Reconstitution of GDP-mannose Transport Activity with Purified Leishmania LPG2 Protein in Liposomes*

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Activated nucleotide sugars required for the synthesis of glycoconjugates within the secretory pathway of eukaryotes are provided by the action of nucleotide sugar transporters (NSTs). Typically, NSTs are studied in microsomal preparations from wild-type or mutant lines; however, in this setting it can be difficult to assess NST properties because of the presence of glycosyltransferases and other interfering activities. Here we have engineered Leishmania donovani to express high levels of an active LPG2 Golgi GDP-Man transporter bearing a C-terminal polyhistidine tag. The functional LPG2-HIS was solubilized, purified by metal affinity chromatography, and reconstituted into phosphatidylcholine-containing liposomes using polystyrene SM-2 beads. The proteoliposomes exhibited robust GDP-Man transport activity with an apparent $K_m$ of 6.6 $\mu$M. Transport activity was enhanced by preloading of GMP and showed specificity for multiple substrates (GDP-Ara and GDP-Fuc). In contrast to the activity in crude microsomes, transport was not dependent on the presence of divalent cations. Thus, reconstitution of transport activity using purified LPG2 protein in liposomes provides firm experimental evidence that a single polypeptide is solely required for NST activity and is able to mediate the uptake of multiple substrates. These studies are relevant to the study of NST structure and function in both protozoan parasites as well as their higher eukaryotic hosts.

Many glycosylation reactions in eukaryotes require the synthesis of nucleotide sugar substrates in the cytoplasm and their subsequent translocation into the lumen of the endoplasmic reticulum or Golgi apparatus. The membrane proteins that facilitate the translocation are termed nucleotide sugar transporters (NSTs) and work in concert with glycosyltransferases to assemble a variety of specific oligosaccharide structures. NST genes have been identified in many eukaryotes, including yeast (1–6), protozoa (7), worms (8), insects (9–11), plants (12, 13), and mammals (14–21). NST genes generally encode for multi-transmembrane-spanning proteins (30–45 kDa) that function in oligomeric complexes, ranging from dimers to hexamers (22–24).

NST activities occur in both the endoplasmic reticulum and Golgi apparatus, and specificities for a variety of the nucleotide sugars described include UDP-Gal, UDP-Glc, UDP-GlcA, UDP-GalNAc, UDP-GalNAc, CMP-sialic acid, GDP-Man, GDP-Ara, and GDP-Fuc (25–27). Although originally thought to be specific for a single nucleotide sugar substrate, more recent studies have shown that some NSTs can transport several different nucleotide sugars (9–11, 13, 20, 24, 28), with the principal determinant for specificity residing in the nucleotide portion (29). Although most attention has been given to NST substrate specificity, the quantity of nucleotide sugars supplied by the respective NSTs has important consequences. Under conditions of limited substrate availability due to down-regulation of NST activity, glycosyltransferases with low $K_m$ values may prevail, whereas under substrate-saturating conditions glycosyltransferases with higher $K_m$ values are activated. For example, elevated expression of a UDP-Gal NST in colonic cancers, in turn, adversely affects the synthesis of several important carbohydrate determinants such as sialyl Lewis X (30). Thus, modifications of the activity of NST may have profound consequences on glycoconjugate structures.

NSTs are thought to act through an antiporter mechanism involving a one-for-one exchange between the external nucleotide sugar and the corresponding luminal nucleotide monophosphate (26). The NMPs arise directly following the action of glycosylphosphotransferases (7) or by the hydrolysis of NDPs arising from glycosyltransferases through the action of nucleotide diesters (31, 32). Most biochemical characterizations of NSTs have been performed using crude microsomes, and these microsomes have been hampered by the presence of the glycosyltransferases and diesters mentioned earlier as well as by the limiting quantities of NSTs naturally occurring in microsomal membranes. Several NSTs have been solubilized from rat liver Golgi microsomes, and then the transport activities were reconstituted in proteoliposomes (22, 23). Only the rat liver Golgi transporters for UDP-N-acetylgalactosamine (22) and GDP-fucose (23) have been highly purified and characterized in an active form.

