Phosphomannose isomerase (PMI) catalyzes the reversible interconversion of fructose 6-phosphate and mannose 6-phosphate, which is the first step in the biosynthesis of activated mannose donors required for the biosynthesis of various glycoconjugates. Leishmania species synthesize copious amounts of mannose-containing glycolipids and glycoproteins, which are involved in virulence of these parasitic protozoa. To investigate the role of PMI for parasite glycoconjugate synthesis, we have cloned the PMI gene (lmexpmi) from Leishmania mexicana, generated gene deletion mutants (Δlmexpmi), and analyzed their phenotype. Δlmexpmi mutants lack completely the high PMI activity found in wild type parasites, but are, in contrast to fungi, able to grow in media deficient for free mannose. The mutants are unable to synthesize phosphoglycan repeats [6-Galβ1-4Manα1-PO4] and mannose-containing glycoinositolphospholipids, and the surface expression of the glycosylphosphatidylinositol-anchored dominant surface glycoprotein leishmanolysin is strongly decreased, unless the parasite growth medium is supplemented with mannose. The Δlmexpmi mutant is attenuated in infections of macrophages in vitro and of mice, suggesting that PMI may be a target for anti-Leishmania drug development. L. mexicana Δlmexpmi provides the first conditional mannose-controlled system for parasite glycoconjugate assembly with potential applications for the investigation of their biosynthesis, intracellular sorting, and function.

Leishmania are protozoan parasites and the causative agents of a spectrum of animal and human diseases. Their life cycle includes flagellated promastigote stages that reside in the midgut lumen of the sandfly vector and a nonmotile amastigote stage that lives within the mammalian macrophage, where it colonizes the phagolysosomal compartment (1). Leishmania species synthesize large amounts of glycoconjugates that include the unusual glycoinositolphospholipids (GIPLs), the con-
Phosphomannose Isomerase of Leishmania mexicana

Materials and Methods

Parasite Culture and Experimental Infections of Mice and Peritoneal Macrophages—Promastigotes of the L. mexicana wild type (WT) strain MNYC/BZ/62/M379 and derived mutants were grown at 27 °C in defined medium 79 (SDM) supplemented with 4% heat-inactivated fetal calf serum (iFCS) as described previously (18). Infection of mice with 10⁷ stationary phase promastigotes and infection of mouse peritoneal macrophages were performed as outlined earlier (19). Growth curves of L. mexicana WT and mutants were obtained by seeding SDM/iFCS containing 10–50 μg/ml G418 (Roche Molecular Biochemicals). Alternatively, the lmxexpmi gene was expressed under the control of the rRNA promoter, which is known to lead to high level expression not only in promastigotes but also in amastigotes (24). For the construction of an integration vector, pFW31 (25) was first linearized by digestion with AgeI. The purified DNA fragment (9347 bp) was then subjected to partial digestion with AflIII generating digestion products with lengths of 7769, 6720, 2627, 1578, and 1049 bp. The largest fragment lacking the lmexpmi open reading frame, but preserving the following spliced leader addition and polyadenylation sites, was then ligated with the annealed primers GG(A/G/C/T)AC(A/G/C/T)CA(C/T)CC and GCCAT(C/T)TC(A/G/C/T)GG-CA with annealing temperatures of 60 °C. The ligated DNA fragment was inserted into the BamHI restriction site of pUC18 and the resulting plasmid was named pUC18 + lmexpmi. The plasmid was then linearized with NotI and KO1 (CTTAAGTGGATCCCCGAGTTTCCTTCAACATTG) and KO2 (CTTAAGTGGATCCCCGAGTTTCCTCAAATCT) generating plasmid-containing Km resistance cassette, a lmxexpmi-containing gene replacement fragment that was then ligated into the SpeI/BamHI restriction sites of pUC18 + lmexpmi and transformed into E. coli DH1 competent cells (19). The ligated plasmid was then isolated and used in conjugation experiments with the wild-type strain L. mexicana Ns15 (26) as described by the manufacturer (Qiagen). Rabbits were immunized with 200 μg of purified recombinant protein, which was dissolved in PBS 0.15 M NaCl, 1% NaHCO₃, pH 7.2, and emulsified with 50% (v/v) incomplete Freund’s adjuvant for primary immunizations and with 50% (v/v) complete Freund’s adjuvant for all subsequent booster immunizations. Serum was obtained 10–14 days after each booster immunization.

