

***Leishmania* LPG3 encodes a GRP94 homolog required for phosphoglycan synthesis implicated in parasite virulence but not viability**

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***Leishmania* promastigotes express an abundant cell surface glycoconjugate, lipophosphoglycan (LPG). LPG contains a polymer of the disaccharide-phosphate repeat unit Gal β 1,4Man α 1-PO₄, shared by other developmentally regulated molecules implicated in parasite virulence. Functional complementation of a *Leishmania donovani* LPG-defective mutant (OB1) accumulating a truncated LPG containing only the Man α 1-PO₄ residue of the first repeat unit identified *LPG3*, the *Leishmania* homolog of the mammalian endoplasmic reticulum (ER) chaperone GRP94. *LPG3* resembles GRP94, as it localizes to the parasite ER, and *lpg3*⁻ mutants show defects including down-regulation of surface GPI-anchored proteins and mild effects on other glycoconjugates. *LPG3* binds cellular proteins and its *Leishmania infantum* GRP94 ortholog is highly immunogenic, suggesting a potential role in directing the immune response. However, null *lpg3*⁻ mutants grow normally, are completely defective in the synthesis of phosphoglycans, and the *LPG3* mRNA is regulated developmentally but not by stress or heat. Thus the role of *LPG3/GRP94* in *Leishmania* metabolism differs significantly from other eukaryotes. Like the other glycoconjugate synthetic pathways in this parasite, its activity is focused on molecules implicated in virulence rather than viability.**

Keywords: glycoconjugate biosynthesis/GPI-anchored molecules/lipophosphoglycan/parasite virulence/trypanosomatid protozoan

Introduction

During its life cycle, the protozoan parasite *Leishmania* alternates between two distinct developmental stages and hosts. Promastigotes replicate in the midgut of sand flies as the procyclic stage, and upon entry into stationary phase concomitant with the digestion of the blood meal by the fly, differentiate into the infective metacyclic form that is transmitted by the next fly bite. In the mammal, metacyclic

promastigotes avoid destruction by immune defenses and ultimately are ingested by macrophages, where they differentiate and proliferate as amastigotes within an acidified vacuole. Amongst the mechanisms employed for survival within the hosts, *Leishmania* relies on the expression of specialized, developmentally regulated glycoconjugates (Ilg *et al.*, 1999; Ilgoutz and McConville, 2001; Turco *et al.*, 2001). In promastigotes, the most abundant cell surface glycoconjugate is lipophosphoglycan (LPG), a complex glycolipid consisting of a phosphoglycan backbone structure of Gal(β 1,4)Man α 1-PO₄ repeat units (or PG repeats), anchored to the membrane via a GPI anchor containing a unique, conserved glycan core (Figure 1). LPG has been implicated in many important steps in the *Leishmania* life cycle (Turco and Descoteaux, 1992; McConville and Ferguson, 1993; Handman, 1999), and a *Leishmania major* mutant lacking LPG alone in an otherwise virulent background is attenuated in sand fly, mouse and macrophage infections (Sacks *et al.*, 2000; Späth *et al.*, 2000).

In addition to LPG, *Leishmania* parasites abundantly synthesize a variety of complex glycoconjugates. These include a heterogeneous class of small glycosylinositol-phospholipids (GIPLs), GPI anchored proteins such as gp63, gp46 and proteophosphoglycans (PPGs), and a variety of secreted proteins including acid phosphatase (sAP) and other forms of PPG (Handman, 1999; Ilg *et al.*, 1999; McConville *et al.*, 2002). Some of these are developmentally regulated, while others are constitutive, and like LPG, many have been proposed to be important to the execution of various steps of the parasite infectious cycle. One challenge in studying the biosynthetic pathways of these glycoconjugates and their role in virulence has been the finding that they share common structural domains. For example, the PG repeats of LPG are also added to secreted and membrane proteins including sAP and PPGs, and the LPG and GPI anchors of proteins have features in common with the GIPLs (references cited above).

Thus, we and others have made use of *Leishmania* mutants selected for specific defects in glycoconjugate expression to dissect out structure–function relationships and to identify LPG biosynthetic genes by functional rescue approaches (Ilg, 2001; Turco *et al.*, 2001). These have proven useful in delineating the LPG biosynthetic pathway and its relationship to that of other glycoconjugates, and for generating clean ‘knockout’ mutants in a virulent background with defined effects on glycoconjugate synthesis. For example, mutants in the *LPG1* gene affect only LPG, whereas mutations in *LPG2* affect all phosphoglycan-containing glycoconjugates (Descoteaux *et al.*, 1995; Ilg, 2000; Späth *et al.*, 2000; Ilg *et al.*, 2001; Turco *et al.*, 2001). Interestingly, this role(s) appears to differ substantially amongst *Leishmania* species, with

LPG and PG repeats playing important roles in survival in both the sand fly and macrophage stages in *L. major* and other species, but having little effect on macrophage survival in *Leishmania mexicana*, whereas viable mutants in other glycoconjugate synthetic pathways have strong virulence defects (Ilg, 2000; Späth *et al.*, 2000; Garami and Ilg, 2001a,b; Garami *et al.*, 2001; Ilg *et al.*, 2001; Turco *et al.*, 2001). These studies suggest that further study of the biosynthetic pathways leading to glycoconjugate assembly and expression could lead to the development of novel and more specific chemotherapeutic treatments against *Leishmania*.

