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Lipophosphoglycan is not required for infection of macrophages or mice by *Leishmania mexicana*

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Cell surface lipophosphoglycan (LPG) is commonly regarded as a multifunctional *Leishmania* virulence factor required for survival and development of these parasites in mammals. In this study, the LPG biosynthesis gene *lpg1* was deleted in *Leishmania mexicana* by targeted gene replacement. The resulting mutants are deficient in LPG synthesis but still display on their surface and secrete phosphoglycan-modified molecules, most likely in the form of proteophosphoglycans, whose expression appears to be up-regulated. LPG-deficient *L.mexicana* promastigotes show no significant differences to LPG-expressing parasites with respect to attachment to, uptake into and multiplication inside macrophages. Moreover, in Balb/c and C57/BL6 mice, LPG-deficient *L.mexicana* clones are at least as virulent as the parental wild-type strain and lead to lethal disseminated disease. The results demonstrate that at least *L.mexicana* does not require LPG for experimental infections of macrophages or mice. *Leishmania mexicana* LPG is therefore not a virulence factor in the mammalian host.

Keywords: *Leishmania*/lipophosphoglycan/proteophosphoglycan/macrophage/virulence

Introduction

Leishmania are protozoan parasites that cause a number of important human diseases ranging from cutaneous lesions to fatal visceral infection. These pathogens have a digenetic life cycle that alternates between the colonization of the vector sandfly digestive tract by extracellular flagellated promastigotes, and intracellular parasitism of mammalian macrophages by non-motile amastigotes. Transmission occurs during the bite of a mammal by a sandfly, where infectious metacyclic promastigotes are injected into the skin. After uptake by macrophages, the metacyclic promastigotes transform to amastigotes which multiply within a phagolysosome and, after release by an unknown mechanism, infect other macrophages (Alexander and Russell, 1992). *Leishmania* are most remarkable in their ability to circumvent or resist various innate and acquired immune mechanisms of the mammalian host (Reiner and Locksley, 1995). Both metacyclic promastigotes and amastigotes are largely resistant to complement lysis (Mosser *et al*., 1985; Sacks, 1989). Inside the macrophage phagolysosome, the two *Leishmania* mammalian forms avoid or inhibit the production of toxic NO and/or O_2^- (Liew *et al*., 1990; Murray and Nathan, 1999), they resist acidic pH (Antoine *et al*., 1990) and the action of hydrolytic enzymes (Prina *et al*., 1990) and other microbiocidal activities of their host cell.

Molecules and mechanisms allowing these parasites not only to survive, but actually to thrive in such hostile environments have been a main topic of interest in *Leishmania* reseach. In particular, the major cell surface glycoconjugate ($1–5 \times 10^6$/cell) of all *Leishmania* promastigotes, a unique molecule called lipophosphoglycan (LPG), has been a focus of intense research over the past 15 years. This complex glycolipid is organized in four domains: a conserved 1-*O*-alkyl-2-*lyso*-phosphatidyl(*myo*)inositol membrane anchor, a conserved diphosphoheptasaccharide core structure, repeating phosphodisaccharide units carrying species-specific side chains and variable, often mannose-rich cap structures (McConville and Ferguson, 1993; see Figure 1A). In the mammalian host, LPG has been proposed to mediate promastigote complement resistance (Sacks, 1989) as well as attachment and uptake of promastigotes by macrophages (Handman and Goding, 1985; Da Silva *et al*., 1989; Talamas-Rohana *et al*., 1990). It has been suggested that LPG may protect the invading promastigotes against macrophage phagolysosome hydrolases (Turco and Descoteaux, 1992) and against the respiratory burst (Chan *et al*., 1989). LPG modulates macrophage signal transduction pathways by inhibition of protein kinase C (reviewed in Descoteaux and Turco, 1993; Giorgione *et al*., 1996) and, possibly, by chelation of intracellular calcium (Eilam *et al*., 1985). The marked suppression of interleukin (IL)-1β and tumour necrosis factor-α gene expression in LPG-treated macrophages (Hatzigeorgiou *et al*., 1996) may be a consequence of these effects. Furthermore, LPG inhibits phagosome–endosome fusion in the macrophage after invasion of promastigotes (Desjardins and Descoteaux, 1997) and reduces endothelial adhesion and transendothelial migration of monocytes (Ho *et al*., 1996; Lo *et al*., 1998), processes believed to be involved in survival and spreading of the parasites. Finally, it was shown that LPG down-regulates expression of inducible NO synthase (iNOS) in macrophages and synthesis of macrophage IL12, which are both agents crucial for control of *Leishmania* infections (Proudfoot *et al*., 1996; Piedrafita *et al*., 1999). This large number of functional studies led to the concept of LPG as a multifunctional virulence determinant required for establishment of *Leishmania* infections in the mammalian host (Turco and Descoteaux, 1992; Beverley and Turco, 1998; Descoteaux and Turco, 1999). The fact that LPG-deficient mutants either identified fortuitously (*L.major* LRC-L119, Handman *et al*., 1986) or obtained by chemical mutagenesis (various *L.donovani* and *L.major* lines; King and Turco, 1988; Elhay *et al*., 1990; McNeely

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and Turco, 1990) were non-infective to macrophages or mice strengthened this view further. However, the exact defects in some of these mutants are not known, and chemical mutagenesis is likely to lead to multiple mutations. Furthermore, some LPG-deficient *L.donovani* mutants (Descoteaux *et al*., 1995, 1998) have defects that affect not only LPG biosynthesis, but also the synthesis of *Leishmania* proteophosphoglycans (PPGs; reviewed in Ilg *et al*., 1999a). In addition, it has not yet been reported for any of these mutants that restoration of LPG expression leads to restoration of infectivity to macrophages or mice (Beverley and Turco, 1998).

The biosynthesis of LPG has attracted considerable interest, and several enzymes and transporters have been identified biochemically, genetically or both (reviewed in Mengeling *et al*., 1997). One of the enzymes essential for LPG biosynthesis is LPG1, a putative β-galactofuranosyl (β-Gal_f) transferase localized in the Golgi apparatus, that is crucial for the addition of an unusual internal β-Gal_f residue to the LPG diphosphoheptasaccharide core (Huang and Turco, 1993). The gene encoding LPG1 (*lpg1*) was isolated by genetic complementation of the *L.donovani* mutant R2D2 (King and Turco, 1988; Ryan *et al*., 1993), which partially restored LPG expression in this LPG-deficient parasite line. In *L.mexicana*, no β-Gal_f-containing compounds other than the LPG core are known (McConville and Ferguson, 1993). Therefore, targeted deletion of *lpg1* in this species is expected to block LPG biosynthesis selectively, while leaving other glycan biosynthesis pathways unaffected. Such a defined mutant should allow a more definite assessment of the significance of LPG for virulence of *L.mexicana* promastigotes to cultured macrophages and to mice.

In this study, *L.mexicana lpg1* mutants were generated by targeted gene deletion. These mutants were unable to synthesize LPG, but still displayed a variety of phosphoglycan structures on their surface, most likely PPGs. Surprisingly, the LPG-deficient mutants were impaired neither in binding and uptake by macrophages, nor in their survival, transformation and multiplication in host cell phagolysosomes. Despite the lack of LPG expression, large amounts of phosphoglycan-modified compounds were still synthesized and released by the parasites in infected host cells. Moreover, in mouse infection experiments, the LPG-deficient *L.mexicana* mutants were as virulent or even more virulent than their parental wild-type *L.mexicana* strain. The results suggest that in experimental infections with *L.mexicana*, LPG is not a virulence factor.

