Evidence That Intracellular β1-2 Mannan Is a Virulence Factor in Leishmania Parasites*

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The protozoan parasite Leishmania mexicana proliferates within macrophage phagosomes in the mammalian host. In this study we provide evidence that a novel class of intracellular β1–2 mannan oligosaccharides is important for parasite survival in host macrophages. Mannan (degree of polymerization 4–40) is expressed at low levels in non-pathogenic promastigote stages but constitutes 80 and 90% of the cellular carbohydrate in the two developmental stages that infect macrophages, non-dividing promastigotes, and lesion-derived amastigotes, respectively. Mannan is catabolized when parasites are starved of glucose, suggesting a reserve function, and developmental stages having low mannan levels or L. mexicana GDMP mutants lacking all mannose molecules are highly sensitive to glucose starvation. Environmental stresses, such as mild heat shock or the heat shock protein-90 inhibitor, geldanamycin, that trigger the differentiation of promastigotes to amastigotes, result in a 10–25-fold increase in mannan levels. Developmental stages with low mannan levels or L. mexicana mutants lacking mannan do not survive heat shock and are unable to differentiate to amastigotes or infect macrophages in vitro. In contrast, a L. mexicana mutant deficient only in components of the mannose-rich surface glycoalkalyx differentiates normally and infects macrophages in vitro. Collectively, these data provide strong evidence that mannann accumulation is important for parasite differentiation and survival in macrophages.

Leishmania species are sandfly-transmitted protozoan parasites that cause a number of human diseases, ranging from self-healing cutaneous lesions to fatal visceral infections, affecting more than 12 million people worldwide (www.who.int/inf-fs/en/fact116.html). These parasites develop within the midgut of the sandfly vector, initially as rapidly dividing promastigotes that synthesize a mannan oligosaccharide layer of the phagolysosome as the coat components, are expressed at much higher levels in pathogenic parasites than in non-pathogenic ones. This coat layer is required for survival of parasites in the phagolysosome as well as for evasion of host immune responses. Recently, a DNA microarray screen revealed that the expression of all GPI-anchored molecules exhibited only a partial loss of virulence under the same experimental conditions (4). The surface expression of GPI-anchored glycoproteins was as virulent as wild type parasites (3, 8–10), whereas mutants with defects in the surface expression of all GPI-anchored molecules exhibited only a partial loss of virulence under the same experimental conditions (4). The surface expression of LPG and GPI-anchored glycoproteins is not essential for survival within macrophages.

These genetic studies raise the possibility that mannose-containing molecules other than the major cell surface glycoconjugates are required for Leishmania infectivity. Studies by Blum and colleagues (13, 14) more than a decade ago showed that Leishmania donovani promastigotes synthesize a mannose-rich polysaccharide, termed mannan, although the precise structure of this material was not determined. In this study we show that L. mexicana mannan comprises a family of β1-2 mannan oligosaccharides that, in contrast to the major surface coat components, are expressed at much higher levels in pathogenic parasites. This work was supported by the Australian National Health and Medical Research Council, and equipment grants from the Wellcome Trust. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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ogenic developmental stages than in non-pathogenic stages. We present evidence that these intracellular oligosaccharides are required for differentiation and may protect *Leishmania* from starvation and/or stress conditions encountered in the macrophage phagolysosome. Our results provide strong evidence that these intracellular oligosaccharides constitute a new class of virulence factor.

**MATERIALS AND METHODS**

**Parasite Culture and Experimental Infection of Mice—**Promastigotes of the *L. mexicana* WT strain MNYS/BZ/62/M379 and the *L. mexicana* glycosylation mutant DIG1 (15) were grown in RPMI supplemented with 10% fetal bovine serum, and the infected fetal bovine serum was harvested at day 1 or day 5 to obtain log phase (LP) or stationary phase (SP) promastigotes, respectively. The day 5 cultures contained ~40% metacyclic promastigotes consistent with previous observations (16). The *L. mexicana* mutant, GDPMP, was routinely cultivated in semi-defined medium 79 supplemented with 4% iFBS (3). Mice were infected with either 5 × 10⁶ SP or freshly isolated LA by subcutaneous injection near the tail.

