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Disruption of mannose activation in *Leishmania mexicana*: GDP-mannose pyrophosphorylase is required for virulence, but not for viability

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In eukaryotes, the enzyme GDP-mannose pyrophosphorylase (GDPMP) is essential for the formation of GDP-mannose, the central activated mannose donor in glycosylation reactions. Deletion of its gene is lethal in fungi, most likely as a consequence of disrupted glycoconjugate biosynthesis. Furthermore, absence of GDPMP enzyme activity and the expected loss of all mannose-containing glycoconjugates have so far not been observed in any eukaryotic organism. In this study we have cloned and characterized the gene encoding GDPMP from the eukaryotic protozoan parasite *Leishmania mexicana*. We report the generation of GDPMP gene deletion mutants of this human pathogen that are devoid of detectable GDPMP activity and completely lack mannose-containing glycoproteins and glycolipids, such as lipophosphoglycan, proteophosphoglycans, glycosylphosphatidylinositol protein membrane anchors, glycoinositolphospholipids and *N*-glycans. The loss of GDPMP renders the parasites unable to infect macrophages or mice, while gene addback restores virulence. Our study demonstrates that GDP-mannose biosynthesis is not essential for *Leishmania* viability in culture, but constitutes a virulence pathway in these human pathogens.

Keywords: GDP-mannose pyrophosphorylase/*Leishmania*/mannose/metabolism/virulence

Introduction

In eukaryotes, mannose (Man) is a key monosaccharide for the glycosylation of proteins and lipids. Man-containing glycoconjugates, such as protein *N*- and *C*-glycans, some *O*-glycans, glycosylphosphatidylinositol (GPI) protein membrane anchors, as well as some glycolipids, are considered to have a variety of important functions. These include the promotion of correct folding, solubility, stability and intracellular sorting of proteins. Furthermore, Man-containing glycans have been shown to be essential for the enzymatic, hormonal or receptor activity of many proteins, the formation of cell surface glycocalyces, extracellular matrices and protective cell walls, and they are also known to play major roles in cell–cell interactions. It is generally assumed that the biosynthesis of Man-containing glycoconjugates is of vital importance to eukaryotic organisms (summarized in Varki, 1999). In the absence of exogenous Man, eukaryotic cells synthesize this hexose by phosphomannose isomerase (PMI)-catalysed conversion of fructose (Frc)-6-PO_4 to Man-6-PO_4 (Smith *et al*., 1992). The consecutive action of phosphomannomutase (PMM) and GDP-mannose pyrophosphorylase (GDPMP) transforms Man-6-PO_4 to GDP-Man (reviewed in Freeze and Aebi, 1999; see also Figure 1A), which is the critical metabolite of the Man activation pathway for glycoconjugate synthesis in eukaryotes. GDP-Man is utilized directly or indirectly as a Man donor for all mannosylation reactions, it is a precursor for GDP-fucose synthesis (Kaufman and Ginsburg, 1968) and acts as a substrate for ascorbic acid synthesis in plants (Wheeler *et al*., 1998). The crucial role of GDP-Man is supported by the finding that, in *Saccharomyces cerevisiae* and *Candida albicans*, deletion of the structural gene for GDPMP is lethal (Hashimoto *et al*., 1997; Warit *et al*., 2000), as are deletions of other genes of the Man pathway (Kepes and Schekman, 1988; Orlean *et al*., 1988). The absence of GDPMP activity has not yet been reported for any uni- or multicellular eukaryote, and the available data suggest that complete disruption of the Man pathway and the resulting absence of Man-containing glycoconjugates are incompatible with eukaryotic life.

Eukaryotic parasitic protozoa of the genus *Leishmania* are the causative agents of several human diseases ranging from self-healing skin ulcers to fatal visceral infections. These parasites exhibit a dimorphic life cycle comprising extracellular promastigotes that colonize the midgut of the sandfly vector and intracellular amastigotes that reside within the phagolysosomal compartment of mammalian macrophages (Alexander and Russell, 1992). *Leishmania* produce large amounts of unusual Man-rich cell surface-associated and secreted glycoconjugates, which include *N*-glycans, GPI anchors, *O*-phosphoglycosylated proteophosphoglycans (PPGs), lipophosphoglycan (LPG) and glycoinositolphospholipids (GIPLs), and whose biosynthesis depends directly or indirectly on the availability of GDP-Man (Figure 1A). A large number of studies suggest that glycoconjugate synthesis is required by *Leishmania* to resist the hostile conditions of their habitats as well as to maintain virulence (reviewed in Descoteaux and Turco, 1999; Ferguson, 1999; Ilg, 2000a), and it is expected that, like in yeast and humans, a functional Man pathway (and in particular, GDPMP), is of essential importance to these parasites (Ilgoutz *et al*., 1999).

In this study, we report for the first time GDPMP gene deletion mutants in a eukaryotic organism. *Leishmania mexicana* parasites lacking the GDPMP gene (Δ*GDPMP*) due to gene replacement are still viable in culture, but are completely unable to synthesize Man-containing glycoconjugates. However, *Leishmania* Δ*GDPMP* mutants have lost the capacity to infect macrophages and mice, which establishes GDPMP as a virulence factor.

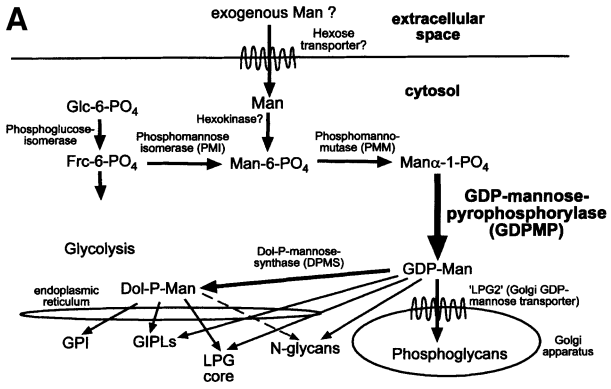
Results

Isolation of the L.mexicana LmxGDPMP gene

For the cloning of the *Leishmania* GDPMP gene, a degenerate PCR primer pair was constructed from the partially conserved peptide sequences PMILHQIE and WMDVGQPKDY/F (Figure 1B). PCR was performed using *L.mexicana* genomic DNA as a template. The resulting PCR product was sequenced and an open reading frame (ORF) was identified with high homology to known GDPMPs (data not shown). The digoxigenin (DIG)-labeled PCR fragment was used to screen a λ-DashII library of genomic *L.mexicana* DNA. Sequencing of an *LmxGDPMP* gene-containing DNA fragment revealed an ORF of 1266 bp (Figure 2A) encoding a protein of ~46.4 kDa (Figures 1B and 3A), which showed between 50 and 54% identity to GDPMPs from other eukaryotes like *S.cerevisiae*, *Arabidopsis thaliana* and *Homo sapiens*, and 92.4% identity to a gene annotated as being a potential GDPMP, which has been identified in the *Leishmania major* genome project (Figure 1B). Southern blot analysis of *L.mexicana* genomic DNA digested with different restriction enzymes and hybridized with an ORF probe suggests that *LmxGDPMP* is a single-copy gene (Figure 2C and data not shown). Northern blotting and RT–PCR analysis suggest that *LmxGDPMP* mRNA is present in both parasite life stages, but is more abundant in the forms occurring in the mammalian host, the amastigotes. However, immunoblots of total parasite cell lysates probed with affinity-purified antibodies raised against recombinant *L.mexicana* GDPMP indicate equal abundance of this enzyme in both life stages (Figure 3D). *Leishmania mexicana* GDPMP activity is largely (>90%) soluble after disruption of promastigotes followed by ultracentrifugation (data not shown), which is in agreement with immunoblotting studies on membrane fractions (Figure 3E). Immunofluorescence experiments on permeabilized promastigotes using anti-*Lmx*GDPMP serum yielded a diffuse signal throughout the cell body, which suggests a cytoplasmic localization of the enzyme (Figure 5C).

