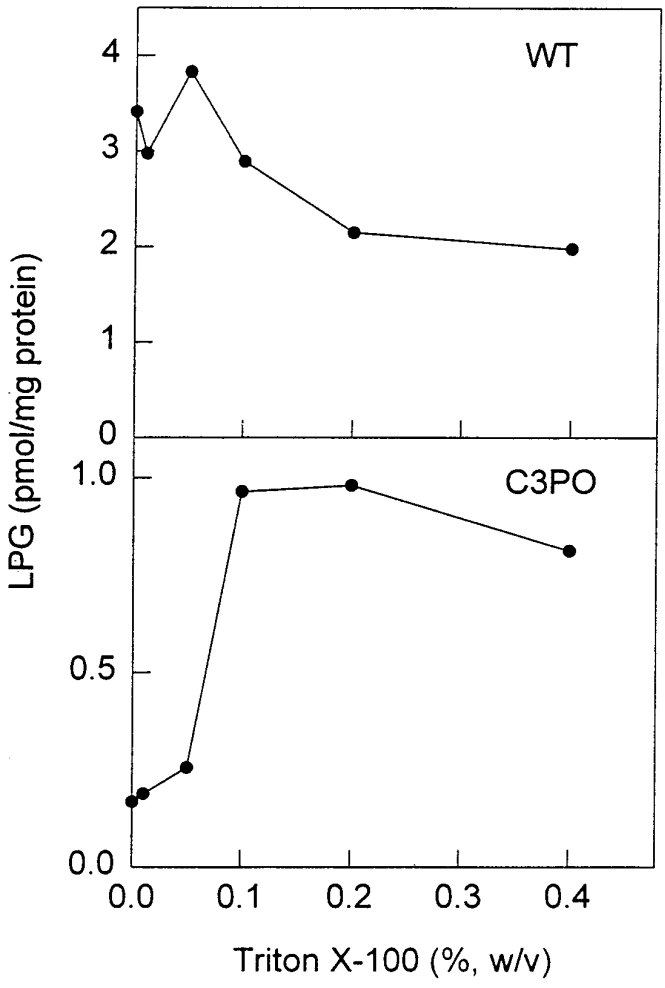

FIG. 5. **Sensitivity of LPG synthesis in vesicles to the presence of Triton X-100.** Sealed microsomal vesicles were prepared from wild type or C3PO cells and incubated with 0.4 μCi of GDP-[^{14}C]Man (2 μM) at 28 °C and the indicated concentration of Triton X-100 in a final volume of 250 μl. The amount of LPG synthesized was determined as described under "Experimental Procedures." *Upper panel*, wild type. *Lower panel*, C3PO.

TABLE II

Transport of nucleotide-sugars in vesicles from wild type, C3PO, or LPG2-transfected C3PO cells

Microsomal vesicles (5 mg/ml) were prepared from the designated cells and were incubated with 0.16 μCi of GDP-[^{14}C]Man (3 μM) or 0.3 μCi of UDP-[^{14}C]Gal (3 μM) at 28 °C for 6 min in a final volume of 200 μl. After incubation the amount of GDP-Man or UDP-Gal transported into the vesicles and LPG synthesis were determined as described under "Experimental Procedures." Values shown are an average of three experiments and are the percentage transported relative to wild-type. "C3PO(*LPG2*)-50" refers to LPG2-transfected C3PO cells that were maintained in 50 μg/ml hygromycin B. Similarly, the "100" and "250" designations refer to transfected cells maintained in 100 and 250 μg/ml hygromycin B, respectively.

Vesicles	GDP-Man	UDP-Gal	LPG synthesis
	%	%	%
Wild type	100	100	100
C3PO	3 ± 0.1	72 ± 9.2	4.4 ± 0.5
C3PO(*LPG2*)-50	13 ± 0.2	90 ± 6.4	31.6 ± 1.0
C3PO(*LPG2*)-100	41 ± 2.8	78 ± 12.0	41.6 ± 1.3
C3PO(*LPG2*)-250	52 ± 3.9	71 ± 5.0	40.0 ± 0.8

nose from GDP-[^{14}C]Man into LPG could only occur when the vesicles were unsealed with concentrations of at least 0.1% Triton X-100. In contrast, vesicles from wild type cells were able to carry out LPG synthesis using GDP-[^{14}C]Man, in the absence or presence of the detergent (Fig. 5, *upper panel*), as anticipated with a functional GDP-Man transporter. The higher concentrations of detergent were found to have a partial inhibitory effect on LPG synthesis activity as reported previously (10).

