Leishmania mexicana are parasitic protozoa that express a variety of glycoconjugates that play important roles in their biology as well as the storage carbohydrate β-mannan, which is an essential virulence factor for survival of intracellular amastigote forms in the mammalian host. Glucose transporter null mutants, which are viable as insect form promastigotes but not as amastigotes, do not take up glucose and other hexoses but are still able to synthesize these glycoconjugates and β-mannan, although at reduced levels. Synthesis of these carbohydrate-containing macromolecules could be accounted for by incorporation of non-carbohydrate precursors into carbohydrates by gluconeogenesis. However, the significantly reduced level of the virulence factor β-mannan in the glucose transporter null mutants compared with wild-type parasites may contribute to the non-viability of these null mutants in the disease-causing amastigote stage of the life cycle.

Leishmaniae are pathogenic protozoa of the order Kinetoplastida that cause important diseases in humans and other vertebrates (1). There are two principal stages in the parasite life cycle, (i) extracellular flagellated promastigotes that live within the alimentary tract of the sand fly vector and (ii) non-flagellated amastigotes that reside within the acidified phagolysosomal vesicles of vertebrate host macrophages. Both life cycle stages of Leishmania mexicana can be cultivated in vitro (2) facilitating biochemical and genetic studies. Uptake and metabolism of glucose in Leishmania species has been of considerable interest, as this sugar provides a major source of carbon and energy to these parasites (3). In L. mexicana, glucose catabolism is 10–20-fold higher in the promastigotes compared with amastigotes (4). Furthermore, amastigotes transport glucose at a much lower rate than promastigotes and derive metabolic energy primarily from fatty acid oxidation (4, 5). The higher rates of glucose uptake and utilization by promastigotes in relation to amastigotes is consistent with the high concentrations of sugars that promastigotes experience from the plant sap ingested by the sand fly vector (6) compared with the apparently sugar-limited environment that amastigotes experience (7) in the macrophage phagolysosome.

The L. mexicana genome encompasses a cluster of three glucose transporter genes, LmGT1, LmGT2, and LmGT3, which encode closely related isoforms (8). A L. mexicana glucose transporter “knock-out” line (Δlmgt) (9), in which the entire cluster of glucose transporter genes was eliminated by targeted gene replacement, exhibited no detectable glucose transport activity. The Δlmgt promastigotes were able to grow, although at a reduced rate compared with that of wild-type cells. In contrast, Δlmgt cells exhibited dramatically reduced viability inside macrophages compared with wild-type parasites and were unable to grow as axenic amastigotes, demonstrating that glucose transporters are essential for amastigote viability. Furthermore, axenically cultured wild-type amastigotes required glucose for viability (9).

The observation of the essential nature of glucose transporters in amastigotes originally posed a conundrum. Unexpectedly, the glucose carriers were essential in the life cycle stage in which they were least used for energy production. This suggested that glucose and the other hexoses (fructose, mannose, and galactose) that these permeases transport may be building blocks for essential macromolecules in the mammalian stage of the parasite life cycle. Leishmania parasites synthesize a large number of abundant glycoconjugates, many of which are expressed on the parasite membrane or are secreted, that require hexoses for their biosynthesis. These include a major plasma membrane glycoconjugate lipophosphoglycan (LPG) (3) (10), a high molecular weight proteophosphoglycan (PPG) (11), a family of small neutral glycosylinositolphospholipids (GIPLs) (12), the major cell surface glycoprotein, a metalloproteinase designated gp63 (13), and a secreted glycoprotein enzyme designated secreted acid phosphatase (sAP) (14). Several such glycoconjugates have been shown to play important functions in the biology of the parasite. Leishmania parasites also elaborate an intracellular storage carbohydrate, β1–2 mannann, that is an important virulence factor required for viability of the amastigote stage of the life cycle, possibly because it protects against nutrient deprivation and other stresses encountered in the macrophage (15).
Because hexoses are necessary for biosynthesis of these carbohydrate-containing macromolecules, it is possible that genetic ablation of the glucose (hexose) transporters would also prevent their biosynthesis, thus explaining the lethal phenotype of the Δmgt mutation in amastigotes. Indeed, evidence for an essential role of glycoconjugates and β-mannan emerged from previous studies on the gene encoding the enzyme GDP-mannose pyrophosphorylase (15, 16). Consequently, we have studied the ability of the Δmgt null mutants to synthesize these carbohydrate-containing molecules. We have determined that Δmgt parasites are still able to synthesize glycoconjugates and β-mannan using gluconeogenesis to generate hexose-phosphate precursors. However, the reduced level of the essential virulence factor β-mannan in Δmgt parasites may contribute to the inability of the glucose transporter null mutants to survive as amastigotes, despite the operation of gluconeogenesis in both promastigotes and amastigotes.