The transport of nucleotide sugars into the secretory pathway is particularly important for pathogenic microbes, such as the protozoan parasite Leishmania, because of the synthesis of considerable quantities of glycoconjugates that play critical

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1 The abbreviations used are: NST, nucleotide sugar transporter; CHAPS, 3-[3-cholamidopropyl]dimethylammoniom-1-propanesulfonic acid; CHAPSO, 3-[3-cholamidopropyl]dimethylammoniom-2-hydroxy-1-propanesulfonic acid; GDA, glycodeoxycholic acid; HRP, horseradish peroxidase; LPG, lipopolysaccharide; Ni-NTA, nickel-nitrioltriacetic acid; PBS, phosphate-buffered saline.
roles in their infectious cycle. *Leishmania* synthesizes a variety of mannose-containing glycoconjugates including glycoproteins and phosphoglycans, and genetic studies show that many of these are required for the ability of the parasite to successfully survive and induce disease in the mammalian host (33-37). By forward genetic approaches we identified the *Leishmania* LPG2 gene, which is required for the synthesis of the abundant surface glycoconjugate lipophosphoglycan (LPG) and Golgi GDP-Man uptake (7, 38). LPG2 encodes a ~37 kDa protein containing up to nine transmembrane domains (7, 38). When examined in cellular preparations, LPG2 proteins existed as a homo-hexameric complex that mediates the translocation of several GDP-sugars into the Golgi apparatus of the parasite (24). In this report, we purified LPG2, reconstituted the transporting complex in an active form in proteoliposomes, and characterized its properties relative to those observed previously in *situ*.

**EXPERIMENTAL PROCEDURES**

**Materials**—GDP-D-[3H]mannose (60 Ci/mmol) was kindly provided from Dr. Jeff Rush (University of Kentucky). GDP-D-[3H]fucose (15 Ci/mmol) was synthesized as described elsewhere (39). Materials were obtained as follows: GDP-D-[3H]fucose (5.1 Ci/mmol) was from American Radiolabeled Chemicals; Complete EDTA-free protease inhibitor mixture came from Roche Applied Science (catalog number 873 580), Ni-NTA resin was obtained from Qiagen; TALON resin came from Clontech; Spin Column Biogel P-6 and Precision protein standards were from Bio-Rad; Super Signal West Pico detection reagents and India ink were from Pierce; glycodeoxycholic acid (GDA), taurocholic acid, and G-418 were from Calbiochem; the nitrocellulose membrane came from Schleicher & Schuell BioScience; x-ray film was from Kodak; anti-His monoclonal antibody came from Invitrogen; Alexa Fluor 488-conjugated anti-mouse IgG was from Molecular Probes; Per-Fluoro mounting medium came from Beckman-Coulter; brain-heart infusion medium was from DIFCO; and the PlusOne silver stain kit was from Amersham Biosciences. All other chemicals were obtained from Sigma.

**Cells and Cell Culture**—A *Leishmania donovani* (Ld4 clone) lpg2^-/-^ knock-out strain (7) transfected with the expression construct pXG-LPG2cHis6 (24) was cultivated at 25 °C in M199 medium supplemented with 10% heat-inactivated fetal bovine serum, biotin (1 μg/ml), and G-418 (50 μg/ml). For microsomal preparations, cells were grown in brain-heart infusion medium supplemented with adenine (2.67 mM), sodium phosphate, pH 8., 0.5% GDA, and 0.25 mM imidazole, and then washed with 6-column volumes of 10 mM imidazole containing 10 μM HEPEs, pH 7.4, 0.5% GDA, and 0.25 mM succrose. The LPG2-HIS protein was eluted with 250 mM imidazole containing 10 μM HEPEs, pH 7.4, 0.5% GDA, and 0.25 mM succrose.