An examination of GDP-[^{14}C]Man transport using vesicles prepared from C3PO cells transfected with *LPG2* resulted in restoration of approximately 13% of wild type transport levels (Table II). We reasoned that the low level of transport reflect relatively weak expression of *LPG2*, which was carried on a large cosmid vector. Since elevated drug pressure is known to increase episomal plasmid copy number (26), the hygromycin B concentration was increased from 50 to 100 and then 250 μg/ml, and the cells grown for several passages. Microsomal vesicles were prepared from these transfectants, and assayed for GDP-[^{14}C]Man transport. As shown in Table II, there was a proportional increase in *in vitro* GDP-[^{14}C]Man transport activity using vesicles from *LPG2*-transfected cells selected with elevated amounts of hygromycin B.

Significantly, alterations in GDP-Man uptake were specific, as no alterations in uptake of UDP-[^{3}H]Gal were observed in sealed vesicles from wild type, C3PO, and C3PO cells transfected with the *LPG2* gene and maintained in various concentrations of hygromycin B (Table II).

Subcellular Localization of the Protein Encoded by LPG2

In an earlier study (17), an epitope-tagged *LPG2* protein was detected in a region of the parasite between the nucleus and the kinetoplast, which is where the parasite Golgi apparatus lies. We used immunoelectron microscopy to confirm whether the *LPG2* protein was localized to the Golgi apparatus. The LPG2-HA construct described previously was transfected into the C3PO mutant, where it restored the LPG^+ phenotype (17). By electron microscope-gold immunostaining techniques utilizing an antibody that recognizes the HA tag, particles were observed only in the Golgi cisternae that are located near the flagellar pocket (Fig. 6*B*). In all trypanosomatids the Golgi is represented as a stack of flattened membraneous vesicles that abut the flagellar pocket (Fig. 6*A*), which is the sole demonstrated site of secretion of proteins and carbohydrate complexes from these organisms (7, 27). Examination of areas where the vesicles of the Golgi are distended indicate that the majority of the label (the C terminus of the *LPG2* protein bearing the HA tag in this construct) is associated with the lumenal face of the membrane, (Fig. 6*C*).

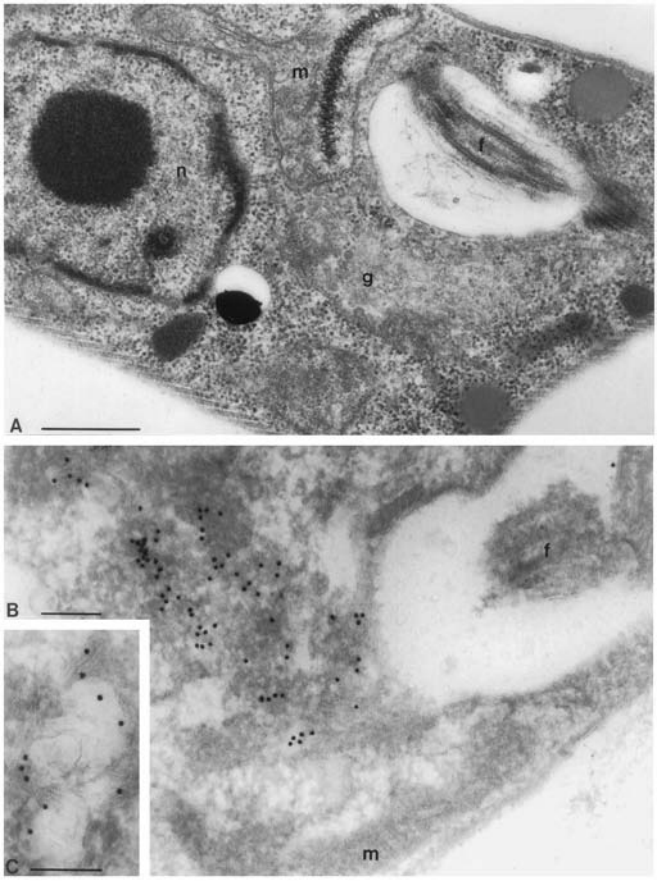

FIG. 6. **Immunogold labeling of the fusion protein encoded by the *LPG2*(HA) construct.** *A*, an electron micrograph of a promastigote cell revealing its cellular organization. The Golgi apparatus (*g*) is a relatively amorphous, vesicular network that lies anterior of the nucleus (*n*), alongside the mitochondrion (*m*) and the flagellar (*f*) pocket. *Scale bar* = 0.5 μm. *B*, immunoelectron micrographs of promastigotes expressing *LPG2*(HA) probed with affinity-purified rabbit anti-HA and goat anti-rabbit IgG (18 nm gold). The label is associated primarily with the Golgi stacks that lie close to the flagellar pocket of the parasite. *C*, close examination of the Golgi apparatus indicates that the majority of the label lies within the lumen of the membraneous stacks. *Scale bars* = 0.2 μm.