EXPERIMENTAL PROCEDURES

Parasite Cell Lines and Culture Conditions—Promastigotes of L. mexicana wild-type and null mutant cell lines were cultured at 26°C in RPMI 1640 medium, pH 7.2, supplemented with 10% heat-inactivated fetal bovine serum (iFBS). The Δlpq1 and Δgdpmp null mutants were donated by Dr. Steven Beverley (Washington University, St. Louis, MO) and Dr. Thomas Ilg (Max-Planck-Institut für Biologie, Tübingen, Germany). Axenic amastigotes of wild-type parasites were obtained by incubating stationary phase promastigotes at 32.5°C in Dulbecco's modified Eagle's medium—Leishmania (17), pH 5.5, supplemented with 20% iFBS and 10 mM glucose. Continuous amastigote cultures were maintained by periodic dilution in Dulbecco's modified Eagle's medium—Leishmania.

Isolation of LPG, mPPG, gp63, and sAP—To detect LPG, parasites were lysed in 0.1% Triton X-100 and treated with DNase I for 1 h at 37°C (18) and then with proteinase K for 1 h at 55°C to eliminate proteins containing carbohydrate epitopes that are also recognized by the mAb CA7AE (19). In a complementary approach, LPG was purified from delipidated cells as described below. For detection of mPPG, total cell lysates were obtained using 0.1% Triton X-100 as described previously (20). To detect gp63, amphilastic membrane proteins were extracted by Triton X-114 phase separation as detailed previously (21). The sAP and soluble gp63 (gp63 that is shed from the cell surface) were obtained as described previously (22) by washing the promastigotes twice in phosphate-buffered saline, pH 7.4, followed by resuspension in serum-free medium. After 24 h, the cells were removed by centrifugation and the supernatants were filtered and concentrated with a Centricon filter unit (Millipore). For each sample, the amount of protein was determined using the detergent-compatible DC protein assay (Bio-Rad).

Evaluation of N-Glycosylation in gp63 and sAP—To evaluate N-glycosylation of gp63 and sAP, heat-denatured Triton X-114 preparations or soluble fractions (see above) from wild-type and the null mutant cell lines were treated with PNGase F (BioLabs) (which specifically cleaves N-linked oligosaccharides) in a phosphate buffer as suggested by the manufacturer. The loss of N-linked glycans present in the proteins was detected as a reduction in the molecular mass by SDS-PAGE/immunoblotting.

Detection of LPG, mPPG, gp63, and sAP by Western Blot—Equal amounts of protein (for cell lysates) or equivalent numbers of cells (for delipidated cells) were separated by SDS-PAGE, transferred to nitrocellulose or polyvinylidene fluoride membrane, and analyzed by immunoblotting. For immunodetection of LPG and mPPG, the mAb CA7AE ascites fluid (CLP003A, Cedarlane Laboratories), which reacts with the disaccharide repeat PO4-6Galβ1,4Manα1 (25) was used as the primary antibody, as this epitope is shared between both glycoconjugates (11, 25), and mPPG is detected in the stacking gel. The presence of gp63 and sAP in Δmgt was determined by Western blot of the Triton X-114 extract (membrane-bound gp63) or cell supernatant (secreted gp63 and sAP), using a polyclonal serum against Leishmania infantum gp63 (21) (donated by Dr. Mary Wilson), which cross-reacts with L. mexicana gp63, and the hybridoma supernatant of mAb LT8.2 against sAP (25) (donated by Drs. Stephen Beverley and Thomas Ilg), respectively. Secondary antibodies coupled to horseradish peroxidase were subsequently applied as described by the commercial provider (Pierce). Blots were developed using the Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences) and Kodak BioMax MR film.