Targeted gene replacement of LmxGDPMP

Deletion of the single-copy GDPMP gene in *S.cerevisiae* or *C.albicans* is lethal, and it is generally assumed that this is also the case in other eukaryotes (Hashimoto *et al*., 1997; Warit *et al*., 2000). However, when two rounds of targeted *LmxGDPMP* gene replacement using the antibiotic resistance markers *HYG* and *BLE* were performed on *L.mexicana* (Figure 2A), remarkably, several clones were obtained that lacked both alleles of the *LmxGDPMP* ORF (Figure 2C, lane 2), but were viable in standard culture medium and showed only a mild growth defect compared with wild-type parasites (Figure 4H). The absence of the gene products in *L.mexicana* Δ*GDPMP::HYG* Δ*GDPMP::BLE* (Δ*GDPMP*) was confirmed by immunoblots of total cell lysates (Figure 3A, lane 2). GDPMP activity was detected in the soluble fraction of *L.mexicana* wild-type promastigote total-cell lysate, but the complexity of the GDPMP assay system results in strong GDP-Man-independent background reactions, probably due to metabolites and/or enzymes present in crude extracts. Therefore, for comparison of GDPMP activity in wild-type and Δ*GDPMP* promastigotes, concentrated ultracentrifugation supernatants were fractionated by Superose 6 gel filtration to remove interfering compounds from GDPMP fractions. Equivalent loading of



B

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           1                                                          50
                    *    ***** ** ******* * **** * * *    ** ***
LmxGDPMP   MSASDGQGMR AVILVGGFGT RLRPLTLTTP KPLVPFCNKP MIIHQIEALK
LmaGDPMP   MSSSDGQGMR AVILVGGFGT RLRPLTLTTP KPLVPFCNKP MIIHQIEALK
AthGDPMP   ~~~~~~~~MK ALILVGGFGT RLRPLTLSFP KPLVDFANKP MILHQIEALK
ScerGDPMP  ~~~~~~~~MK GLILVGGYGT RLRPLTLTVP KPLVEFGNRP MILHQIEALA
HsapGDPMP  ~~~~~~~~MK ALILVGGYGT RLRPLTLSTP KPLVDFCNKP ILLHQVEALA

           50                                                         100
             *  *      **  *                        * *   ********
LmxGDPMP   AVG..VTEVI LAVAYRPEAM KEQMDEWSRK LGVSFVFSVE EDPLGTAGPL
LmaGDPMP   AVG..VTEVI LAVAYRPEAM KEQMDEWSRK LGVLFVFSVE EEPLGTAGPL
AthGDPMP   AVGFTVDEVV LAINYQPEVM LNFLKDFETK LEIKITCSQE TEPLGTAGPL
ScerGDPMP  NAGFTVTDIA LAVNYRPEVM VETLKKYEKE YGVNITFSVE TEPLGTAGPL
HsapGDPMP  AAGFTVDHVI LAVSYMSQVL EKEMKAQEQR LGIRISMSHE EEPLGTAGPL

           101                                                        150
             ** *  *      **  *** ****    *       **     *    *
LmxGDPMP   ALARD.ILMQ DDKPF..FVL NSDVTCMFPL QELLDFHKAR GGEGTIMVSQ
LmaGDPMP   ALARD.ILMQ DDKPF..FVL NSDVTCPFPM QELLDFHKAH GGEGTIMVSQ
AthGDPMP   ALARDKLLDG SGEPFTFFVL NSDVISEYPL KEMLEFHKSH GGEASIMVTK
ScerGDPMP  KLAED.VLKK DNSPFFTFVL NSDVICEYPF KELADFHKAH GGKGTIVATK
HsapGDPMP  ALARD.LLSE TADPFFTFVL NSDVICDFPF QAMVQFHRHH GQEGSILVTK

           151                                                        200
           *    ****  *             ****           ***  *
LmxGDPMP   VTQWEKYGVV VYSQQSY.QI ERFVEK..PS SFLGDRVNAG IYIFNKSILD
LmaGDPMP   VTQWEKYGVV VYSPQNY.QI ERFVEK..PS SFLGDRINAG IYIFNKSILD
AthGDPMP   VDEPSKYGVV VMEEST.GRV EKFVEKFTPK LYVGNKINAG IYLLNPSVLD
ScerGDPMP  VDEPSKYGVI VHDIATPNLI DRFVEKFTPK EFVGNRINAG LYILNPEVID
HsapGDPMP  VEEPSKYGVV VCEADT.GRI HRFVEKPFTQ VFVSNKINAG MYILSPAVLR

           201                                                        250
             *    **** ** **        **   * * **** **     *   *
LmxGDPMP   RIPPCRTSIE KEIFPAMAAE GELYAFNLEG FWMDVGQ..P KDYILGMTKF
LmaGDPMP   RIPPCRTSIE KEIFPSMAAE GQLYAFNLEG FWMDIGQ..P KDYILGMTKY
AthGDPMP   KIELRPTSIE KETFPKIAAA QGLYAMVLPG FWMDIGQFTP RDYITGLRLY
ScerGDPMP  LIEMKPTSIE KETFPILVEE KQLYSFDLEG FWMDVGQFTP KDFLSGTVLY
HsapGDPMP  RIQLQPTSIE KEVFPIMAKE GQLYAMELQG FWMDIGQPFT KDFLTGMCLF

           251                                                        300
             *           *                    *  * * *  *    ***
LmxGDPMP   IPSLLDGDRK TEQLHTEATE HQHGGRFTVV GASLIDPSAK IGDGAVIGPC
LmaGDPMP   IPFLVHSNRE TEKLHTEATK HQRGGRFTVI GASLIDPSAK IGDGAVIGPY
AthGDPMP   LDSL..RKKS PAKLTSGP.. .......HIV GNVLVDETAT IGEGCLIGPD
ScerGDPMP  LNSL..AKRQ PKKLATGA.. .......NIV GNALIDPTAK ISSTAKIGPD
HsapGDPMP  LQSL..RQKQ PERLCSGP.. .......GIV GNVLVDPSAR IGQNCSIGPN

           301                                                        350
             *                                    * * *    * *
LmxGDPMP   ASIGANCVI. .GESCRIDNA AILENSKVGK GTMVSRSIVG WNNRIGSWCH
LmaGDPMP   ASIGANCVI. .GESCRIDNA AILENSKVGK GSMVSRSIVG WNNRIGSWCH
AthGDPMP   VAIGPGCIVF TESGVRLSRC TVMRGVRIKK HACISSSIIG WHSTVGQWAR
ScerGDPMP  VVIGPNVTIF TGDGVRITRS VVLCNSTIKN HSLVKSTIVG WNSTVGQWCR
HsapGDPMP  VSLGPGVVVE FTDGVCIRRC TVLRDARIRS HSWLESCIVG WRCRVGQWVR

           351                                                393
                 ** * * * *           *** *                * *
LmxGDPMP   IEDISVLGDD VEVKDGVVL. .IGTKVLPNK DVGEHHFQAG IIMZ
LmaGDPMP   IKDISVLGDD VEVEDGVVL. .IGTKVLPNK DVGEHHFEPG IIMZ
AthGDPMP   IENMTILGED VHVSDEIYSN FTGGVVLPHK EIKSNILKPE IVMZ
ScerGDPMP  LEGVTVLGDD VEVKDEIYIN FTGGKVLPHK SISDNVPKEA IIMZ
HsapGDPMP  MENVTVLGED VIVNDELYLN GFTASVLPHK SIGESVPEPR IIMZ
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Fig. 1. (**A**) Putative Man activation pathways and glycoconjugate biosynthesis in *L.mexicana*. The indication of GDP-Man and Dol-P-Man as Man donors for the biosynthesis of different *Leishmania* glycoconjugates is based on earlier studies (Ilgoutz *et al*., 1999). (**B**) Alignment of *L.mexicana* GDPMP (*LmxGDPMP*) with GDPMP amino acid sequences from various organisms: *L.major* (*LmaGDPMP*); *A.thaliana* (*AthGDPMP*); *S.cerevisiae* (*ScerGDPMP*; Hashimoto *et al*., 1997); *H.sapiens* (*HsapGDPMP*). Amino acids conserved in GDPMP of all five species are indicated by asterisks.