DISCUSSION

A topological need for a transporter of GDP-Man, as well as UDP-Gal, in the Golgi apparatus of *Leishmania* is understandable based upon our current knowledge of repeating unit assembly of LPG and other phosphoglycan-containing glycoconjugates. Biosynthesis occurs in the Golgi, and consistent with this finding, the protein encoded by the *LPG2* gene was clearly localized by immunoelectron microscopy to the Golgi apparatus with the C terminus of the protein being lumenally oriented. Other LPG biosynthetic genes have also been localized to the Golgi apparatus (28).

Evidence for the existence of a transporter for GDP-Man in *Leishmania* resulted from the development of an *in vitro* transport assay involving sealed microsomal vesicles. GDP-Man transport exhibited the normal properties of a protein-mediated system, including selectivity of solutes and sensitivity to protease. Furthermore, transport was inhibited by DIDS, a commonly used inhibitor of nucleotide-sugar transport in mammalian Golgi vesicles. Of particular importance, GDP-Man was discovered within the sealed *Leishmania* vesicles following a timed incubation. Once inside, the nucleotide-sugar was used as a substrate for LPG synthesis. Identically prepared vesicles from C3PO cells neither transported the substrate nor incorporated the sugar into LPG unless the vesicles were unsealed with detergent.

In mammalian Golgi vesicles, nucleotide-sugar transporters are classified as antiporters since transport is always coupled to the exit of the corresponding nucleoside monophosphate (25). The GDP-Man transporter in yeast is believed to operate via a coupled antiporter reaction (29). A guanosine diphosphatase has been shown to be required for protein and sphingolipid mannosylation in the Golgi lumen of *Saccharomyces cerevisiae*, implicating the significance of GDP-Man/GMP exchange (30). In the *Leishmania* system, coupling of entry of GDP-Man into the Golgi compartment to exit of GMP is not known nor has a guanosine diphosphatase been demonstrated. The latter enzyme may not be necessary in *Leishmania* because GMP presumably is one product produced in abundance by the mannosyl-phosphate transferase reaction during assembly of the repeat units of LPG (11).

We showed that the *Leishmania* gene *LPG2* is required for transport of GDP-Man into the lumen of the Golgi apparatus. Although we favor the idea that *LPG2* encodes the GDP-Man

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Majority     VLTYLLISGLCGFGCVYGVGWCASVTSALTLSMVGTLRKLVSLLLSVIVFSNPVSVL----GFLGIFIVFLGGILY

Sc YEM9      SLAAMVISGLMSVGISMCSGWQVRVTSSTTYSMVGALNKLPIALAGLVFFDAPKNFL----SFFSTFLGFLSGLLY
Sc VRG4      SLTAMILSGVASVGISMCSGWQVRVTSSTTYSMVGALNKLPIALSGLIFFDAPRNFL----SILSIFTGFLSGIIY
Ld LPG2      KLV-LTFSVLVSSVMTFSVFWCMSITSPTTMSVVGSLNKIPLTFLGMLVFHQFPTAT----GYLGIMIALSAGFLY
Ce C50F14.14 FWILMTLGGVFGFMMGMVTGWQIQATSPLTHNISGTAKAAAQTVMAVVWYSELKTLLWWTSNF----VVLFGSGMY
Ce C52E12.3  VWICFLLSCICGFVLNMSLVLCTHHNSALTTTCVGPIKNLFVTYVGM--FSSGDYVFQWA-NFTGINVSVFGSILY
KI MNN22     KLFMLIANNVTQFIGIKGVNMLASNTDALTLSVVLLVRKFVSLLLSVYIYKNVLSVT----AYLGTITVFLGAGLY
Sc YEA4      EETLLLFNVLTQYFCVKGVNILASKTNALTLSITLLVRKFISLLLSVRLFDNNLSYT----GYIGVYLVFFGAFIM
Ce C53B4.6   AWVMIFAICLFQFAQTKGVYMLSAVTTSLNVTMVLTLRKFFSLLISFIVFENVFNMF----HIIGAAFVFIGTILF
Sc 38KCS.11  VWGYLMLYSFCGAMGQCFIFYTLEQFGSLVLIMITVTRKMVSMILSIIVFGKSVR----FQQWVGMFIVFGG--IT
Ce F54E7-6   TFGYALLLSCLGYLGVNVVLTHIKVFGALVAVTMTTLRKALTIILSFMLFSKPFTIEYVYAGSVVMLAIYLN--EY
Bo PL-TPT    IYTKSLIAALGFHAYQQVSYMILARVSPVTHSVGNCVKRVMVIVSSVIFFKTPVSPV----NAFGTGIALAGVFLY
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FIG. 7. **Amino acid alignment of proteins encoded by *LPG2* and related genes.** Protein sequences were aligned by the Clustal algorithm (34). *Shaded* amino acid residues match the consensus sequence (*Majority*) exactly.