Analytical Procedures—Production of SDS-cell lysates, discontinuous SDS-PAGE, immunoblotting using the monoclonal antibodies (mAbs) LT6 and 1.7.25 (directed against P0, 60 kDa, 1–4Man₁, β = 1→6Man₁, β = 1→6Man₂, β = 1→6Man₃, β = 1→6Man₄, β = 1→6Man₅, and Man₁–2, 4Gal and Man₁–2Gal and Man₁–3Gal), and P0-reactive antigenic determinants on affinity-purified rabbit anti-L. mexicana SAP antibodies (26) and affinity-purified rabbit anti-L. mexicana PMI antibodies, as well as acid phosphatase enzyme assays (27) were performed as described earlier (19). Total lipids from washed L. mexicana promastigotes were obtained by two extractions with chloroform/methanol/water (4:8:3, v/v). High performance thin layer chromatography (HPTLC, silica gel 60, Merck, Darmstadt, Germany).

Cloning of the L. mexicana lmexpmi Gene, Generation of Gene Knock-out and Gene Addback Mutants, Heterologous Expression of PMI, and Generation of Antibodies—DNA techniques were performed as described previously (20). A 300-bp fragment of the lmxexpmi gene was obtained from L. major LRC-L137/V121 genomic DNA by polymerase chain reaction (PCR) using the degenerate primers TT(A/G)TGGATG-CA(A/G/C/T)CA(C/T)CC and GCCAT(C/T)TC(A/G/C/T)GG-CA with annealing temperature of 60 °C. The PCR product was then subcloned into pCR2.1 (Invitrogen) and sequenced. The digoxigenin-labeled PCR product was used to screen a λ-Dash II library (21) derived from genomic L. mexicana DNA. Positive clones were subcloned into pBSK + (Stratagene) or pGEM-5z (Promega) and sequenced on both strands by the dideoxy chain termination method using an ALFExpress automated sequencer (Amersham Pharmacia Biotech) as described earlier (20). The open reading frame corresponding to lmexpmi was identified by homology to known PMI genes in the data base and by determination of the spliced leader site (20). Double-targeted gene replacement was performed by PCR amplification of the 5′-untranslated region (5′-UTR) of lmexpmi using the primers K01 (AAAGGCGCAATGATCCTTGGTGTC- TC) and K02 (AGTACTGATGTCATGCTGCGTC) as described previously (18). Selection in 96-well microtiter plates and analysis of positive clones were performed as outlined earlier (19). Lmxexpmi 5′-UTR- and open reading frame DNA probes were generated by PCR using a DIG-labeling kit (Roche Molecular Biochemicals) using the primer pairs AGAGGGGAAATGGTGAGGTCGATAACATCCCTCGTCA and TTACACTTGGCAGACCCCTCGAAGGTTTGGCCAGAAGTCG, respectively. The gene addback and heterologous expression studies, the open reading frame of lmexpmi was amplified from a L. major genomic DNA fragment, ligated into pBSK +, and sequenced. Positive clones were subcloned into pBluescript II (Stratagene) or pGEM-5z (Promega) and sequenced on both strands by the dideoxy chain termination method using an ALFExpress automated sequencer (Amersham Pharmacia Biotech) as described earlier (20).

Glycoprotein degradation?

FIG. 1. Putative pathways of mannose 6-phosphate and glycoconjugate biosynthesis in L. mexicana. Glc-T, glucose transporter; Man-T, mannose transporter; HK, hexokinase; PGI, phosphoglucone isomerase; PMI, phosphomannose isomerase. Glc-6-P, glucose 6-phosphate; Fru-6-P, fructose 6-phosphate; Man-6-P, mannose 6-phosphate. The indication of GDP-Man and Dol-P-Man as Man donors for Man-T.

Glycosyltransferases and Glycosylation of Polysaccharides—Glycosyltransferases and glycosylation of polysaccharides were performed as described earlier (20). O-linked glycosylation experiments were performed as described previously (20). Addition of the lmxexpmi gene was determined by homology to known PMI genes in the data base and by determination of the spliced leader site (20). Double-targeted gene replacement was performed by PCR amplification of the 5′-untranslated region (5′-UTR) of lmexpmi using the primers K01 (AAAGGCGCAATGATCCTTGGTGTC- TC) and K02 (AGTACTGATGTCATGCTGCGTC) as described previously (18). Selection in 96-well microtiter plates and analysis of positive clones were performed as outlined earlier (19). Lmxexpmi 5′-UTR- and open reading frame DNA probes were generated by PCR using a DIG-labeling kit (Roche Molecular Biochemicals) using the primer pairs AGAGGGGAAATGGTGAGGTCGATAACATCCCTCGTCA and TTACACTTGGCAGACCCCTCGAAGGTTTGGCCAGAAGTCG, respectively. The gene addback and heterologous expression studies, the open reading frame of lmexpmi was amplified from a L. major genomic DNA fragment, ligated into pBSK +, and sequenced. Positive clones were subcloned into pBluescript II (Stratagene) or pGEM-5z (Promega) and sequenced on both strands by the dideoxy chain termination method using an ALFExpress automated sequencer (Amersham Pharmacia Biotech) as described earlier (20).