Extraction of GIPLs, LPG, and β-Mannan and Detection by High Performance Thin Layer Chromatography (HPTLC)—GIPLs and β-mannan were purified by extraction of promastigotes with chloroform/methanol/water ((C/M/W) 1:2:0.8, v/v) as described previously (15) with modifications. The cell pellet was extracted by vortexing with glass beads for 10 min instead of sonication for 2 h. Insoluble material was removed by centrifugation (2,000 × g for 10 min) and re-extracted with C/M/W, twice. The pooled C/M/W fractions were dried and partitioned by biphasic separation in 1-butanol/water (1:1 v/v). Phospholipids, such as GIPLs, were recovered in the upper organic phase, dried, resuspended in 40% 1-propanol for HPTLC analysis. The lower aqueous phase containing β-mannan was extracted twice with water-saturated butanol, desalted using AG501-X8 resin (Bio-Rad), and dried before separating by HPTLC. LPG was extracted from delipidated pellets using 1-butanol-saturated water followed by octyl-Sepharose chromatography, as described in detail (26). The different carbohydrate fractions were analyzed on glass-backed silica 60 plates (Merck) by HPTLC and selectively stained by spraying with orcinol/H2SO4, and heating to 100°C for 10 min. For each cell line, extracts prepared from the same number of cells were applied to the HPTLC plates and developed as described previously (12, 15, 27). LPG was also detected on Western blot as described above.

Quantification of Glycoconjugates—To determine the expression levels in wild-type and Δmgt cell lines, β-mannan was quantified using the phenol-sulfuric acid method, which detects the carbohydrate present in the samples extracted from the same number of wild-type and Δmgt cells (28). The sugar concentration of each sample was obtained using a standard curve for D-mannose. To quantify LPG and glycoconjugates containing the disaccharide-phosphate epitope recognized by the mAb CA7AE, a standard indirect enzyme-linked immu-
Labeled with sugars by incubating the cells with 10 μCi/ml D-[2-3H]mannose (Moravek Biochemicals), D-[6-3H]glucose (PerkinElmer Life Sciences), and D-[6-3H]galactose (American Radiolabeled Chemicals, Inc.) in sugar-free RPMI 1640 medium supplemented with 10% iFBS. For gluconeogenesis studies, incubations of wild-type and Δlmgt promastigotes and wild-type axenic amastigotes were performed in the respective medium (see above) supplemented with dialyzed iFBS and containing 0.5 mM (10 μCi/ml) of the following radiolabeled precursors: L-[14C(U)]alanine (128 mCi/mmol; Moravek Biochemicals), [1-14C]acetic acid (56 mCi/mmol; American Radiolabeled Chemicals, Inc.), [1,3,14C]glycerol (55 mCi/mmol; American Radiolabeled Chemicals, Inc.), and L-[14C(U)]aspartic acid (171 mCi/mmol; Moravek Biochemicals). After incubation for 18 h, the cells were washed twice in phosphate-buffered saline, pH 7.4, and used to purify LPG (promastigotes) and β-mannan (promastigotes and amastigotes) as described above. The phospholipid fraction was also recovered from each sample and used as the control for the metabolic labeling. Beta-mannan samples were separated on HPTLC plates and sprayed with EN3HANCE (DuPont) followed by fluorography. LPG was subjected to SDS-PAGE and detected by immersion of the polycrylamide gel in Amplify (Amersham Biosciences), followed by drying and fluorography. In addition, LPG was subjected to mild acid hydrolysis using 0.02N HCl at 100 °C for 5 min (30), to release the carbohydrate domain of the purified LPG. After hydrolysis, LPG was again passed through the octyl-Sepharose column. In this way, the label incorporated in the lipidic moiety of LPG remained bound to the column and the octyl-Sepharose column. In this way, the label incorporated in the lipidic moiety of LPG remained bound to the column and was separated from the carbohydrate portion of LPG, which did not bind to the column. The radioactivity present in the carbohydrate moiety of each sample (unbound fraction) was counted on a liquid scintillation counter.

Fluorescence Microscopy—Wild-type Δlmgt, Δpgl1, and Δgdppm promastigotes were fixed and stained for immunofluorescence as described previously (31). The mAb CA7AE was used at a dilution of 1:500. Alexa Fluor 488 goat anti-mouse IgM (μ chain) was used as a secondary antibody at a dilution of 1:1500 following the manufacturer’s instructions (Molecular Probes). Negative controls were only incubated with the secondary antibody. Cells were washed in phosphate-buffered saline, pH 7.4, followed by embedding in Fluoromount G (Southern Biotechnology Associates, Inc.) and inspection by fluorescence microscopy using a Nikon Microphot-FX phase contrast microscope. Images were captured using MagnaFire software (Optronics, Goleta, CA).