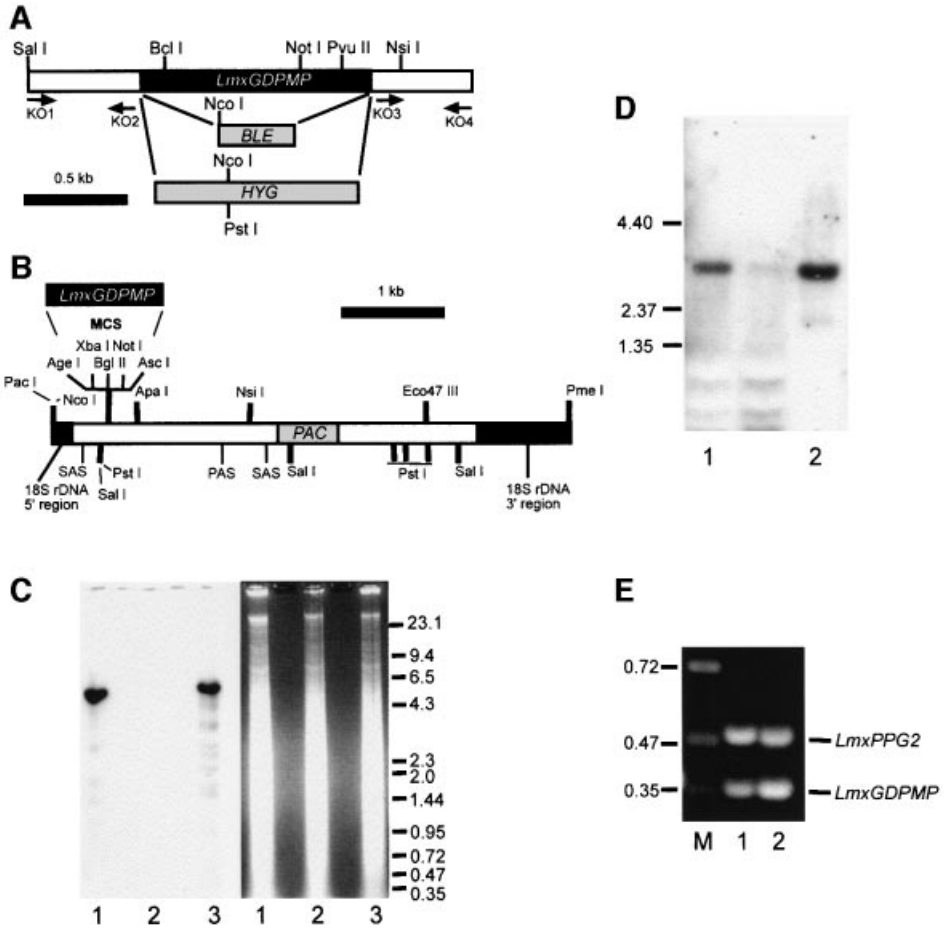

Fig. 2. Targeted gene replacement and gene addback of the *LmxGDPMP* alleles and analysis of mRNA expression. (**A**) Restriction maps of the *LmxGDPMP* locus. The resistance genes *BLE* and *HYG* and the primer binding sites (KO1–4) used for the construction of gene deletion cassettes are indicated. (**B**) Restriction map of the gene addback cassette for genetic rescue of the *L.mexicana* Δ*GDPMP* mutant. (**C**) Southern blot analysis of *Nco*I–*Pst*I-digested chromosomal DNA (10 μg) from *L.mexicana* wild type (lane 1), a Δ*GDPMP* mutant (lane 2) and a Δ*GDPMP* + cRIB*LmxGDPMP* gene addback mutant (lane 3). DNA was separated on an ethidium bromide-containing 0.7% agarose gel (right panel), blotted onto a nylon membrane and incubated with a DIG-labeled *LmxGDPMP* ORF probe (left panel). The sizes of DNA standards are indicated in kilobases. (**D**) Northern blot analysis of total RNA (10 μg) isolated from *L.mexicana* log-phase promastigotes (lane 1) and amastigotes (lane 2). RNA was separated on a 0.7% formaldehyde-containing agarose gel, blotted onto a nylon membrane and incubated with a DIG-labeled *LmxGDPMP* ORF probe. The sizes of RNA standards are indicated in kilobases. (**E**) Amplification of *LmxGDPMP* cDNA from *L.mexicana* log-phase promastigote (lane 1) and amastigote (lane 2) by RT–PCR from total RNA. The loading was normalized to the co-amplified cDNA fragment derived from the *LmxPPG2* gene whose mRNA is roughly equally abundant in *L.mexicana* promastigotes and amastigotes. The sizes of DNA standards are indicated in kilobases.

the Superose 6 column was confirmed by measuring the enzyme activity of UDP-glucose pyrophosphorylase (UDPGP) in both chromatography runs (Figure 4A). While in the wild-type fractions a prominent GDPMP activity peak eluting between 240 and 300 kDa was observed, no GDPMP activity was detected in the Δ*GDPMP* mutant (Figure 4A).

Absence of Man-containing glycoproteins and glycolipids in L.mexicana ΔGDPMP

Earlier studies have suggested that in *L.mexicana*, as in other eukaryotes, GDP-Man is directly or indirectly the sole Man donor for glycoconjugate synthesis (Mengeling *et al*., 1997; Ilgoutz *et al*., 1999; Moss *et al*., 1999 and references therein; see Figure 1A). In agreement with the expected inability of the GDPMP-deficient mutant to synthesize GDP-Man, expression of all known lipid- and protein-bound Man-containing glycoconjugates was found to be defective in *L.mexicana* Δ*GDPMP*: (i) LPG and phosphoglycans on PPGs were absent, as judged by the lack of specific bands on immunoblots of total cell lysates probed with the anti-phosphoglycan repeat monoclonal antibodies (mAbs) LT6 and LT17, even after extensive overexposure (Figure 3B and C); the complete absence of surface and flagellar pocket signals in immunofluorescence experiments (Figure 5A, compare I and II, V and VI) and of fluorescence-activated cell sorter (FACS) signals in labelings of live cells with LT6 and LT17 (Figure 5B); the lack of detectable LPG in metabolic [^{3}H]inositol labelings; the absence of LPG in attempted purifications by a standard protocol (data not shown and McConville *et al*., 1990); and the complete lack of binding sites for anti-phosphoglycan cap mAb L7.25 (Figure 4D and E) and other anti-phosphoglycan mAbs (not shown) on the normally highly phosphoglycosylated secreted acid phosphatase (SAP). (ii) Δ*GDPMP* promastigotes did not express the normally dominant GPI-anchored surface protein gp63 on their surface (Figure 5A, IX and X),