Results

Targeted gene replacement of the L.mexicana lmexlpg1 gene

The *lmexlpg1* gene was isolated by homology cloning from *L.mexicana* genomic DNA using a fragment as a probe that was amplified from the same DNA by PCR with primers derived from the known sequence of *L.donovani lpg1* (Ryan *et al*., 1993). The deduced protein sequence of LPG1 from both species is 83% identical and 87% similar, with conservation of the putative 20 residue membrane-spanning sequence, the region with similarity to mannose-binding proteins and three out of four N-glycosylation sites (Figure 1B; Ryan *et al*., 1993). Southern blot analysis

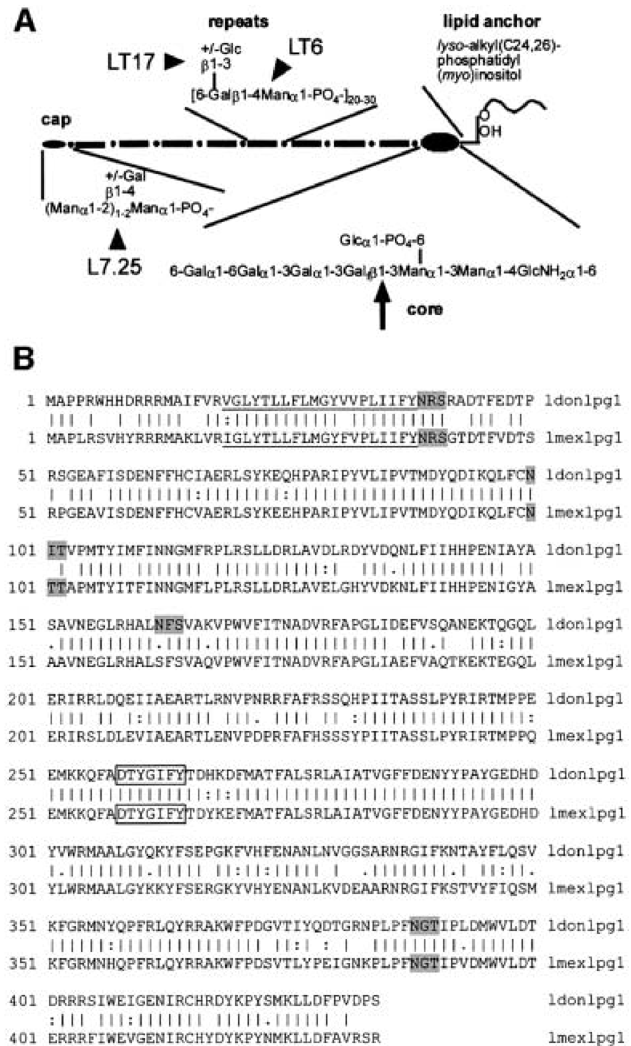

Fig. 1. (**A**) Structure of *L.mexicana* LPG (Ilg *et al*., 1992). The site of the defect caused by the deletion of *lpg1* (Huang *et al*., 1993) and the putative binding sites of anti-phosphoglycan mAbs used in this study (Ilg *et al*., 1993) are indicated by an arrow and arrowheads, respectively. (**B**) Alignment of *L.mexicana* LPG1 with *L.donovani* LPG1 (Ryan *et al*., 1993). The putative membrane anchor sequence is underlined, the region similar to mannose-binding proteins boxed and the potential N-glycosylation sites are shaded in grey.

showed that *lmexlpg1* is a single copy gene (Figure 2B), which is advantageous for efficient generation of gene deletion mutants. Two rounds of targeted gene replacement (Figure 2A) resulted in several clones lacking the *lmexlpg1* open reading frame (ORF) (Figure 2C and D; data not shown). All clones showed normal growth behaviour in culture compared with their parental wild-type strain (data not shown).

Expression of phosphoglycans by lmexlpg1 gene deletion mutants

Immunoblots of total cell lysates from three independent *lmexlpg1* gene deletion mutants using the anti-$[PO_4\text{-}6Gal\beta1\text{–}4Man\alpha1\text{-}]_x$ repeat monoclonal antibody (mAb) LT6 suggested that they lacked LPG (Figure 3A). This was confirmed by attempts to purify LPG from such mutants, which resulted in no enrichment of this glycolipid, as judged by SDS–PAGE/immunoblots (Figure 3E and F) and chemical staining after SDS–PAGE with the

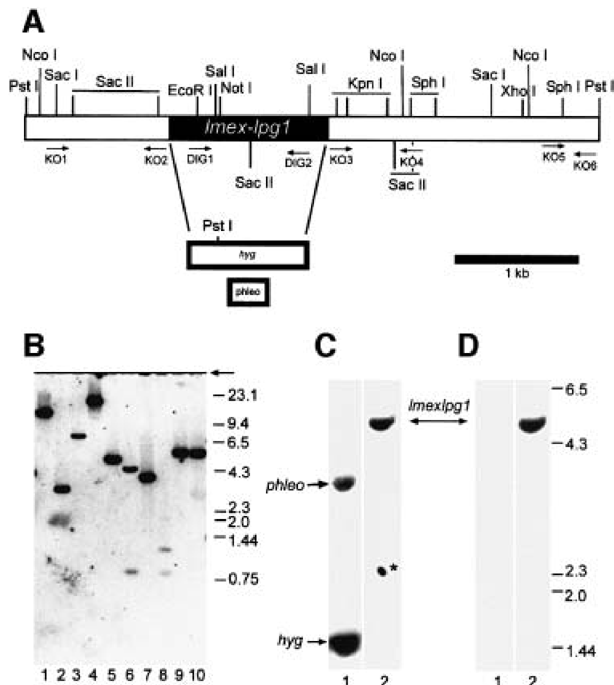

Fig. 2. Targeted gene replacement of the *lmexlpg1* alleles. (**A**) Restriction map of the *lmexlpg1* locus. Restriction sites relevant for Southern blot analysis, the resistance genes and the primer-binding sites for the construction of gene deletion cassettes and for the generation of DIG-labelled probes are indicated. (**B**) Southern blot analysis of restriction enzyme-digested *L.mexicana* chromosomal DNA (5 μg) resolved on a 0.7% agarose gel using a labelled *lmexlpg1* gene probe. Lane 1, *Hin*dIII; lane 2, *Nco*I; lane 3, *Kpn*I; lane 4, *Pvu*II; lane 5, *Pst*I; lane 6, *Sal*I; lane 7, *Sac*I; lane 8, *Sac*II; lane 9, *Sph*I; lane 10, *Xho*I. The size of DNA standards is indicated in kb. (**C** and **D**) Southern blot analysis of *L.mexicana* Δ*lmexlpg1* mutants. Genomic DNA was digested with *Pst*I, separated on a 0.7% agarose gel, blotted and probed with a labelled *lmexlpg1* 5′-UTR fragment (C) or an *lmexlpg1* ORF probe (D). Lanes 1, *L.mexicana* Δ*lmexlpg1*, clone I/8D; lanes 2, *L.mexicana* wild type. The asterisk depicts a background spot due to antibody aggregates.