**Reconstitution**—Egg yolk phosphatidylcholine was suspended under nitrogen gas in 10 μM HEPEs, pH 7.4, 0.25 mM succrose, and 1 mM GMP at a concentration of 20 mg/ml and 0.5% GDA using a water bath sonicator. The lipid mixture was added to protein at a ratio of 4:1 (v/v). SM-2 beads (30 mg), pre-wetted with 10 μM HEPEs, pH 7.4, and containing 0.25 mM succrose, were added to the protein-lipid mixture with gentle agitation for 30 min at 25 °C. Another 30 mg of pre-wetted SM-2 beads was added and incubated for an additional 30 min. Finally, 100 mg of pre-wetted beads were added to the reaction mixture and incubated at 4 °C for 16 h with gentle agitation. After filtering the suspension through a glass wool-plugged plastic syringe, the suspension was frozen in liquid nitrogen and thawed at room temperature. The reconstituted proteoliposomes were obtained by passage through a BioSpin P-6 column at 700 × g for 4 min.

**Immunofluorescence Staining—Leishmania cells (10^6 grown to 10^7/ml) were harvested by centrifugation at 700 × g for 10 min and wash one more with ice-cold PBS. The cells were fixed in 1 ml of PBS containing 2% formaldehyde and 0.05% glutaraldehyde for 30 min. The fixed cells were washed once with PBS and resuspended in 1 ml of PBS. A 50-μl aliquot of cell suspension was spotted onto each well of an 8-well slide, which was pre-coated with 1 mg/ml poly-l-lysine. After incubation for 10 min, unbound cells were removed by washing with PBS. The bound cells were blocked for 30 min in 50 mM ammonium chloride containing 3% bovine serum albumin in PBS and stained for 1 h with anti-polyhistidine monoclonal antibody (1:200) in PBS containing 0.1% Triton X-100 and 3% bovine serum albumin. The bound antibody was detected with 1:400 diluted Alexa Fluor 488-conjugated anti mouse IgG. The cells were observed with a Nikon ECLIPSE E600 fluorescence microscope at a 1000-fold magnification and photographed with a SPOT digital camera (Diagnostic Instruments Inc., Sterling Heights, MI).

**RESULTS**

**Expression of LPG2 Bearing a C-terminal His Tag**—To express high levels of the *L. donovani* LPG2 GDP-Man transporter, we made use of the *Leishmania* episomal expression vector pXG, as the insertion of protein open reading frames and transfection into *Leishmania* typically induces overexpression of the cognate protein up to 100-fold (40). To facilitate the detection of the LPG2 transporter during its purification, the LPG2 gene was modified with a C-terminal epitope polyhistidine-dye (LPG2cHis6 or LPG2-HIS), as previously we showed that LPG2 activity tolerated a variety of C-terminal tags (24). As a recipient cell we used the *L. donovani* lpg2^-/-^ knockout line described previously; these cells thus lack endogenous, unmodified LPG2. By using a homologous *Leishmania* expression system, we ensured that the purified LPG2 protein would...
The protein was now pelleted (Fig. 3) and the C terminus of LPG2-HIS accessible to the Ni-NTA, rendered the C terminus of LPG2-HIS accessible to the Ni-NTA, and transporter by treatment with 1% GDA (see below) rendered the C terminus of LPG2-HIS accessible to the Ni-NTA, and the protein was now pelleted (Fig. 3, fourth and fifth lanes from the left). These results provide further evidence regarding the topology of the GDP-Man NST; in contrast to other known NSTs, the C terminus of LPG2 is lumenally oriented in the Golgi apparatus.

