The *Leishmania* GDP-Mannose Transporter Is an Autonomous, Multi-specific, Hexameric Complex of LPG2 Subunits†

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Received October 11, 1999; Revised Manuscript Received November 29, 1999

ABSTRACT: *LPG2* (a gene involved in **l**ipo**p**hospho**g**lycan assembly) encodes the Golgi GDP-Man transporter of the protozoan parasite *Leishmania* and is a defining member of a new family of eukaryotic nucleotide-sugar transporters (NSTs). Although NST activities are widespread, mammalian cells lack a GDP-Man NST, thereby providing an ideal heterologous system for probing the LPG2 structure and activity. *LPG2* expression constructs introduced into either mammalian cells or a *Leishmania lpg2*$^-$ mutant conferred GDP-Man, GDP-Ara, and GDP-Fuc (in *Leishmania* only) uptake in isolated microsomes. LPG2 is the first NST to be associated with multiple substrate specificities. Uptake activity showed latency, exhibited an antiport mechanism of transport with GMP, and was susceptible to the anion transport inhibitor DIDS. The apparent K_m for GDP-Man uptake was similar in transfected mammalian cells (12.2 μM) or *Leishmania* (6.9 μM). Given the evolutionary distance between protozoans and vertebrates, these data suggest that LPG2 functions autonomously to provide transporter activity. Using epitope-tagged LPG2 proteins, we showed the existence of hexameric LPG2 complexes by immunoprecipitation experiments, glycerol gradient centrifugation, pore-limited native gel electrophoresis, and cross-linking experiments. This provides strong biochemical evidence for a multimeric complex of NSTs, a finding with important implications to the structure and specificity of NSTs in both *Leishmania* and other organisms. Inhibition of essential GDP-Man uptake in fungal and protozoan systems offers an attractive target for potential chemotherapy.

The ability of the Golgi apparatus to serve as the major site for many different glycosylation reactions requires the provision of lumenal nucleotide-sugars, via transport from their cytoplasmic site of synthesis (*1, 2*). This requirement is carried out by a group of proteins termed nucleotide-sugar transporters (NSTs)[1] which have specificity for both the nucleotide and the sugar. Some NST activities are found in all organisms [e.g., UDP-Gal (*3*)], some are restricted to certain lineages [e.g., GDP-Man in yeast and *Leishmania* (*4, 5*)], and some have been found in other subcellular compartments such as the ER [e.g., UDP-Glc (*6*)], Golgi apparatus [e.g., UDP-GlcA (*7*)], or possibly even the nucleus [e.g., CMP-NANA (*8*)]. Since NST activity is absolutely required for proper glycosylation, there has been a great interest in questions concerning NST specificity, regulation, and cellular targeting (*1, 2, 9*). NSTs are currently believed to transport a single kind of nucleotide-sugar, although the possibility exists that where some of these transporter activities are absent or diminished, other transporters may partially compensate for lost function (*9, 10*).

The study of NSTs has benefited in the last several years from the isolation of genes required for NST activity. These include the NST genes for UDP-Gal, CMP-NANA, and UDP-GlcNAc in mammals (*11*−*14*); UDP-Gal, UDP-GlcNAc, and GDP-Man in yeast (*4, 15*−*17*); and GDP-Man in *Leishmania* (*5*). The predicted proteins comprise a large family of structurally similar integral membrane proteins of about 35−45 kDa, bearing from 5 to 10 transmembrane regions (*1, 2*). Several of these proteins were expressed in distantly related species, leading to the proposal that the identified genes encoded functionally autonomous NST proteins (*13, 18*). At present, there is relatively little information on the structure of the NST protein complex, the basis for NST specificity, and the targeting and organization of NSTs within in the cell. Some information has emerged along this line since the rat liver Golgi membrane UDP-GalNAc transporter has been recently purified to apparent homogeneity and shown to be a homodimer of 43 kDa subunits (*10*).

The C3P0 mutant of *L. donovani* lacks a surface glycolipid required for virulence, the lipophosphoglycan (LPG), and genetic complementation of this mutant resulted in the identification of the single-copy *LPG2* gene (*19*). Transfection of C3P0 with *LPG2* restored GDP-Man uptake and, thus, LPG expression (*5, 19*). Although GDP-Man NST activity was not required for *Leishmania* growth *in vitro*, it was essential for the synthesis of LPG and related molecules

† This investigation received financial support from National Institutes of Health Grant AI31078. S.M.B. and S.J.T. are Burroughs Wellcome Scholars in Molecular Parasitology.

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[1] Abbreviations: NST, nucleotide−sugar transporter; LPG, lipophosphoglycan; PBS, phosphate-buffered saline; ORF, open reading frame; DIDS, 4,4-diisothiocyanatostilbene-2,2′-disulfonic acid; TLCK, 1-chloro-3-tosylamido-7amino-2-heptone; HA, hemagglutinin; His_6, hexa-histidine epitope tag; HYG, hygromycin; DSP, dithiobis(succimidylpropionate).

10.1021/bi992363l CCC: $19.00
Published on Web 00/00/0000 PAGE EST: 9.5

thought to be required for parasite virulence (*19*, *20*). This lead to the suggestion that this pathway may be suitable for chemotherapeutic intervention, since humans and other mammalian hosts of this parasite lack the GDP-Man NST activity gene (*5*).

LPG2 encodes a protein of 341 amino acids, containing up to 10 transmembrane domains (*19*). Epitope tagging experiments localized the LPG2 protein to the parasite Golgi apparatus, with the C-terminus located on the lumenal side (*5*). In this work, we took advantage of the distant evolutionary null background of mammalian cells to provide an insert platform for the study of LPG2 function. GDP-Man transport activity was obtained following transient transfection of *LPG2* expression constructs, suggesting that LPG2 acts autonomously as the GDP-Man transporter. We further established that LPG2 occurs in a hexameric complex in *Leishmania* and also showed that GDP-Man, GDP-Ara, and GDP-Fuc can be transported by this NST. These findings have important implications to the structure and function of the NST family in both *Leishmania* and other eukaryotes.

EXPERIMENTAL PROCEDURES

Materials. Materials were obtained as follows: UDP-[^{3}H]-Gal (10.2 Ci/mmol) from American Radiolabeled Chemicals; Dulbecco's modified Eagle's medium, penicillin, and streptomycin from GIBCO Life Technologies; G418 from Calbiochem; protein G−agarose, *Pfu* DNA polymerase, and all restriction enzymes from Boehringer Mannheim; Western Blot SuperSubstrate System and DSP from Pierce; thin-layer chromatography cellulose plates and X-ray films from Kodak; anti-*c-Myc* monoclonal antibody from Invitrogen; thermocycling sequencing kit from Amersham; and PVDF membranes from Bio-Rad. Anti-HA monoclonal antibody was a generous gift from Dr. Neta Dean (SUNY at Stony Brook). GDP-[^{14}C]-Man (150 mCi/mmol) was prepared as described (*21*). GDP-D-[^{3}H]-arabinopyranose (15 Ci/mmol) and GDP-L-[^{3}H]-Fuc (60 Ci/mmol) were prepared as described (*22*). All other chemicals were from Sigma.

Cell Cultures. HEK 293 and COS 7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 50 units/mL penicillin, and 50 μg/mL streptomycin.

Construction of pcDNA3-LPG2HA. The cDNA for *LPG2* tagged in the C-terminus with 33 nucleotides coding for the HA epitope (1.1 kb fragment) was released from pX63HYG-LPG2HA (*19*) by digesting with *Xho*II. The mammalian expression vector pcDNA3 (Invitrogen) was digested with *Bam*HI, dephosphorylated, and ligated to the 1.1 kb fragment containing LPG2HA.