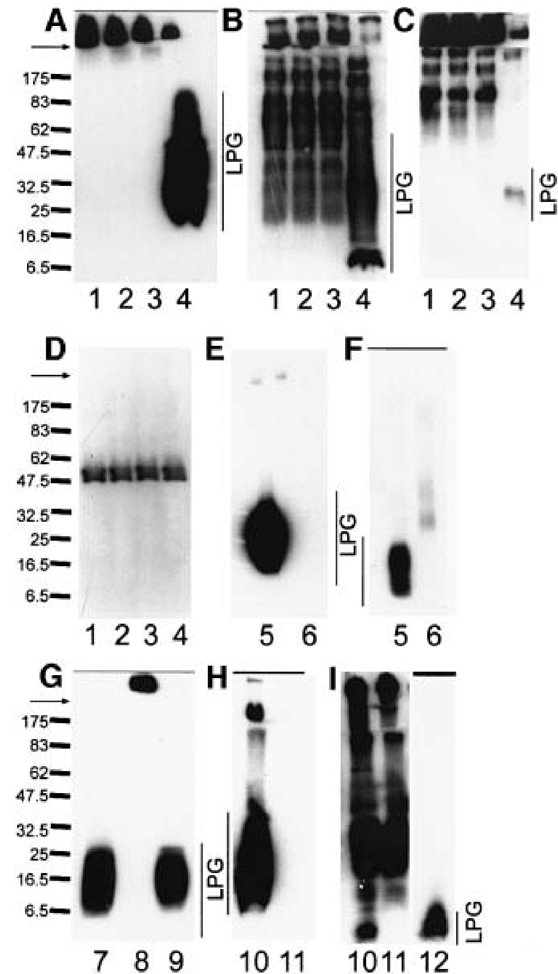

Fig. 3. SDS–PAGE/immunoblot analysis of *L.mexicana* and *L.donovani* LPG-deficient mutants. (**A**–**D**) Total promastigote lysates (100 μg protein). Lane 1, *L.mexicana* Δ*lmexlpg1*, clone I/8D; lane 2, *L.mexicana* Δ*lmexlpg1*, clone II/5C; lane 3, *L.mexicana* Δ*lmexlpg1*, clone IV/2B; lane 4, *L.mexicana* wild type. The blots were probed with the mAbs LT6 (A), L7.25 (B) and LT17 (C). Equal loading of lanes 1–4 was confirmed by probing a blot with the anti-leishmanolysin/gp63 mAb L3.8 (D). (**E**–**F**) LPG from 10^8 promastigotes enriched by solvent extraction (McConville *et al*., 1990). Lane 5, *L.mexicana* wild type; lane 6, *L.mexicana* Δ*lmexlpg1*, clone I/8D. The blots were probed with LT6 (E) and L7.25 (F). (**G**) Total promastigote lysates (100 μg protein) digested with proteinase K in SDS–PAGE sample buffer (200 μg/ml final concentration, 15 min, 37°C). Lane 7, *L.mexicana* wild type; lane 8, *L.mexicana* Δ*lmexlpg1*, clone I/8D; lane 9, *L.mexicana* Δ*lmexlpg1*, clone I/8D + *pXlmexlpg1*. The blot was probed with mAb LT6. (**H** and **I**) Total promastigote lysates (100 μg of protein). Lane 10, *L.donovani* wild type; lane 11, *L.donovani* R2D2; lane 12, purified *L.donovani* LPG (2.5 μg), for reference. The blots were probed with mAb LT6 (H) or L7.25 (I). Arrows mark the border between the stacking and the separating gel. The molecular masses of standard proteins are indicated in kDa.

dye Stains-all (not shown). Likewise, the strong *L.mexicana* promastigote surface labelling by LT6 that can be observed in immunofluorescence was drastically diminished (Figure 4A and B) and, in fluorescence activated cell sorting (FACS) analysis of live cells, the fluorescence intensity was decreased at least 100-fold (Figure 5B). Similar results were obtained in labellings with ricin (not shown), a lectin known to bind strongly to the β-Gal-containing cap structures of many LPGs (King and Turco, 1988; T.Ilg, unpublished results). Episomal addback of the *lmexlpg1* gene to mutant promastigotes showed that the LPG-negative phenotype was due to the gene deletion, because it resulted in parasites that re-expressed LPG (Figure 3G) that was displayed on the cell surface at wild-type levels (Figures 4D and 5E). Cell to cell variability of LPG expression in the complemented mutants was higher than in wild-type cells (Figure 4D), a phenomenon previously also observed in pX-plasmid-driven expression of a membrane-bound acid phosphatase in *L.major* (Ilg *et al*., 1999b). Possibly, the copy number of the expression vector pX is not uniform in a *Leishmania* population. However, the LPG-negative mutants were not completely devoid of LT6 epitopes. A compound strongly reactive with this mAb was present in SDS–PAGE/immunoblots in the stacking gel region and appeared to be up-regulated in the LPG-deficient mutants compared with the wild-type parasites (Figure 3A). The electrophoretic migration of this compound was reminiscent of filamentous PPG (fPPG) secreted via the flagellar pocket (Ilg *et al*., 1999a) and of membrane-bound PPG (mPPG) present on the surface of *L.major* promastigotes (Ilg *et al*., 1999b). Indeed, a strong flagellar pocket signal was observed in immunofluorescence labelling of LPG-deficient *L.mexicana* promastigotes with LT6 (Figure 4B and C), and longer photographic exposure revealed a spotty