**Extraction of Mannan—**Promastigote and amastigote stages were extracted in CHCl₃/CH₃OH:H₂O (1:2.0:8.8 v/v) at a density of 2–4 × 10⁶ cells/ml (2 h at room temperature) with regular sonication and vortex mixing. Insoluble material was removed by centrifugation (15,000 × g for 5 min) and the supernatant was dried under N₂ before being partitioned within a biphasic mixture of water (200 μl) and 1-butanol (400 μl) to separate the major class of glycolipids (upper organic phase) from the mannan (lower aqueous phase). The lower phase was subsequently desalted by passage down a small column (400 μl) of AG 50-X12 (H⁻) over AG 3-X8 (OH⁻) and freeze-dried to give a neutral non-lipidic (NNL) fraction. The diglycosylated mannan was further extracted with 9% 1-butanol (2–12 h at 4°C with sonication and vortex mixing) to extract LPG and additional mannan. After centrifugation, the extract was freeze dried, suspended in 5% 1-propanol, 0.1M NH₄OAc, and loaded onto a small column of octyl-Sepharose (1 ml) equilibrated in the same buffer. Material eluted in the unbound fraction (4 ml) was desalted and combined with the NNL fraction, whereas LPG was eluted with 30% 1-propanol (4 ml) and freeze-dried prior to further analyses.

**HPLC, HPTLC, and Bio-Gel P-4 Chromatography—**The NNL fraction was analyzed on a Dionex HPLC system equipped with an online pulsed amperometric detector and a CarboPac PA-100 column. Samples were loaded onto a column pre-equilibrated with 0.1M NaOH, 7.5 mM NaOAc at a flow rate of 0.6 ml/min, then eluted with 2 linear gradients of NaOAc (7.5–175 mM from 60 to 70 min) to 0.5M NaOH. Glycans were detected with the pulsed amperometric detector and, if radiolabeled, by neutralization of aliquot of each fraction with 1M acetic acid and scintillation counting. The NNL fractions were also analyzed on Silica Gel 60 aluminum-backed HPTLC sheets developed twice in 1-butanol/ethanol/water (4:3:3 v/v). Radiolabeled bands were detected with a Bertold LB 2821 automatic thin layer chromatography linear scanner or by fluorography after spraying HPTLC sheets with EN'HANCE spray (PerkinElmer Life Sciences) and exposing them to Biomax MR film (Eastman Kodak Co.) at ~70°C. High resolution gel filtration of the NNL fraction was performed on a column (2 × 100 cm) of Bio-Gel P-4 (200–400 mesh) held at 55°C and eluted with water at a flow rate of 12 ml h⁻¹, and 1 ml fractions were collected. NNL fractions containing radiolabeled glycans were co-injected with a mixture of α1–6 glucose oligomers generated by partial acid hydrolysis of dextran (1 M trifluoroacetic acid at 100°C for 2 h) to define the degree of polymerization (dp) of each chain. Unlabeled mannose and glucose oligomers were detected using an online refractometer (Erna), whereas radiolabeled mannan was detected by scintillation counting.

**Gas Chromatography-Mass Spectrometry (GC-MS)—**The monosaccharide composition of each subcellular fraction was determined after solvolysis in 0.5M methanolic HCl (50 μl at 80°C for 12 h), trimethylsilylation of the resulting monosaccharide methyl esters, and analysis of the trimethylsilylation derivatives by GC-MS (17). Crude and purified mannnan fractions were permethylated, and the partially permethylated alditol acetates were analyzed by GC-MS as previously described (17).

**Metabolic Labeling—**Promastigotes were harvested by centrifugation (800 × g for 10 min) and resuspended in glucose-free RPMI medium containing 1% BSA (100 μl) for 10 min (2°C) and resuspended in various media (RPMI, RPMI minus glucose, or phosphate-buffered saline (PBS)) containing 1% BSA. Aliquots were removed at the indicated times and sequentially extracted in CHCl₃/CH₃OH:H₂O (1:2.0:8.8 v/v) and 9% 1-butanol as described above.