Construction of pcDNA3-LPG2Myc. The cDNA for LPG2Myc, in which the encoded LPG2 protein was tagged in the C-terminus with the Myc epitope tag, was obtained by PCR using the oligonucleotides 5′-GCGGGATCCCATATGAACCATACTCGCTC and 5′-CCGGAATTCTACAGATCCTCTTCTGAGATGAGTTTTTGTTCCTCAGATTTGGAAGT. Using pXG-LPG2HA as the template, LPG2Myc was amplified by PCR (30 cycles of 94 °C for 1 min, 49 °C for 1 min, and 72 °C for 1 min). The 1.1 kb product was digested with *Bam*HI and *Eco*RI, and inserted between the *Bam*HI and *Eco*RI sites of pcDNA3, yielding pcDNA3-LPG2Myc. The LPG2Myc sequence was confirmed by DNA sequencing, using a thermocycling sequencing kit (Amersham). Plasmid DNAs were purified by poly(ethylene glycol) precipitation.

Construction of pXG-LPG2His$_6$. pXG-LPG2His$_6$, in which the encoded LPG2 protein was tagged in the C-terminus with the His$_6$ epitope tag, was constructed by PCR using the oligonucleotide primers 5′-GGGGATCCCATATGAACCATA and 5′-CCGGAAT TCCTAATGATGATGATGATGATGCTCAAGATTTGGAAGTGTC. pXG-LPG2 was used as the template, and LPG2His$_6$ was amplified using *Pfu* polymerase (30 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min). The 1.1 kb product was inserted into the *Sma*I site of pXG. The sequence was confirmed by DNA sequencing, using a thermocycling sequencing kit (Amersham). The purified pXG-LPG2His$_6$ construct was transfected into the *L. donovani lpg2*$^-$ knock-out strain (*19*) using an electroporator (0.45 kV, 500 μF) and selected with G418 (50 μg/mL) as described (*23*).

Expression of LPG2HA and LPG2Myc in HEK Cells. HEK cells were seeded at a density of (1−2) × 10^6/100 mm dish. Purified pcDNA3, pcDNA3-LPG2HA, or pcDNA3-LPG2Myc DNA was transfected into cells by a calcium phosphate method (*24*) with minor modifications. After 48 h, cells were harvested and washed once with PBS (*25*). To determine the efficiency of transfection, pSV-β-galactosidase contol vector (Promega) was cotransfected with pcDNA3-LPG2HA and pcDNA3-LPG2Myc. After transfection (48 h), the cells were fixed with 2% formaldehyde and 0.2% glutaraldehyde, stained with X-gal (Invitrogen) (*26*), and observed under the light microscope.

Standard GDP-Man Transport Assay. Transfected HEK cells were washed twice with PBS, harvested, and resuspended in homogenization buffer (10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 0.1 mM TLCK, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM 2,3-dimercaptopropanol). Cells were disrupted by homogenization (5 strokes) in a Dounce homogenizer and freeze−thawing twice; microsomal vesicles were isolated by the procedure of Goud et al. (*27*). *Leishmania* microsomal vesicles were prepared as described earlier (*5*). Transport assays (conducted in duplicate per data point) were started by mixing 100 μL of vesicle suspension with 100 μL of reaction buffer [homogenization buffer containing 10 mM $MnCl_2$, 4 mM $MgCl_2$, and 16 μM (0.16 μCi) of GDP-[^{14}C]-Man]. After incubation at 28 °C for 6 min, the samples were placed on ice, diluted with 1.5 mL of washing buffer (10 mM Tris-HCl, pH 7.4, 0.25 M sucrose), and applied to a filtration apparatus (Millipore 1225 Sampling Manifold) containing HAWP filters (24 mm diameter; 0.45 μm pore size; Millipore). The filters were washed with 25−30 mL of washing buffer, and the 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 0 time of incubation of vesicles with solute. Vesicle integrity was determined by measuring the latency of galactosyltransferase-catalyzed transfer of [^{3}H]-galactose from UDP-[^{3}H]Gal to GlcNAc (*28*).

In experiments probing antiport mechanisms, 5 mM aliquots of the appropriate nucleotides were added to homogenization buffer prior to disruption, allowing nucle-

otides to be trapped within the vesicles. The vesicles were thoroughly washed with washing buffer prior to use.

Western Blotting. To detect the expression of LPG2HA and LPG2Myc proteins, the transfected cells were resuspended in SDS gel-loading buffer containing 5% mercaptoethanol (*24*), incubated for 30 min at 37 °C, and subjected to SDS−PAGE gel electrophoresis under reducing conditions (*24*). Proteins were electrotransferred to poly(vinylidene difluoride) membranes, blocked with 5% milk, and incubated with monoclonal antibody anti-HA (1:500; ascites fluid) or anti-Myc (1:5000) (Invitrogen). Detection was performed using horseradish peroxidase-conjugated goat anti-mouse IgG (Gibco) followed by chemiluminescence using the Super-Substrate System from Pierce. For detection of LPG2His_6, the membranes were blocked with 2% BSA and incubated with India Hisprobe horseradish peroxidase (1:5000) (Pierce) and detected by incubation with the SuperSubstrate for 5 min.

Immunoprecipitation of LPG2HA and LPG2Myc Proteins. HEK cells, cotransfected with pcDNA3-LPG2HA and pcDNA3-LPG2Myc, were harvested 2 days after transfection. The cells were washed once with PBS, resuspended at a density of 1×10^7 cells/mL in prechilled lysis buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.5% Triton X-100, 1% NP-40, 0.1 mM TLCK, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, 0.5 mM phenylmethylsulfonyl fluoride), and incubated on ice for 30 min. The cell lysate was centrifuged at 10000*g* for 10 min at 4 °C to remove debris, followed by centrifugation at 137000*g* for 1 h at 4 °C. The supernatant was used as follows: After preabsorption with protein G−agarose (Boehringer Mannheim), antibody (2 μL of anti-HA or 1 μL of anti-Myc) was added to aliquots (1 mL), and then incubated for 1 h at 4 °C with shaking. Then 80 μL of protein G−agarose suspension was added to the samples and further incubated overnight at 4 °C with shaking. The complexes were collected by centrifugation for 20 s at 12000*g*, and the pellet was washed 5 times: twice with lysis buffer, twice with 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.1% NP-40, and once with 50 mM Tris-HCl, pH 7.5, 0.1% NP-40. SDS−polyacrylamide gel loading buffer was added to the protein G−agarose pellets, and the preparations were then sonicated for 5 min, incubated an additional 20 min at 37 °C, and centrifuged for 20 s at 12000*g*. The supernatant was analyzed by Western blotting using anti-HA or anti-Myc antibodies. Anti-HA and anti-Myc antibodies do not cross-react with Myc- and HA-epitope tags, respectively (data not shown).

Pore-Limited Native Gel Electrophoresis and Glycerol Gradient Fractionation. Leishmania lpg2$^-$ cells (*19*), transfected with pXG-LPG2His_6, were disrupted in lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1 mM TLCK, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, 0.5 mM phenylmethylsulfonyl fluoride, and the appropriate detergent) for 30 min on ice. Samples were centrifuged in a Type 90 Ti rotor (Beckman) for 1 h at 137000*g* to remove aggregates and debris. For pore-limited native gel electrophoresis, an aliquot of the 1% digitonin-solubilized supernatant was resolved on 4−24% polyacrylamide gradient gels in the presence of 0.01% SDS in running buffer (*29*).