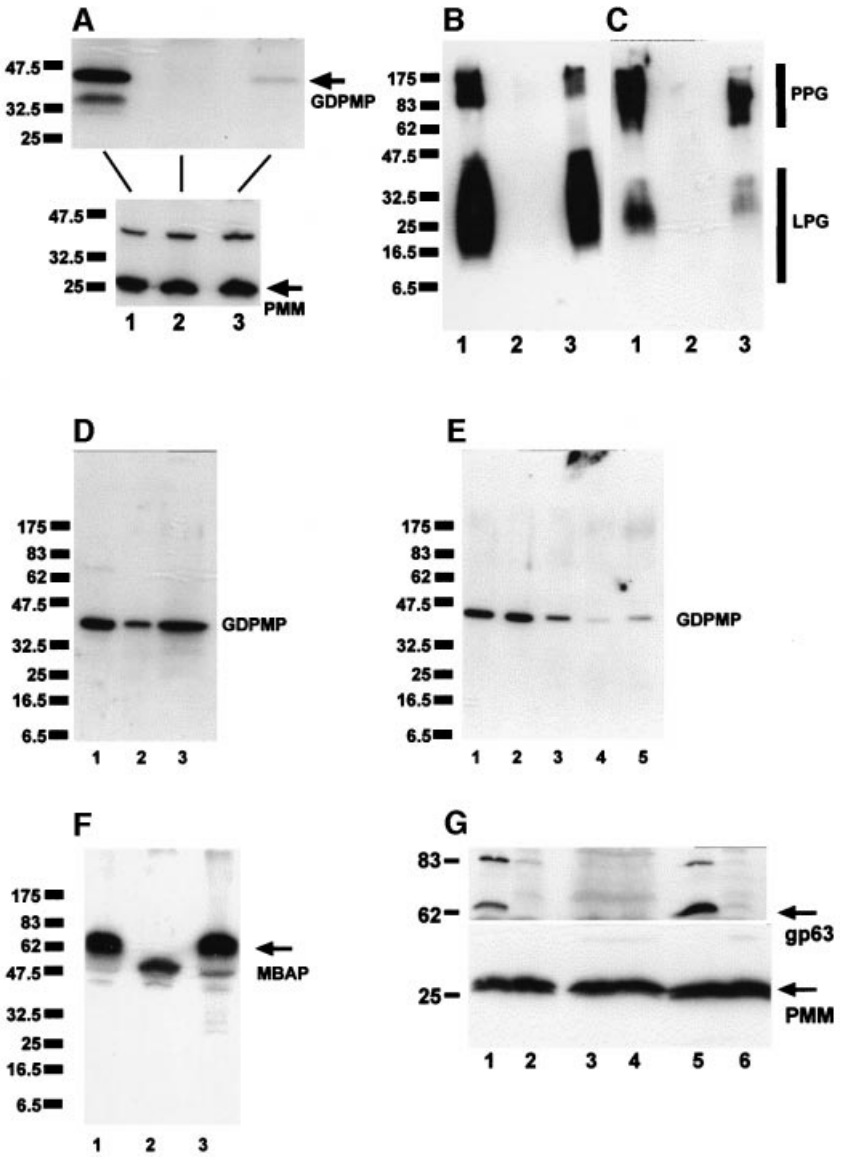

Fig. 3. SDS–PAGE/immunoblotting of *L.mexicana* total cell lysates. (A–C) Lane 1, WT; lane 2, Δ*GDPMP*; lane 3 Δ*GDPMP* + cRIB*LmxGDPMP*. Each lane was loaded with 1×10^7 promastigotes (~40 μg of protein). (**A**) Blot probed with affinity-purified rabbit anti-*L.mexicana* GDPMP antibodies (upper panel) and an identically loaded blot was probed with affinity-purified rabbit anti-*L.mexicana* PMM antibodies (lower panel) as a loading control. The same or identically loaded blots were then stripped and probed with mAb LT6 (anti-6-Galβ1-4Manα1-PO_4-) (**B**) and mAb LT17 [anti 6-(Glcβ1-3)Galβ1-4Manα1-PO_4-] (**C**). (**D**) SDS–PAGE/immunoblotting of total cell lysates of *L.mexicana* promastigotes (lane 1, 2.5×10^6 parasites, corresponding to ~10 μg of protein) and lesion-derived amastigotes (lane 2, 2.5×10^6 parasites, corresponding to ~3.5 μg of protein; and lane 3, 7×10^6 parasites, corresponding to ~10 μg of protein). The blots were probed with affinity-purified rabbit anti-*L.mexicana* GDPMP antibodies. (**E**) SDS–PAGE/immunoblotting of total cell lysates of *L.mexicana* promastigotes fractionated by ultracentrifugation. Lane 1, total cell lysate of 2.5×10^6 parasites, corresponding to ~10 μg of protein; lane 2, first ultracentrifugation supernatant; lane 3, first ultracentrifugation pellet; lane 4, second ultracentrifugation supernatant; lane 5, second ultracentrifugation pellet. Equivalent sample volumes were loaded and the blots were probed with affinity-purified rabbit anti-*L.mexicana* GDPMP antibodies. (**F**) SDS–PAGE/immunoblotting of promastigote lysates (2×10^7 parasites, corresponding to ~80 μg of protein) from WT (lane 1), Δ*GDPMP* (lane 2) and Δ*GDPMP* + cRIB*LmxGDPMP* (lane 3) probed with affinity-purified rabbit anti-*L.mexicana* MBAP antibodies. (**G**) SDS–PAGE/immunoblotting of promastigote lysates with (lanes 1, 3 and 5) or without (lanes 2, 4 and 6) prior GPI-PLC treatment. Lanes 1 and 2, WT; lanes 3 and 4, Δ*GDPMP*; lanes 5 and 6, Δ*GDPMP* + cRIB*LmxGDPMP*. The blot was probed with rabbit anti-CRD antibodies (upper panel). Equal loading was confirmed by stripping and reprobing with rabbit anti-PMM antibodies (lower panel).

although it could be detected within permeabilized cells, particularly in the perinuclear region, which suggested its retention in the endoplasmic reticulum (Figure 5A, XI). Furthermore, the cross-reactive determinant (CRD), which is indicative of GPI-anchored proteins in many eukaryotes, including *L.mexicana* (Zamze *et al*., 1988; Ilg *et al*., 1993), was not present in Δ*GDPMP* promastigote total cell lysates after GPI-phospholipase C (GPI-PLC) digestion (Figure 3G, lanes 3 and 4), and no [^{3}H]inositol-labeled gp63 was detected after metabolic labeling (Figure 4C), suggesting that protein GPI anchor synthesis is severely downregulated in the mutant parasites. (iii) Evidence for a defect in *N*-glycosylation was obtained by immunoblots of *L.mexicana* wild type and Δ*GDPMP* total cell lysates, where a mobility shift of 15–20 kDa for the normally heavily *N*-glycosylated, but neither GPI-anchored nor phosphoglycosylated, membrane-bound acid phosphatase (MBAP) (Menz *et al*., 1991; Wiese *et al*., 1996) was