Glycoprotein degradation?
Immunofluorescence microscopy and FACs of Leishmania Promastigotes and Infected Macrophages—Immunofluorescence microscopy and fluorescence-activated cell sorting (FACS) studies on Leishmania promastigotes and infected macrophages were performed as described and fluorescence-activated cell sorting (FACS) studies on Leishmania promastigotes and Infected Macrophages—were estimated according to Peterson (29). The predicted involvement of Zn$^{2+}$ in the catalytic activity of L. mexicana PMI was corroborated by its complete inhibition by 5 mM o-phenanthroline (data not shown). Southern blots of L. mexicana genomic DNA indicated the presence of a single gene copy (Fig. 3B and data not shown). Gene replacement cassettes containing the resistance markers pheleo and hyg were constructed, and two rounds of targeted gene replacement were performed (Fig. 3A). Plating of transfected cells on SDM medium with or without additional Man (0.5 mM) yielded about 10$^7$ cells/ml overnight at 27 °C. Plating of transfected cells on SDM medium with or without additional Man (0.5 mM) yielded about 10$^7$ cells/ml overnight at 27 °C. Plating of transfected cells on SDM medium with or without additional Man (0.5 mM) yielded about 10$^7$ cells/ml overnight at 27 °C.
Phosphomannose Isomerase of Leishmania mexicana 6569

FIG. 3. Targeted gene replacement of the *lmexpmi* alleles. A, restriction map of the *lmexpmi* locus. The resistance genes *phleo* and *hyg* and the primer binding sites (KO1–4) for the construction of gene deletion cassettes are indicated. B, Southern blot analysis of *PstI* restriction enzyme-digested chromosomal DNA (5 µg) from *L. mexicana* wild type (lanes 1) and a *Δlmexpmi* mutant (lanes 2). DNA was separated on an ethidium bromide-containing 0.7% agarose gel (right panel), blotted onto a nylon membrane, and incubated with either a DIG-labeled *lmexpmi* open reading frame (ORF) probe (middle panel) or a DIG-labeled *lmexpmi* 5'-untranslated region (UTR) probe (left panel). The sizes of DNA standards are indicated in kilobases.

at very similar levels in both parasite life stages (Fig. 4A). Ultracentrifugation experiments on sonicated cell lysates demonstrated that >95% of *L. mexicana* PMI activity is soluble (data not shown). The PMI protein band was completely absent in overexposed immunoblots of *L. mexicana Δlmexpmi* mutants (Fig. 4B), and PMI enzyme assays revealed that these mutants were completely deficient in PMI activity, whereas the activities of other metabolic enzymes like phosphoglucomutase or hexokinase showed little change or were even elevated (Fig. 4C). *L. mexicana Δlmexpmi* mutant promastigotes exhibited slowed growth in standard SDM medium (Fig. 5B) compared with wild type parasites (Fig. 5A). This growth defect could be overcome partially by the addition of 20 µM Man to the medium (Fig. 5B), but 2 mM Man led again to slow growth and a bloated shape of the cells, whereas 10 mM Man inhibited growth and ultimately killed most of the parasites (Fig. 5B). In contrast, neither growth nor cell shape of *L. mexicana* wild type cells and of *Δlmexpmi* mutant promastigotes carrying episomal copies of the *lmexpmi* gene were affected by high Man concentrations (Fig. 5C).