Glycerol gradient fractionation was performed according to the procedure of Martin and Ames (*30*). An aliquot of the detergent-solubilized supernatant (0.5 mL) was subjected to centrifugation in a linear glycerol gradient, consisting of 5−25% glycerol (10 mL total) in 25 mM HEPES (pH 7.4), 150 mM NaCl, and the appropriate detergent. Gradients were centrifuged at 285000*g* at 4 °C for 18 h in a SW40 Ti rotor (Beckman), and fractions of 0.5 mL were taken from the bottom under gravity flow. An aliquot of each fraction was analyzed by Western blotting. Standards were β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa).

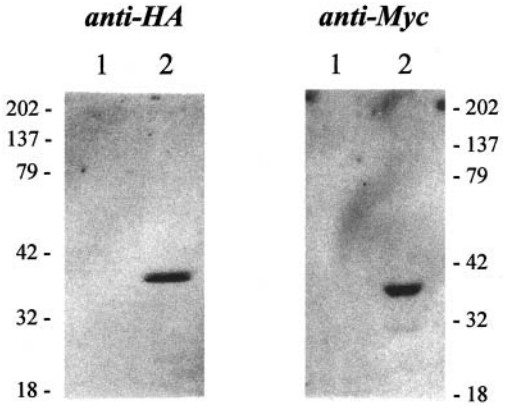

FIGURE 1: Western blotting of HEK cells transfected with either *LPG2HA* or *LPG2Myc*. HEK cells were transfected with plasmids containing either *LPG2HA* or *LPG2Myc*. Expression of the proteins was detected by Western blotting using anti-HA or anti-Myc monoclonal antibodies. Left Panel: Expression of the LPG2HA protein. Lane 1, cell lysate from cells transfected with the vector alone; lane 2, cell lysate from *LPG2HA*-transfected cells. Right Panel: Expression of the LPG2Myc protein. Lane 1, cell lysate from cells transfected with the vector alone; lane 2, cell lysate from *LPG2Myc*-transfected cells.

SDS Gel Electrophoresis of Cross-Linked LPG2. Cross-linking was performed using the homobifunctinal cross-linker DSP (Pierce). *Leishmania* microsomes were diluted to 2.5 mg/mL in PBS (pH 7.4) containing 0.2% NP-40 to mildly permeabilize the microsomes without solubilizing the transporter. The permeabilized microsomes were incubated with the designated concentration of DSP for 30 min at 25 °C. The cross-linking reaction was terminated by the addition of Tris-HCl (pH 7.5) at a final concentration of 0.1 M and 1% SDS. The cross-linked samples were subjected to gel electrophoresis (4−15% polyacrylamide gradient containing 1% SDS) in the absence of reducing reagent.

RESULTS

Expression of Epitope-Tagged LPG2 Proteins in Mammalian Cells. In earlier work, we showed that LPG2 bearing a C-terminal epitope tag (LPG2HA) was active and correctly targeted to the parasite Golgi apparatus when introduced into *Leishmania* (*5*, *19*). We generated a second version carrying the Myc epitope at the C-terminus (LPG2Myc), and introduced both epitope-tagged genes into the mammalian expression vector pcDNA3 (Invitrogen). These constructs were combined with a pSV-β-galactosidase control plasmid to monitor transformation efficiency, and transfected into HEK cells. β-Gal staining showed that about 40% of the cells had taken up DNA. The ~38 kDa LPG2HA (Figure 1, left panel) and LPG2Myc (Figure 1, right panel) proteins were detected by Western blotting, using monoclonal anti-HA and anti-

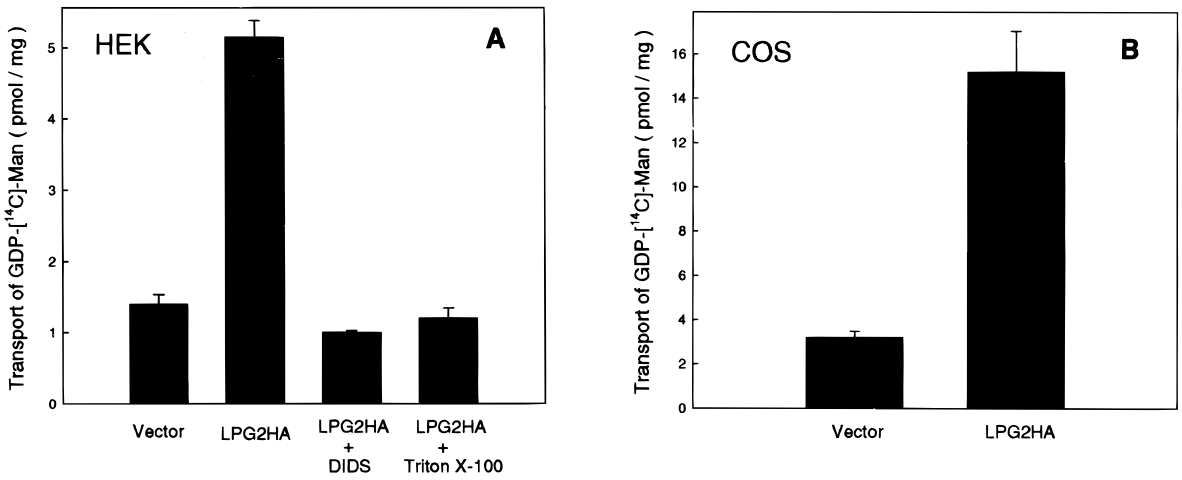

FIGURE 2: Transport of GDP-[^{14}C]-Man into *LPG2HA*-transfected mammalian microsomal vesicles. Microsomal vesicles from pcDNA3-LPG2HA-transfected or pcDNA3 vector-transfected cells (HEK cells, panel A; COS 7 cells, panel B) were incubated with GDP-[^{14}C]-Man at 28 °C for 6 min in a final volume of 200 μL. After incubation, the amount of GDP-[^{14}C]-Man incorporated into the vesicles was determined as described under Experimental Procedures. In selected assays, the final concentration of DIDS was 4 mM and of Triton X-100 was 0.1%.