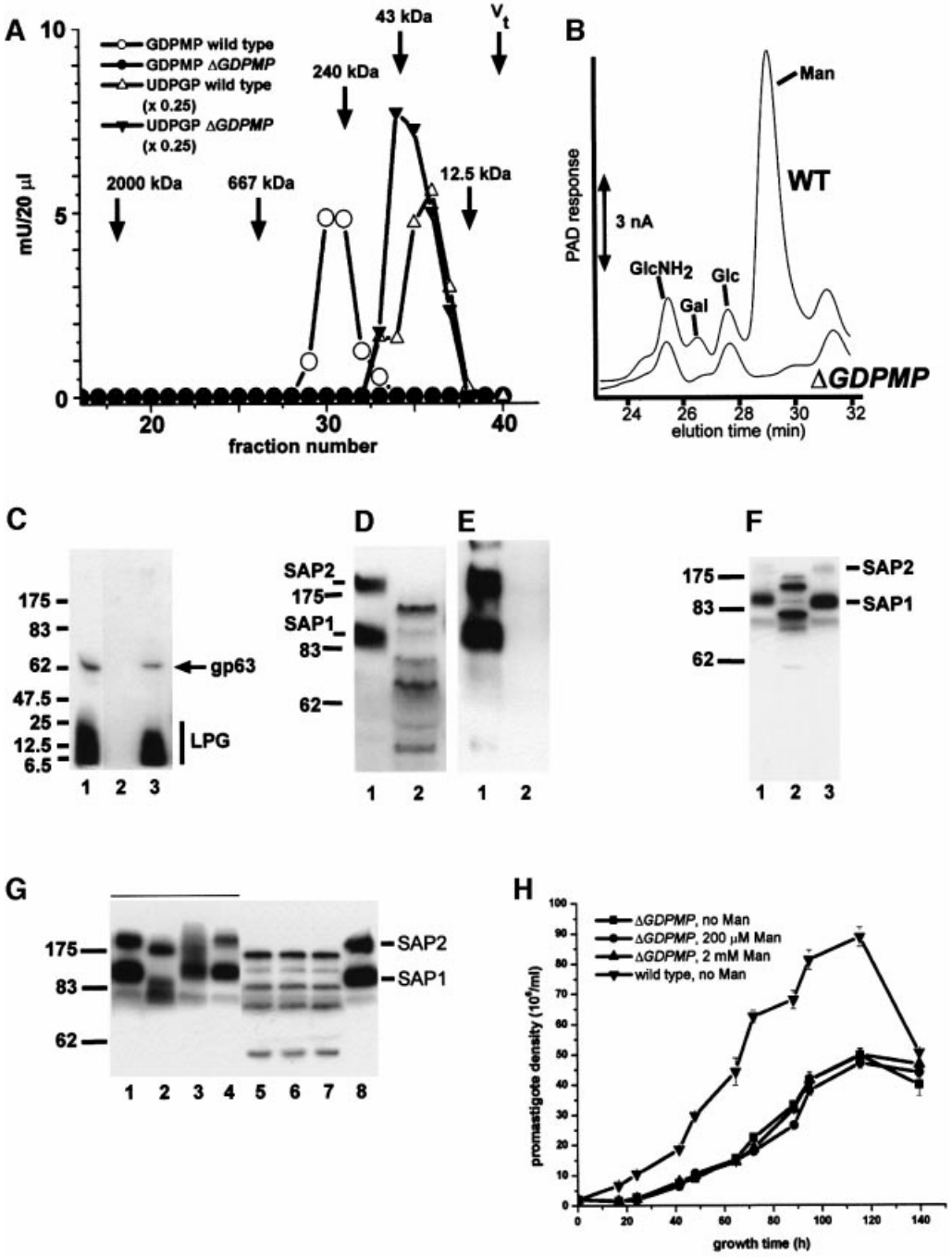

Fig. 4. (**A**) Superose 6 gel filtration of soluble fractions from *L.mexicana* wild type (WT) and Δ*GDPMP* promastigote lysates. The enzymatic activities of GDPMP and UDPGP were determined in each fraction. The elution positions of size standards are indicated by arrows. (**B**) High-pH anion-exchange HPLC of 2 M TFA hydrolysates of *L.mexicana* WT and Δ*GDPMP* promastigote membranes. The hexose fraction of equal amounts of cells (~1 × 10^8) was loaded onto a Carbo-Pac PA10 column. (**C**) SDS–PAGE/fluorography of delipidated total promastigote lysates from [^{3}H]*myo*-inositol-labeled *L.mexicana* WT (lane 1), *L.mexicana* Δ*GDPMP* (lane 2) and *L.mexicana* Δ*GDPMP* + cRIB*LmxGDPMP* (lane 3). Each lane was loaded with 2.5 × 10^7 delipidated promastigotes labeled overnight with [^{3}H]*myo*-inositol. The positions of LPG and the major GPI-anchored surface metalloproteinase gp63 are indicated by a bar and an arrow, respectively. (**D**–**G**) SDS–PAGE/immunoblot of *L.mexicana* culture supernatants. Loading was normalized to SAP activity (2 mU). (D and E) Lane 1, WT; lane 2, Δ*GDPM*P. (F) Lane 1, WT; lane 2, Δ*GDPMP*; lane 3, Δ*GDPM* + cRIB*LmxGDPMP*. (G) Lane 1, WT; lane 2, Δ*PMI*; lane 3, Δ*PMI* + 20 µM Man; lane 4, Δ*PMI* + 200 µM Man; lane 5, Δ*GDPMP*; lane 6, Δ*GDPMP* + 200 µM Man; lane 7, Δ*GDPMP* + 2 mM Man; lane 8, WT. (**H**) Growth curves of *L.mexicana* WT and of *L.mexicana* Δ*GDPMP* in the presence of various Man concentrations. (D), (F) and (G) were probed with affinity-purified rabbit anti-*L.mexicana* GDPMP antibodies, while (E) was probed with mAb L7.25. The molecular masses and relative positions of standard proteins are indicated on the gels and blots in (C–G).

observed in the mutant (Figure 3F, lanes 1 and 2), which is indicative of the loss of *N*-glycans in this molecule. (iv) The Man-containing GIPLs are undetectable in *L.mexicana* Δ*GDPMP* in high-performance thin-layer chromatography (HPTLC)-separated total lipids either by orcinol staining or by fluorography after metabolic labeling with [^{3}H]Man, [^{3}H]GlcNH_2 and [^{3}H]*myo*-inositol (Figure 6). (v) Δ*GDPMP* promastigotes also did not

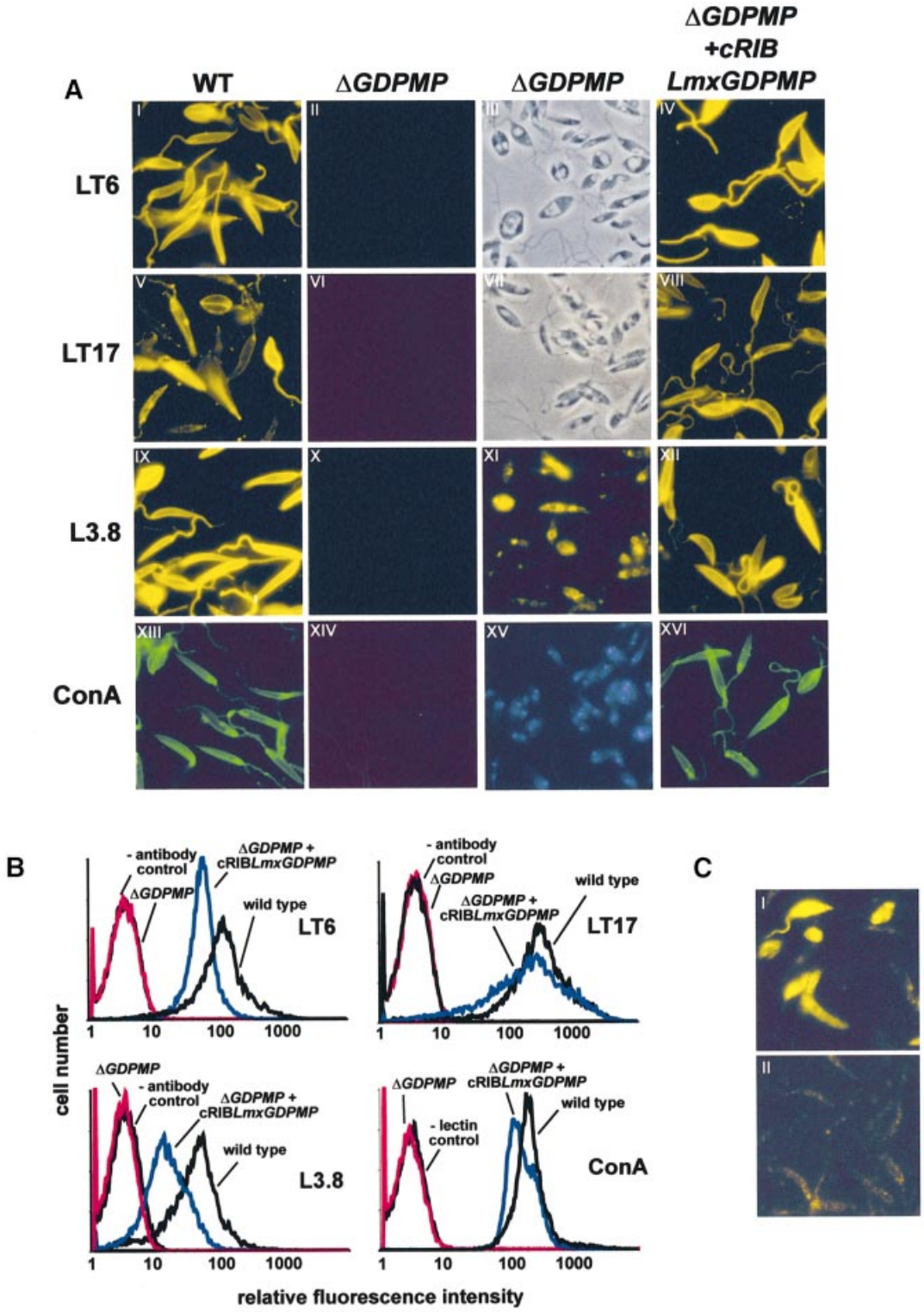

Fig. 5. Immuno-/lectin fluorescence microscopy and FACS analysis of *Leishmania* WT and mutant promastigotes. (**A**) Immuno-/lectin fluorescence microscopy of fixed *L.mexicana* promastigotes. The cells were not permeabilized, except for XI. The parasite lines and the mAbs/lectins used are indicated by the labeling of columns and rows. Exposure times within rows (A) are identical, except for XI, which is ~20× overexposed compared with IX. III and VII in (A) are phase-contrast light microscopy images of II and VI, respectively. (**B**) FACS analysis of live *L.mexicana* promastigotes. The parasite lines and the mAbs/lectins used are indicated in each panel. (**C**) IFM of saponin-permeabilized *L.mexicana* WT promastigotes labeled with rabbit anti-GDPMP serum (1:200) (I) and pre-immune serum (1:200) (II). Exposure times were identical in I and II.

show any signal, in fluorescence microscopy or FACS analysis, with concanavalin A, a lectin that strongly binds to α-Man residues present in *L.mexicana N*-glycans, LPG, PPGs and GIPLs (Ilgoutz *et al*., 1999; Ilg, 2000a,b; Ilg *et al*., 2001) (Figure 5A, XIII and XIV, and B). In contrast to the situation in *L.mexicana* Δ*PMI* promastigotes that

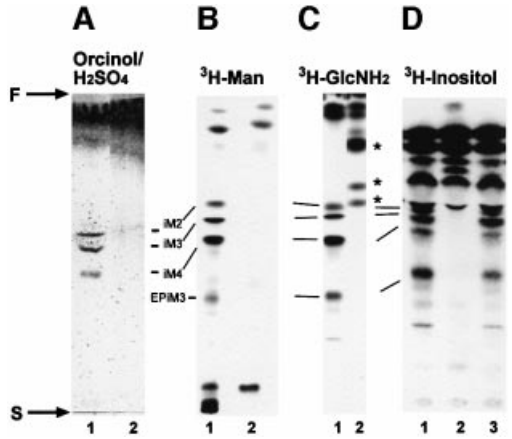

Fig. 6. Silica gel 60 HPTLC analysis of the predominant promastigote glycolipids of *L.mexicana* in WT (lane 1), Δ*GDPMP* (lane 2) and Δ*GDPMP* + cRIB*LmxGDPMP* (lane 3) parasites. (**A**) Total lipids from 2×10^8 promastigotes visualized by orcinol/H_2SO_4 spraying. (**B**) Fluorography of total lipids from 2.5×10^7 [^{3}H]Man-labeled promastigotes (~100 000 c.p.m.). (**C**) Fluorography of total lipids from 5×10^6 [^{3}H]GlcNH_2-labeled promastigotes (~100 000 c.p.m.). (**D**) Fluorography of total lipids from 5×10^6 [^{3}H]*myo*-inositol-labeled promastigotes (~100 000 c.p.m.). The positions of the abundant *L.mexicana* GIPLs (McConville *et al*., 1993) are indicated by the bars, and the start and front of the TLCs are marked by S and F, respectively. Asterisks mark new [^{3}H]GlcNH_2-labeled compounds accumulating in the Δ*GDPMP* mutant.