Purification of LPG2-HIS—The microsomal fraction was taken as the starting material, and first a variety of detergents were surveyed for their ability to solubilize the LPG2-HIS protein in this fraction. Although Triton X-100 (1.1%) and Nonidet P-40 (0.5%) have been used successfully to extract active nucleotide metabolite transporters from rat liver Golgi (22, 23, 42), neither these nor cholic acid, MEGA8, or MEGA9 were effective in solubilizing the LPG2-HIS protein from the Leishmania microsomes (Fig. 4A). As shown in Fig. 4A, deoxy-
cholic acid and its conjugates GDA and taurodeoxycholic acid at concentrations of 1% solubilized the Leishmania microsomes efficiently. GDA was chosen for further usage because of its tolerance of high concentrations of salt. GDA solubilized the LPG2-HIS protein from Leishmania microsomes in amounts as low as 0.2% (Fig. 4B), but GDA was used for LPG2-HIS purification at a concentration of 0.5%, corresponding to 5-fold above its critical micelle concentration.

To purify the GDA-solubilized LPG2-HIS, the extract was applied sequentially to two resins that interact with the His6 tag, and purification was monitored by SDS-PAGE followed by silver staining and anti-His tag Western blotting. The solubilized microsomal LPG2-HIS extract was first applied to a column of the cobalt chelate resin TALON. The large majority of solubilized microsomal proteins did not bind, whereas LPG2-HIS was efficiently bound by the TALON resin (Fig. 5). Following elution of LPG2-HIS with imidazole, the eluent was diluted 2-fold by loading buffer and loaded onto a column of Ni-NTA resin. The few remaining protein contaminants were removed in the loading and washing steps. Upon elution with 250 mM imidazole containing buffer, nearly homogeneous LPG2-HIS was obtained with a minor contaminant of ~75 kDa (Fig. 5). In a separate experiment using a comparable strategy of metal affinity chromatographic purification, similar results were obtained with LPG2-HIS again being the predominant band; in this experiment, the only contaminant evident by silver staining migrated at a position corresponding to ~50 kDa instead of ~75 kDa (data not shown). As both preparations showed similar behavior in the tests described below, we thus attribute NST activity exclusively to the purified LPG2-HIS protein.

We estimated that ~1 μg of purified LPG2-HIS was obtained from the starting 500 mg of microsomes. The extremely low amounts of purified protein from limited quantities of Leishmania microsomes precluded an accurate assessment of fold purification. At various stages of purification the LPG2-HIS was quickly frozen in liquid nitrogen and stored at -80 °C.

Reconstitution of GDP-Man Transport Activity—In crude Leishmania Golgi microsomes, translocation of GDP-Man into the lumen was previously demonstrated to be dependent on latency of the vesicles, exhibited an antiport mechanism of transport with GMP, and had an apparent \( K_m \) of 0.3 μM for GDP-Man (7). To demonstrate activity of the purified GDP-Man transporter, the transport activity was reconstituted in phosphatidylcholine liposomes using a freeze-thaw system described by Hirschberg and co-workers (43) and with SM-2 polystyrene beads to remove the GDA solubilizing detergent. Lipids and purified LPG2-HIS proteins were mixed in a ratio of 4:1 (v/v) containing 1 mM GMP and incubated at 25 °C, with several additions of small amounts of the beads to begin removing the detergent. After an hour, excess beads were added to the mixture and incubated at 4 °C overnight. The beads were removed by filtration, and the reconstituted LPG2-HIS liposomes were frozen in liquid nitrogen and thawed at room temperature. The reconstituted vesicles were active through at least three freeze-thaw cycles and did not show significant differences in transport activity among these cycles. The LPG2-HIS

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**Fig. 4.** Detergent survey to solubilize LPG2-HIS in Leishmania microsomes. A, microsomes (1 mg) were prepared from pXG-LPG2cHis6 transfected lpg2Δ/Δ cells and suspended in 1 ml of solubilization buffer containing 1% (w/v) of the indicated detergents. After incubation for 1 h on ice, microsomes were centrifuged at 100,000 × g to separate soluble (S) and pellet (P) fractions. Pellet fractions were resuspended in the same volume as the soluble fraction; 20-μl aliquots of each fraction were subjected to electrophoresis with 12% SDS-polyacrylamide gels. LPG2-HIS was detected with 5000-fold diluted India HisProbe-HRP. The detergents used in this experiment were deoxycholic acid (DOC), GDA, taurodeoxycholic acid (TDA), CHAPS, CHAPSO, MEGA8, MEGA9, cholic acid (CA), Triton X-100 (TX100), and Nonidet P-40 (NP40). Det indicates the buffer without detergent. B, 5 mg of microsomes were suspended in solubilization buffer containing 0, 0.1, 0.2, 0.5, and 1% GDA and 1% deoxycholic acid.
liposomes preloaded with GMP were passed through a BioSpin P-6 column spin column to remove excess amounts of GMP.