**Subcellular Fractionation—**The *L. mexicana* LP and SP were harvested by centrifugation (800 × g for 10 min), washed twice in PBS, and hypotonically permeabilized under conditions known to preserve the latency of intracellular organelles (18). Permeabilized promastigotes were pelleted by centrifugation (2000 × g for 5 min at 4°C), and the cell pellet (1) was washed three times with 75 mM triethanolamine/HCl buffer, pH 7.6. The cytoplasmic supernatant and P1 washes were centrifuged at 100,000 × g (for 30 min at 4°C) to yield a cytosolic supernatant and a microsomal pellet (P2). The P1 and P2 fractions were suspended in 75 mM triethanolamine/HCl buffer, pH 7.6, and all fractions were adjusted to contain 0.1% Triton X-100. The mannan content of the NNL fraction was determined by GC-MS. Hexose and 6-phosphate dehydrogenase were used as markers for the glycosome and cytosol, respectively, (19). Hexose kinase was assayed in 75 mM triethanolamine/HCl buffer, pH 7.6, containing 10 mM glucose, 2.5 mM MgCl₂, 1.5 mM NAD, 1 mM ATP, and 2.5 μg of *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase (Sigma), whereas endogenous glucose-6-phosphate dehydrogenase was assayed in 75 mM triethanolamine/HCl buffer, pH 7.6, containing 10 mM glucose 6-phosphate, 2.5 mM MgCl₂, and 1.5 mM NADP. Enzyme reactions (1 ml) were carried out at 25°C, and the rate of reduction of NAD⁺ or NADP⁺ was monitored at 340 nm.

**Growth Assays and Macrophage Infections—**WT, DIG1, and GDPMP promastigotes were harvested, washed twice with PBS, then resuspended in PBS (1 × 10⁶ cell/ml), and incubated at 27°C for 20 h. Parasite viability was tested by harvesting cells and resuspending them in RPMI medium containing 10% iFBS. Aliquots were removed at the indicated time points, parasites were harvested by centrifugation (15,000 × g for 1 min), washed twice with PBS, and solubilized by sonication. 1% SDS, and cellular protein was determined with the BCA assay (20). Data shown are representative of three separate experiments.

Promastigotes were induced to differentiate to amastigotes by suspension in RPMI medium, pH 5.5, containing 20% iFBS and incubation at 33°C (21). In some experiments, promastigotes were subjected to heat shock (53°C alone or with 1% SDS) for 10 min. In other experiments, the growth of parasites grown under these conditions was monitored by measuring cellular protein. Cell viability was assessed after 48 h by resuspending parasites in fresh RPMI medium, pH 7.5, containing 10% iFBS at 27°C and measuring cellular protein at the times indicated. SP were also induced to differentiate to amastigotes by adding the heat shock protein 90 inhibitor, GA (Sigma), to promastigote medium (1). A concentration of 200 ng of GA/ml (from a 1 mg/ml Me₂SO stock) was used, and aliquots were removed at the indicated times for microscopy and mannan analysis.

**For the in vitro macrophage infectivity experiments, J774A.1 macrophages (2 × 10⁶) were grown on 10-mm coverslips in RPMI medium, supplemented with 10% iFBS, 4 mM glucose, 100 μg/ml penicillin, 100 μg/ml streptomycin at 37°C in 5% CO₂.** Confuent macrophage cultures were overlaid with stationary phase *L. mexicana* promastigotes (2 × 10⁶) and incubated for 4 h at 33°C in 5% CO₂. Coverslips were washed three times with RPMI medium to remove unattached parasites and then incubated in macrophage medium for 120 h. Infectivity was assessed by washing coverslips with PBS (three times), and the number of infected macrophages was determined using fluorescence microscopy.