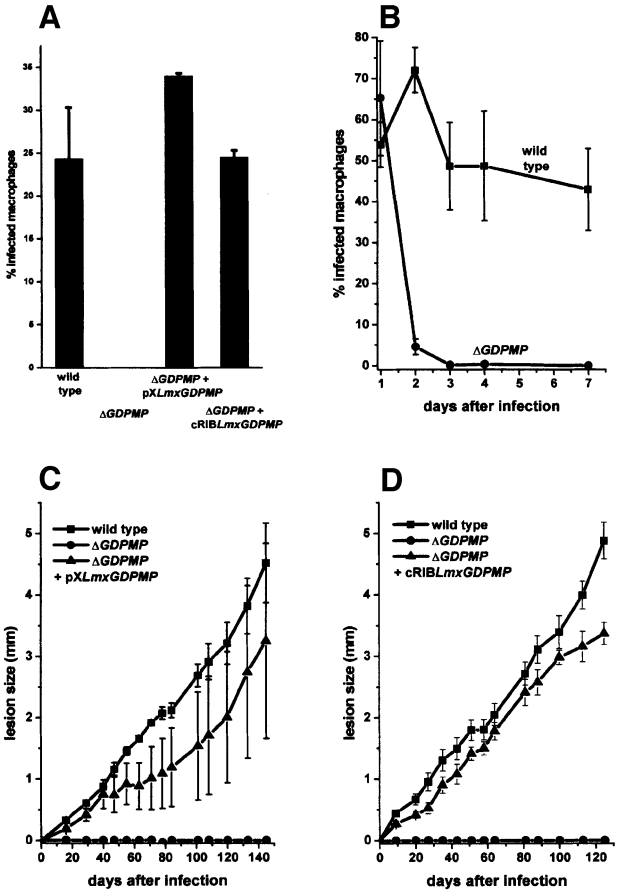

Fig. 7. Analysis of macrophage and mouse infections by *L.mexicana* WT, Δ*GDPMP* and *LmxGDPMP* gene addback mutants. (**A** and **B**) Infection of peritoneal macrophages by *L.mexicana* WT, Δ*GDPMP*, Δ*GDPMP* + pX*LmxGDPMP* and Δ*GDPMP* + cRIB*LmxGDPMP*. Peritoneal macrophages were infected at a ratio of two stationary phase promastigotes per cell. The percentage of infected macrophages (sample size 300) was counted 6 days (A) or at day 1, 2, 3, 4 and 7 (B) after the infection. The standard errors of duplicate experiments are indicated. In *in vivo* infection experiments, Balb/c mice were challenged with 1×10^7 *L.mexicana* promastigotes in the right hind footpad. The swelling caused by *L.mexicana* WT, Δ*GDPMP* and Δ*GDPMP* + pX*LmxGDPMP* (**C**), and Δ*GDPMP* + cRIB*LmxGDPMP* (**D**) was recorded. The infection experiments were performed in quadruplicates and the standard errors are indicated.

lack PMI activity (Garami and Ilg, 2001), the growth delay and the profound lipid and protein glycosylation defects in the Δ*GDPMP* mutant could not be rescued by addition of Man to the culture medium (Figure 4F and G, and data not shown). Finally, hexose analysis of trifluoroacetic acid (TFA)-hydrolyzed promastigote membranes by high-pH anion-exchange high-pressure liquid chromatography (HPLC) showed that Man is absent (<1%) from macromolecules of the *L.mexicana* Δ*GDPMP* mutant compared with wild-type parasites (Figure 4B), a result confirmed by hexose analysis using gas chromatography–mass spectrometry (<0.5% of wild-type levels; T.Ilg, A.Mehlert and M.A.J.Ferguson, unpublished results). The profound glycosylation defects in *L.mexicana* Δ*GDPMP* promastigotes were caused by the absence of the GDPMP gene. *LmxGDPMP* addback, either by gene integration into an rRNA gene locus (Figure 2B and C) or by introduction of episomal gene copies (pX, not shown), reconstituted the synthesis of all glycoconjugates investigated in this study (Figure 3A–C and F, lanes 3; Figure 3G, lanes 5 and 6; Figure 5A, IV, VIII, XII, XVI and B), although the level of enzyme re-expression was only ~10–20% of the wild-type levels (Figure 3A and data not shown). The reason for this lower level of GDPMP re-expression is not known. Possibly upstream or downstream elements of *LmxGDPMP* not present in the DNA constructs are required for optimal enzyme production.

Loss of virulence in L.mexicana ΔGDPMP mutants

Leishmania mexicana Δ*GDPMP* promastigotes were totally unable to establish an infection in cultured macrophages (Figure 7A). This inability was not due to a lack of attachment to and invasion of host cells, where the Δ*GDPMP* mutant proved to be as efficient as *L.mexicana* wild type (Figure 7B, 1 day after infection), but, as demonstrated by time course experiments of macrophage infections, the Δ*GDPMP* parasites were rapidly killed by the host cells, whereas wild-type parasites survived (Figure 7B) and proliferated (data not shown). Furthermore, *L.mexicana* Δ*GDPMP* promastigotes proved to be completely avirulent to Balb/c mice, even at a high parasite dose (1×10^7/mouse) (Figure 7C and D). Furthermore, repeated attempts to re-isolate Δ*GDPMP* parasites from the site of injection, the draining lymph nodes or the spleen of challenged animals were unsuccessful, which suggests the absence of persistent parasites. Virulence of Δ*GDPMP* mutants to macrophages and mice could be completely restored by the *LmxGDPMP* gene expressed from an episome or integrated into the rRNA locus (Figure 7A, C and D).

Discussion

Mutations that lead to a complete block in the Man activation pathway are invariably lethal in fungi, because *N*-glycosylation, *O*-mannosylation and GPI protein membrane anchor synthesis are essential processes in these organisms (Kapteyn *et al*., 1999; Knauer and Lehle, 1999; Strahl-Bolsinger *et al*., 1999). In mammalian cell lines, only dolicholphosphate mannose synthase (DPMS) mutants are known, which lack GPI protein membrane anchors and the outer Man residues in *N*-glycans, but still synthesize and transfer $Man_5GlcNAc_2$ to proteins (Chapman *et al*., 1980; Stoll *et al*., 1982; Sugiyama *et al*., 1991). Complete lack of any of the other Man pathway enzymes has never been observed in mammals and is expected to be lethal, mainly because a basal level of *N*-glycosylation appears to be essential in multicellular organisms (Stanley and Ioffe, 1995; Knauer and Lehle, 1999). This work is the first demonstration that a eukaryotic organism exists in which the complete disruption of the Man activation pathway and the concomitant loss of all known Man-containing glycoconjugates are compatible with viability in culture. *Leishmania mexicana* Δ*GDPMP* down-regulates expression of LPG, PPGs, Man-containing GIPLs, GPI-anchored proteins and Man-containing *N*-glycans to levels undetectable by a variety of methods, including immunoblotting, immunofluorescence microscopy (IFM), FACS analysis, metabolic labelings with [^{3}H]Man, [^{3}H]$GlcNH_2$ and [^{3}H]*myo*-inositol, and hexose analysis by two different methods. GDPMP activity is not detectable, and there is no evidence at present for the existence of an alternative pathway for GDP-Man synthesis. If such a hypothetical pathway exists in *L.mexicana*, it is extremely inefficient.