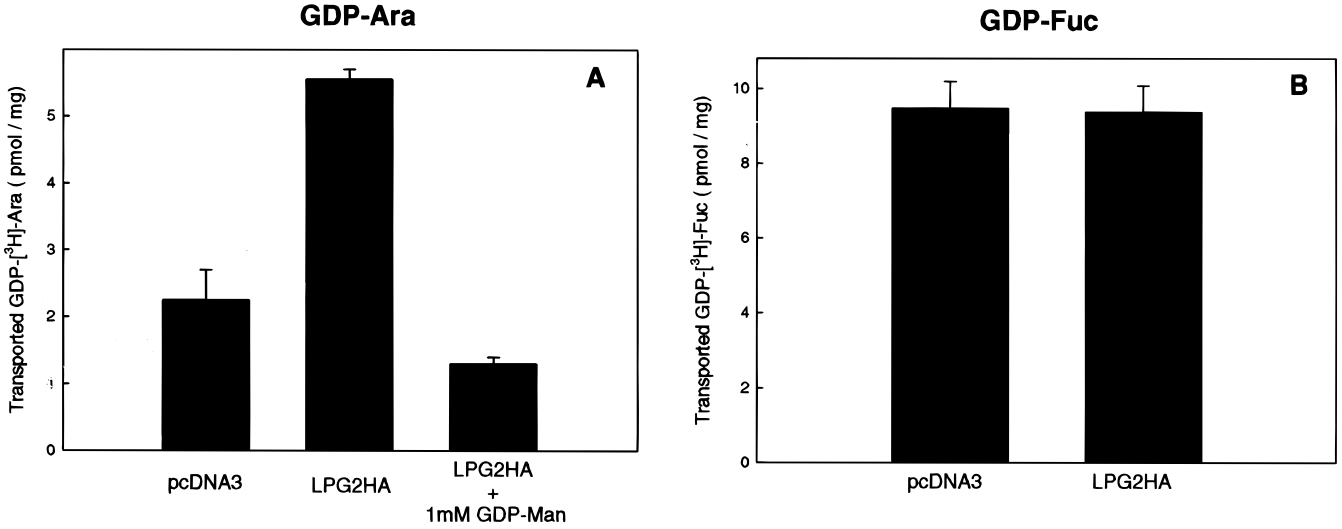

FIGURE 3: Transport of GDP-[^{3}H]-Ara or GDP-[^{3}H]-Fuc into *LPG2HA*-transfected HEK microsomal vesicles. Vesicles from pcDNA3-LPG2HA-transfected or pcDNA3 vector-transfected HEK cells were examined for the uptake of either GDP-D-[^{3}H]-Ara or GDP-L-[^{3}H]-Fuc using the identical conditions described in the standard GDP-[^{14}C]-Man transport assay.

Myc antibodies, respectively. Since this protein was not present in control cells (Figure 1), these experiments demonstrated the expression of the epitope-tagged LPG2 proteins in mammalian cells.

Transport of GDP-Man by the LPG2 Protein. To determine if LPG2 expressed in HEK cells functioned as a GDP-Man transporter, microsomal vesicles from HEK cells transfected with pcDNA3-LPG2HA were prepared and incubated with GDP-[^{14}C]-Man. Vesicular incorporation increased with time and vesicular protein concentration (data not shown). Incorporation was about 3−4-fold higher after transfection with pcDNA3-LPG2HA than pcDNA3 (Figure 2A). Similar observations were made using vesicles derived from pcDNA3-LPG2Myc-transfected HEK cells (data not shown), or with vesicles from pcDNA3-LPG2HA-transfected COS cells (Figure 2B). We attribute the background in the control cells to mannosylation reactions, including the synthesis of [^{14}C]mannosylphosphoryldolichol using GDP-[^{14}C]-Man as a substrate on the outer surface of microsomal vesicles (*31*, *32*).

As shown previously using microsomal vesicles from *Leishmania* (*5*), treatment of the LPG2HA-containing vesicles with DIDS, an inhibitor of nucleotide-sugar transport (*32*, *33*), reduced ^{14}C incorporation to background levels (pcDNA3 vector alone; Figure 2A). Triton X-100 treatment inhibited incorporation, showing that vesicle integrity was required and that GDP-Man uptake represented authentic transport (Figure 2A).

In addition to GDP-Man uptake, the LPG2HA-containing vesicles from HEK cells were capable of transporting GDP-D-Ara, which could be competed by 1 mM GDP-Man (Figure 3A). This result was remarkable since nucleotide-sugar transporters previously were thought to transport a single nucleotide-sugar (*9*, *10*). As shown in Figure 3B, there was no notable difference in the transport of GDP-L-Fuc in LPG2HA-containing vesicles compared to the pcDNA3 vector control. However, using *Leishmania* microsomes which lack an endogenous GDP-L-Fuc transporter, transfection of the LPG2 transporter resulted in significant transport of this nucleotide-sugar (described below).

Two separate experiments were used to localize the LPG2 product in HEK cells. Immunofluorescence of fixed pcDNA3-LPG2Myc-transfected cells with anti-Myc antibodies coupled to FITC showed that the LPG2Myc protein was dispersed throughout the cell. Subcellular fractionation of LPG2HA-transfected HEK cells followed by GDP-Man transport assays or Western blotting with anti-HA antibodies revealed the presence of LPG2HA protein in all membrane fractions (data not shown). These results indicated that, unlike its localization in *Leishmania* (*5*), the distribution of the LPG2 protein was not confined to the Golgi apparatus in the heterologous system. Similar observations were reported in the heterologous expression of the murine CMP-NANA transporter in *Saccharomyces cerevisiae* (*18*). While the broad distribution of the LPG2 protein in mammalian cells likely is due to inappropriate localization motifs, another possibility may be due to overexpression.

Kinetic Comparison of LPG2-Mediated GDP-Man Uptake in Leishmania and Transfected Mammalian Cells. We reported previously that GDP-Man uptake into isolated *Leishmania* microsomes showed a specific activity of 25 pmol mg^{-1} $(6\ min)^{-1}$ with an apparent K_m of 0.3 μM (*5*). In the present studies, we used a modified transport assay, which yielded an activity of 94 pmol mg^{-1} $(6\ min)^{-1}$ with a K_m of 6.9 ± 0.3 μM for GDP-Man. Following transfection of HEK and COS cells with pcDNA3-LPG2HA, we obtained a microsomal uptake activity of 10 and 33 pmol mg^{-1} $(6\ min)^{-1}$ over background, corresponding to 26 and 82 pmol mg^{-1} $(6\ min)^{-1}$, respectively, after correction for transfection efficiency. These values are 28% and 87% that of *Leishmania*, indicating that expression was significant.

While comparisons of the V_{max} can be obscured by differences in the efficacy of expression systems in transfected cells, the apparent K_m for GDP-Man uptake should solely reflect the intrinsic features of the protein. We found that the apparent K_m for microsomal GDP-Man uptake in LPG2HA-transfected cells was 12.2 ± 0.5 μM, similar to the value of 6.9 μM seen with *Leishmania*. Thus, the kinetic properties of LPG2-dependent GDP-Man uptake in mammalian cells closely resemble those seen in the parasite.

Identification of GDP-Man Transported Inside Vesicles. We established that vesicular ^{14}C incorporation was due to GDP-[^{14}C]-Man uptake. Following a 6 min incubation of the vesicles with GDP-[^{14}C]-Man, microsomal vesicles from pcDNA3- or pcDNA3-LPG2HA-transfected HEK cells were washed by filtration and then extracted with organic solvents to remove mannolipids. No radioactivity was recovered from the water-soluble fraction obtained from pcDNA3 vector-transfected cells. TLC analysis of the water-soluble solutes from the pcDNA3-LPG2HA-transfected cells showed that the majority of the label comigrated with GDP-Man (Figure 4A), similar to results observed earlier (*5*). Our studies indicated that more than two-thirds of the radiolabel associated with microsomal vesicles from pcDNA3-LPG2HA cells was authentic GDP-Man. Similar studies in *L. donovani* showed that approximately 20% of the incorporation represented GDP-Man (*5*). The lower percentage of the latter is attributed to GDP-Man utilization in phosphoglycan synthetic reactions (*20*) upon uptake. Most importantly, these data demonstrated that heterologous expression resulted in a functional LPG2 that was capable of transporting GDP-Man.