**RESULTS**

**Man-containing Oligosaccharides Accumulate in Pathogenic Stages of Leishmania—**Recent genetic studies in *L. mexicana* have suggested that mannose-containing molecules, other than the major cell surface components, are potential virulence factors (3). We therefore determined the monosaccharide composition of different subcellular fractions obtained from wild type (WT) *L. mexicana* by sequential solvent extraction, 1-butanol/water partitioning, and octyl-Sepharose chromatography. In addition to fractions containing cell surface G1PLs and LPG, this fractionation procedure generated a NNL fraction. The major sugar in the NNL fraction was mannose, and subsequent analyses (see below) showed that this hexose was associated exclusively with a family of mannosyl oligosaccharides. The
The mannose content of the NNL fraction increased from 25 nmol of Man/10^8 cells in log phase promastigotes to 80 nmol of Man/10^8 cells and 325 nmol of Man/10^8 cells in stationary phase promastigotes and amastigotes derived from mouse lesions, respectively (Fig. 1, A–C). The cellular levels of GIPLs remained unchanged in LP and SP (10 nmol of hexose/10^8 cells) but increased 3-fold in LA (30 nmol of hexose/10^8 cells) (Fig. 1, A–C). In contrast, the cellular levels of LPG decreased from 16 nmol of hexose/10^8 cells in LP to 2 and 1 nmol of hexose/10^8 cells in SP and LA, respectively. Hexose levels in the final residue fraction, corresponding to insoluble glycoproteins and proteophosphoglycans, accounted for 5% of the total cellular carbohydrate in all developmental stages. Thus the NNL fraction comprised 25, 80, and 90% of the total carbohydrate content of LP, SP, and LA, respectively.

To investigate whether the changes in the size of the steady-state pools of mannans, GIPL, and LPG were because of changes in the rate of synthesis of these molecules, each developmental stage (LP, SP, and LA) was pulse-labeled with [3H]Man for 10 min and fractionated as described above. Although the uptake of [3H]Man into all developmental stages was similar (550,000, 600,000, and 300,000 cpm/10^8 cells in LP, SP, and LA, respectively), the rate of [3H]Man incorporation into the GIPL, LPG, and NNL fractions varied markedly (Fig. 1, D–F). In rapidly dividing LP, ~65% of the [3H]Man was incorporated into the GIPL and LPG fractions (Fig. 1D). However, in non-dividing SP and in LA, most of the label (80 and 95% of total label, respectively) was incorporated into the mannan fraction (Fig. 1, E and F). The low rate of synthesis of GIPL and LPG in SP most likely reflects a low requirement for new plasma membrane components in these non-dividing cells. LA also retain relatively large steady-state pools of GIPLs despite very low rates of synthesis, implying that LA in mature lesions are growing very slowly.

The NNL Fraction Contains a Family of β1-2 Mannan Oligosaccharides—HPLC analysis of the NNL fraction revealed the presence of a heterogeneous population of oligosaccharides that were metabolically labeled with [3H]Man (Table I). Gel filtration on a Bio-Gel P-4 column showed that these oligosaccharides comprised 4–40 hexose residues. Compositional and methylation-linkage analysis of Bio-Gel P-4 purified peaks indicated that each of these oligosaccharides contained only terminal or 2-O-substituted mannose.

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<FIG. 1. Mannan is the major carbohydrate in pathogenic stages of L. mexicana. A–C, L. mexicana WT-LP (A), -SP (B), and -LA (C) were sequentially extracted as described under “Materials and Methods,” and the monosaccharide composition of the GIPL, LPG, NNL, and final cell pellet (Res) fractions was determined by GC-MS. D–F, WT-LP, -SP, and -LA were pulse-labeled with [3H]Man for 10 min and labeled in the individual subcellular fractions determined by scintillation counting.

<FIG. 2. Characterization of the L. mexicana β1-2 mannan. A, WT-SP were metabolically labeled with [3H]Man, and the NNL fraction was analyzed by HPLC. Unlabeled and labeled mannan oligosaccharides were detected with an amperometric detector (solid line) or by scintillation counting (dotted line), respectively. The elution times of dextran oligomers (degrees of polymerization of 1–20) are indicated at the top of the profile. B, analysis of the [3H]Man-labeled polysaccharides in the NNL fraction by gel filtration on a Bio-Gel P-4 column. The elution positions of co-injected dextran oligomers (dp 1–15) are indicated at the top of the profile. C, HPTLC analysis of [3H]Man-labeled oligosaccharides in the NNL fraction before or after treatment with jack bean α-mannosidase (JBAM) or snail β-mannosidase (SBM). Radiolabeled glycans were detected by fluorography. The migration positions of Man and dextran oligomers (dp 4–10) are indicated.

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TABLE I

| GU       | Peak | 1-Man | 2-Man | dp |
|----------|------|-------|-------|----|
| 3.6      | 1.0  | 2.1   | 4     |
| 4.5      | 1.0  | 2.8   | 5     |
| 5.2      | 1.0  | 3.4   | 6     |
| 6.0      | 1.0  | 4.7   | 7     |
| 6.7      | 1.0  | 6.1   | 8     |
| 7.7      | 1.0  | 7.5   | 9     |

* Values shown are molar ratios.
* Relative glucose units.