In agreement with the results of this study, we have succeeded in generating *L.mexicana* gene deletion mutants for all other known enzymes and transporters of the *L.mexicana* Man biosynthesis and activation pathway, which include the PMI (Garami and Ilg, 2001), PMM, DPMS (A.Garami, A.Mehlert and T.Ilg, submitted) and the GDP-mannose transporter LPG2 (Descoteaux *et al*., 1995; Ilg *et al*., 2001) (Figure 1A). These results suggest that, again in contrast to other eukaryotes (Kapteyn *et al*., 1999; Knauer and Lehle, 1999; Strahl-Bolsinger *et al*., 1999), in *L.mexicana* none of the mannosyltransferases involved in glycoconjugate synthesis is expected to be essential for viability in culture. Therefore, *L.mexicana* could be used as a unique model system for investigation of the biosynthesis and function of *N*-glycans, GPI protein anchors, phosphoglycans and GIPLs by a reverse genetics approach.

Deletion of the gene encoding UDP-GlcNAc:Dol-P-GlcNAc-1-P transferase has been reported to be lethal in several fungi, mammals and *L.mexicana* (Chen *et al*., 1999; Knauer and Lehle, 1999), indicating that a complete lack of *N*-glycosylation is not tolerated by eukaryotes. Therefore, the question arises whether Δ*GDPMP* parasites possibly transfer $(GlcNAc)_{1–2}$ from a truncated *N*-glycan precursor [e.g. Dol-PP-$(GlcNAc)_{1–2}$] to their proteins. The latter process has been observed in *in vitro N*-glycosylation assays, but has so far not been detected in living organisms due to the lethality of the mutations required for this phenotype (Knauer and Lehle, 1999).

While the simultaneous absence of LPG, PPGs, GPI-anchored proteins as well as Man-containing GIPLs and *N*-glycans does not grossly affect viability in culture, it appears likely that the capacity of *L.mexicana* Δ*GDPMP* to infect sandflies will be strongly impaired, as parasites with less severe glycosylation defects were unable to colonize these vector insects (Sacks *et al*., 2000). Based on the observation that deletion of the *lmx*DPMS gene is apparently lethal, it has been suggested recently that Man-containing GIPLs are essential for *L.mexicana* viability in culture (Ilgoutz *et al*., 1999). The results of the current study and our recent success in generating Δ*DPMS* mutants by double targeted gene replacement (A.Garami, A.Mehlert and T.Ilg, submitted) argue against this hypothesis. However, it will be important to determine whether Man-free GIPL biosynthetic intermediates like $GlcNH_2$-phosphatidylinositol accumulate in Δ*GDPMP* mutants, as suggested by our radioactive metabolic labeling experiments with [^{3}H]$GlcNH_2$ (Figure 6C), and whether these truncated GIPLs are displayed on the parasite cell surface.

Our observation that a lack of GDPMP (and the consequent absence of Man-containing glycoconjugates) leads to complete loss of *L.mexicana* virulence to macrophages and mice may be medically relevant. To our knowledge, a compound is considered a virulence factor if it is not essential for viability of a pathogen but is indispensable for its infectivity to the respective host organism. By this definition, GDPMP is a virulence factor. Our results define the Man metabolism of *Leishmania* as a virulence pathway, and suggest that the enzyme GDPMP may be a potential target for the design of new anti-*Leishmania* drugs.

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 semi-defined medium 79 (SDM) supplemented with 4% heat-inactivated fetal calf serum (iFCS) as described previously (Ilg *et al*., 1993). Infection of mice with 1×10^7 stationary phase promastigotes and infection of mouse peritoneal macrophages were performed as outlined earlier (Ilg, 2000b). Growth curves of *L.mexicana* WT and mutants were obtained by seeding SDM/4% iFCS with 2×10^6 promastigotes and counting the parasite numbers at 8–24 h intervals. In some experiments, the medium was supplemented with various concentrations of Man (20 μM–2 mM).

Cloning of the L.mexicana LmxGDPMP gene, generation of gene knockout and gene addback mutants, heterologous expression of GDPMP and generation of antibodies

DNA techniques were performed as described previously (Ilg *et al*., 1999). A 568 bp fragment of the *L.mexicana* GDPMP gene (*LmxGDPMP*) was obtained from *L.mexicana* genomic DNA by PCR using the degenerate primers CC(A/G/C/T)ATGAT(A/C/T)(CT)T(A/G/C/T)CA(C/T)CA(A/G)(AG)T(A/G/C/T)GA and A(AG)TC(CT)TT(A/G/C/T)GG(A/G/C/T)CC(A/G/C/T)AC(AG)TCCATCCA, which were derived from the conserved *S.cerevisiae* (DDBJ/EMBL/GenBank accession No. U24437) and *C.albicans* (DDBJ/EMBL/GenBank AF030299) GDPMP peptide sequences PMILHQIE and WMDVGQPKDY/F. The PCR product was subcloned into pGEM-T (Promega) and sequenced. The DIG-labeled PCR product was used to screen a λ-DashII library (Wiese *et al*., 1996) 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) as described earlier (Ilg *et al*., 1999). The ORF corresponding to *LmxGDPMP* was identified by homology to known GDPMP genes in

the database and by determination of the spliced leader site (Ilg *et al*., 1999). The sequence data for the *LmxGDPMP*-containing genomic DNA fragment have been submitted to the EMBL database under accession No. AJ292039. Double targeted gene replacement was performed by PCR amplification of the 5′-untranslated region (5′-UTR) of *LmxGDPMP* using the primers KO1 (AATGCGGCCGCGTTAACGACTCAAC-CAAAATG) and KO2 (AGTACTAGTCTTGTCGGTTGAGATAGAG), and by amplification of the 3′-UTR of *LmxGDPMP* using the primers KO3 (AGTACTAGTAGATCTTGCTGCATCGACGGGG) and KO4 (CTTAAGCTTGTTAACAGAAGGGAGATGGG). The *Not*I–*Spe*I-cut *LmxGDPMP* 5′-UTR PCR DNA fragment, the *Bgl*II–*Hin*dIII-cut *LmxGDPMP* 3′-UTR PCR DNA fragment and a *Spe*I–*Bam*HI DNA fragment containing a hygromycin phosphotransferase gene (*HYG*) (Cruz *et al*., 1991) were ligated consecutively into pBSK$^+$. For the second *LmxGDPMP* gene replacement cassette, a *Spe*I–*Bam*HI fragment encoding bleomycin binding protein gene (*BLE*) was used (Ilg, 2000b). The *HYG*- and *BLE*-containing *LmxGDPMP* gene replacement cassettes were excised from the plasmids by *Hpa*I digestion, and transfected into *L.mexicana* promastigotes as previously described (Ilg *et al*., 1999). Selection on 96-well microtiter plates and analysis of positive clones were performed as outlined earlier (Ilg, 2000b). *LmxGDPMP* 5′-UTR DNA and ORF probes were generated either by PCR using a PCR-DIG labeling kit (Roche) or by random priming (DIG-high prime; Roche). For gene addback and heterologous expression studies, the ORF of *LmxGDPMP* was amplified from an *LmxGDPMP* gene-containing plasmid using the primers GDPMPORF1 (ACCGGTACCGCGGATGTCTGCATCCGA-TGGCCAGG) and GDPMPORF2 (CCGCGGTACCCGGGTTACAT-GATGATCCCAGCCTGC), and cloned into pGEM-T (Promega). The accuracy of the cloned PCR amplicon was checked by sequencing. Episomal gene addback was achieved by cloning the *Sac*II–*Xma*I-cut *LmxGDPMP* ORF into pX (LeBowitz *et al*., 1990) and transfection of *L.mexicana* ΔGDPMP promastigotes with this construct as described earlier (Ilg *et al*., 1999). Transfectants were selected by growth in SDM/4% iFCS containing 10–50 μg/ml G418 (Roche). Alternatively, the *LmxGDPMP* gene was expressed under the control of the rRNA promoter by first cloning it into pRIB (Garami and Ilg, 2001). The *LmxGDPMP* ORF (see above) was excised with *Age*I and *Xma*I, and ligated into *Age*I-cut pRIB, yielding pRIBLmxGDPMP. For chromosomal integration into the ribosomal locus of *L.mexicana*, the integration cassette was excised by digestion with *Pac*I and *Pme*I (Figure 2B), gel purified and transfected into *L.mexicana*. Recombinant clones were isolated by limiting dilution on 96-well plates in SDM medium containing 20 μg/ml hygromycin, 2.5 μg/ml bleomycin and 20 μM puromycin. Isolation of total RNA from *L.mexicana* promastigotes and mouse lesion-derived amastigotes, as well as northern blot analysis, were performed as described earlier (Göpfert *et al*., 1999). RT–PCR was performed with the Titan one tube system (Roche) using the primer pairs CCGTACTCGTTTTTTCAGCAGCAAC/AGTGGAGCGGTAAAGTGAACTTCTC for reverse transcription/amplification of the control *LmxPPG2* mRNAs (Göpfert *et al*., 1999) and TCACCAAATTGAGGCATTGAAGGCG/ATCTGGTAACTCTGCTG-CGAGTAGA for the *LmxGDPMP* mRNAs.