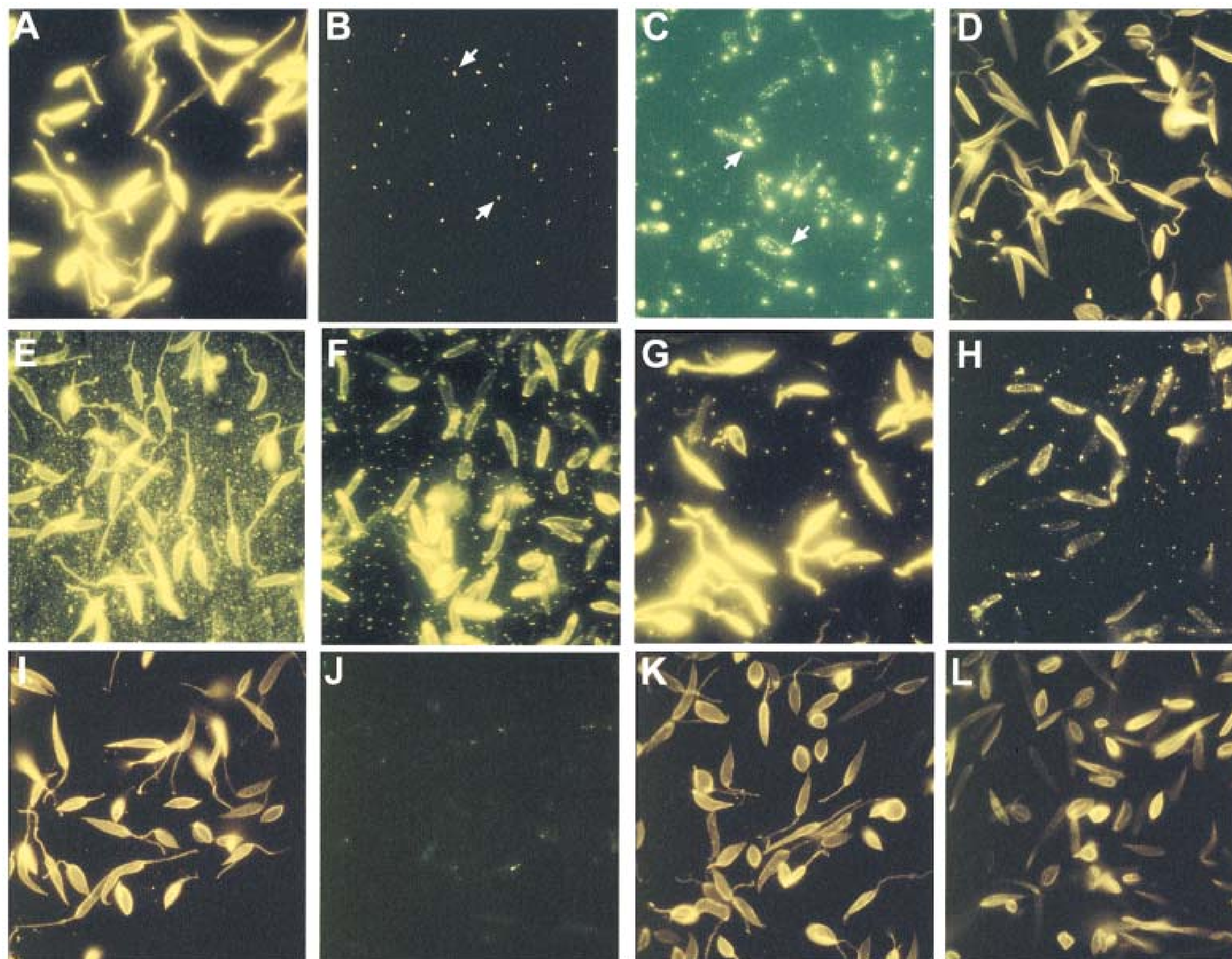

Fig. 4. Immunofluorescence labelling of *L.mexicana* and *L.donovani* LPG-deficient mutants using anti-phosphoglycan mAbs. (**A**–**D**) mAb LT6. (A) *L.mexicana* wild type; (B) *L.mexicana* Δ*lmexlpg1*, clone I/8D at the same exposure time as in (A). (C) *L.mexicana* Δ*lmexlpg1*, clone I/8D exposed 10 times longer than in (A). Flagellar pockets are indicated by arrows. (D) *L.mexicana* Δ*lmexlpg1*, clone I/8D + pX*lmexlpg1* exposed for half the time in (A). (**E** and **F**) mAb L7.25. (E) *L.mexicana* wild type; (F) *L.mexicana* Δ*lmexlpg1*, clone I/8D at the same exposure time as in (E). (**G** and **H**) mAb LT17. (G) *L.mexicana* wild type; (H) *L.mexicana* Δ*lmexlpg1*, clone I/8D at the same exposure time as in (G). (**I** and **J**) mAb LT6. (I) *L.donovani* wild type; (J) *L.donovani* R2D2 at 10 times the exposure time in (I). (**K** and **L**) mAb L7.25. (K) *L.donovani* wild type; (L) *L.donovani* R2D2 at the same exposure time as in (K).

cell surface signal on many promastigotes that was intense on 10–20% of the cells (Figure 4C). Likewise, in FACS analysis with mAb LT6, the surface signal on LPG-deficient *L.mexicana* promastigotes was markedly above background fluorescence for a subfraction of the cells (Figure 5E). The high molecular weight phosphoglycan compounds present in LPG-deficient *L.mexicana* promastigotes could also be detected with the anti-[Manα 1–2]$_{0-2}$Manα1-PO_4] mAb L7.25 (Figure 3B) as well as with mAb LT17 (Figure 3C), most likely directed against [PO_4-6(Glcβ1–3)Galβ1–4Manα1-]$_x$ epitopes (Figure 1A). These anti-phosphoglycan antibodies, however, also recognized a variety of other *L.mexicana* proteins whose expression was either unaffected or even up-regulated in *lmexlpg1* deletion mutants (Figure 3B and C). At least some of these phosphoglycosylated proteins are displayed on the surface of *L.mexicana* LPG-deficient promastigotes (Figure 4F and H). FACS analyses suggest that the surface epitope density for L7.25 (Figure 5C) is similar to or even slightly elevated compared with *L.mexicana* wild type, while for LT17, a decrease of mAb binding by a factor of 5, but still well above background, was noted (Figure 5D). This decrease in LT17 binding could be reversed to wild-type levels by episomal expression of *lmexlpg1* (Figure 5F). Taken together these results suggest that in the absence of LPG, *L.mexicana* promastigotes still display abundant phosphoglycan structures on their surface, in particular manno-oligosaccharide caps, but also, to a lesser degree, repeats. Except for the putative fPPG/mPPG in the stacking gel (Figure 3G), the bulk of these non-LPG phosphoglycans are sensitive to digestion with proteinase K (not shown), suggesting that they are phosphoglycosylated proteins. In FACS analysis, the anti-leishmanolysin/gp63 mAb L3.8 (Ilg *et al.*, 1993) recognizes more epitopes on LPG-deficient *L.mexicana* promastigotes (Figure 5A) despite the fact that expression levels on immunoblots appeared to be unaltered compared with the wild type (Figure 3D). The absence of the bulky glycolipid LPG may improve the accessiblity of the parasite surface to antibodies (Karp *et al.*, 1991). A remarkable observation is the restriction of surface-exposed phosphoglycosylated proteins to the cell body of the parasites while they appear to be absent from the flagella (Figure 4C, F and H), in contrast to the distribution

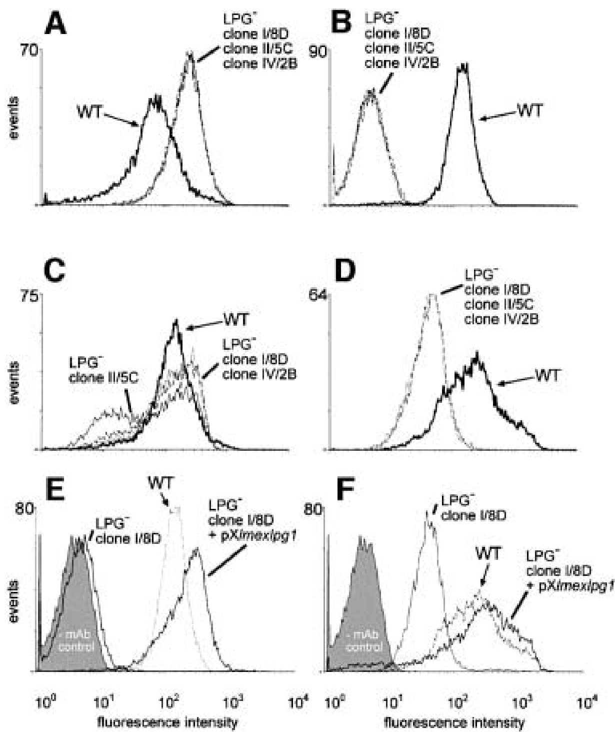

Fig. 5. FACS analysis of live *L.mexicana* wild-type (WT), LPG-deficient mutant (LPG⁻) and episomal *lmexlpg1* addback (LPG⁻ + pX*lmexlpg1*) promastigotes. (**A**) mAb L3.8; (**B**) mAb LT6; (**C**) mAb L7.25; (**D**) mAb LT17; (**E**) mAb LT6; (**F**) mAb LT17. The fluorescence signal of the three LPG-deficient mutants overlaps completely in (A), (B) and (D), and partially in (C). The control lacking the primary antibody (–mAb control, shaded grey) is only shown in (E) and (F), but was identical in (A–D).

of LPG (Figure 4A, D, E, G, I and K). Phosphoglycosylation of secreted products such as secretory acid phosphatase (SAP) and fPPG (Ilg *et al.*, 1999a) appeared to be normal in *L.mexicana* LPG-deficient strains, as shown by monoclonal antibody binding in two-site enzyme-linked immunosorbent assay (ELISA) (Figure 6A) and by immunofluorescence of fPPG-containing promastigote aggregates (not shown).