BLAST Searches—The Leishmania major genome sequence (32) was searched with the amino acid sequences of the human and Saccharomyces cerevisiae pyruvate carboxylase (EC 6.4.1.1), phosphoenolpyruvate carboxylase or PEPCK (EC 4.1.1.49), fructose-1,6-bisphosphatase or FBPase (EC 3.1.3.11), glucone-6-phosphatase (EC 3.1.3.9) using the BLAST server at GenDB and the default settings. For pyruvate, phosphate dikinase or PPDK (EC 2.7.9.1), the amino acid sequence and glucose-6-phosphatase (EC 3.1.3.9) using the BLAST server at GenDB and the default settings. For pyruvate, phosphate dikinase or PPDK (EC 2.7.9.1), the amino acid sequence

RESULTS

Effect of Glucose Transporter Null Mutation on the Biosynthesis of Glycoconjugates—Because Δlmgt cells are unable to transport hexoses 4 (9), we investigated whether the inability of the Δlmgt mutant to proliferate as amastigotes may be due to the lack of expression of glycoconjugates by determining these glycoconjugates in the Leishmania major genome sequence (32) was searched with the amino acid sequences of the human and Saccharomyces cerevisiae pyruvate carboxylase (EC 6.4.1.1), phosphoenolpyruvate carboxylase or PEPCK (EC 4.1.1.49), fructose-1,6-bisphosphatase or FBPase (EC 3.1.3.11), and glucose-6-phosphatase (EC 3.1.3.9) using the BLAST server at GenDB and the default settings. For pyruvate, phosphate dikinase or PPDK (EC 2.7.9.1), the amino acid sequence of the Trypanosoma brucei enzyme was used.

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or β-mannan (33). LPG and mPPG were present in Δlmgt promastigotes, as judged by immunoblots of delipidated cells (Fig. 1A, LPG (bottom)) or cell lysates (Fig. 1A, mPPG (top)); Fig. 1B) probed with the mAb CA7AE. Similar results were obtained when GIPPs isolated from wild-type and Δlmgt promastigotes were analyzed by HPTLC. Δlmgt promastigotes synthesize detectable levels of the polar GIPPs, iM3 and iM4, and the apolar GIPPL iM2 (Fig. 1C). As was expected, no LPG was detected in the Δlpg1 and Δgdpmp mutants (Fig. 1A). In contrast, mPPG and GIPPLs were detected in the Δlpg1 mutant but not in the Δgdpmp mutant (Fig. 1). The presence of the glycoproteins gp63 and sAP in Δlmgt were confirmed by immunoblots of the Triton X-114 extraction (surface gp63, data not shown) or cell supernatant.

A similar reduction in the molecular weight of sAP and gp63 was observed in both wild-type and Δlmgt samples after digestion with PNGase F, indicating that the Δlmgt null mutant can properly N-glycosylate glycoproteins. These results were supported further by immunolocalization studies using the mAb CA7AE ascites fluid, demonstrating the presence of disaccharide-phosphate-containing glycoconjugates on the surface of Δlmgt cells (Fig. 2).

To quantitate the level of glycoconjugates containing the PO4-6Galβ1,4Man epitope between wild-type and Δlmgt cells, we carried out a standard indirect enzyme-linked immunosorbent assay using the whole parasite cell as described previously (29). Our results indicated that the epitope recognized by the mAb CA7AE is slightly reduced in logarithmic phase Δlmgt promastigotes (1.55 ± 0.35-fold (n = 10)) compared with wild-type promastigotes. Although Δlmgt promastigotes are smaller in volume than wild-type promastigotes, the modest reduction in disaccharide-phosphate repeat levels was not due to size, because the value reported above was normalized to account for the 1.5-fold volume difference between the two strains.2 Summarizing, our results indicated that glycoconjugates continue to be made by Δlmgt cells even in the absence of hexose uptake, although at slightly reduced levels.

Expression of β-Mannan by Δlmgt Promastigotes—We also tested whether Δlmgt promastigotes are able to synthesize β-mannan, because failure to make sufficient β-mannan (15) could explain why the glucose transporter null mutant does not survive as amastigotes. The results (Fig. 3A) showed that β-mannan was synthesized by Δlmgt promastigotes but apparently at lower levels than in wild-type parasites. To confirm this observation, we compared the levels of β-mannan between the same number of wild-type and Δlmgt late logarithmic phase cell lines. These experiments indicated that β-mannan was 3.7 ± 0.7-fold (n = 5) less abundant in Δlmgt than in wild-type promastigotes (Fig. 3C). Similar results were obtained using cells in the stationary phase of growth (data not shown). Moreover, wild-type cells growing without glucose (24-h sugar starvation) had similar levels of β-mannan compared with Δlmgt cells (Fig. 3B); in contrast, production of

(Fig. 1D, secreted gp63 and sAP).
β-mannan did not change when Δlmgt cells grew in the presence or absence of glucose.