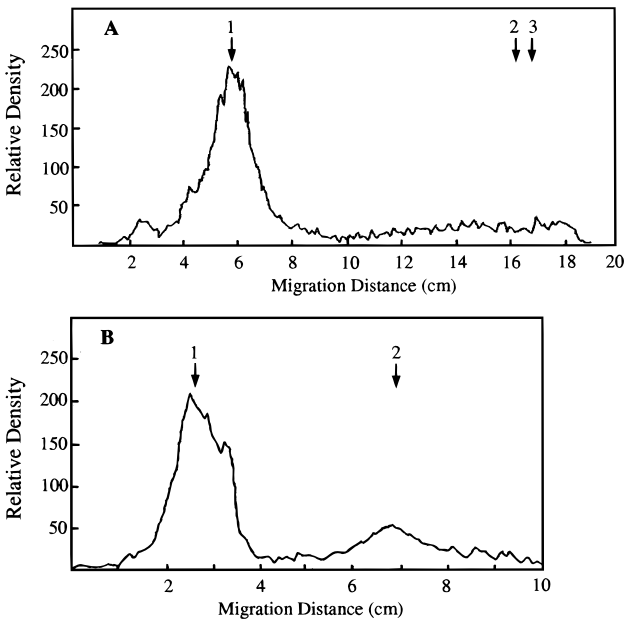

FIGURE 4: TLC of aqueous soluble material in microsomal vesicles. Following a 6 min incubation of vesicles with GDP-[^{14}C]-Man (panel A) or GDP -[^{3}H]-Ara (panel B) at 28 °C, an aliquot of the desalted, aqueous-soluble sample was applied to a thin-layer cellulose plate developed in methanol/formic acid/water (80:15:5) along with appropriate standards as designated in the figure: panel A, (1) GDP-Man, (2) Man-1-P, (3) Man; and panel B, (1) GDP-Ara and (2) Ara. The radioactive samples were scanned by a Bioscan detector.

Furthermore, following transport assays using radioactive GDP-D-Ara instead of GDP-Man, GDP-D-Ara could be isolated from washed vesicles as well (Figure 4B).

Effect of Nucleotides on the Transport of GDP-Man. NSTs have been shown to function by an antiport mechanism, with the simultaneous uptake of the nucleotide-sugar and the exit of the corresponding nucleoside monophosphate (*3*). We confirmed the antiport mechanism of the *Leishmania* GDP-Man transporter. GDP-Man translocation activity was analyzed by measuring transport in vesicles that were prepared from pcDNA3-LPG2HA-transfected HEK cells and subsequently preloaded with selected nucleotides. GMP-loaded vesicles possessed approximately twice the GDP-Man uptake activity ($P < 0.03$) compared to unloaded vesicles (Figure 5). With GDP-, AMP-, or ADP-preloaded vesicles, the GDP-Man transport activity was lower than unloaded vesicles (Figure 5). These observations were consistent with the GDP-Man NST reported in yeast Golgi in which GDP-Man uptake was coupled to exit of GMP (*34*, *35*). In a related experiment, inhibition of GDP-Man transport by selected nucleotides was measured. Using GMP-preloaded vesicles, GDP-Man transport was analyzed in the presence of 5 mM GMP, GDP, AMP, and ADP. As shown in Figure 6, only GDP almost completely abolished transport of the nucleotide-sugar ($P < 0.01$). This suggested the likelihood that GDP-Man and GDP may be competing for binding to the transporter. The partial inhibitory effect of externally added GMP is understandable since the GMP concentration gradient, established during the preloading step, would be lessened and, consequently, would decrease the rate of GMP exit.

Nature of the LPG2 Transporting Complex Examined by Immunoprecipitation. Some members of the NST family possess small leucine zipper motifs, suggesting the possibility

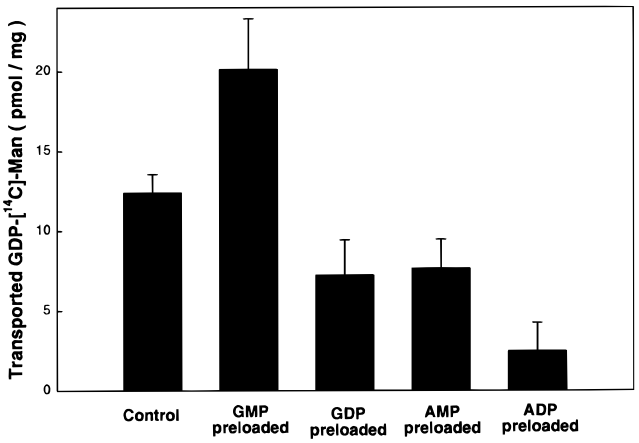

FIGURE 5: Effect of preloading vesicles with nucleotides on GDP-Man transport. Microsomal vesicles were preloaded with a 5 mM aliquot of the designated nucleotides and assayed for GDP-[^{14}C]-Man transport activity.

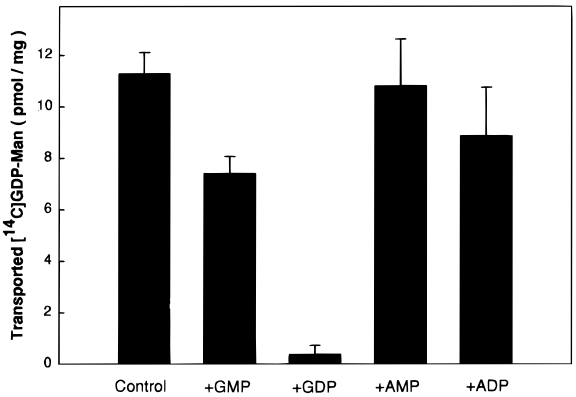

FIGURE 6: Effect of nucleotide addition on GDP-Man transport into microsomal vesicles. Microsomal vesicles, preloaded with 5 mM GMP, were prepared and assayed for GDP-[^{14}C]-Man transport activity in the presence of 5 mM of the designated nucleotides.

of protein−protein interactions. This finding has not been tested rigorously, and LPG2 itself lacks the leucine zipper motif (*19*). To investigate the potential of LPG2 to form homo-multimeric complexes, pcDNA3-LPG2HA and pcDNA3-LPG2Myc were cotransfected into HEK cells. Two days after transfection, the cells were harvested, and proteins were obtained by solubilization with detergents. Aliquots were then immunoprecipitated with either anti-HA or anti-Myc monoclonal antibodies. Immunoprecipitated proteins were resolved by SDS−PAGE, and the epitope-tagged proteins were detected by Western blotting (Figure 7, "Co-transfected" lanes).

Both anti-HA and anti-Myc antibodies detected the expected ~38 kDa protein in the anti-HA immunoprecipitates (Figure 7, lanes 2 and 4). We estimated that approximately two-thirds of the total Myc-tagged protein was present in the anti-HA immunoprecipitate. Similarly, the HA- and Myc-tagged LPG2 proteins were detected by the antibodies in the anti-Myc immunoprecipitates (Figure 7, lanes 6 and 8). No proteins were detected with either antibody in the pcDNA3-transfected controls (Figure 7, lanes 1, 3, 5, and 7). The ability of antibodies directed against either epitope-tagged LPG2 to coprecipitate the other epitope-tagged LPG2 in solubilized protein preparations implied the existence of multimeric LPG2 protein complexes. Identical results were

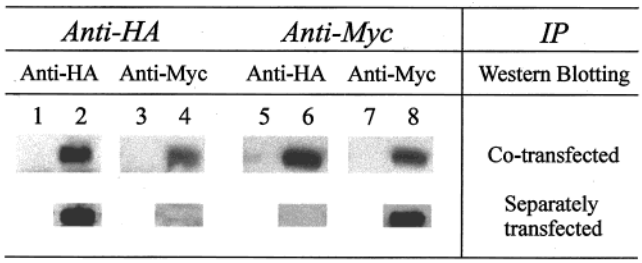

FIGURE 7: Western blotting of immunoprecipitates obtained with anti-HA or anti-Myc antibodies. HEK cells were "Co-transfected" or "Separately transfected" with pcDNA3-LPG2HA and pcDNA3-LPG2Myc, or with vector alone. As detailed under Experimental Procedures, detergent-solubilized proteins were immunoprecipitated with either anti-HA (lanes 1−4) or anti-Myc (lanes 5−8) antibodies, and Western blotting was performed. In lanes 1, 2, 5, and 6, the immunoprecipitates (IP) were blotted with anti-HA antibodies, and in lanes 3, 4, 7, and 8, the immunoprecipitates were blotted with anti-Myc antibodies.

obtained using pX63HYG-LPG2HA- and pXG-LPG2Myc-cotransfected *Leishmania* (data not shown).