To investigate whether other *Leishmania* species also display phosphoglycans on their surface in the absence of LPG, immunofluorescence experiments were performed on the LPG-deficient *L.donovani* mutant strain R2D2 generated by chemical mutagenesis (King and Turco, 1988) that carries an unknown defect in the *lpg1* gene (Ryan *et al.*, 1993). mAb LT6, which produces a strong surface signal on the parental wild-type strain (Figure 4I), showed only an occasional weak flagellar pocket labelling in R2D2, presumably due to phosphoglycosylated SAP (Bates *et al.*, 1990; Ilg *et al.*, 1993). This result is in agreement with the virtual absence of staining with this mAb in immunoblots of washed cells (Figure 3H). In contrast, a large number of proteins are recognized by the mAb L7.25 (Figure 3I). This antibody binds to the surface of R2D2 promastigotes and produces a fluorescence signal only slightly weaker than that obtained with wild-type parasites (Figure 4K and L). Up-regulation of PPG expression, as in *lpg1*-deficient *L.mexicana*, was not observed.

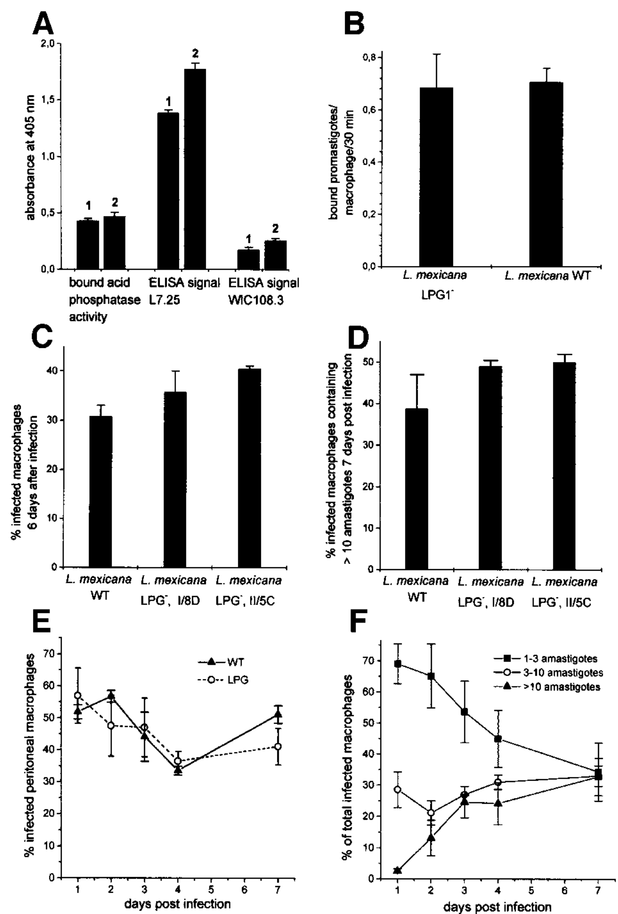

Fig. 6. Analysis of *L.mexicana* SAP phosphoglycosylation, macrophage binding and macrophage infection by *L.mexicana* wild-type and LPG-deficient mutant (LPG⁻) promastigotes. (**A**) ELISA of LT8.2-bound SAP released from *L.mexicana* wild-type (bar 1) and *L.mexicana* Δ*lmexlpg1*, clone I/8D (bar 2) using the anti-phosphoglycan cap mAb L7.25 and the anti-repeat mAb WIC108.3. (**B**) Binding of *L.mexicana* wild-type and an LPG-deficient *L.mexicana* mutant (clone II/5C) to peritoneal macrophages. The bars represent the average of three experiments. The standard error is indicated. (**C** and **D**) Infection of peritoneal macrophages by *L.mexicana* wild type and LPG-deficient *L.mexicana* mutants (clones I/8D and II/5C). The ratio of infected to uninfected macrophages 6 days after a challenge with two promastigotes/cell is shown in (C), while (D) shows the percentage of infected macrophages with an amastigote burden >10 after a challenge with two promastigotes/cell of *L.mexicana* wild-type or LPG-deficient *L.mexicana* mutants (clone I/8D and clone II/5C) after 7 days in culture. The bars represent the average of duplicate determinations and the standard error is indicated. A representative of three separate experiments with similar results is shown. (**E**) Time course of infection of peritoneal macrophages after a challenge with two promastigotes/cell of *L.mexicana* wild-type or an LPG-deficient *L.mexicana* mutant (clone I/8D). (**F**) Time course of parasite burden after infection of peritoneal macrophages with two promastigotes/cell of an LPG-deficient *L.mexicana* mutant (clone I/8D). (E and F) Each time point was determined in duplicate experiments. The standard error is indicated. Identical experiments with clone II/5C gave the same results.

Binding of LPG-deficient L.mexicana promastigotes to macrophages and multiplication inside their host cell

Earlier publications have reported an inability of LPG-deficient mutants to establish infections in macrophages (Handman *et al.*, 1986; McNeely and Turco, 1990) that was paramount to the proposition that LPG is a key factor

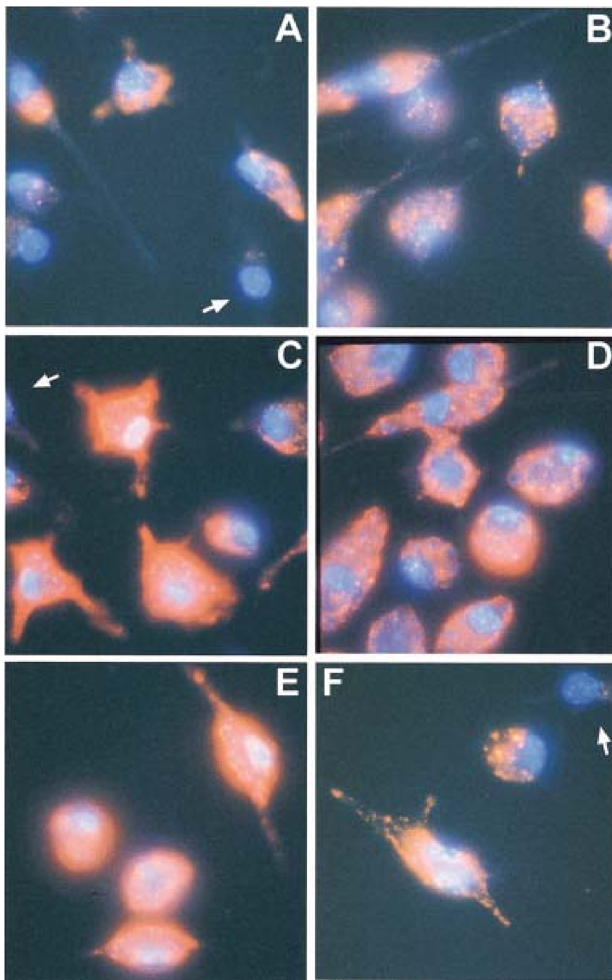

Fig. 7. Immunofluorescence of saponin-permeabilized peritoneal macrophages infected with *L.mexicana* wild-type (WT) and LPG-deficient mutant (LPG^-) promastigotes. Peritoneal macrophages were infected with five promastigotes of *L.mexicana* wild-type (**A**, **C** and **E**) or of *L.mexicana* Δ*lmexlpg1*, clone I/8D per cell (**B**, **D** and **F**). Infected macrophages were labelled after 2 days in culture with the mAbs LT6 (A and B), L7.25 (C and D) and LT17 (E and F) (orange fluorescence). Macrophages and *Leishmania* nuclei and kinetoplasts were stained with DAPI (blue fluorescence). Uninfected macrophages showing only background fluorescence are indicated by arrows.

for successful parasitism of mammalian host cells by *Leishmania*.