transporter itself, strictly speaking it is possible that *LPG2* may be an essential component of a multi-subunit NST transporter complex. Similar reservations pertain to other putative NST genes recently identified (2–4). This issue might be resolved by reconstitution experiments of the protein in artificial liposomes or by expression of the *LPG2* gene in a heterologous system, such as mammalian cells, that do not possess a Golgi GDP-Man transporter.

The LPG2 Gene Family—We reported previously that *LPG2* showed strong homology to the yeast Golgi protein encoded by *VRG4/VAN2* (17, 31). Since that time this gene family has grown to include three more from *S. cerevisae*, one from *K. lactis*, and four from *Caenorhabditis elegans*, all of which form a clearly related group of hydrophobic membrane proteins (Figs. 7 and 8, Table III). This family also shows similarity to the *Brassica* triose phosphate translocator as well (Figs. 7 and 8, Table III). Moreover, searches of the EST sequence data bases additionally revealed at least two human and one plant gene (Table III). The group termed "NST1" includes 23 different ESTs and yields a contiguous overlap of more than 1323 base pairs, which is sufficient to encode a protein similar in size to *LPG2* or its relatives (Table III). Interestingly, the putative CMP-sialic acid and UDP-galactose translocators recently identified in mammalian cells (3, 4) shows little sequence relationship to the *LPG2* family. However, the UDP-Gal translocator shows weak similarity to the *Brassica* plastid triose-phosphate translocator (data not shown), as does the *LPG2* family (Table III, Figs. 7 and 8), suggesting that all of these genes may share a common ancestor.

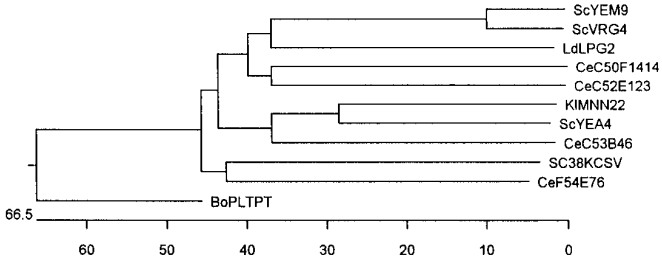

FIG. 8. **Evolutionary tree depicting relationships among *LPG2* and potential NSTs.** The tree was constructed using amino acid sequences for the genes shown in Table III. The evolutionary tree was constructed with the neighbor-joining algorithm (35) using programs implemented in the DNAStar Lasergene package. The *numbers* in the *abscissa* represent the percent amino acid sequence divergence among the descendant species.

Within the *LPG2* family, the properties of the *Kluyveromyces MNN2–2* gene show interesting similarities to those of *LPG2*; *mnn2-2* mutants exhibit decreased uptake of a nucleotide-sugar, UDP-GlcNAc (32), and hydrophilicity analysis predicts a very hydrophobic protein with multiple putative transmembrane domains (2). Preliminary data have suggested that yeast *vrg4* mutants have reduced GDP-Man uptake as well.[2] Thus, it seems reasonable that the members of this gene family may share the common feature of involvement in NST. The finding of a large and expanding gene family meshes well with current views, that eukaryotes exhibit numerous and varied NST activities with different substrate specificities in the Golgi apparatus or endoplasmic reticulum. Significantly, the identification of *LPG2*-related mammalian ESTs will permit rapid cloning of the full-length cDNA, and ultimately, functional characterization of the encoded proteins of mammalian cells (1). Thus, studies of the protozoan *Leishmania* have provided important new insights for the function of genes in other eukaryotic organisms such as *S. cerevisae* and humans. Moreover, the amenability of the *Leishmania* system to biochemical and genetic manipulation and the relative ease in preparing intact vesicles could be exploited in the functional characterization of NST activities from other eukaryotes.