Here we report on *LPG3*, a gene identified by functional complementation of the *Leishmania donovani* mutant OB1, which accumulates a truncated LPG that was characterized as Man α 1-PO₄-glycan core-PI. Remarkably, *LPG3* encodes a homolog of the mammalian GRP94, a member of the HSP90 family of chaperones localized in the endoplasmic reticulum (ER) (Lee *et al.*, 1984; Mazzarella and Green, 1987). In mammalian cells, GRP94 is a general chaperone associated with a variety of processes including antigen presentation, protein folding and secretion, and is required for viability (Li and Srivastava, 1993; Little *et al.*, 1994; Nicchitta, 1998;

Argon and Simen, 1999). Recently, a GRP94 homolog from *Leishmania infantum* was described, and was shown to be highly immunogenic similar to many HSP family members (Larreta *et al.*, 2000), although the cellular role of this protein was not investigated. Our data show that while *LPG3* shares some properties with *GRP94*, its functions within *Leishmania* appear to be highly divergent, as it is not required for normal growth and its role(s) and regulation differ considerably.

Results

Selection and characterization of the LPG-deficient mutant OB1

Wild-type (WT) *L. donovani* promastigotes were chemically mutagenized and selected for loss of agglutination by the β -galactose-binding lectin ricin agglutinin (King and Turco, 1988). From this population a clonal line was recovered that failed to react with ricin, named OB1. OB1 also was unreactive with the monoclonal antibody CA7AE, which recognizes the Gal(β 1,4)Man(α 1-PO₄) PG repeating units from LPG (McNeely *et al.*, 1990).

Glycoconjugate synthesis in OB1 was examined by metabolic labeling followed by extraction with organic