*L. mexicana Δlmexpmi* Mutant Promastigotes Show Impaired Synthesis of Glycoconjugates That Can Be Reversed by Man Complementation of the Growth Medium—Immunoblots of *L. mexicana* WT total cell lysates probed with the anti-PO4-6Galβ1–4Manα-1-repeat mAb LT6 showed a strong signal in the low molecular weight range that corresponds to LPG, and a weaker signal in the stacking gel and the top of the separating gel due to the presence of membrane-bound proteophosphoglycan (mPPG) (mPPG (20, 31)) (Fig. 6A, lane 1). The *L. mexicana Δlmexpmi* null mutants investigated showed any reaction with mAb LT6 on immunoblots. This result was confirmed by FACS analysis of live promastigotes, where the *Δlmexpmi* mutant clones examined showed only negligible surface fluorescence, in contrast to *Δlmexpmi* promastigotes, which displayed at least some LT6 epitopes on their surface, most likely due to mPPG expression (Fig. 7A). Likewise, surface binding sites for LT17, an mAb most likely recognizing glycosylated disaccharide phosphate repeats (20), were down-regulated by a factor of 10–20 (data not shown). The binding of the anti-[Man1–2]1–3Galβ1–4Manα-1-repeat mAb L7.25 on blots to proteins of *L. mexicana Δlmexpmi* promastigote total cell lysates was only slightly affected compared with wild type parasite lysates, but a shift of all antibody-recognized proteins to lower apparent molecular mass was clearly visible, which may indicate decreased glycosylation (Fig. 6C). A lower level of glycosylation on surface molecules in *L. mexicana*...
\( \Delta m \text{expmi} \) was also detected in FACS analyses with the lectin concanavalin A, where a strongly decreased signal intensity was observed compared with wild type parasites (Fig. 7C). This decrease in concanavalin A labeling was not due to the loss of LPG and mPPG in \( \Delta m \text{expmi} \) mutants, as \( L. \text{mexicana} \ \Delta m \text{exp} 1 \) promastigotes, which lack both molecules on the surface, display more binding sites for this lectin than wild type parasites (32). The surface binding of mAb L3.8 (33, 34) directed against the unglycosylated gp63 was decreased on \( \Delta m \text{expmi} \) parasites compared with wild type parasites (Fig. 7B). The absence or decrease of LT6, LT17, L3.8, and concanavalin A binding sites on \( \Delta m \text{expmi} \) parasites revealed a shorter, rounded cell shape in comparison to wild type parasites (Fig. 8, I–L). Likewise, these GIPLs were largely absent from the total lipid fraction of wild type parasites (Fig. 9A). HPTLC analysis of a total lipid fraction of \([3H] \text{GlcNH}_2\)-labeled \( L. \text{mexicana} \) revealed a shorter, rounded cell shape in comparison to wild type parasites (Fig. 8, I–L). Likewise, these GIPLs were largely absent from the total lipid fraction of \([3H] \text{GlcNH}_2\)-labeled \( L. \text{mexicana} \) parasites (Fig. 9A). HPTLC analysis of a total lipid fraction of \([3H] \text{GlcNH}_2\)-labeled \( L. \text{mexicana} \) revealed a shorter, rounded cell shape in comparison to wild type parasites (Fig. 8, I–L). Likewise, these GIPLs were largely absent from the total lipid fraction of \([3H] \text{GlcNH}_2\)-labeled \( L. \text{mexicana} \) parasites (Fig. 9A). HPTLC analysis of a total lipid fraction of \([3H] \text{GlcNH}_2\)-labeled \( L. \text{mexicana} \) revealed a shorter, rounded cell shape in comparison to wild type parasites (Fig. 8, I–L). Likewise, these GIPLs were largely absent from the total lipid fraction of \([3H] \text{GlcNH}_2\)-labeled \( L. \text{mexicana} \) parasites (Fig. 9A). HPTLC analysis of a total lipid fraction of \([3H] \text{GlcNH}_2\)-labeled \( L. \text{mexicana} \) revealed a shorter, rounded cell shape in comparison to wild type parasites (Fig. 8, I–L). Likewise, these GIPLs were largely absent from the total lipid fraction of \([3H] \text{GlcNH}_2\)-labeled \( L. \text{mexicana} \) parasites (Fig. 9A). HPTLC analysis of a total lipid fraction of \([3H] \text{GlcNH}_2\)-labeled \( L. \text{mexicana} \) revealed a shorter, rounded cell shape in comparison to wild type parasites (Fig. 8, I–L). Likewise, these GIPLs were largely absent from the total lipid fraction of \([3H] \text{GlcNH}_2\)-labeled \( L. \text{mexicana} \) parasites (Fig. 9A). HPTLC analysis of a total lipid fraction of \([3H] \text{GlcNH}_2\)-labeled \( L. \text{mexicana} \) revealed a shorter, rounded cell shape in comparison to wild type parasites (Fig. 8, I–L). Likewise, these GIPLs were largely absent from the total lipid fraction of \([3H] \text{GlcNH}_2\)-labeled \( L. \text{mexicana} \) parasites (Fig. 9A). HPTLC analysis of a total lipid fraction of \([3H] \text{GlcNH}_2\)-labeled \( L. \text{mexicana} \) revealed a shorter, rounded cell shape in comparison to wild type parasites (Fig. 8, I–L). Likewise, these GIPLs were largely absent from the total lipid fraction of \([3H] \text{GlcNH}_2\)-labeled \( L. \text{mexicana} \) parasites (Fig. 9A). HPTLC analysis of a total lipid fraction of \([3H] \text{GlcNH}_2\)-labeled \( L. \text{mexicana} \) revealed a shorter, rounded cell shape in comparison to wild type parasites (Fig. 8, I–L). Likewise, these GIPLs were largely absent from the total lipid fraction of \([3H] \text{GlcNH}_2\)-labeled \( L. \text{mexicana} \) parasites (Fig. 9A). HPTLC analysis of a total lipid fraction of \([3H] \text{GlcNH}_2\)-labeled \( L. \text{mexicana} \) revealed a shorter, rounded cell shape in comparison to wild type parasites (Fig. 8, I–L). Likewise, these GIPLs were largely absent from the total lipid fraction of \([3H] \text{GlcNH}_2\)-labeled \( L. \text{mexicana} \) parasites (Fig. 9A). HPTLC analysis of a total lipid fraction of \([3H] \text{GlcNH}_2\)-labeled \( L. \text{mexicana} \) revealed a shorter, rounded cell shape in comparison to wild type parasites (Fig. 8, I–L). Likewise, these GIPLs were largely absent from the total lipid fraction of \([3H] \text{GlcNH}_2\)-labeled \( L. \text{mexicana} \) parasites (Fig. 9A). HPTLC analysis of a total lipid fraction of \([3H] \text{GlcNH}_2\)-labeled \( L. \text{mexicana} \) revealed a shorter, rounded cell shape in comparison to wild type parasites (Fig. 8, I–L). Likewise, these GIPLs were largely absent from the total lipid fraction of \([3H] \text{GlcNH}_2\)-labeled \( L. \text{mexicana} \) parasites (Fig. 9A).
pmi mutant parasites (Fig. 9B). Instead, three new major labeled glycolipid species not present in the wild type parasites were observed, which migrated faster on the TLC indicative of a less hydrophilic nature (Fig. 9B). Like LPG and mPPG synthesis, GPI synthesis could also be partially reconstituted by Man complementation of the growth medium, although, even at 200 μM, synthesis of the largest GPL species iM4 was not observed (Fig. 9C).