Since the LPG2 protein is predicted to be extremely hydrophobic, the possibility existed that the cotransfected LPG2 subunits aggregated during solubilization. This possibility was examined by an experiment involving separate transfections of pcDNA3-LPG2HA and pcDNA3-LPG2Myc into HEK cells. After the addition of detergents, the solubilized proteins were mixed, immunoprecipitated with either anti-HA or anti-Myc monoclonal antibodies, and subjected to gel electrophoresis and Western blotting. As shown in Figure 7 ("Separately transfected" lanes), HA- and Myc-tagged LPG2 were detected by Western blotting only in anti-HA and anti-Myc immunoprecipitates, respectively. Thus, the multimeric complex of LPG2 observed in the cotransfection experiments was unlikely to arise from nonspecific aggregation.

To further confirm that the coimmunoprecipitation of LPG2HA and LPG2Myc was not due to aggregation, coimmunoprecipitation experiments with another hydrophobic protein were conducted. The glucose transporter D2 in the plasma membrane of *Leishmania* has 12 transmembrane domains (*36*). The D2 gene was removed from the *Eco*RI and *Xba*I sites of pAlt-D2 (*36*) and inserted into the *Eco*RI and *Xba*I sites of pcDNA3. HEK cells were cotransfected with pcDNA3-LPG2HA and pcDNA3-D2, solubilized with lysis buffer containing 0.5% Triton X-100 and 1% NP-40, immunoprecipitated with either anti-HA or anti-D2 antibodies, and subjected to Western blotting with either anti-HA or anti-D2 antibodies. Immunoprecipitates of LPG2HA or D2 with their respective antibodies resulted in no coimmunoprecipitation (data not shown).

Nature of the LPG2 Complex Examined by Glycerol Gradient Centrifugation. The native state of the LPG2 transporter complex was determined in *Leishmania* as the appropriate source. The *Leishmania lpg2* null mutant was transfected with pXG-LPG2His$_6$, and the solubilized proteins were obtained from a stably transfected line with selected detergents. The protein extracts were subjected to centrifugation using a linear glycerol velocity gradient, and the LPGHis$_6$ was monitored by immunoblotting. As shown in Figure 8, the LPG2His$_6$ complex was sensitive to detergents. When solubilized in the presence of 1% SDS and centrifuged

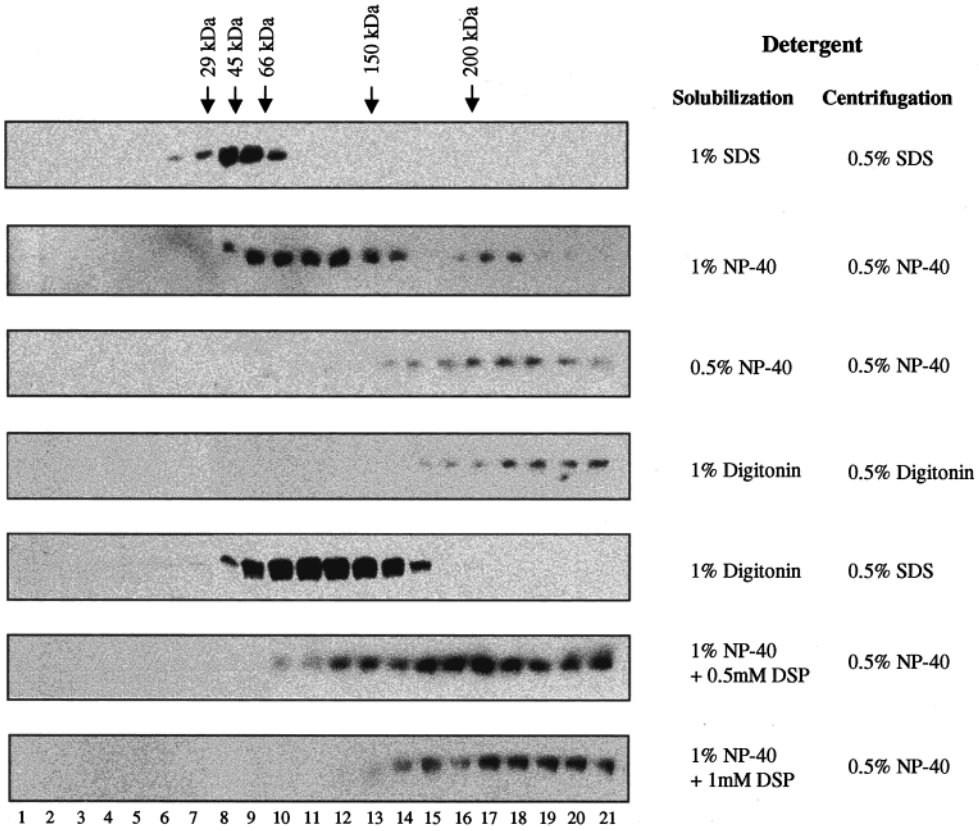

FIGURE 8: Analysis of the LPG2His$_6$ complex by glycerol gradient centrifugation and Western blotting. *Leishmania* mutant parasites (*lpg2*$^-$) were transfected with pXG-LPG2His$_6$ and solubilized with the designated detergent. After centrifugation to remove debris, the supernatant was centrifuged in a linear 5−25% gradient of glycerol containing the designated detergent. Shown are immunoblots of the fractions from the gradient. When DSP was used, the cross-linker at the designated concentration was added to the NP-40 (1%) solubilized proteins and incubated for 30 min at 25 °C before centrifugation. The cross-linking reaction was terminated by the addition of 0.1 M Tris-HCl, pH 7.5, prior to centrifugation. Arrows at the top indicate the elution positions of protein molecular mass markers. Numbers at the bottom indicate the numbered fractions collected from the glycerol gradient.

in 0.5% SDS, LPG2His$_6$ appeared in a low molecular mass, mostly monomeric form (approximately 30−50 kDa). The highest molecular mass of the LPG2His$_6$ (approximately 200−300 kDa) was seen when the complex was solubilized with either 0.5% NP-40 or 1% digitonin and centrifuged with 0.5% NP-40 or 0.5% digitonin. Solubilization with a higher concentration of NP-40 (1%) or solubilization with 1% digitonin and centrifugation with 0.5% SDS resulted in partial disassembly of the LPG2His$_6$ complex. Importantly, two different concentrations of the cross-linker DSP yielded the LPG2His$_6$ complex as the highest molecular mass complex (approximately 200−300 kDa).