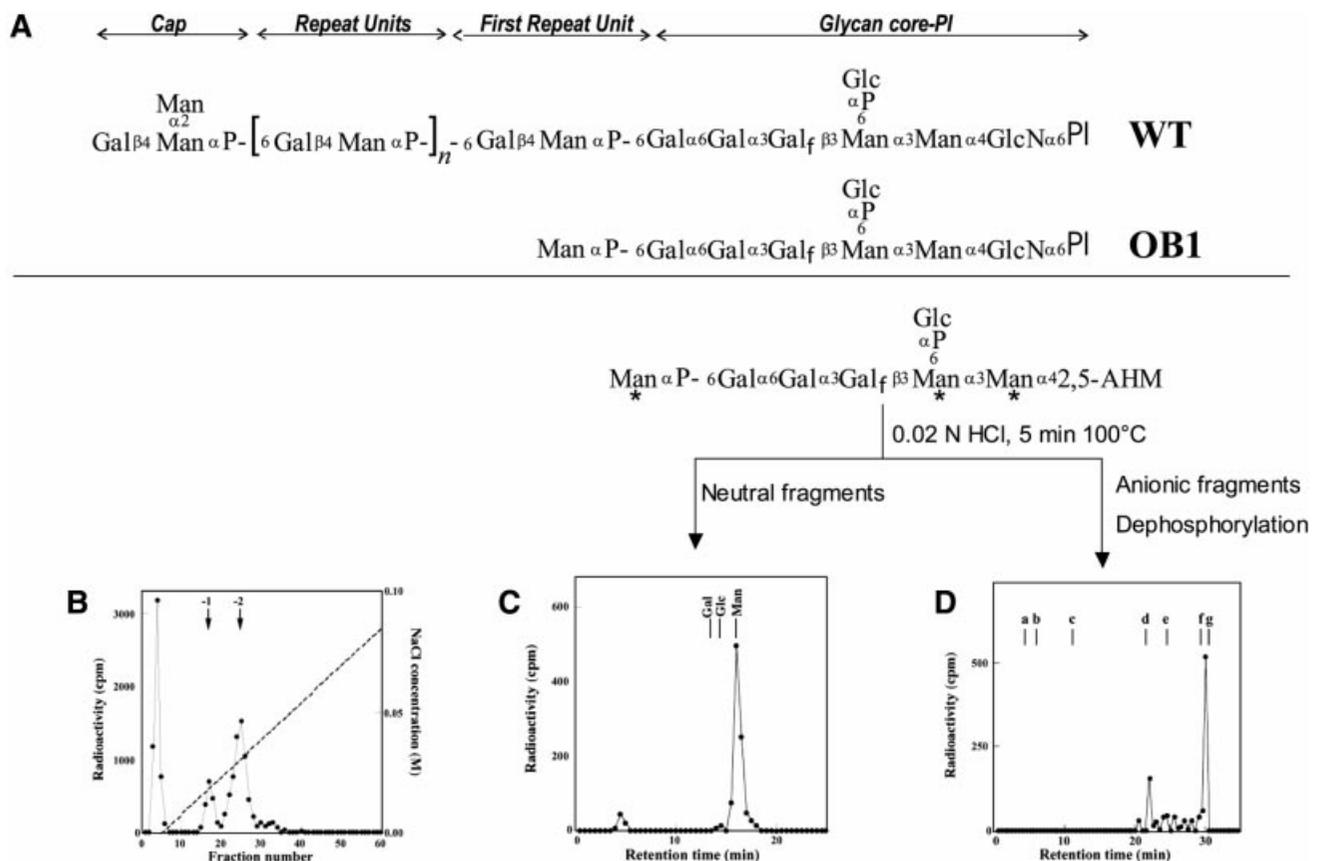


Fig. 1. Analysis of the truncated LPG accumulated in the *L. donovani* mutant OB1. (A) Structure of the LPG from *L. donovani* and OB1. (B) The [³H]mannose-labeled truncated LPG from OB1 was subjected to nitrous acid deamination and then applied to DEAE cellulose as described in Materials and methods. (C) The (−2) charged, [³H]mannose-labeled glycan from (B) (fractions 21–28) was hydrolyzed with mild acid into neutral and (−4) charged fragments, as depicted in the reaction scheme with the radioactive mannose residues indicated with asterisks, which were separated by anion exchange chromatography. The graph shows Dionex HPLC chromatography of the neutral fragment as described previously (Mahoney *et al.*, 1999). The elution positions of standard monosaccharides are shown. (D) The (−4) charged fragment described in (C) was further treated with alkaline phosphatase and then analyzed by Dionex HPLC as described previously (Mahoney *et al.*, 1999). Standards: a, Glc; b, Gal-Man; c, maltose; d, maltotriose; e, maltopentaose; f, maltohexaose; g, maltoheptaose.

solvents. As shown in Table I, low levels of [³H]mannose were incorporated into the LPG fraction (1.25% of WT), confirming that OB1 had lost the ability to synthesize LPG and that the loss of CA7AE reactivity was not due to conformational changes in LPG structure (Cappai *et al.*, 1994). In contrast to the loss of LPG, [³H]mannose incorporation into GIPL fractions was only slightly reduced in OB1, and incorporation into glycoproteins was reduced by 50% (Table I).

OB1 accumulates a truncated form of LPG

To determine the LPG biosynthetic defect in OB1, cells were metabolically labeled with [³H]mannose for 16 h. Glycolipids were extracted and delipidated with nitrous acid, and the deaminated products were separated by anion exchange chromatography. The ³H-labeled (−2) charged material (Figure 1B, fractions 21–28) was treated with mild acid hydrolysis and separated into two fragments, one of which co-migrated with standard mannose (Figure 1C). The other ³H fragment was found to be (−4) charged by anion exchange chromatography (data not shown). Following treatment with alkaline phosphatase, this fragment migrated with a retention time close to standard maltohexaose (Figure 1D, fraction 30) and identical to that of the dephosphorylated and deaminated glycan core of LPG (Gal-Gal-Gal_f-Man-Man-2,5-anhydromannose (Mahoney *et al.*, 1999). A minor [³H]mannose-labeled

peak (Figure 1D, fraction 24) co-migrating with standard maltotriose is probably the trisaccharide Man-Man-2,5-anhydromannose arising from partial hydrolysis at the labile Gal_f residue. These results indicated that the structure of the (−2) charged material (Figure 1B) corresponds to that arising from Man(α1-PO₄)-glycan core-PI (Figure 1A). The neutral ³H-labeled material (Figure 1B, fractions 3–5) probably corresponds to the mannosylated GIPLs of *L.donovani* characterized previously (McConville and Blackwell, 1991). The (−1) charged ³H-labeled material (Figure 1B, fractions 15–20) was not studied further; it is most likely to be the same as the (−2) charged peak discussed above except for the lack of the Glc(α1-PO₄) branch off the glycan core [Figure 1A; the LPG glycan core is known to be heterogeneous for this branch (McConville and Blackwell, 1991)]. Taken together, the data are consistent with the truncated form of LPG in OB1 as Man(α1-PO₄)-glycan core-PI (Figure 1A).

OB1 cells lack GalT activity required for PG repeating unit synthesis

The results above suggest that OB1 has a defect in galactose attachment to the mannose residue within the PG repeating unit. We used a cell-free system to assay β1,4-GalT activity in OB1 membrane preparations, in which the transfer of radioactive UDP-Gal to endogenous LPG acceptors is measured (Carver and Turco, 1991; Huang and Turco, 1993). OB1 membranes completely lacked GalT activity; this was not restored by the addition of 0.1% Triton X-100 to disrupt membrane vesicles (Figure 2A), suggesting that the OB1 defect was not due to sequestration of the GalT activity within the microsome lumen. An equal mixture of WT and OB1 microsomal membranes (Figure 2A, OB1+WT) yielded 50% GalT activity, indicating that the OB1 defect was not due to the presence of an inhibitor or the deficiency of an activator. Collectively, these results suggested that OB1 lacked the GalT activity responsible for β-galactose addition to Man(α1-PO₄)-glycan core-PI.

Western blot analysis of total cell lysates from OB1 using a general anti-PG repeating unit antibody CA7AE showed a complete absence of LPG, as well as other PGs

Table I. [³H]mannose incorporation into glycoconjugates from WT and OB1 mutant *L.donovani*

Cell line	LPG fraction (%)	GIPLs fraction (%)	Glycoprotein fraction (%)
WT	100	100	100
OB1	1.25 ± 0.05	92 ± 34	51 ± 1.5

The average and range of two replicate experiments is shown, normalized to the parental WT *L.donovani* line (100%). In WT parasites the total c.p.m. incorporated into each fraction was: LPG, 146 000; GIPLs, 5000; and glycoproteins, 77 000. Parasites were labeled with [³H]mannose and the LPG, GIPL and glycoprotein fractions were prepared and incorporation quantitated as described in Materials and methods.