Role of LPG2 in Glycoconjugate Synthesis—Despite their functional and sequence similarities, disruption of *Leishmania LPG2* has profoundly different consequences than disruption of *S. cerevisae VRG4* (31, 33). *LPG2* deletion mutants are deficient in a very specific biochemical step: the synthesis of the repeating units normally present in LPG and related glycoconjugates (5, 6). Otherwise, *lpg2*$^-$ *Leishmania* are completely normal in protein *N*-glycosylation, secreted or GPI-anchored protein expression and localization, glycolipid profile, and growth rate. In contrast, *VRG4* is an essential gene, and partial loss of function mutants show severe defects in protein targeting, glycosylation, Golgi structure, and growth (31). These differences led to the proposal that *LPG2* belonged to a pathway of genes related to those of other eukaryotes, but whose members had specialized toward the synthesis of LPG and related

[2] N. Dean, D. Ma, S. Beverley, and S. Turco, unpublished observations.

TABLE III
A family of related genes known or potentially implicated in NST activity

Name	Organism	Data base	Function
		The *LPG2* gene family	
LPG2	*L. donovani*	U26175	Golgi GDP-Man transport
VRG4	*S. cerevisae*	L33915	Golgi GDP-Man transport?
YEM9	*S. cerevisae*	Z48639	
C52E12.3	*C. elegans*	U50135	
C50F4.14	*C. elegans*	Z70750	
MNN2–2	*K. lactis*	U48413	Golgi UDP-GlcNAc transport
YEA4	*S. cerevisae*	U18530	
C53B4.6	*C. elegans*	Z68215	
F54E7.6	*C. elegans*	U00067	
Sc38KCSV.i11	*S. cerevisae*	Z67751	
Plant plastid	*B. oleracea*	U13632	Triose-phosphate translocator
"NST1"	Human	23 ESTs (including W80625, H65599, W02480)[a]	
"NST2"	Human	4 ESTs (including R24922, R86287)[b]	
"NST3"	Plant	D24450, T45513[c]	
		Other NSTs	
CMP-SA-Tr	CHO cells	Z71268	CMP-sialic acid transport
UGT	Had-1 cells	D84454	UDP-Gal transport

[a] 1337-bp contig., BLASTX $p = 10^{-8}$ with U00067, Z67751.
[b] 713-bp contig., BLASTX $p = 10^{-10}$ with U50135.
[c] 784-bp contig, BLASTX $p = 10^{-9}$ with Z48639.

molecules, most of which are believed to play important roles unique to the parasite infectious cycle (5, 6).

The finding that *LPG2* participates in what might be viewed as a "housekeeping" function, provision of Golgi lumenal GDP-Man, suggests that the postulated specialization evolved, not by duplication and divergence as originally proposed, but in another way. One possibility is that the LPG pathway occurs in a separate compartment, thereby necessitating a dedicated GDP-Man transporter. This seems unlikely as the crude organellar preparations studied here would be expected to contain most if not all intracellular compartments, yet the *lpg2*$^{-}$ mutant C3PO exhibits essentially no uptake of GDP-Man suggestive of a second NST activity. More likely is the possibility that specialization indeed has occurred in the *Leishmania* secretory pathway, not by gene duplication and divergence, but by differential expression and/or usage. Although eukaryotic cells exhibit numerous and diverse NST activities, most cell types feature only a subset of these (1), a salient example being the lack of GDP-Man uptake activity in mammalian cells. The minor consequences of loss of UDP-GlcNAc uptake in *mnn2-2 Kluyveromyces* (32) further suggest that, even when present, these activities may often be associated with dispensable, specialized functions rather than essential metabolic roles. Thus, loss of *LPG2* may be less severe solely because the only role for GDP-Man involves LPG-related pathways, which are dispensable for growth in culture but indispensable for parasite virulence. In contrast, yeast *VRG4* has taken on roles required for normal viability, perhaps in cell wall or glycosphingolipid biosynthesis (31, 33).

Mammalian cells have no use for a Golgi GDP-Man transporter, in contrast to its obligate need in the pathway of assembling the structurally complex glycoconjugates of *Leishmania*, yeast, and other lower eukaryotes. The extreme difference in this requirement suggests that Golgi GDP-Man transport may represent a potential target for effective chemotherapeutic intervention. Further characterization and purification of the *Leishmania* GDP-Man transporter may lead to the identification of specific inhibitors of this pathway in the future.

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Note Added in Proof—Recently, another member of the *LPG2* family was reported (GenBank™ Accession number D87449). This human sequence is related to the *C-elegans* C52E12.3 sequence (32% identity) and illustrates the rapidity of expression of this gene family.

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