Δlmgt Promastigotes Do Not Use External Sources of Sugar for Glycoconjugate Biosynthesis—To confirm that the null mutant was not using external sugar sources for biosynthesis of glycoconjugates and β-mannan, we metabolically labeled wild-type and Δlmgt promastigotes with [3H]hexoses and monitored the incorporation of label into β-mannan and LPG. The results showed that, although [3H]mannose was robustly incorporated into β-mannan, no such incorporation could be detected in Δlmgt promastigotes. Similar results were obtained with [3H]glucose (Fig. 4, left) and [3H]galactose (data not shown), confirming the inability of Δlmgt promastigotes to use external sugar sources. Thus, the presence of sugars in the glycoconjugates synthesized by Δlmgt parasites cannot be explained by hexose acquisition during long-term culture simply by diffusion across the plasma membrane, vesicular transport, or some other processes that might result in low uptake or in the absence of hexosyl carriers. Consequently, glycoconjugates made in Δlmgt parasites must derive hexoses from a biosynthetic pathway rather than from the external medium.

Gluconeogenesis Supplies Sugars for Glycoconjugate Biosynthesis in Δlmgt Promastigotes—The most likely way that the Δlmgt parasites could obtain sugars for synthesis of carbohydrate-containing molecules would be to synthesize them de novo by gluconeogenesis, the pathway whereby lactate, pyruvate, acetate, glycerol, and certain amino acids are converted to glucose (34). We studied whether gluconeogenesis functions in Δlmgt promastigotes to provide precursors for glycoconjugate biosynthesis by incubating wild-type and Δlmgt promastigotes with radiolabeled glucogenic precursors in the presence of 10 mM glucose, a condition that prevents gluconeogenesis from occurring in wild-type promastigotes where glycolysis is active. As a positive control, we also incubated wild-type cells in sugar-deficient medium, where gluconeogenesis should be activated. Fig. 5A shows that label from [14C]alanine was readily incorporated into β-mannan by Δlmgt promastigotes grown in the presence of 10 mM glucose. In contrast and as predicted, wild-type parasites did not significantly incorporate radiolabel from this substrate into the β-mannan backbone. However, removal of glucose from the culture medium of wild-type promastigotes allowed incorporation of label from [14C]alanine into β-mannan (Fig. 5B).

Similar results were obtained when LPG purified from Δlmgt and wild-type promastigotes was analyzed (Fig. 5C). In principle, the label from this precursor might be incorporated into both the lipidic and the carbohydrate moiety of LPG. Thus, we confirmed the incorporation of label from [14C]alanine into the carbohydrate moiety of LPG purified from Δlmgt cells, but not from wild-type cells, by purifying the carbohydrate component (Fig. 5D). Radiolabel incorporation into the phospholipid fractions of wild-type and Δlmgt parasites was similar (e.g. $-3.9 \times 10^8$ versus $-3.2 \times 10^8$ counts/min/3.2 x 10^6 cells, respectively), confirming that wild-type cells employed here were metabolically active. Similar results were obtained using [14C]aspartic acid, [14C]acetate, and [14C]glycerol (Fig. 5A and data not shown).

Overall, our results demonstrated that wild-type promastigotes can activate gluconeogenesis under conditions of glucose starvation, and Δlmgt parasites, which are unable to take up hexoses, up-regulated gluconeogenesis to obtain sugars necessary for glycoconjugate and β-mannan biosynthesis, even in the presence of extracellular glucose.