In macrophage binding experiments, the LPG-deficient *L.mexicana* promastigotes generated in this study were found attached to the same proportion of host cells as the wild-type promastigotes (45 ± 5% and 42 ± 8%, $n = 3$, respectively) and also the total number of bound parasites was very similar (Figure 6B). After uptake by their host cells, the *L.mexicana* Δ*lmexlpg1* mutants were at least as successful in colonizing macrophages as the parental wild-type strain with respect to both the percentage of infected host cells (Figure 6C) and the fraction of infected host cells carrying >10 parasites (Figure 6D). It has been argued that the presence of LPG in *Leishmania* may be particularly crucial during the initial phase of macrophage invasion by promastigotes. However, we could not detect any disadvantage of LPG-deficient *L.mexicana* promastigotes compared with the parental wild-type strain within the first 4 days of infection, because the percentage of parasitized peritoneal macrophages was very similar in both cases (Figure 6E). In addition, the LPG-deficient mutants started to proliferate inside the macrophages within 1 day with no sign of a lag phase (Figure 6F). Similar results were obtained with bone marrow-derived macrophages (data not shown).

An important argument for the hypothesis that LPG functions as a key molecule in the successful colonization of macrophages by promastigotes is the observation that LPG appeared to be transfered to the host cell plasma and internal membranes during invasion and the early stages of infection (Handman, 1990; Tolson *et al.*, 1990), where it was predicted to have a variety of effects on signal transduction events (reviewed in Descoteaux and Turco, 1993, 1999). Immunofluorescence experiments on macrophages infected 2 days earlier with *L.mexicana* wild-type promastigotes confirmed the presence of phosphoglycan epitopes. Three anti-phosphoglycan mAbs of different specificity (Figure 1A) all recognized intracellular structures and often also the surface of parasitized cells (Figure 7A, C and E). However, macrophages infected with LPG-deficient Δ*lmexlpg1* promastigotes also displayed abundant epitopes for all three mAbs, often intracellularly in compartments apparently devoid of parasites; only fluorescence on the host cell surface was observed less frequently than in infections with wild-type strains (Figure 7B, D and E). These data demonstrate that LPG is not the only phosphoglycan-modified compound that is released by the invading promastigotes and distributed in the host macrophage in the early stages of infection.

Experimental infection of mice with L.mexicana LPG-deficient mutants

It is widely believed that LPG is required by *Leishmania* promastigotes for successful infection of mammals. This is largely due to the fact that previously characterized LPG-deficient mutants proved to be unable to infect mice or hamsters and because LPG was thought to protect the parasites against complement lysis in mammalian tissue (reviewed in Sacks, 1989; Turco and Descoteaux, 1992). However, stringent experimental proof for a role of LPG as an essential virulence factor in an animal model has not yet been provided. To investigate this open question for *L.mexicana*, Balb/c mice were infected with three independent LPG-deficient *L.mexicana* Δ*lmexlpg1* mutants and their parental wild-type strain. At a challenge dose of 10^7 parasites/mouse, footpad lesions developed immediately in all four cases, with extensive swelling visible after 14 days. Disease progressed rapidly in infections with all three LPG-deficient *L.mexicana* mutants and more slowly in the case of the parental wild-type strains (Figure 8A, C, E and G). It could be argued that the high parasite load swamped the innate defence mechanisms of the mice, such as the complement system. However, a similar picture was obtained when the challenge dose was lowered to 10^5 parasites/mouse. A lag phase of ~40 days was followed by rapid disease progression for all three LPG-deficient mutants, while the infection with the parental wild-type strain showed the same lag phase and then a slow increase in footpad swelling (Figure 8B, D, F and H). In sacrificed mice, LPG-deficient parasites could be recovered not only from the lesion and the draining lymph nodes, but also in

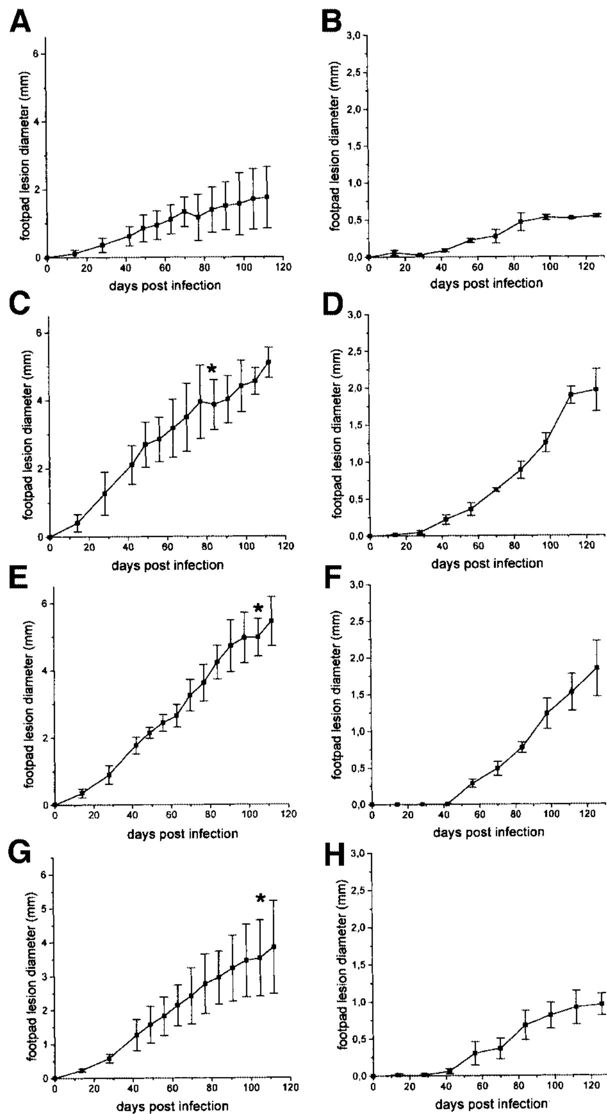

Fig. 8. Infection of Balb/c mice with *L.mexicana* wild-type and LPG-deficient mutant promastigotes. Mice were challenged with either 10^7 (A, C, E and G) or 10^5 (B, D, F and H) *L.mexicana* promastigotes in the right hind footpad. The swellings caused by *L.mexicana* wild type (**A** and **B**), *L.mexicana* Δ*lmexlpg1*, clone I/8D (**C** and **D**), *L.mexicana* Δ*lmexlpg1*, clone II/5C (**E** and **F**) or *L.mexicana* Δ*lmexlpg1*, clone IV/2B (**G** and **H**) were measured. The infection experiments were performed in quadruplicate (10^7 parasites) or triplicate (10^5 parasites) and the standard error is indicated. In (C), (E) and (G), at the time points indicated by *, the mouse with the largest lesion was killed for ethical reasons.

each case from the spleen, indicating that the Δ*lmexlpg1* mutants retained the capability to disseminate and to form metastases. The absence of LPG was confirmed in these re-isolated parasites by immunofluorescence using mAb LT6. The three LPG-deficient mutants were also capable of causing lesions in C57/BL6, a mouse strain that is more resistant to *L.mexicana* infections than Balb/c (Alexander and Kaye, 1985). Disease development was similar or faster than that observed in infections with wild-type *L.mexicana* and led variably to rapid progression and ulceration, or to a cessation of lesion growth after ~8 weeks (data not shown). Taken together, these results suggest that LPG is not essential for efficient experimental infections of mice with *L.mexicana* promastigotes.