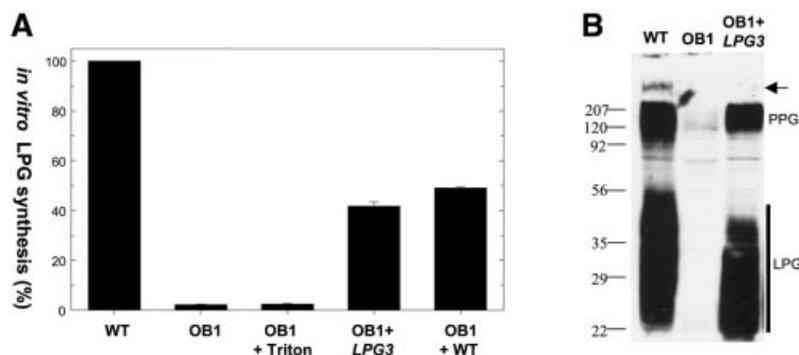


Fig. 2. Galactosyltransferase activity and LPG biosynthesis in the OB1 mutant. (A) Microsomal membranes were prepared from promastigotes of WT, OB1 and OB1 transfected with an *LPG3* expression construct (OB1 + *LPG3*; cosOB1-Δ*HindIII*; see Figure 3C). Galactosyltransferase activity was assayed using endogenous LPG acceptors and UDP-[³H]galactose donor as described in Materials and methods, either directly, or in the presence of 0.1% Triton (OB1 + Triton), or of an equal mixture of WT and OB1 membranes (OB1 + WT). (B) Lysates corresponding to 10⁶ log phase promastigotes were analyzed by western blotting with the anti-PG antibody CA7AE. The samples were WT, OB1 or OB1 transfected with an *LPG3* expression construct (pXG-LPG3-GFP+). The arrow marks the interface between the stacking and separating gel, and the positions of PPG and LPG are marked.

including those added to proteophosphoglycan (PPG) (Figure 2B; data not shown).

Functional rescue of OB1

To identify the gene defective in OB1, mutant parasites were transfected with a WT *L. donovani* cosmid library, containing ~40 kb random segments of genomic DNA inserted in the episomal shuttle cosmid vector cLHYG (Ryan *et al.*, 1993a). Ten thousand independent transfectants were obtained and LPG⁺ transfectants were selected from these by two rounds of ricin panning. Four cosmids with maps overlapping by ~17 kb were recovered (Figure 3A and B). Upon transfection into OB1, these cosmids were able to restore reactivity with ricin and CA7AE (data not shown). Microsomal membrane fractions from an OB1 transfectant bearing a cosOB1-A derivative showed 40–50% of WT GalT activity (Figure 2A, lane OB1+LPG3). Lastly, western blot analysis with CA7AE antibody showed restoration of LPG and PPG synthesis upon re-expression of *LPG3* in OB1 (Figure 2B, lane OB1+LPG3).

Identification of the *LPG3* gene

We tested a series of deletions of cosOB1-A, or of subcloned segments inserted into the *Leishmania* shuttle vector pSNBR (Callahan and Beverley, 1991). These data mapped the active gene to a segment spanning an *EcoRV* site adjacent to the cLHYG backbone, which was contained on a 5.4 kb *NotI*–*SpeI* fragment (Figure 3C, pSNAR 5.4 kb *NotI*–*SpeI*; this fragment additionally contained 1.9 kb of the cLHYG vector). Sequence analysis of the relevant region revealed an 813 amino acid reading frame, the last 43 amino acids of which arose from fusion to the vector cLHYG sequences. The remaining portion of this open reading frame (ORF) was obtained by sequencing the relevant region derived from cosOB1-B. Ultimately, we sequenced a 3.7 kb region that contained a 771 amino acid ORF, designated *LPG3* (DDBJ/EMBL/GenBank accession No. AF369892; Figure 4). As shown below, this reading frame alone was able to restore LPG synthesis of OB1. In contrast, expression of mouse GRP94 in OB1 did not restore LPG synthesis (Materials and methods; data not shown).

Database comparisons showed that the predicted *LPG3* protein was closely related to the GRP94/HSP90 protein family, with 42% identity and 60% similarity to human GRP94 (Figure 4), and 97.7% identity to a GRP94 homolog recently described in *L. infantum* (Maki *et al.*, 1990; Larreta *et al.*, 2000). GRP94, a molecular chaperone belonging to the HSP90 family, is an abundant resident protein of the ER (Little *et al.*, 1994; Argon and Simen, 1999). The predicted *LPG3* protein exhibited many hallmarks of the GRP94 protein family, including an N-terminal signal peptide (Box I; residues 1–21), nucleotide binding domain (Box II; residues 154–159), several N-linked glycosylation sites in the mature protein (residues 63, 306 and 402) and the GRP94 dimerization domain located at positions 673–716 in *LPG3* (Box III; Figure 4).