Gluconeogenesis Operates in Wild-type Axenic Amastigotes—We also investigated whether gluconeogenesis operates in amastigotes. Because the Δlmgt null mutant does not grow as amastigotes (9), we probed the capacity of wild-type axenic amastigotes (2) starved for glucose to carry out gluconeogenesis by incubating the cells with the glucogenic precursors, both in the absence or in the presence of 10 mM glucose. Radiolabel incorporation into the phospholipid fractions of amastigotes was similar or higher in cells incubated without sugar (data not shown), confirming that glucose-starved amastigotes were metabolically active for the time period used in these experiments. Our results demonstrated that amastigotes, similar to promastigotes, were able to incorporate label from the four substrates assayed into β-mannan (Fig. 6). We also compared the relative incorporation of radiolabeled precursors into the β-mannan fraction in both promastigotes and amastigotes incubated in the presence or absence of sugar in the medium and normalized the results to protein content. Both life cycle stages incorporated similar levels of radiolabeled precursors under conditions of sugar starvation (Table 1; sugar depleted), suggesting that both amastigotes and promastigotes have similar capacities to carry out gluconeogenesis. However, one striking difference between promastigotes and amastigotes is that the latter exhibited significant incorporation of radiolabel into β-mannan in the presence of glucose in the medium (Fig. 6), suggesting that gluconeogenesis is less regulated by external glucose in amastigotes, compared with the promastigotes. This is likely possible because both glucose transport (5) and the glycolytic pathway (4, 35) are down-regulated in amastigotes,
that would shut down gluconeogenesis.

**DISCUSSION**

**Factors Affecting Viability of Δlmgt Parasites**—Expression of complex and unique carbohydrate-containing molecules appears to be crucial for the survival and development of *Leishmania* parasites in the sand fly vector and the mammalian host (15, 16, 36). One way for these parasites to obtain sugars required for glycoconjugate biosynthesis is via uptake from the external medium. It has been demonstrated previously that wild-type promastigotes incorporate label from radiolabeled sugars, such as D-glucose, D-mannose, D-galactose, and D-glucosamine, into various glycoconjugates (12, 19, 33, 37) and β-mannan (15, 38). Thus, it is likely that a major role of sugar uptake is to provide glucose and other hexoses for biosynthesis of glycoconjugates and β-mannan. The genetic ablation of the glucose transporters in the parasite could affect the biosynthesis of these molecules and thus might affect parasite development and survival. Because the Δlmgt null mutant is deficient in transport of four hexoses used as building blocks for the biosynthesis of glycoconjugates and β-mannan, we studied whether Δlmgt promastigotes were able to synthesize these macromolecules. Notably, we found that Δlmgt promastigotes were able to make all glycoconjugates studied. Furthermore, the modest reduction of the glycoconjugate expression levels observed is unlikely to explain by itself why this null mutant lost the capacity to survive as amastigotes (16). On the other hand, Δlmgt parasites expressed the storage carbohydrate β-mannan at a ~3.7-fold lower level than wild-type cells. The importance of β-mannan for survival of amastigotes of *L. mexicana* parasites has been established (15). Furthermore, wild-type amastigotes increase robustly the expression of β-mannan compared with promastigotes (15), suggesting that the deficit in the ability to make β-mannan observed in Δlmgt promastigotes will be amplified in the amastigote stage.

**Gluconeogenesis as a Source of Carbohydrates**—In many organisms, gluconeogenesis meets the requirements of the cell for glucose when carbohydrate is not available in sufficient amounts from the extracellular environment (34), and it has been suggested that gluconeogenesis may operate in *Leishmania* species (39). Results reported here confirm that when *Leishmania* parasites are unable to obtain sufficient hexoses from the external medium because of starvation for sugars or genetic ablation of the glucose transporter genes, they activate the gluconeogenic pathway to synthesize sugars. Thus, classical gluconeogenic metabolites such as alanine, aspartate, and glycerol can serve as precursors for the synthesis of complex carbohydrates such as β-mannan or glycoconjugates such as LPG. The ability of these glucogenic precursors to promote synthesis of polysaccharides argues that a *bona fide* gluconeogenic pathway exists in *Leishmania* parasites. Furthermore, a recent study (40) reveals that this pathway is essential for the survival of amastigotes of *L. major*, because a null mutant in the gene encoding the gluconeogenic enzyme FBPase is not viable in the amastigote stage of the life cycle.