Discussion

It is commonly accepted that *Leishmania* promastigote LPG is a multifunctional virulence factor that is required for parasite survival and development both in sandflies and in mammals. In the latter host organism, LPG has been implicated in promastigote complement resistance, attachment and uptake of promastigotes by macrophages, resistance to and inhibition of several host cell defence mechanisms, and manipulation of signal transduction pathways and of gene expression in macrophages (reviewed in Sacks, 1989; Turco and Descoteaux, 1992; Descoteaux and Turco, 1993, 1999; Beverley and Turco, 1998). The results obtained in this study show that in experimental infections with *L.mexicana*, all potential functions of LPG listed above are dispensable for the parasites, because LPG-deficient lines are at least as efficient in colonizing macrophages and appear to be even more virulent to mice than their parental wild-type strain. We currently are performing mouse infection studies with re-isolated wild-type and mutant parasites to determine whether this increased virulence of LPG-deficient *L.mexicana* is a stable phenomenon. In summary, at least for *L.mexicana*, LPG is not a virulence factor in the mammalian host.

Currently, it is not known whether LPG is required in other *Leishmania* species for infectivity to mammals. It will be interesting to investigate *lpg1*-negative mutants in *L.major* or *L.donovani*, which were the model organisms for many of the experiments on LPG function. In the case of *L.major*, it is possible that deletion of *lpg1* would affect not only LPG synthesis, because, in contrast to *L.mexicana* and *L.donovani*, this organism contains several β-Gal$_f$-containing glycosylinositolphospholipids (McConville and Ferguson, 1993). However, it should be noted that there is evidence already that LPG is not absolutely required for infectivity of *L.major* to mice. Some virulent ricin-resistant *L.major* mutants previously were believed to synthesize a high molecular weight LPG that replaced wild-type LPG (Cappai *et al*., 1994). Our recent results suggest that this modified *L.major* LPG may in fact be the novel surface molecule mPPG (Ilg *et al*., 1999b; Piani *et al*., 1999). In this light, it is interesting that a similar molecule appears to be up-regulated in the Δ*lmexlpg1* mutants generated in this study. It should also be noted that for the disease-causing amastigotes, the findings of this report are not too surprising, because this main mammalian parasite form down-regulates LPG expression to very low or even undetectable levels in *L.mexicana*, *L.donovani* and *L.major* (McConville and Blackwell, 1991; Bahr *et al*., 1993; Moody *et al*., 1993).

A series of earlier studies indicated that species-specific LPG has a crucial role in successful colonization of the insect host (reviewed in Sacks *et al*., 1994). This suggestion has been confirmed recently by the investigation of LPG-deficient *L.major* mutants that were generated by the same approach as in this study (Sacks *et al*., 2000). Promastigotes of these mutants showed only a slight reduction of survival and growth in the early stages of development in the sandfly, which suggested that LPG is not required for protection against digestive hydrolases in the bloodfed midgut. However, after bloodmeal excretion, the LPG-deficient mutants were completely lost from the

midgut, most likely due to lack of binding to the walls of the insect's digestive tract. These results demonstrate unequivocally that LPG is a virulence factor for the sandfly stages of the Old World *Leishmania* species *L.major* (Sacks *et al.*, 2000). The LPG-deficient mutants and the corresponding *lmexlpg1* addback lines generated in this study will now allow the investigation of a similar role for LPG in sandfly infections by the New World species *L.mexicana*.

It is possible that part of the biological effects identified for LPG may still be of importance for *Leishmania* parasites in the mammal. In a number of reports, it was shown that the pharmacologically active phosphoglycan repeats and caps of LPG are also attached to a variety of secreted and membrane-bound PPGs of both *Leishmania* life stages (reviewed in Ilg *et al.*, 1999a). In particular, some of these PPGs are present in macrophages at both an early (Piani *et al.*, 1999; this study) and late stage of *Leishmania* infection (Ilg *et al.*, 1999a), where they may mediate effects previously attributed to LPG. It is also possible that the up-regulation of PPGs observed in *lpg1*-deficient *L.mexicana* promastigotes compensates for the loss of LPG. However, it should also be pointed out that so far, it remains unclear whether PPGs are virulence factors. Deletion mutants for phosphoglycan-modified SAP, for example, retain their infectivity to macrophages and mice (Wiese, 1998). To solve this question, it may be necessary to perform targeted deletions on multiple PPG genes or on genes of biosynthetic enzymes that are selectively involved in assembly of the PPG glycans, such as the GDP-mannose:serine-protein mannose-1-phosphotransferase that has been characterized recently in *L.mexicana* promastigotes (Moss *et al.*, 1999). Further studies will be required to define the role of the PPG family for *Leishmania* virulence in mammals.

Materials and methods

Parasites and experimental infections of mice and cultured peritoneal macrophages

Promastigotes of the *L.mexicana* wild-type strain MNYC/BZ/62/M379 and of derived gene knockout 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). For mouse infection studies, 50 µl of phosphate-buffered saline (PBS) containing either 10^7 or 10^5 stationary phase promastigotes were injected into the right hindleg footpad of either Balb/c or C57/BL6 mice. The course of infections was followed by measuring the swelling relative to the uninfected left hindleg footpad at 7–14 day intervals. Parasites were re-isolated from infected animals by homogenizing footpad lesion tissue, draining lymph nodes and spleens in SDM/5% iFCS and culturing at 27°C. Peritoneal cells were isolated from either Balb/c or C57/BL6 mice by peritoneal lavage, seeded onto glass coverslips (10 mm diameter, 8×10^5 cells each) and incubated overnight in Dulbecco's modified Eagle's medium (DMEM), 10% iFCS at 37°C and 5% CO_2 in air. Approximately 50% of the cells adhered to the coverslips and were peritoneal macrophages as judged by their morphology. For promastigote binding studies, macrophages were washed twice with serum-free DMEM and incubated with stationary phase promastigotes resuspended in serum-free DMEM at a parasite to macrophage ratio of 5:1 for 30 min at 33°C. Non-adherent parasites were removed by three washings with phosphate-buffered saline (PBS), followed by a fixation step [0.1 M PIPES–NaOH pH 7.2, 2% para-formaldehyde (PAF), 0.05% glutaraldehyde (GA), 30 min, 22°C]. After three washes with PBS and a 20–40 min incubation with 1 µg/ml 4′,6-diamidino-2-phenylindole (DAPI) in PBS, 2% bovine serum albumin (BSA), 50 mM NH_4Cl, followed by an additional three washes with PBS, the coverslips were embedded in Mowiol (Stierhof *et al.*, 1999). Macrophages with bound parasites and the total number of bound parasites were counted by inspection with a fluorescence microscope. For macrophage infection studies, peritoneal macrophages were incubated overnight at 33°C with stationary phase promastigotes resuspended in DMEM/10% iFCS at a parasite to macrophage ratio of 2:1, followed by three washes with DMEM/10% iFCS to remove residual free promastigotes. After 6–7 days, the infected macrophages were washed, fixed and stained with DAPI as described above. The number of infected macrophages and the number of *L.mexicana* amastigotes per host cell were counted by inspection with a fluorescence microscope. In some experiments, a time course of macrophage infection was performed by counting parasitized host cells and intracellular amastigotes at days 1, 2, 3, 4 and 7.