Cellular localization of *LPG3*

Notably, the predicted *L. donovani* *LPG3* protein has a C-terminal EGDG sequence, which differs from the canonical KDEL ER retrieval signal of GRP94 and other

ER resident proteins of other eukaryotes (Mazzarella and Green, 1987). The related *L. infantum* GRP94 gene contains EDDL at its C-terminus (Larreta *et al.*, 2000). While the requirements for the ER retrieval signal have not been studied in *Leishmania*, data from the related species *Trypanosoma brucei* suggest that the requirements may be more relaxed than in higher eukaryotes, with sequences such as MDDL or KQDL showing ER retention activity (Bangs *et al.*, 1996).

We created an *LPG3*–GFP fusion protein gene and expressed it in the OB1 mutant; these transfectants synthesized LPG, as judged by restoration of ricin and CA7AE reactivity and western blot analysis (Figure 2B). Fluorescence microscopy showed that the *LPG3*–GFP fusion protein was localized to the *Leishmania* ER, as seen by the colocalization of the *LPG3*–GFP with BIP/GRP78, a known ER resident protein (Bangs *et al.*, 1993; Jensen *et al.*, 2001; Figure 5). Western blot and immunoprecipitation studies with anti-GFP antisera described below revealed that the *LPG3*–GFP fusion protein was intact. An ER localization of the native *L. infantum* GRP94 protein has also been suggested using anti-GRP94 antisera (J.Requena, personal communication), in agreement with the GFP-tagged *LPG3/GRP94*.

In the active, ER-localized *LPG3*–GFP fusion protein, the terminal EGDG sequence was placed internal to the C-terminal GFP tag. Similarly, several other active *LPG3* expressing constructs have truncated termini and/or heterologous C-terminal extensions (cosOB1-A is one example; Figure 3A and B). Since the KDEL ER retrieval signal must be C-terminal for its function (Munro and Pelham, 1987), these data suggest *LPG3* may utilize other signals to achieve ER localization in addition to the EGDG. Recently, COP-I-independent (e.g. non-KDEL) ER retrieval mechanisms have been described in mammalian cells (Girod *et al.*, 1999; White *et al.*, 1999).

Identification of a mutant *LPG3* allele in OB1

Southern blot analysis of digested DNAs or *Leishmania* chromosomes separated by pulsed-field gel electrophoresis showed that *LPG3* was a single copy gene located on a 1.2 Mb chromosome, and that there was no difference between OB1 and WT *L. donovani* (Figure 6B; data not shown). We did not detect other *LPG3*-related sequences in these experiments, nor did we detect more than one candidate *LPG3* ortholog in the *L. major* or trypanosome genome project data (in contrast, many *HSP90* family members were found). Given the close evolutionary relationship of the two *Leishmania* species (classified within the same species complex), these data indicate that *L. donovani* *LPG3* is orthologous to *L. infantum* GRP94.

The similar Southern blot patterns between WT and OB1 suggested the possibility that rescue of the OB1 *LPG* defect was due to overexpression of *LPG3* from the multicopy cosmid/plasmid vectors used, rather than by complementation. To test this, one of the OB1 *LPG3* alleles was recovered from an OB1 genomic DNA cosmid library (cos*LPG3*^{OB1}). When transfected into OB1, cos*LPG3*^{OB1} failed to restore LPG expression (data not shown), suggesting this was a loss-of-function allele. Sequence analysis of the *LPG3*^{OB1} gene revealed a single point mutation at nucleotide 476 (G→A), resulting in the substitution of Gly159 for Asp159 (Figure 4). Gly159 is