Mannitol was absent from the monosaccharide analysis unless the NNL fraction was reduced with NaBD₄, indicating that all these oligosaccharides contain mannose at their reducing termini. The mannan oligosaccharides were resistant to prolonged digestion with jack bean α-mannosidase but were slowly hydrolyzed by snail β-1–4-mannosidase with release of mannose (Fig. 2C). Further additions of β-1–4-mannosidase increased the proportion of mannan digested, suggesting that the incomplete digestion reflected the linkage specificity of this enzyme. Collectively these analyses suggest that Leishmania mannan comprises a family of linear β1–2-linked mannose oligosaccharides. Interestingly, promastigote mannan comprised chains up to 40 mannose residues long, whereas most of the LA mannan chains were between 4 and 10 residues long (Fig. 3).

The subcellular distribution of mannan was investigated by differential centrifugation of hypotonically lysed L. mexicana SP (18). Intracellular organelles such as glycosomes and endoplasmic reticulum retain their latency under these conditions, as shown by the recovery of the glycosomal marker, hexose kinase (Fig. 4) and the endoplasmic reticulum marker, binding protein (18), in the P1 and P2 fractions. In contrast, the cytosolic marker glucose-6-phosphate dehydrogenase and mannan were largely recovered in the S1 cytosolic fraction (Fig. 4). Based on the mannan content of each developmental stage and estimates of cell volume (45 and 4 µm³ for promastigotes and amastigotes, respectively), the cytosolic concentration of mannan in LP, SP, and LA was estimated to be 0.5, 1.8, and 6.4 µM, respectively.

**Newly Synthesized Mannan Is Rapidly Catabolized under Starvation Conditions.—**To investigate whether mannan is a reserve material, WT-SP were pulse-labeled with [³H]Man and then resuspended in either RPMI plus 1% BSA with or without glucose or in PBS. Although [³H]mannan was slowly catabolized when promastigotes were suspended in glucose-containing medium, incubation in glucose-free medium or in PBS dramatically increased the turnover of [³H]Man label in the mannan fraction (Fig. 5, A and B). The [³H]Man label from the mannan fraction was not chased into the GIPLS (Fig. 5B), or other cellular fractions (data not shown), suggesting that the majority of the [³H]Man released from mannan was being converted to other hexoses with loss of the label as [³H₂O.

Similar results were observed when LA were metabolically labeled and chased under the same conditions (data not shown). Prolonged starvation of both SP and LA also resulted in a decrease in the total cellular pools of mannan detected by both GC-MS and HPLC analyses (data not shown), indicating that the turnover of the radiolabeled mannan reflected increased turnover of the total mannan pool and not just newly synthesized oligosaccharides.

**Mannan without Leishmania Are Unable to Tolerate Nutrient Starvation.—**The pulse-chase labeling studies suggested that mannan was a dynamic reserve material. We therefore investigated the capacity of LP and SP to recover after incubation in the absence of a carbon source for 20 h. L. mexicana LP, containing low levels of mannan, recovered slowly with a lag of 1–2 days after being resuspended in full medium (Fig. 6). In contrast, L. mexicana SP, containing high levels of mannan, recovered rapidly when transferred from the starve medium to complete medium (Fig. 6). We also tested the sensitivity of the L. mexicana GDPMP mutant to nutrient starvation. This mutant lacks the enzyme GDP-Man pyrophosphorylase, which catalyzes the syn-
thesis of GDP-Man and is therefore deficient in all surface mannose-containing molecules (3), as well as detectable levels of mannan (Fig. 3). The GDPMP mutant was found to be extremely susceptible to starvation, with none of the cells surviving 20 h of incubation in PBS (Fig. 6). As the GDPMP promastigotes are deficient in all mannose-containing molecules, we also tested the sensitivity of a second L. mexicana mutant, termed DIG1, to starvation. This mutant synthesizes wild type levels of mannan (Fig. 3) but not the major surface components such as LPG, the secreted PPGs, GPI-anchored proteins, and polar GIPLs (15). DIG1-SP rapidly recovered from starvation with the same kinetics as WT-SP (Fig. 6) suggesting that the sensitivity of the GDPMP mutant to starvation most likely reflects the absence of mannan in these cells.