Immunofluorescence microscopy and FACS analysis of Leishmania promastigotes and infected macrophages

Leishmania promastigotes were washed with PBS, fixed to poly-L-lysine-coated glass slides by incubation (30 min, 22°C) with PBS, 2% FA and 0.05% GA, washed again with PBS and incubated with blocking buffer [2% (w/w) BSA, 50 mM NH_4Cl in PBS, 30–60 min, 22°C]. The cells were then incubated (60 min, 22°C) with the mAbs (Ilg *et al.*, 1993) LT6, L7.25, LT17 and L3.8 diluted 1:2–1:10 (hybridoma supernatant) or 1:500–1:2000 (ascites fluid) in PBS, 2% BSA, followed by four washings with PBS. Bound mAbs were detected by incubation (45 min, 22°C) with Cy3-labelled goat anti-mouse IgG/IgM (Dianova) diluted 1:500 in PBS, 2% BSA. Subsequently, the cells were incubated for 10 min with 1 µg/ml DAPI in PBS, washed again four times with PBS, embedded in Mowiol and inspected by fluorescence microscopy.

Peritoneal macrophages were infected with *L.mexicana* promastigotes as described above. After 2 days, the cells were washed three times with PBS, fixed with 2% PAF, 0.05% GA in PBS and subjected to mAb labelling as described above, except for adding 0.1% saponin to all buffers for permeabilization of the host cell membranes.

For FACS analysis, all steps were performed at 0°C with ice-cold and sterile-filtered solutions. Promastigotes were washed three times with PBS, incubated at 5×10^7 cells/ml in PBS, 2% BSA for 1 h and then resuspended for 20 min in PBS, 2% BSA containing hybridoma culture supernatant (LT17 and LT6, at 1:2 dilutions) or ascites fluid (L7.25 and L3.8, diluted 1:1000 and 1:500, respectively). The cells were washed twice with PBS, resuspended in PBS to 5×10^7/ml and then fixed for 45 min by the addition of an equal volume of 0.1 M PIPES–NaOH pH 7.2, 4% PAF, 0.1% GA followed by two washings with PBS, once with PBS, 2% BSA, and a 15 min incubation in PBS, 2% BSA. The fixed samples were incubated for 1 h with fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG/IgM antibodies (Dianova, 1:250 in PBS, 2% BSA), washed twice with 2% BSA in PBS, once with PBS, then resuspended to 2×10^7 cells/ml in PBS and subjected to FACS analysis (FACScan, Becton Dickinson).

Cloning of the L.mexicana lpg1 gene and generation of gene knockout mutants

DNA techniques were performed as described previously (Ilg *et al.*, 1999b). Part of the *L.mexicana lpg1* gene (*lmexlpg1*) was obtained from *L.mexicana* genomic DNA by PCR using the primers AAT**GGATCC**AATCGCTCGCGCGCAGACAC and AAT**AAGCTT**TCTTCGCCGTAGGCGGGGTA derived from the *L.donovani lpg1* gene (Ryan *et al.*, 1993). The PCR product was subcloned into *Bam*HI–*Hin*dIII-cut pQE30 (Qiagen). The digoxygenin-labelled PCR product was used to screen a dedicated pBSK+ plasmid library of 4–6 kb *Pst*I fragments derived from *L.mexicana* genomic DNA. Positive clones were sequenced on both strands by the dideoxy chain termination method using an ALFexpress automated sequencer (Amersham-Pharmacia) as described earlier (Ilg *et al.*, 1999b) and the ORF corresponding to *lmexlpg1* was identified by homology to *L.donovani lpg1*. Double targeted gene replacement was performed by PCR amplification of the 5′-untranslated region (5′-UTR) of *lmexlpg1* using the primers KO1 (AAT**GCGGCCGCAACGTT**CAGGAGTGACGAG) and KO2 (AGT**ACTAGT**GATGCGCTCTGTTTCT) and by amplification of the 3′-UTR of *lmexlpg1* using the primers KO5 (TCACTAGT**GGATCC**AGCGCGACATGCCAGT) and KO6 (TG**AATTCAACGTT**TACGGTCCTGCTCCAG). The *Not*I–*Spe*I-cut *lmexlpg1* 5′-UTR PCR DNA fragment, the *Bam*HI–*Eco*RI-cut *lmexlpg*1 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 *lmexlpg1* gene replacement cassette, the phleomycin-binding protein gene (*phleo*) was amplified from pHM-PHLEO (Freedman and Beverley, 1993) using the primers AGT**ACTAGT**CATCCGGGTCCGAGC and AT**GGATCC**TTGGT-

CGGCGTCGGTCA. For this construct, a DNA fragment was amplified from the *lmexlpg1* 3′-UTR region by PCR using the primers KO3 (AGTACTAGT**GGATCC**CGTTAAGCATTCTGG) and KO4 (TTC-**GAATTC**GGAAGCGCTGTGATGAA). The ligation strategy into pBSK$^+$ was as outlined above. The *hyg*- and *phleo*-containing gene replacement cassettes were excised from the plasmids by *Acl*I or *Acl*I–*Eco*RI digestion, respectively, and transfected into *L.mexicana* promastigotes as previously described (Ilg *et al*., 1999b). Selection on 96-well microtitre plates was initiated by the addition of 20 μg/ml hygromycin B (Roche), 2.5 μg/ml phleomycin (Sigma) or both to the growth medium. Positive clones were analysed by Southern blotting using PCR-DIG-labelled DNA fragments (PCR DIG labelling kit, Roche) containing parts of the ORFs of *lmexlpg1* (DIG1, GAACACCCA-GCCCGAATTCC, and DIG2, ATGACAGCGAATATTCTCGC) or the *lmexlpg1* 5′-UTR (primers see above) as probes. For gene addback studies, the complete *lmexlpg1* ORF was PCR amplified using the primers CCCG**GGATCC**TCGTAGAAACAGAGAGC and AGA**TCTAGA**-ATGCTTAACGGGAGCGA and the *lmexlpg1* gene-containing *Pst*I fragment as a template. The *Bam*HI–*Xba*I-cut PCR fragment was then cloned into pX (LeBowitz *et al*., 1990). *Leishmania mexicana* Δ*lmexlpg1* promastigotes were transfected with this construct as described earlier (Ilg *et al*., 1999b) and transfectants were selected by growth in SDM/5% iFCS containing 10 μg/ml G418 (Roche). The sequence data for the *lmexlpg1*-containing *Pst*I fragment have been submitted to the DDBJ/EMBL/GenBank database under accession No. AJ271080.

Analytical procedures

For the production of lysates, late log phase *L.mexicana* and *L.donovani* promastigotes were washed twice in PBS and then resuspended at 2×10^9 cells/ml in 50 mM Tris–HCl pH 8.0 containing 10 mM *o*-phenanthroline, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 20 μM leupeptin, 0.1% Triton X-100 and 100 U/ml benzonuclease (Merck, Darmstadt, Germany) and sonicated briefly. After a 15 min incubation at 37°C to digest nucleic acids, 1/5 volume 10% SDS was added. Total protein was estimated by the method of Peterson (1983). Discontinuous SDS–PAGE was performed on 4% stacking gels over 7.5–20% separating gels. For electrotransfer of proteins from SDS–PAGE, polyvinylidene difluoride membranes (Millipore) were used (Ilg *et al*., 1993). Immunodetection of antigens was performed as outlined earlier (Ilg *et al*., 1999a) using the mAbs LT6, L7.25, LT17 and L3.8. SAP binding experiments with subsequent two-site ELISA detection from culture supernatants from *L.mexicana* promastigotes were performed using flexibe polvinylchloride ELISA plates (Becton-Dickinson) coated (50 μl/well 10 μg/ml, 50 mM $NaHCO_3$, 100 mM NaCl pH 9.6, 1 h at 25°C) with mAb LT8.2 (directed against a peptide epitope of *L.mexicana* SAP; Ilg *et al*., 1993). Bound acid phosphatase activity and the two-site ELISA signal obtained after subsequent incubation with the biotinylated mAbs L7.25 and WIC108.3 were determined as described earlier (Ilg *et al*., 1999b).

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