Properties of Reconstituted LPG2-HIS Liposomes—As shown in Fig. 6A, GDP-Man uptake into the reconstituted proteoliposomes containing purified LPG2-HIS leveled off after ~2 min of incubation at 28 °C. By comparison, uptake of the nucleotide sugar into intact microsomal preparations of Leishmania was linear for longer times, and kinetic information was typically obtained using transport incubations of 6 min (7). This finding reflects the fact that unlike normal sealed Golgi membranes, transporters reconstituted into vesicles are likely to be randomly oriented, and, thus, the uptake of GDP-Man would be followed by its eventual efflux. Accordingly, based on the data shown in Fig. 6A, 30-s incubations at 28 °C were used in subsequent analyses of transport activity to measure initial rates of influx before intravesicular GDP-Man concentrations would be significant. The reconstituted LPG2 liposomes showed saturable uptake activity (Fig. 6B) with an apparent $K_m$ of 6.6 μM for GDP-Man, calculated by a double-reciprocal plot and similar to the value obtained with intact Leishmania microsomal vesicles. The $V_{max}$ of GDP-Man transport was 20 nmol/mg/min in the LPG2-HIS liposomes compared with the less active $V_{max}$ of 4.2 pmol/mg/min in crude microsomes. Furthermore, the addition of 4 mM 4,4′-diisothiocyanatostilbene-2,2′-disulfonate, a widely used inhibitor of nucleotide sugar transport (44), to the NST assay inhibited uptake of GDP-Man (data not shown).

NSTs are believed to function by an antiport mechanism with the uptake of the nucleotide sugar and the simultaneous exit of the corresponding nucleotide monophosphate. The antiport mechanism for the purified GDP-Man transporter was examined by using reconstituted LPG2-HIS liposomes preloaded with or without 1 mM GMP. LPG2-HIS liposomes reconstituted in the presence of GMP exhibited 3-fold higher initial rates of transport activity than vesicles reconstituted without GMP (3.2 ± 0.3 nmol/min/mg versus 1.1 ± 0.2 nmol/min/mg, respectively). Furthermore, preloading of the LPG2-HIS liposomes with 1 mM UMP or 1 mM GDP did not stimulate GDP-Man uptake. The ~3-fold enhancement of GDP-Man transport in the presence of GMP being preloaded into vesicles is virtually identical with the ~3-fold stimulation reported by Hirschberg and co-workers (23) with the purified rat liver GDP-fucose transporter that was reconstituted in proteoliposomes. Because low levels of transport are observed in the absence of luminal GDP, these results indicate that the antiport mechanism is not a strict requirement of NSTs but rather that the corresponding NMP enhances the translocation process. In a related experiment, the LPG2-HIS liposomes were preloaded with 1 mM GDP and 0.002 units of snake venom phosphodiesterase and then preincubated for 30 min at 37 °C to allow conversion of the luminal GDP to GMP. Consistent with simply preloading the liposomes with GMP, the transport activity in the presence of both GDP and the enzyme was 1.4 nmol/min/mg compared with 0.2 ± 0.1 nmol/min/mg without the phosphodiesterase.