Mannan Accumulates in Response to Stress—Intracellular polysaccharides have been shown to confer resistance to stress in diverse eukaryotic systems (22). Leishmania parasites are exposed to both elevated temperature and reduced pH when introduced into the mammalian host and internalized by macrophages, and these conditions have been shown to trigger differentiation of promastigotes to amastigotes in vitro (Fig. 7A). Incubation of WT-SP at 33°C in RPMI medium induced a 25-fold increase in intracellular mannan levels (Fig. 7B). Heat shock, combined with acidification of the medium, also triggered the accumulation of mannan although to a lesser extent than heat shock alone (Fig. 7A). In contrast, mannan levels in cells incubated in RPMI medium, pH 7.5, at 27°C did not increase over the same time period (Fig. 7A). Inhibitors of L. donovani heat shock protein 90, such as the drug geldanamycin (GA), also induce a heat shock response and the differentiation of promastigotes to axenic amastigotes (1). GA induced L. mexicana SP to differentiate to amastigote-like forms,
although differentiation took twice as long compared with when heat shock and pH reduction were used (Fig. 7B). Remarkably, incubation with GA also triggered WT-SP to accumulate mannan (Fig. 7B). These data suggest that mannan accumulation may be part of the heat stress response of these parasites.

**Mannan May Be Required for Differentiation of Promastigotes to Amastigotes**—We next compared the capacity of wild type, GDPMP, and DIG1 promastigotes to differentiate to amastigotes by incubating them at 33°C in RPMI medium, pH 5.5. Parasites containing high levels of mannan (i.e. WT- and DIG1-SP) continued to grow slowly under these conditions (Fig. 8A). In contrast, parasites containing low levels of mannan (i.e. WT-LP) or no mannan (GDPMP-SP) failed to grow under these conditions. The decrease in biomass of GDPMP reflected a rapid loss of viability, as these cells did not grow when resuspended in promastigote medium (Fig. 8B). Although GDPMP-SP were taken up by J774 macrophages, they did not differentiate into amastigotes or survive within this macrophage cell line (Fig. 8C), as previously reported for mouse peritoneal macrophages (3). In contrast, both WT-SP and DIG1-SP established an infection in J774 macrophages (Fig. 8C). Collectively, these data suggest that environmental or pharmacological triggers of promastigote-amastigote differentiation induce the accumulation of mannan and that the lack of mannan synthesis in the GDPMP mutant is responsible for the loss of virulence of this parasite line.

**DISCUSSION**

A variety of studies have shown that mannos-containing molecules in the surface glycoconlyx of *L. major* and *L. donovani*, including LPG and the PPGs, are important for parasite virulence in both the sandfly vector and the mammalian host (5, 7, 23). Mannose metabolism is also essential for the virulence of *L. mexicana* in animal models (3, 4). However, targeted deletion of genes involved in the assembly of LPG, PPG, GPI-anchored proteins, and GPIPLs has little or no effect on the infectivity of this species in macrophage or the mammalian host suggesting that other mannose-containing molecules may be important for virulence (6, 8, 10). In this study we show that intracellular β1-2 mannann oligosaccharides accumulate to very high levels in pathogenic stages of *L. mexicana* and provide strong evidence that these intracellular oligosaccharides are essential for parasite survival within mammalian macrophages.