In *L. mexicana* parasites, the enzymatic activities of the key
enzymes in gluconeogenesis PEPCK and FBPase have been detected (41, 42), and PEPCK has been purified and partially characterized (43); however, reports concerning pyruvate carboxylase activity are contradictory (41, 42, 44, 45). Searching the genome data base from L. major using the BLAST algorithm, we found homologous genes for only FBPase (LmjF04.1160) and PEPCK (LmjF27.1810 and LmjF27.1805), the same enzymes whose activities have been clearly detected experimentally, but not for pyruvate carboxylase or glucose-6-phosphatase. However, one potential shortcoming of any sequence similarity-based search is that proteins with highly divergent sequences could be missed. Nonetheless, the absence of glucose-6-phosphatase in Leishmania parasites is not unexpected, because unicellular organisms do not need to generate and export free glucose. In contrast, the absence of pyruvate carboxylase could, in principle, present an obstacle for incorporation of various precursors, such as pyruvate, alanine, and lactate, into carbohydrates. However, the existence of the enzyme PPDK, which catalyzes the reversible production of phosphoenolpyruvate directly from pyruvate in many plants, bacteria, and fungi (46) would circumvent the need for pyruvate carboxylase (Fig. 7). The gene encoding PPDK has been isolated and molecularly characterized in T. brucei, a parasite closely related to Leishmania species, and the recombinant protein has been expressed and shown to exhibit PPDK enzymatic activity (47). Furthermore, we were able to detect a homologous gene (LmjF11.1000) for this enzyme in the L. major genome data base using the T. brucei PPDK amino acid sequence and the BLAST algorithm.

Amino acids present in the phagolysosome could serve as gluconeogenic precursors to support the synthesis of glycoconjugates and β-mannan. Thus, alanine can enter to the gluconeogenic pathway via pyruvate using PDPK, whereas aspartic acid can enter via oxaloacetate using PEPCK. Similarly, glycerol generated in the macrophage lysosomes from the degradation of triacylglycerols could serve as a gluconic precursor for amastigotes. Because Leishmania parasites express aquaglyceroporins (48) and putative glycerol transporters (49) as well as the enzymes glycerol kinase and glycerol 3-phosphate dehydrogenase (35, 41, 50), they can import glycerol and convert it to dihydroxyacetone phosphate for entry into the gluconeogenic pathway. On the other hand, amastigotes up-regulate β-oxidation of fatty acids, a pathway that produces two carbon substrates, such as acetyl-CoA, to satisfy energy requirements (35). In some organisms, including various plants and bacteria, two carbon substrates are also used as carbon sources to generate sugars (34). The L. mexicana parasites incorporate label from [14C]acetate into β-mannan (Figs. 5 and 6). However, it is not clear whether acetate acts as a gluconic precursor that can generate net synthesis of carbohydrates in these parasites.

The pathway we propose for gluconeogenesis in Leishmania species is shown in Fig. 7. This pathway is supported by the existence of genes encoding the central enzymes of gluconeogenesis (Fig. 7, boxes) and the detection of the corresponding enzyme activities in L. mexicana or related kinetoplastid para-
sites. Furthermore, this pathway explains the ability of alanine, aspartate, and glycerol to serve as precursors for synthesis of β-mannan.

**Requirement for Glucose Transporters in Amastigotes**—An essential role for glucose transporters in amastigotes is supported by the observation that Δlgmt promastigotes enter macrophages as efficiently as wild-type parasites and transform into amastigotes; however, the transformed Δlgmt amastigotes are unable to replicate inside the macrophage phagolysosome and die over the course of several days, whereas the wild-type amastigotes replicate (9). Furthermore, amastigotes require exogenous glucose, because wild-type axenic amastigotes die if they are transferred to growth medium lacking glucose (9). Despite the ability of Δlgmt promastigotes to synthesize glyco-conjugates at levels similar to wild-type parasites, the significantly reduced level of the essential virulence factor β-mannan in the null mutant parasites likely contributes to their inability to survive as amastigotes, a life cycle stage that is highly dependent upon reserves of this complex carbohydrate (15). There are probably additional factors that contribute to the non-viability of Δlgmt amastigotes, including our recent observation that Δlgmt promastigotes are significantly more susceptible to killing by reactive oxygen species such as H₂O₂ that they encounter inside the macrophage (51), possibly as a result of reduced generation of NADPH (52) via glucose metabolism through the pentose phosphate pathway (53).

A recent publication by Naderer et al. (40) has demonstrated that a null mutant (Δlgmt) in the gene encoding the gluconeogenic enzyme FBPase in *L. major* is also unable to survive as amastigotes, and in this regard, has a phenotype quite similar to that of the glucose transporter null mutant. Together, these results underscore the importance of carbohydrates in amastigotes and imply that the intracellular stage of the life cycle requires both glucose transporters and gluconeogenesis for viability.

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