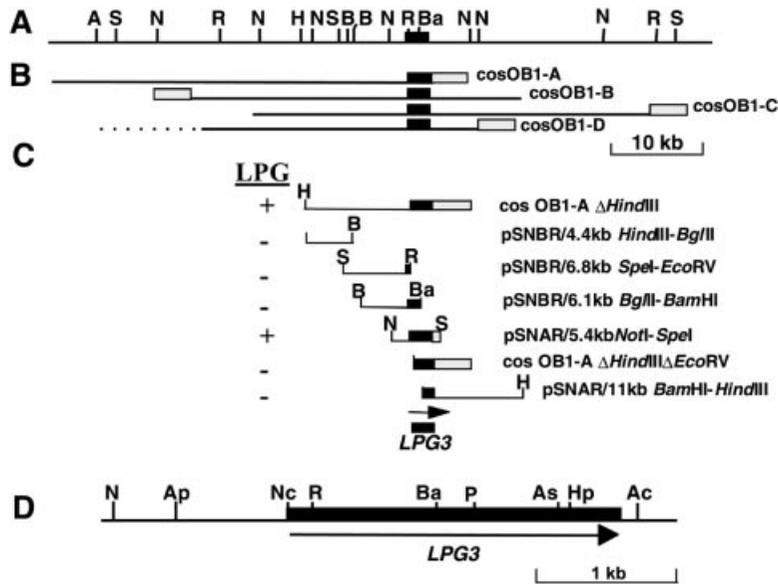


Fig. 3. Functional mapping of the *LPG3* locus. (A) Restriction map of the *LPG3* genomic region. The *LPG3* coding sequence is represented by the black box. (B) Position of four overlapping cosmids obtained by functional complementation of OB1. The shaded box represents the vector portion of cLHYG. (C) Localization of *LPG3*. CosOB1-A was subjected to deletional analysis to localize the region encoding the OB1-complementing gene (cosOB1- Δ HindIII and cosOB1- Δ HindIII Δ EcoRV). Various restriction fragments from cosOB1- Δ HindIII were subcloned in either *Leishmania* shuttle vectors pSNBR or pSNAR (Callahan and Beverley, 1991) and tested for their ability to restore LPG repeat unit expression in OB1 cells. An 11 kb *Bam*HI–*Hind*III fragment from cosOB1-C subcloned into pSNAR was also tested. (D) Organization of the *LPG3* locus. The *LPG3* ORF is represented by a black box. Restriction enzymes: A, *Afl*III; Ac, *Acc*I; Ap, *Apa*I; As, *Asc*I; B, *Bgl*II; Ba, *Bam*HI; H, *Hind*III; Hp, *Hpa*I; N, *Not*I; Nc, *Nco*I; P, *Pvu*I; R, *Eco*RV; S, *Spe*I.

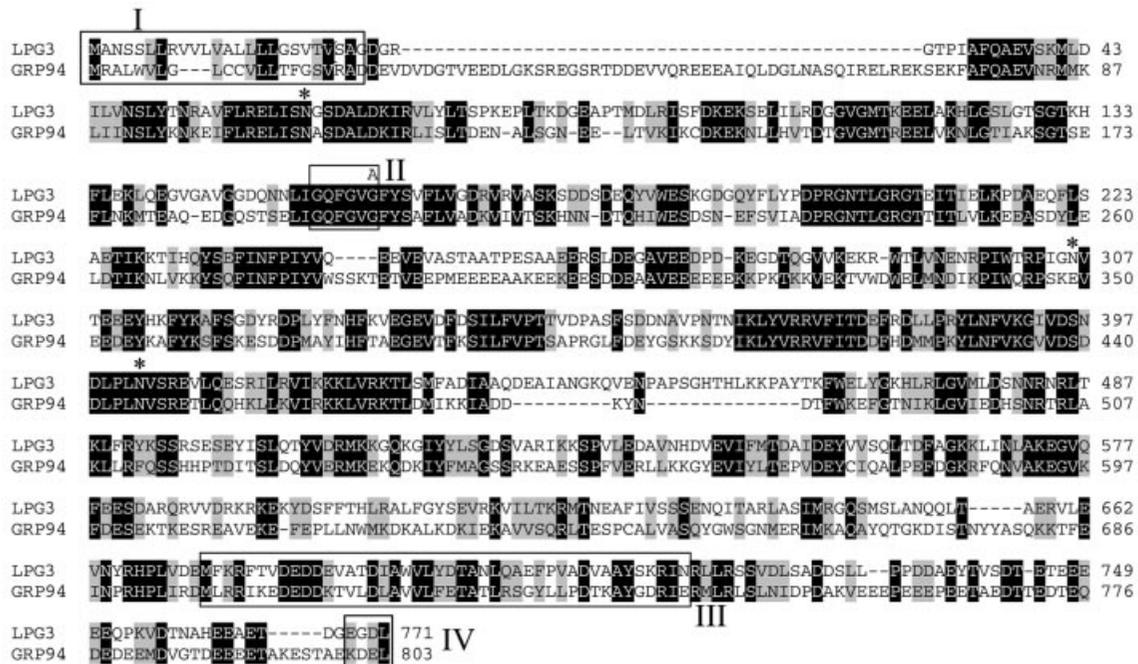


Fig. 4. Sequence of *LPG3* and alignment with human *GRP94*. The sequences of *LPG3* and human *GRP94* proteins (Maki *et al.*, 1990) were aligned using the ClustalW program. Black boxes indicate identity and shaded boxes indicate similarity. In one OB1 *LPG3* allele, guanine 476 has been mutated into an adenine, resulting in the substitution of Gly159 for Asp159 (shown above *LPG3* sequence). The *GRP94* (Argon and Simen, 1999) and *LPG3* signal sequences [box I; predicted by the SignalP algorithm; (Nielsen *et al.*, 1997)] are underlined. The GXXGXX motif (box II) present within the putative nucleotide-binding region (amino acids 35–274) is conserved in all Hsp90 family proteins (Schulte *et al.*, 1999). The dimerization domain of *GRP94* (Argon and Simen, 1999) is shown in box III. The C-terminal KDEL tetrapeptide (box IV) is required for ER targeting of *GRP94* (Munro and Pelham, 1987). Potential N-linked glycosylation sites in the mature *LPG3* protein are marked by asterisks.