Many enzymes involved in synthesizing glycoconjugates require metal ions as a cofactor. Whereas glycosyltransferases require magnesium and/or manganese for their activities, the metal ion requirements of nucleotide sugar transporters are obscure. We tested the effect of magnesium and manganese on GDP-Man transport activity in the LPG2-HIS-containing liposomes. As shown in Table I, crude microsomes incubated with the divalent cations showed a substantial 4-fold enhancement of “uptake activity” compared with microsomes that were not incubated with the metals. In contrast, GDP-Man uptake activities of reconstituted proteoliposomes were basically unchanged when assayed in the presence or absence of the metal ions in the assay buffer. These results indicate that the GDP-Man transporter does not require Mg$^{2+}$ or Mn$^{2+}$ for its activity. Furthermore, these data were consistent with earlier conclusions that a large degree of so-called uptake activity using crude microsomes is due to the presence of microsomal glycosyltransferases that use GDP-Man as a substrate and Mg$^{2+}$ or Mn$^{2+}$ as cofactors to glycosylate endogenous acceptors.

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**Fig. 5. Purification of LPG2-HIS on metal chelate columns.** Microsomes prepared from pXG-LPG2cHis, transgenic 1pg2−/− cells (4 × 10^8) were solubilized in 20 ml of lysis buffer and purified using a series of metal affinity column chromatographies. 20-μl aliquots of each fraction were subjected to electrophoresis on 12% SDS-polyacrylamide gels and analyzed by silver staining (A) and Western blotting (B) using India HisProbe-HRP. Depicted are crude and unbound fractions (100 μl), TALON wash (2 ml), TALON elution (2 ml), Ni-NTA unbound (4 ml), Ni-NTA wash (1 ml), and Ni-NTA elution (1 ml).
We reported previously that LPG2-containing microsomes transported GDP-arabinose and GDP-fucose in addition to GDP-Man. We tested whether the purified LPG2-HIS-containing liposomes retained this multi-substrate specificity by examining the GDP-sugars as substrates in the transport assay. As shown in Table II, the reconstituted microsomes transported GDP-arabinose and GDP-fucose as well as GDP-Man, confirming that LPG2 is a GDP-sugar transporter. Importantly, no uptake of UDP-[3H]Gal was observed in the LPG2-HIS-containing liposomes preloaded with either 1 mM GMP or 1 mM UMP, consistent with the notion that the transporter is specific for GDP-sugars (data not shown).

**DISCUSSION**

Definitive characterization of NSTs in transport assays involving microsomal preparations is complicated by competing endogenous glycosyltransferases, which also use nucleotide sugars as substrates. Separating glycosylation and the nucleotide sugar transport activities is challenging yet paramount in our fundamental understanding of the cellular steps required for glycosylation. NST characterization can best be accomplished by purification of the NST, but this approach is hampered by an expected low abundance of the transporter. Our overall strategy involved the preparation of an expression construct of the gene for an NST (LPG2) fused with an epitope tag for easy detection purposes. Our goal was to purify the LPG2 protein that comprises the GDP-Man transporting complex in *Leishmania* and find that the general transport characteristics were basically the same as in crude microsomes with some notable differences.

GDP-Man transporter genes have been identified from *Saccharomyces cerevisiae* (2), *Candida* (6), plants (12), and *Leishmania* (7), and the amino acid sequences among the encoded proteins are very similar. Because mammalian cells lack the gene, the GDP-Man transporter may be an attractive target for the development of drugs for pathogenic organisms such as *Candida* and *Leishmania*, and, thus, knowledge of their molecular properties is warranted. The LPG2 GDP-Man NST from *Leishmania* was purified to near homogeneity along with the UDP-GalNAc and GDP-Fuc NSTs from the rat liver Golgi apparatus (22, 23), now constituting a remarkably short list of three. The mammalian NSTs were purified by more conventional protein purification procedures, including ion exchange and hydrophobic affinity chromatographic steps. Purification of the LPG2 transporter was achieved by preparing an expression construct containing the LPG2 gene fused with a poly-His epitope tag and then purifying the expressed protein effectively using metal chelate columns. Because of the extremely low amounts of NSTs, purity of the two rat liver NSTs was monitored by photoaffinity radiolabeling of proteins with [32P]nucleotide triphosphates and demonstrating a single radioactive peptide band of the proper molecular mass, whereas purity of the LPG2 transporter was assessed by silver staining after SDS gel electrophoresis. Another distinction between the *Leishmania* LPG2 and the rat liver transporter purification protocols is the detergents that were effective in their solubilization. Deoxycholic acid-related detergents solubilized the *Leishmania* GDP-Man transporter efficiently. Non-ionic detergents that easily solubilized the rat liver NSTs were ineffective with LPG2-HIS. Importantly, all three purified NSTs were judged to be active following solubilization, purification, and reconstitution as proteoliposomes.