Nature of the LPG2 Complex Examined by Gel Electrophoresis. Aliquots of the 1% digitonin-solubilized LPG2His$_6$ complex were subjected to either SDS or pore-limited native gel electrophoresis using a polyacryamide gel gradient. Under the reducing conditions of SDS−PAGE, the LPG2His$_6$ (Figure 9A, lane 2) migrated as a monomer (∼38 kDa). Under pore-limited native gel conditions, in which the electrophoresis was run to equilibrium, LPG2His$_6$ migrated as a hexameric complex with approximate molecular mass of 220 kDa (Figure 9B, lane 2). Additional support for the hexameric nature of the transporter complex was obtained by cross-linking experiments. The LPG2His$_6$-containing vesicles were permeabilized with 0.2% NP-40, conditions that do not solubilize LPG2. The permeabilized vesicles were incubated with increasing concentrations of the cross-linker DSP and the samples examined by Western blotting. With increasing amounts of DSP, intermediate-sized bands were visualized that corresponded to multimers of the ∼38 kDa monomeric LPG2 protein (Figure 9C, lanes 1−4). At the highest concentrations of DSP (Figure 9C, lanes 4 and 5), the main band was 215 kDa, corresponding to the hexameric complex.

GDP-Sugar Specificity of the LPG2 Transporting Complex. We examined the GDP-sugar specificity of the LPG2 transporting complex in vesicles isolated from wild-type, *lpg2*$^-$, and *lpg2*$^-$ transfected with pXG-LPG2. As expected, GDP-Man uptake was minimal in vesicles from *lpg2*$^-$ cells and was restored to almost wild-type levels using vesicles from pXG-LPG2-transfected *lpg2*$^-$ cells (Figure 10A). These results are similar to the transport of GDP-Man in vesicles isolated from the LPG2-defective mutant C3PO and *LPG2*-transfected C3PO (*5*). Either GDP-D-Ara or GDP-L-Fuc was transported in vesicles from wild-type cells and could be inhibited by the addition of 1 mM GDP-Man (Figure 10B,C). Importantly, uptake of either GDP-D-Ara or GDP-L-Fuc also was diminished in vesicles from *lpg2*$^-$ cells, but restored after *LPG2* transfection (Figure 10B,C). The differences in specific activities in the uptake of the three GDP-sugar likely reflect affinity variances. Arabinose-containing glycolipids in *L. donovani* have recently been reported (*37*) which likely contribute to the background in the GDP-Ara transport assays.

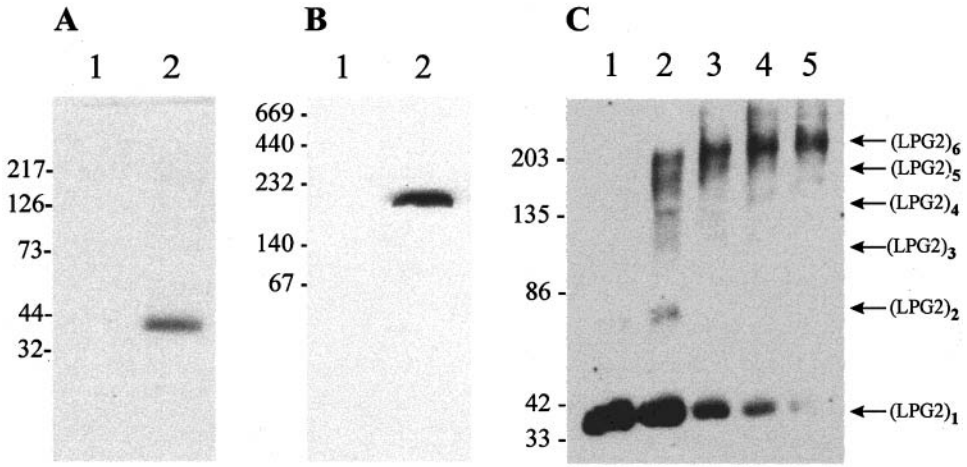

FIGURE 9: Analysis of the LPG2His$_6$ complex by gel electrophoresis and Western blotting. Panel A: Vesicles from vector-transfected (lane 1) or pXG-LPG2His$_6$-transfected *lpg2*$^-$ (lane 2) parasites were solubilized with 1% digitonin. The supernatants were resolved by SDS gel gradient electrophoresis under reducing conditions and Western blotting as described under Experimental Procedures. Panel B: Vesicles from vector-transfected (lane 1) or pXG-LPG2His$_6$-transfected *lpg2*$^-$ (lane 2) parasites were solubilized with 1% digitonin. The supernatants were subjected to pore-limited native gel electrophoresis (4−24% polyacrylamide gradient) and Western blotting. Panel C: Vesicles from pXG-LPG2His$_6$-transfected *lpg2*$^-$ parasites permeabilized with 0.02% NP-40 and then incubated with DSP (0 mM, lane 1; 0.2 mM, lane 2; 0.4 mM, lane 3; 0.6 mM, lane 4; 0.8 mM, lane 5). The cross-linked samples were subjected to SDS−PAGE (4−15% polyacrylamide gradient containing 1% SDS) in the absence of reducing reagent and then analyzed by Western blotting. The multimers of LPG2 listed on the right side of panel C designate the expected migration of multimers of a 38 kDa protein. The molecular mass values stated in the text were determined by plotting the log of molecular mass versus the migration distance of standard proteins. The equations were (panel A) $y = -0.155x + 2.482$ ($r^2 = 0.996$), (panel B) $y = -0.227x + 2.908$ ($r^2 = 0.986$), and (panel C) $y = -0.112x + 2.467$ ($r^2 = 0.999$).

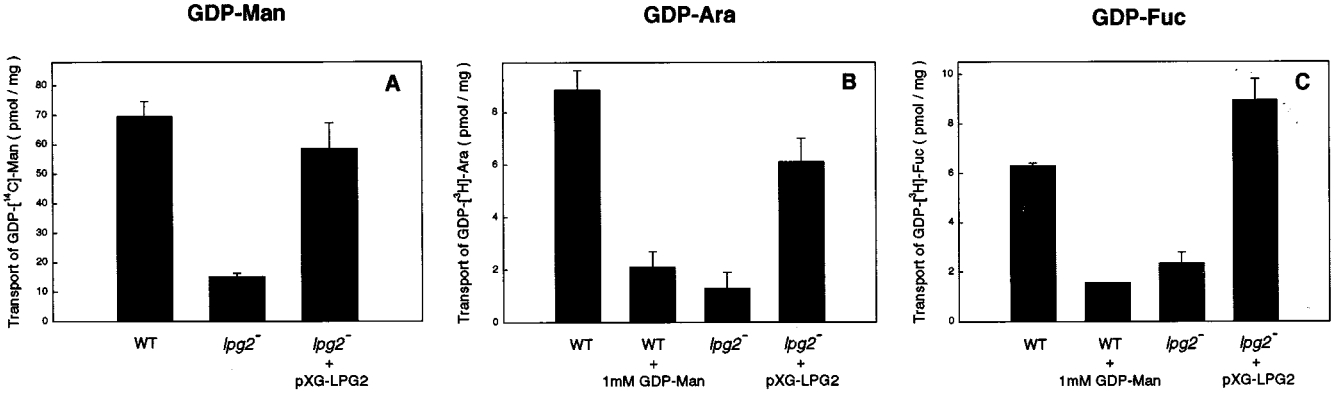

FIGURE 10: Transport of GDP-sugars in vesicles from *Leishmania* cells. Microsomal vesicles from wild-type, *lpg2*$^-$, and pXG-LPG2-transfected *lpg2*$^-$ parasites were tested for uptake of GDP-[^{14}C]-Man (panel A), GDP-[^{3}H]-Ara (panel B), and GDP-[^{3}H]-Fuc (panel C) using the standard GDP-[^{14}C]-Man transport assay.