*Leishmania* mannann comprises a heterogeneous population of oligosaccharides with a dp of 4–40 mannan residues in the promastigote stages and 4–10 mannan residues in amastigotes. Structurally similar mannan oligosaccharides occur in all other species of *Leishmania* examined, including *L. major*, *L. donovani*, *L. tropica*, and *L. enriettii* and have also been reported from other trypanosomatid parasites, such as *Crithidia fasciculata* (24, 25) and *Herpetomonas samuellipsoasi* (26). These oligosaccharides clearly function as a dynamic reserve material. First, metabolically labeled mannan is rapidly catabolized when *L. mexicana* promastigotes and amastigotes are suspended in medium lacking glucose or other carbon sources. The released [3H]Man is not incorporated directly into other mannan-containing glycoconjugates (i.e. GIPs and LPG) but is lost as [3H]H2O, suggesting that it is epimerized to other sugars. The released mannan may be converted to fructose 6-phosphate by the cytosolic phosphomannose isomerase and either transported to the glycosomes, a specialized class of peroxisomes that contain most of the glycolytic enzymes (27), or utilized directly by enzymes in the pentose phosphate pathway that are largely localized in the cytosol (27, 28). Second, *L. mexicana* promastigotes containing low levels of mannan (i.e. WT-SP) or no mannan (i.e. GDPMP-SP) are more sensitive to nutrient starvation than promastigotes containing high levels of mannan (i.e. WT-SP). The sensitivity of the GDPMP mutant to nutrient starvation is unlikely to be because of the absence of surface glycoconjugates as the DIG1 mutant, which lacks most cell surface and secreted glycoconjugates but contains wild type levels of mannan, is as resistant to nutrient starvation as wild type parasites. Oligosaccharides containing three or more β1–2 linked mannan residues appear to adopt a highly constrained helical structure in solution (29). Other storage polysaccharides, such as glycogen, starch, and the algal β1–3 glucans also assume compact conformations (30), which may allow the accumulation of these polysaccharides without perturbing the viscosity of the cytosol.

A direct role of mannan in macrophage survival is strongly supported by several lines of evidence. First, although mannan accounts for ~20% of the cellular carbohydrate of non-pathogenic LP intracellular concentrations increase dramatically in pathogenic stages (up to 10 μM), accounting for 80 and 90% of cellular carbohydrate in pathogenic SP and LA, respectively. Second, mannan levels were found to increase dramatically (up to 25-fold) in response to stress conditions, such as mild heat shock and acidification of the medium, that are associated with macrophage infection and trigger promastigote differentiation to amastigotes. Similarly, inactivation of heat shock protein 90 with GA, which also results in the activation of a heat shock response and the differentiation of *Leishmania* promastigotes to amastigotes (1), increases the mannan steady-state pool, suggesting that mannan
accumulation is part of a coordinated response to stress. Third, the GDPMP mutant was found to be highly sensitive to heat shock and was unable to survive under conditions that normally trigger differentiation to amastigotes. In contrast, the DIG1 mutant, lacking only surface glycolipid components, differentiated to amastigotes with similar kinetics to wild type promastigotes. Apart from expressing wild type levels of mannans, DIG1 parasites differ from the GDPMP mutant in containing a limited number of apolar GILPs and N-glycosylated glycoproteins (15). However, it is unlikely that these molecules contribute to the markedly different phenotypes of the DIG1 and GDPMP lines, as WT-LP containing high levels of GILPs and N-glycosylated proteins but low mannans levels were also sensitive to heat shock. Fourth, only parasite stages containing high mannann levels were able to survive and proliferate within the J774 macrophage-like cell line. Starved WT-SP, like WT-LP, are also poorly infective in macrophages suggesting that the depletion of mannann, rather than other mannose-containing molecules, is responsible for loss of infectivity in macrophages. Finally, the infectivity of *Leishmania* promastigotes in the sandfly midgut is increased if they are exposed to a sugar meal prior to being transmitted to the mammalian host (31). Exposure to a sugar meal would allow metacyclic promastigotes to accumulate mannann, preadapting them to the stress conditions encountered in the mammalian host. Collectively these data provide strong evidence that the accumulation of mannann in *Leishmania* infective stages is required for establishment of infection and continued survival in macrophages.

Mannann could promote survival of *Leishmania* promastigotes in host macrophages in a number of ways. First, as a reserve material it may sustain parasite metabolism in the early stages of infection in the potentially nutrient-poor environment it may sustain parasite metabolism in the goutes in host macrophages in a number of ways. First, as a *L. mexicana* LP reached stationary growth and remained very slow. These data suggest that the dramatic remodeling of the surface glycolipid of *Leishmania*, in particular the loss of the surface LPG coat in amastigote stages (11, 12), reflects growth-dependent changes in the rate of synthesis of all GPI glycolipids and the selective loss of LPG by shedding. Given the reciprocal relationship between GIP and mannann biosynthesis it will be important to determine how these glycosylation pathways are coordinated during parasite development. Finally, the results presented here strongly suggest that enzymes involved in mannann synthesis or catabolism are potential targets for new anti-parasite therapies.

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