\[ \text{\textsuperscript{3}H\textsuperscript{b}}\text{Man Labeling and Swainsonine Treatment of L. mexicana Promastigotes—In} \text{\textsuperscript{3}H\textsuperscript{b}}\text{Man labels of L. mexicana wild type promastigotes, only about 2–3% of the offered radiolabel was incorporated in overnight labeling of parasites and could be detected in cellular and secreted macromolecules like PPGs, leishmanolysin/gp63 and SAP (Fig. 10, A–C) and in GPIs (Fig. 9D). This low incorporation rate was independent of the presence or absence of 1 mM glucose in the labeling medium (Fig. 10A). By contrast, L. mexicana \text{\textsuperscript{D}imexpmi} mutants increased Man incorporation 20- to 30-fold compared with wild type parasites, and more than 60% of the offered radiolabel was found in cellular and secreted macromolecules (Fig. 10, A–C), GPIs, and uncharacterized compounds in the lipid fraction, most likely intermediates of GPL, LPG, and GPI synthesis (Fig. 9D). This dramatic increase in metabolic labeling efficiency is specific for \text{\textsuperscript{3}H\textsuperscript{b}}\text{Man, because radioactivity incorporation rates of \text{\textsuperscript{3}H\textsuperscript{b}}\text{GlcNH}_2 and myo-\text{\textsuperscript{3}H\textsuperscript{b}}\text{inositol labels are very similar in the wild type and the \text{\textsuperscript{D}imexpmi}} mutant.}

Swainsonine is a potent and specific inhibitor of lysosomal α-mannosidase of various eukaryotes (35). This enzyme is essential for the degradation of glycoprotein N-glycans. The addition of 10 μM swainsonine had little effect on the growth of wild type promastigotes, whereas the growth of L. mexicana \text{\textsuperscript{D}imexpmi} is retarded by about 50% (Fig. 11). This result is an indication that, under limiting Man supply, Leishmania may rely partially on the degradation of glycoproteins to acquire this hexose.

**L. mexicana \text{\textsuperscript{D}imexpmi} Mutants Are Attenuated but Remain Infectious to Macrophages and Mice—L. mexicana \text{\textsuperscript{D}imexpmi} was less efficient in establishing and maintaining an infection in mouse peritoneal macrophages than the parental wild type strain (Fig. 12A). Addback of the \text{\textsuperscript{D}imexpmi} gene to the mutant, either on an episome or by insertion into the chromosome at the ribosomal locus, led to increased infectivity that did not, however, reach wild type levels. Phosphoglycan synthesis of intracellular parasites in infected macrophages was down-regulated in L. mexicana \text{\textsuperscript{D}imexpmi} mutants compared with wild type parasites, but both LT6 and LT17 epitopes could be clearly detected (Fig. 13, compare A–D with E–H). This result is remarkable, because cultured promastigotes exhibit no LT6 and very low levels of LT17 epitopes (Fig. 8, B and F). Wild type levels of parasite PG repeat expression in infected macrophages was observed when \text{\textsuperscript{D}imexpmi} mutants with episomal copies of \text{\textsuperscript{D}imexpmi} were used for infection experiments (Fig. 13, I–L). In mouse infection experiments, L. mexicana \text{\textsuperscript{D}imexpmi} led to much smaller lesions compared with wild type parasites, but, surprisingly, this mutant was infectious to these mammals (Fig. 12, B and C). Complementation of \text{\textsuperscript{D}imexpmi} by episomal gene copies or by integration of \text{\textsuperscript{D}imexpmi} into the ribosomal locus led to markedly increased virulence, as indicated by accelerated lesion growth in infected BALB/c mice (Fig. 12, B and C).**