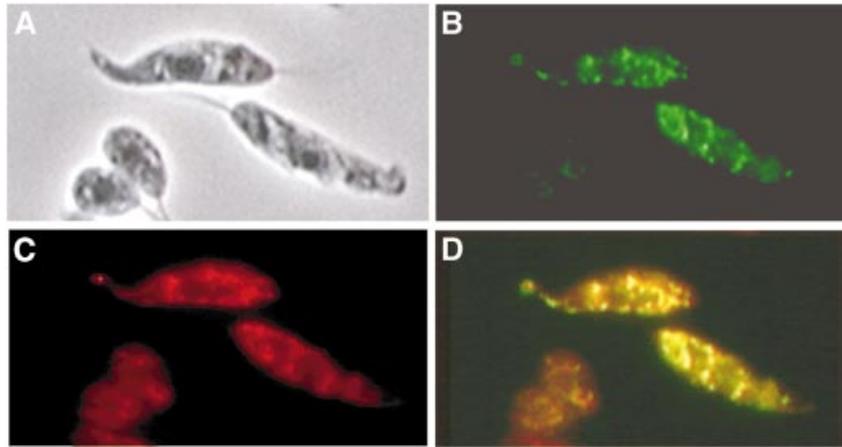


Fig. 5. LPG3-GFP is localized to the ER. WT *L. donovani* transfected with pXG-LPG3-GFP were grown in medium containing 50 μ g/ml G418. (A) Phase contrast; (B) GFP fluorescence; (C) immunofluorescent staining with rabbit anti-*T. brucei* BIP antibody (1:1000) followed by Texas Red-labeled goat anti-rabbit IgG (1:2000). (D) Merge of (B) and (C).

located within the GRP94 nucleotide-binding domain (Argon and Simen, 1999), suggesting that this site is essential for LPG3 function(s) required for GalT activity.

Leishmania mutants frequently arise through a loss of heterozygosity mechanism (Gueiros-Filho and Beverley, 1996). Since the Gly159→Asp mutation also generated a new *SalI* restriction site, we used Southern blot analysis to show that OB1 cells were heterozygous at this site (Figure 6C). These data argue that either this *LPG3^{OB1}* allele was a dominant-negative mutant, or that the other allele contained a different inactivating mutation. Transfection of *cosLPG3^{OB1}* in WT cells did not abolish LPG synthesis (data not shown), implying that *LPG3^{OB1}* was unlikely to be a dominant-negative mutant. While the sequence of the other allele in OB1 was not determined, in light of these data as well as the null mutant analysis below, we concluded that OB1 contains two independent mutations, each of which inactivated *LPG3* function.

***lpg3⁻* null mutants are defective in LPG biosynthesis**

To confirm that *LPG3* was responsible for the LPG biosynthetic defect in OB1, we created an *lpg3⁻* null mutant by homologous gene replacement. A targeting construct in which the first 1.3 kb of the *LPG3* ORF was replaced by a *NEO* cassette (Figure 6A) was introduced into WT parasites, and heterozygous replacements (*LPG3/NEO*) were obtained and confirmed by Southern blot analysis (data not shown). Next, homozygous knockouts were obtained by a loss of heterozygosity protocol as described in Materials and methods. Three clonal lines were obtained, all of which failed to react with CA7AE, and Southern blot analysis confirmed the loss of *LPG3* (Figure 6B). Transfection of *cosOB1-C* into these null *lpg3⁻* mutants restored LPG expression, as revealed by reactivity with CA7AE.

Thus, loss of *LPG3* is responsible for the LPG biosynthetic step defective in OB1 cells. Furthermore, in contrast to *GRP94* in cultured mammalian cells or *Dictyostelium* (Little *et al.*, 1994; Little and Lee, 1995; Morita *et al.*, 2000), *LPG3* was not required for growth

in vitro in *Leishmania*, as the *lpg3⁻* knockouts grew at rates identical to WT.

***LPG3* mRNA is regulated in the infectious cycle but not by stress**

Northern blot analysis revealed that the *LPG3* mRNA is expressed throughout the parasite life cycle as a 4 kb mRNA (Figure 7A). The *LPG3* mRNA was down-regulated in WT stationary phase promastigotes (Figure 7A, lane 1 versus 2), and expressed at higher levels in amastigotes (lane 3). Interestingly, OB1 promastigotes contained increased levels of *LPG3* mRNA in both log phase and stationary phase of growth (Figure 7A, lanes 4 and 5, respectively). This may represent an autoregulatory mechanism in response to loss of active *LPG3* protein expression.

A hallmark of mammalian GRP94 is the induction of its mRNA in the absence of glucose and by stress conditions that interfere with protein modification/folding or damage the ER (Little *et al.*, 1994; Argon and Simen, 1999). However, exposure of WT log phase promastigotes to stress conditions known to induce *GRP94* mRNA in mammalian cells (glucose deprivation, tunicamycin) had no effect on *LPG3* mRNA levels (Figure 7B, lane 1 versus lanes 2 and 3). Moreover, the down-regulation of *LPG3* mRNA observed in heat-shocked log-phase promastigotes (Figure 7B, lane 4) suggested that *LPG3* may not be *Leishmania* HSP, as it is known that both *HSP70* and *HSP83* mRNA levels increase during heat-shock in these parasites [Shapira *et al.*, 1988; this was confirmed by rehybridization of the northern blot shown in Figure 7B (data not shown)]. However, the relationship between mRNA and protein level for several *Leishmania* HSPs is complex (Shapira *et al.*, 2001), and further studies at the protein level will be required to confirm this finding.