For reconstitution, we attempted to insert purified LPG2 into preformed liposomes following the report of Knol et al. (45) but were unsuccessful in measuring any detectable transport activity. Mixing purified LPG2 with phosphatidylcholine and then adding SM-2 beads directly into the protein-lipid mixture resulted in transport activity. This method requires relatively small amounts of buffer, and it is easy to change the reconsti-
tution conditions. The slow removal of the detergent is crucial in this method. When excess amounts of beads were added at one time, or if the protein-lipid mixture was incubated at 4 °C instead of 25 °C in the first step of proteliposome preparation, no GDP-Man transport activity was measured.

In an earlier report, the *Leishmania* LPG2 gene was demonstrated by heterologous expression in mammalian cells to encode the protein responsible for GDP-Man transport activity (24). Because it seemed unlikely that LPG2 would interact with host proteins given the large evolutionary distance between protzoans and mammals, it was proposed that LPG2 functions autonomously to mediate GDP-Man transport. In this paper, purification of LPG2 and the functional reconstitution of nucleotide sugar transport activity with purified LPG2 in artificial phosphatidylcholine liposomes now provides the experimental evidence to confirm this proposal.

For the most part, the properties obtained with the reconstituted GDP-Man NST were similar to those obtained with crude *Leishmania* microsomes. The *Km* for GDP-Man was 6.6 μM, close to the value of transport activity in microsomes (7) and also comparable with those reported for the other purified NSTs (46). The reconstituted LPG2-HIS liposomes showed stimulation with preloaded GMP, consistent with the expected antiport transport mechanism of the nucleotide sugar uptake and the concomitant exit of the corresponding nucleotide monophosphate. However, lower transport activity was also detected in the LPG2-HIS liposomes reconstituted in the absence of GMP, suggesting that the transporter does not have an absolute requirement for its antiporter ligand. Similar results have been reported for the CMP-sialic acid transporter (47), the GDP-Fuc transporter (23), and the adenosine 3′-phosphate 5′-phosphosulfate (PAPS) transporter (42).

It is relevant to note that the requirement for luminal phosphodiesterase may be less in *Leishmania* because of the unique complement of glycosyltransferases, in particular the mannosylphosphoryltransferases used to synthesize the phosphoglycan Galβ1,4Manα1-PO4 repeat units present in the abundant glycoconjugates LPG and proteophosphoglycan. In mammalian and yeast glycosylation, nucleotide diphosphates are the products after the transfer of the appropriate sugar from NDP-sugars by most glycosyltransferases. This requires a luminal phosphodiesterase to hydrolyze the NDP to the corresponding NMP (25, 27). But in phosphoglycan synthesis in *Leishmania*, the assembly of the Galβ1,4Manα1-PO4 repeat units uses a mannosylphosphoryltransferase that transfers Man-P from lumenally oriented GDP-Man and directly generates GDP as a product (7). Thus, although the GDP2-LPG2-GDP-Man transporter does in fact require GMP to enhance its antiport activities of glycosyltransferase instead of NSTs, because this class of enzymes generally requires magnesium and manganese for their activities.

Purification and characterization of the purified GDP-Man-transporter and other NSTs will be invaluable in furthering our understanding of glycosylation pathways in eukaryotes. This is particularly true in the interrelationship of NSTs with glycosyltransferases.

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