**DISCUSSION**

In all eukaryotes investigated so far, the reversible isomerization of Frc-6-PO\textsubscript{4} and Man-6-PO\textsubscript{4} catalyzed by PMI is the first step in the biosynthesis of the activated Man donors GDP-Man and Dol-P-Man (Fig. 1), which are required for the biosynthesis of many glycoproteins, glycolipids, and, in the case of fungi, cell wall components.

In this study, we have cloned, sequenced, and functionally characterized the PMI of the parasitic protozoan L. mexicana as a first step in the investigation of the Leishmania Man pathway. L. mexicana promastigotes express high levels of PMI.
Phosphomannose Isomerase of Leishmania mexicana

Phosphomannose isomerase (PMI) is a soluble enzyme that is free of glycosylation. It is a pathway that occurs in the pathogen Leishmania mexicana, and its activity is crucial for the parasite's growth and survival. PMI activity is essential for the glycolytic pathway, which is responsible for the metabolic conversion of glucose to pyruvate. In Leishmania mexicana, PMI activity is significantly higher than in mammalian tissue, with an average enzyme activity of more than 15-fold (2.6 milliunits/mg of protein, assay at 37 °C) (36). Similarly, high PMI activity as in L. mexicana is found in the pathogenic yeast C. albicans (75 milliunits/mg of protein, assay at 37 °C) (37). L. mexicana PMI is a soluble enzyme that is expressed at the protein level in insect stage promastigotes and mammalian stage amastigotes. Immunofluorescence studies on permeabilized promastigotes suggest a cytoplasmic localization, as indicated by a diffuse fluorescence signal throughout the cell body (data not shown). The gene encoding PMI, lmexpmi, was isolated by a PCR approach using degenerate oligonucleotide primers, and its primary structure is 40% identical to that of S. cerevisiae, C. albicans, or human PMI protein sequences. Two rounds of targeted gene replacement led to lmexpmi null mutants lacking detectable PMI activity. These mutants were able to multiply in standard growth medium, even when PMI activity was measured. However, although PMI is not essential, the absence of this enzyme has several severe consequences for L. mexicana. First, growth of L. mexicana Δlmexpmi promastigotes in standard medium is slower compared with wild type parasites and the cells show a distinctive rounded shape. Both the growth defect and the change in morphology can be corrected by the addition of 200 μM Man to the growth medium or by genetic complementation. Remarkably, addition of 2 mM Man leads again to a growth defect, and 10 mM Man completely inhibits growth and ultimately kills the cells. By contrast, wild type parasites or Δlmexpmi gene addback mutants are unaffected by additions of high Man concentrations to the medium. This observation is reminiscent of the toxicity of nutritional Man to apidae, the so-called honey bee syndrome. It has been suggested that an imbalance between high hexokinase and low PMI activities in honey bees, which leads to an accumulation of potentially toxic Man-6-PO₄ and, possibly, to ATP depletion, is the cause of this syndrome (41).