High-level expression of *L.mexicana* GDPMP in *Escherichia coli* M15 as inclusion bodies was achieved by cloning the *Kpn*I–*Xma*I-cut *LmxGDPMP* PCR fragment (primers: GDPMPORF1/GDPMPORF2) into pQE31, followed by transformation of the bacteria. Inclusion bodies were solubilized in 8 M urea, and denatured GDPMP was purified by Ni-NTA–agarose chromatography as described by the manufacturer (Qiagen). Rabbits were immunized with 200 μg of purified recombinant protein, which was dissolved in 8 M urea, 50 mM NaH_2PO_4 pH 4.8 and emulsified with 50% (v/v) complete Freund's adjuvant for primary immunizations and with 50% incomplete Freund's adjuvant (v/v) for all subsequent booster immunizations. Serum was obtained 10–14 days after each booster immunization. The antiserum was affinity purified on recombinant protein that had been electrotransferred to PVDF membranes after discontinuous SDS–PAGE, as described earlier (Ilg *et al*., 1999).

Analytical procedures

Production of SDS-cell lysates, discontinuous SDS–PAGE, immunoblotting using the mAbs LT6, LT17 and L7.25 {directed against $[PO_4$-6Galβ1-4Manα1-$]_x$, $[PO_4$-6(Glcβ1-3)Galβ1-4Manα1-$]_x$ (x = unknown) and $[Manα1\text{-}2]_{0\text{-}2}Manα1\text{-}PO_4$, respectively} (Ilg *et al*., 1993), affinity-purified rabbit anti-*L.mexicana* SAP antibodies (Garami and Ilg, 2001), anti-*L.mexicana* MBAP antibodies (Wiese *et al*., 1996), anti-CRD antibodies (Oxford Glycosystems), anti-*L.mexicana* PMM antibodies (A.Garami, A.Mehlert and T.Ilg, submitted) and anti-*L.mexicana* GMP antibodies (this study) as well as acid phosphatase enzyme assays were all performed as described earlier (Ilg, 2000b). Stripping of antibodies from immunoblots for reprobing was performed by three 15 min washes in 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 8 M urea, 100 mM 2-mercaptoethanol at 65°C. GPI-PLC digestion of *Leishmania* total cell lysates was performed as described previously (Ilg *et al*., 1999). Total lipids from washed *L.mexicana* promastigotes were obtained by two extractions with $CHCl_3/CH_3OH/H_2O$ (4:8:3). HPTLC (Silica60; Merck, Germany) of total lipids was performed as described earlier (McConville *et al*., 1993) using the solvent $CHCl_3/CH_3OH$/1 M NH_4OH (10:10:3). Glycolipids on HPTLC plates were selectively stained by orcinol/H_2SO_4 spraying. *Leishmania mexicana* promastigotes were metabolically labeled by incubating 5×10^7 cells/ml overnight at 27°C with either 10 μCi/ml [^{3}H]*myo*-inositol, 20 μCi/ml [^{3}H]$GlcNH_2$ or 50 μCi/ml 2-[^{3}H]Man (Hartmann Analytics) in *myo*-inositol-, Glc/$GlcNH_2$- or Glc/Man-free SDM medium, respectively. In labelings with [^{3}H]*myo*-inositol and [^{3}H]$GlcNH_2$, the lipid extracts were further purified by 1-butanol/H_2O phase separation (McConville *et al*., 1993). Radioactively labeled lipids of the 1-butanol phase were separated by HPTLC and detected by spraying with ^{3}H-EnHance (Dupont), followed by fluorography. [^{3}H]*myo*-inositol-labeled delipidated cells were incubated with benzonuclease to cleave nucleic acids (Ilg *et al*., 1999) and then separated by SDS-PAGE. Labeled compounds in acrylamide gels were detected by immersion of the polyacrylamide gel in Amplify™ (Amersham-Pharmacia), followed by drying and fluorography.

For hexose analysis, 5×10^8 promastigotes were washed three times with phosphate-buffered saline, and lysed by resuspension in 1 ml of H_2O and sonication. After centrifugation at 10 000 *g* for 30 min, the membrane-containing pellet was resuspended in 300 μl of 2 M TFA and hydrolysed for 2.5 h at 100°C. After evaporation of the 2 M TFA, the samples were resuspended in 1 ml of H_2O, delipidated by passage through a Sep-Pac C18 column (Waters) and lyophilized. The hexoses of samples equivalent to 1×10^8 promastigotes were analysed by high-pH anion-exchange HPLC using a Carbo-Pac PA10 column equilibrated in 20 mM NaOH at a flow rate of 1 ml/min and pulsed amperometric detection.

Enzyme assays and partial purification of L.mexicana GDPMP

Enzyme assays were performed at room temperature in 1 ml of 50 mM triethylamine–HCl pH 7.0, 0.1 mM EDTA, 2.5 mM $MgCl_2$ and 0.1% bovine serum albumin. For GDPMP assays, this buffer was supplemented with 0.5 mM NADP$^+$ (Roche), 1 mM 2-mercaptoethanol, 1 mM glucose, 1 mM ADP, 1 mM Na-pyrophosphate, 0.5 U/ml nucleoside diphosphate kinase (Sigma), 1 U/ml hexokinase (Sigma) and 2 U/ml glucose-6-phosphate dehydrogenase (Roche). After addition of sample (2.5–20 μl), the rate of background reactions, as indicated by the increase in absorbance at 340 nm, was recorded for 10 min. The specific GDPMP assay was initiated by the addition of GDP-Man to a final concentration of 100 μM and recorded for 2–5 min. UDPGP was measured in assay buffer with the addition of 0.5 mM NADP$^+$ (Roche), 1 mM 2-mercaptoethanol, 1 mM Na-pyrophosphate, 0.5 mM UDP-Glc, 1 U/ml phosphoglucomutase (Roche) and 2 U/ml glucose-6-phosphate dehydrogenase. UDPGP assays were started by the addition of 2.5–10 μl of cell lysates or column fractions and the absorbance at 340 nm was recorded for 1–10 min. One unit of enzyme activity is defined as the amount of enzyme converting 1 μmol substrate/min into the respective product.

To generate *L.mexicana* total cell lysates, 2×10^{10} promastigotes were resuspended in 8 ml of cold homogenization buffer (50 mM triethylamine–HCl pH 7.0, 150 mM NaCl, 1 mM 2-mercaptoethanol, 5 mM *O*-phenanthroline, 20 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride), sonicated on ice and centrifuged at 100 000 *g* for 30 min. The ultracentrifugation supernatant containing GDPMP and UDPGP was concentrated to ~1 ml by Centricon 10 ultrafiltration, and 200 μl aliquots were loaded onto a Superose 6 column (Amersham Pharmacia) pre-equilibrated with 50 mM triethylamine–HCl pH 7.0, 150 mM NaCl, 1 mM 2-mercaptoethanol, eluted in the same buffer at 0.5 ml/min, and collected with a fraction size of 0.5 ml. The Superose 6 column was calibrated with the size markers dextran blue (2000 kDa), thyroglobulin (667 kDa), catalase (240 kDa), ovalbumin (43 kDa), cytochrome *c* (12.5 kDa) and CTP as marker for the total liquid volume (V_t) of the column. The preparation of promastigote lysates, soluble fractions and washed membranes for SDS–PAGE/immunoblot analysis was performed as described earlier (Ilg *et al*., 1999).

IFM and FACS of Leishmania promastigotes and infected macrophages

IFM and FACS studies on *Leishmania* promastigotes and infected macrophages were performed as described previously (Stierhof *et al*.,

1994; Ilg, 2000b) using 4′,6-diamidino-2-phenylindole staining and the mAbs (Ilg *et al*., 1993) LT6, L7.25 and LT17 (see above), mAb L3.8 directed against a polypeptide epitope of *L.mexicana* leishmanolysin/gp63, and the biotinylated lectins concanavalin A and ricin$_{120}$ (Sigma). The mAbs were diluted 1:2–1:10 (hybridoma supernatant) or 1:500–1:2000 (ascites fluid) and the lectins were used at 10 μg/ml. Bound mAbs and the biotinylated lectin were detected by incubation with Cy3-labeled goat anti-mouse IgG/IgM (1:250; Dianova) and fluorescein isothiocyanate-labeled streptavidin (1:250; Sigma), respectively.

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