DISCUSSION

While mammals possess a variety of ER and Golgi NSTs (*1*−*3*, *38*), they lack a transporter for GDP-Man (*6*). Thus, mammalian cells offer an excellent system for heterologous expression of *LPG2*, and in this work we have shown that the *Leishmania* LPG2 GDP-Man transporter can be expressed in an active form in mammalian cells. The kinetic properties of the heterologously expressed LPG2 GDP-Man transport activity closely resembled those observed in *Leishmania* itself, with apparent K_m values of 12.2 μM versus 6.9 μM, respectively. Given the large evolutionary distance between trypanosomatid protozoans and mammals and the rapid rate of evolution of NSTs, it seems unlikely that LPG2 may interact with host proteins. Thus, we propose that LPG2 functions autonomously to mediate GDP-Man uptake.

The heterologously expressed LPG2 exhibited all features expected of an NST. GDP-Man transport activity in the vesicles was dependent on time and protein concentration, showed latency, and was inhibited by the anion transport inhibitor DIDS. The ability of preloaded GMP to enhance GDP-Man uptake suggests that transport occurs by an antiport mechanism, as seen with other NSTs (*34*, *35*). Furthermore, the addition of GDP along with GDP-Man inhibited uptake of the nucleotide-sugar in microsomes, indicating competition of substrate binding to the NST. Last, we showed that these vesicles incorporated authentic GDP-Man.

NSTs are believed to transport only a single type of nucleotide-sugar (*9*, *10*). For example, the purified UDP-GalNAc transporter from rat liver transported only UDP-GalNAc among five different UDP-sugars tested. Using a genetic approach, the LPG2 NST was capable of transporting GDP-D-Ara and GDP-L-Fuc as well as GDP-Man. We had reported previously that LPG2 does not transport UDP-Gal (*5*). Since mammalian cells already have an endogenous GDP-L-Fuc NST (*39*), GDP-L-Fuc uptake in vesicles from vector control or LPG2-transfected HEK cells was identical. Transfecting HEK cells with *LPG2* apparently does not

increase significantly the GDP-L-Fuc transport activity over endogenous levels. Moreover, these observations would imply that the mammalian GDP-L-Fuc NST, in contrast to the *Leishmania* LPG2 NST, is highly restrictive for GDP-L-Fuc. Otherwise, mammalian cells would transport cytoplasmic GDP-Man into the Golgi lumen, where it is not needed and may be potentially detrimental.

The LPG2 GDP-Man NST Exists as a Multimeric Protein Complex. We studied LPG2 proteins bearing different epitope tags expressed simultaneously in *Leishmania* and mammalian cells to demonstrate that LPG2 exists in a multimeric complex. In these experiments, immunoprecipitation with antisera to one tagged LPG2 protein invariably showed coprecipitation of the other tagged LPG2 protein. This multimeric complex was not a result of nonspecific aggregation. First, separate transfections of mammalian cells with HA- and Myc-tagged proteins, followed by mixing, showed no evidence of coimmunoprecipitation. Second, cotransfections of genes encoding LPG2 and an unrelated hydrophobic protein (the glucose transporter) also showed no coimmunoprecipitation. Third, prior to glycerol gradient centrifugation or native gel electrophoresis, the detergent-solubilized LPG2 complex from transfected *Leishmania* was subjected to high-speed centrifugation (137000*g*) that likely would have removed aggregated proteins. Due to the evolutionary distance between mammalian cells and *Leishmania*, we believe that this complex arises solely from LPG2−LPG2 subunit interactions. Since the properties of LPG2-dependent GDP-Man uptake are similar in both *Leishmania* and mammalian cells, we believe that the LPG2 complex in *Leishmania* similarly arises from LPG2−LPG2 subunit interactions.

The estimated size of the detergent-solubilized LPG2 complex expressed in *Leishmania* was approximately 220 kDa, as revealed by glycerol gradient centrifugation or pore-limited native gel electrophoresis. A similarly sized complex was also observed upon cross-linking of the complex, followed by SDS−PAGE. Since the LPG2 monomer is ∼38 kDa, our current picture is that the GDP-Man NST of *Leishmania* consists of a homohexamer complex composed solely of LPG2 protein subunits.

Several NSTs exhibit leucine zipper motifs commonly associated with protein−protein interaction domains, including the human UDP-Gal and CMP-NANA NSTs (*11, 12*) and the yeast UDP-GlcNAc NST (*15*). However, the predictive value of this motif is low for NSTs, as LPG2 lacks the leucine zipper yet exists in a multimeric complex. By radiation target inactivation, the rat Golgi PAPS transporter has a reported molecular mass of 150 kDa, while the purified PAPS transporter polypeptide has an apparent mobility of 75 kDa in SDS−PAGE analysis (*40*). Hirschberg and coworkers (*10*) recently purified the UDP-GalNAc NST from rat liver to apparent homogeneity and showed it to be a homodimer of 43 kDa subunits. Thus, the structural similarity of LPG2 to other NSTs implies that they also exist as multimeric complexes.

Implications to NST Structure and Specificity. The existence of subunit interactions in NST complexes raises the possibility of the formation of heteromeric NST complexes with new specificities. The formation of heteromeric protein complexes with modified specificities is a common theme to expand the diversity of molecular recognition, two examples being the diversity arising from heterodimerization among c-*jun*, c-*fos*, and activating transcription factor (ATF) proteins (*41*), or among steroid receptors (*42*). Conceivably, expression of different NSTs within the same cell could give rise to heteromeric complexes with different affinities and/or specificities. At the present time, our knowledge of the NST family is incomplete, and no studies of NST heteromer formation have been reported. Recently, several isoforms of the mammalian UDP-Gal transporter were identified by their ability to rescue a defect in the Had-1 cell line (*43*), and their close sequence relationship evokes the possibility of heteromer formation. As mammalian cells exhibit a variety of galactosylation reactions with a range of affinities (*44*), it is possible that a range of affinities accompanying heteromer formation may contribute to the maintenance of proper Golgi UDP-Gal levels under all metabolic conditions. Similarly, heteromerization could yield new substrate specificities not yet tested. As our knowledge of the expanding NST gene family grows, we will soon be in a position to test these proposals.

The data presented here and previously (*5, 19*) demonstrate that the *Leishmania LPG2* gene encodes a unique Golgi GDP-mannose transporter. In this regard, *Leishmania* resembles fungi but not mammals (*3, 4, 16*). The requirement for lumenal GDP-Man in the Golgi of lower but not higher eukaryotes (*3*) is understandable in light of the structural complexities of *Leishmania* phosphoglycan-containing glycoconjugates (*20*) and yeast mannans (*3*). Interestingly, yeast has a homologue (*VRG4*) encoding a GDP-Man transporter that is essential to viability (*4, 16*), whereas *LPG2* is required only for *Leishmania* virulence. Thus, inhibitors of this transporter may prove to have a broad applicability to a number of pathogenic organisms. The *Leishmania* LPG2 system offers a powerful system for probing these activities, as the protein is amenable to genetic analysis and there is a diverse array of homologous and heterologous systems and expression tools.

ACKNOWLEDGMENT

We thank Charles Waechter, Jeffery Rush, Wally Whiteheart, and Brenda Jo Mengeling for their thoughtful suggestions during the course of this work. We also thank Scott Landfear and Eric Snapp (Oregon Health Sciences Center) for their generous gift of pAlt-D2 and D2 antibody.

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BI992363L