Second, lack of PMI activity leads to drastic down-regulation of glycoconjugate synthesis in L. mexicana promastigotes: A combination of immunoblot, FACS, and immunofluorescence...
microscopy studies suggest that \( \Delta \text{lmexpmi} \) promastigotes grown in standard medium are unable to synthesize phospho-glycan repeat-modified LPG and PPGs. Furthermore, a decrease in surface binding sites for concanavalin A as well as mobility shifts in SDS-PAGE of many cellular proteins and the dominant secreted glycoprotein SAP suggest a general under-glycosylation of glycoproteins in these mutants. Finally, the synthesis of the dominant promastigote GIPLs iM2, iM3, iM4, and EPiM3 (28) is down-regulated to such an extent that they could not be detected by the methods employed in this study. It has been suggested that Man-containing GIPLs are essential for \( L. \text{mexicana} \) viability (42). The results of this study demonstrate, however, that their biosynthesis can be down-regulated to undetectable levels without affecting parasite viability in culture. Metabolic \([\text{3H}]\text{GlcNH}_2\) labeling revealed new, more hydrophobic GIPL species in the \( \Delta \text{lmexpmi} \) mutants, which are not present in wild type parasites. It is likely that they represent GIPL precursors like GlcNH_2-phosphatidylinositol or GlcNAc-phosphatidylinositol species, although this suggestion requires proof by structure analysis. The defect in glycoconjugate synthesis can be partially or fully restored by \( \text{lmexpmi} \) gene addback or by the addition of 2–200 \( \mu \text{M} \) mannose to the growth medium. In metabolic labeling experiments with \([\text{3H}]\text{Man}\), \( \text{lmexpmi} \) promastigotes incorporate about 30 times more radioactivity into their glycoconjugates than wild type parasites, which use this hexose very inefficiently for biosynthesis. This result suggests that, under normal culture conditions, the majority of Man-6-PO_4 in \( L. \text{mexicana} \) originates from the Glc-6-PO_4/Frc-6-PO_4 pool (Fig. 1) of the promastigote and not from Man. Whether this hexosemonophosphate pool is fed by exogenous Glc or by gluconeogenesis, or both, remains unclear. This is in contrast to the situation in humans, where, in most tissues, the bulk of Man-6-PO_4 utilized for glycoprotein synthesis is not derived from Glc-6-PO_4 but originates from Man in serum, where its concentration is around 50 \( \mu \text{M} \). A specific Man transporter, which is only weakly affected by the large excess of Glc in serum (\(-5 \text{mM}\), ensures efficient uptake of this hexose
Our finding that [3H]Man labeling of *L. mexicana* promastigotes is also only marginally affected by a more than 10,000-fold excess of Glc over [3H]Man suggests that the parasites may also possess a similarly efficient and specific Man uptake system.

A third consequence of the lack of PMI for the parasites is a marked decrease in virulence. In macrophage infection studies, *Dlmexpmi* parasites are less successful in colonizing host cells than the parental wild type line, and in mouse infections, lesion growth is much slower. Episomal and, in particular, chromosomal integration addback of the *lmexpmi* gene to *Dlmexpmi* mutants improves their ability to persist and multiply within macrophages and leads to increased virulence to BALB/c mice. The fact that the *Dlmexpmi* mutants, although severely attenuated, are still infectious to macrophages and mice is at first glance unexpected. However, these mutants can still establish infections in the peritoneal cavity of infected mice and in the footpads of infected mice, albeit at a lower rate than the parental wild type line, and in mouse infections, lesion growth is much slower. Episomal and, in particular, chromosomal integration addback of the *lmexpmi* gene to *Dlmexpmi* mutants improves their ability to persist and multiply within macrophages and leads to increased virulence to BALB/c mice.
unexpected, because the general impairment of glycoconjugate synthesis (2), in particular the defect of GIPL assembly (43), should preclude virulence completely. However, those Δmex

PMI of the pathogenic fungus C. albicans is currently being investigated as a target to combat fungal infections (30). Our results suggest that the inhibition of this enzyme in Leishman
ia amastigotes colonizing a human lesion may lead to drastically slowed parasite growth, which may enable the immune system to eradicate the infection. Therefore, L. mexicana PMI may be a valid target for the development of new anti-Leishman
ia drugs. The ΔmexPMI mutant generated in this study provides the first Man-dependent conditional system for the synthesis of Leishmania phosphoglycans, GIPLs, and possibly GI anchors and N-glycans, which could be exploited for the investigation of biosynthesis, intracellular transport, and biological functions of these parasite glycoconjugates.

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REFERENCES
1. Alexander, J., and Russell, D. G. (1992) Adv. Parasitol. 31, 175–254
2. Descoeuroux, A., and Turco, S. J. (1999) Biochim. Biophys. Acta 1455, 341–352
3. McConville, M. J., and Ferguson, M. A. J. (1993) Biochem. J. 294, 305–324
4. Ilg, T. (2000) Parasitol. Today 16, 489–497
5. Beverley, S. M., and Turco, S. J. (1998) Trends Microbiol. 6, 35–40
6. Ralton, J. E., and McConville, M. J. (1998) J. Biol. Chem. 273, 4245–4257
7. Carver, M. A., and Turco, S. J. (1991) J. Biol. Chem. 266, 10974–10981
8. Schneider, P., McConville, M. J., and Ferguson M. A. J. (1994) J. Biol. Chem. 269, 18332–18337
9. Moss, J. M., Reid, G., Mullin, K. A., Zawadzki, J. L., Simpson, R. J., and McConville, M. J. (1999) J. Biol. Chem. 274, 6678–6688
10. Mahoney, A. B., and Turco, S. J. (1999) Arch. Biochem. Biophys. 372, 367–374
11. Gould, G. W., and Holman, G. D. (1993) Biochem. J. 295, 329–341
12. Panneerselvam, K., and Freeze, H. H. (1996) J. Biol. Chem. 271, 9417–9421
13. Gracy, R. W., and Noltmann, E. A. (1989) J. Biol. Chem. 264, 3161–3168
14. Smith, D. J., Proudfoot, A., Friedli, L., Klig, L. S., Paravic, G., and Payton, M. A. (1992) Mol. Cell. Biol. 12, 2924–2930
15. de Vries, P. J., Dorland, L., van Diggelen, O. P., Boonman, A. M. C., de Jong, G. J., van Noort, W. L., De Schryver, J., Duran, M., van den Berg, I. E. T., Gerwig, G. J., Berger, R., and Poll-The, B. T. (1998) Biochim. Biophys. Res. Commun. 245, 38–42
16. Petersen, R., Hasilik, M., Alton, G., Körner, C., Schiebe-Sukumar, M., Kech, H. G., Zimmer, K.-P., Wu, R., Harms, E., Reiterer, K., von Figura, K., Freeze, H. H., Harms, H. K., and Marquardt, T. (1998) J. Clin. Invest. 104, 1414–1420
17. Jaeken, J., Matthijs, G., Sandruhizzi, J.-M., Dzionie-Vici, C., Bertini, E., de Loury, P., Henri, H., Caron, H., Schollen, E., and van Schaftingen, E. (1998) Am. J. Hum. Genet. 62, 1535–1539
18. Ilg, T., Harbecke, D., Wiese, M., and Overath, P. (1993b) Eur. J. Biochem. 217, 603–615
19. Ilg, T. (2000) EMBO J. 19, 1–11
20. Ilg, T., Montgomery, J., Steriharb, Y.-D., and Handman, E. (1999) J. Biol. Chem. 274, 31410–31420
21. Wiese, M., Ilg, T., Lottspeich, F., and Overath, P. (1995) EMBO J. 14, 1067–1074
22. Cruz, A., Coburn, C. M., and Beverley, S. M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 7170–7174
23. LeBowitz, J. H., Coburn, C. M., McMahon-Pratt, D., and Beverley, S. M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 9736–9740
24. Misslitz, A., Mottram, J. C., Overath, P., and Aebersicher, T. (2000) Mol. Biochem. Parasitol. 107, 251–261
25. Benzel, I., Wiese, F., and Wiese, M. (2000) Mol. Biochem. Parasitol. 111, 77–86
26. Stierhof, Y.-D., Wiese, M., Ilg, T., Overath, P., Hänner, P., and Aeberli, U. (1998) J. Mol. Biol. 282, 137–148
27. Ralton, J. E., Wiese, M., Ilg, T., Overath, P., and Aeberli, U. (1999) J. Biol. Chem. 274, 6678–6688
28. McConville, M. J., Collidge, T. A., Ferguson, M. A. J., and Schneider, P. (1993) J. Biol. Chem. 268, 15595–15604
29. Beverley, S. M. (1992) Methods Enzymol. 219, 95–119
30. Cleasby, A., Wonacott, A., Skarratski, T., Hubbard, R. E., Davies, G. J., Proudfoot, A. E. I., Bernard, A. R., Payton, M. A., and Wells, T. N. C. (1996) Nat. Struct. Biol. 3, 470–479
31. Piani, A., Ilg, T., Elefanty, E. G., Curtis, J., and Handman, E. (1999) Microbes Infect. 1, 589–599
32. Ilg, T., Demar, M., and Harbecke, D. (2001) J. Biol. Chem. 276, 4898–4907
33. Russell, D. G. (1987) Eur. J. Biochem. 164, 213–221
34. Ilg, T., Harbecke, D., and Overath, P. (1993a) FEBS Lett. 327, 103–107
35. Elbein, A. D. (1991) FASEB J. 5, 3055–3063
36. Proudfoot, A. E. I., Turcotte, G., Wells, T. N. C., Payton, M. A., and Smith, D. J. (1995) Eur. J. Biochem. 218, 415–423
37. Proudfoot, A. E. I., Payton, M. A., and Wells, T. N. C. (1994) J. Prot. Chem. 13, 619–627
38. Smith, D. J., and Payton, M. A. (1994) Mol. Cell. Biol. 14, 6030–6038
39. Smith, D. J., Proudfoot, A. E. I., Deianni, M., Wells, T. N. C., and Payton, M. A. (1995) Yeast 11, 301–310
40. Freeze, H. H., and Aebl, M. (1999) Biochim. Biophys. Acta 1455, 167–178
41. Sols, A., Cadenas, E., and Alvarado, F. (1980) Science 197, 297–298
42. Panneerselvam, K., Etchinson, J. R., and Freeze, H. H. (1997) J. Biol. Chem. 272, 23123–23129
43. Ilgoutz, S. C., Zawadzki, J. C., Ralton, J. E., and McConville, M. J. (1999) EMBO J. 18, 2746–2755
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