Role of *LPG3* in the synthesis of secreted and surface *Leishmania* proteins

In mammalian cells, GRP94 is involved in essential processes of protein sorting and secretion (Little *et al.*, 1994; Little and Lee, 1995). As shown earlier, while the *LPG3* mutant OB1 mutant showed severe loss of PG

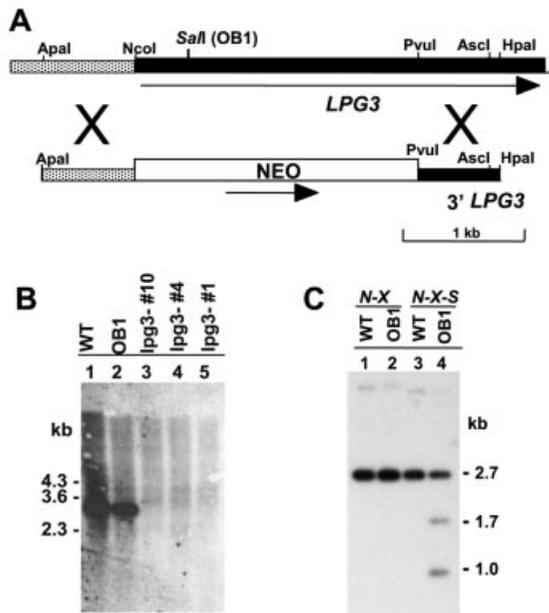


Fig. 6. Disruption of the *LPG3* gene and analysis of OB1 *LPG3* mutations. (A) Targeting construct for the disruption of *LPG3*. The open box represents the *NEO* resistance cassette, dark shading indicates the *LPG3* ORF, and the hatched region represents 5' flanking sequence. (B) Southern blot analysis of genomic DNA digested with *NotI* + *AscI*. The probe consisted of the 0.85 kb *EcoRV*-*BamHI* fragment from the *LPG3* ORF (see Figure 3D). Lane 1, WT; lane 2, OB1; lanes 3–5, *LPG3*-negative *lpg3*⁻ null mutants. (C) Genomic DNA from WT (lanes 1 and 3) or OB1 (lanes 2 and 4) were digested with either *NotI* + *XmaI* (lanes 1 and 2) or *NotI* + *XmaI* + *SalI* (lanes 3 and 4). The restriction fragments were electrophoresed in a 0.85% agarose gel and transferred onto a Gene Screen Plus membrane. The membrane was probed with the ³²P-labeled 1.0 kb *NotI*-*BamHI* fragment from the *LPG3* locus (see Figure 3) spanning the mutated site (nucleotide 476) of the *LPG3*^{OB1} allele.

synthesis involving both LPG and PPG, the effects on the synthesis of total mannosylated GIPLs or glycoproteins were modest (Table I; Figure 2B). We extended these studies to two important classes of *Leishmania* proteins: sAP, which shares the PG repeating units borne by LPG and PPGs, and the surface GPI-anchored proteins gp63 and gp46.

Leishmania sAP is known to be *N*-glycosylated in addition to containing PG repeats, with the former but not the latter modifications being essential for activity (Lovelace and Gottlieb, 1987; Bates *et al.*, 1990; Descoteaux *et al.*, 1995). Culture supernatants of OB1 showed a modest 2-fold reduction in sAP activity; however, this was not reversed following re-expression of *LPG3* and restoration of LPG biosynthesis (Figure 8A). This suggested that the decrease was not related to *LPG3* expression and probably arose from clonal variability associated with long-term *in vitro* culture and mutagenesis. In contrast, *LPG3* was required for PG addition to sAP, as an antigen capture assay with the anti-PG antibody CA7AE showed that the sAP of the OB1 *LPG3* mutant failed to bind at all (Figure 8B). Loss of PG repeating units was also evident in native gel electrophoresis, with OB1 sAP showing an increased mobility relative to WT, approaching that of the sAP of the C3PO mutant, which lacks all PGs (Figure 8C; Descoteaux *et al.*, 1995). The intermediate mobility of OB1 sAP suggests that it is

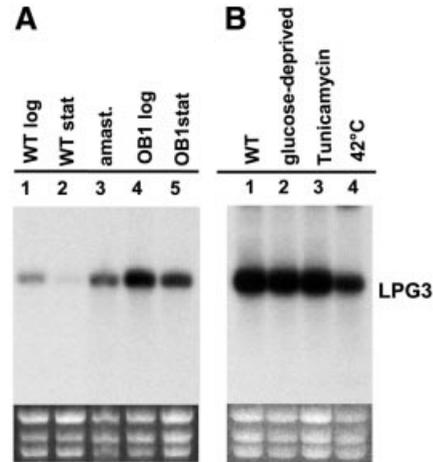


Fig. 7. Expression of *LPG3* mRNA. Northern blot analysis was performed using (A) 5 µg or (B) 15 µg total RNA, and a ³²P-labeled *LPG3* coding region probe (0.85 kb *EcoRV*-*BamHI* or 1.05 kb *NcoI*-*BamHI* fragments, respectively; see Figure 3). The integrity and loading of the RNA is shown by the ribosomal RNA stained with ethidium bromide (bottom). (A) Developmental regulation of *LPG3* mRNA. Lane 1, WT promastigotes in logarithmic phase; lane 2, WT promastigotes in stationary phase; lane 3, splenic amastigotes isolated from infected hamsters; lane 4, OB1 promastigotes in logarithmic phase; lane 5, OB1 promastigotes in stationary phase. (B) Effect of stresses on *LPG3* mRNA. Total RNA was prepared from logarithmic phase WT promastigotes grown for 24 h at 26°C in either complete medium (lane 1), glucose-free medium (lane 2), complete medium in the presence of 5 µg/ml tunicamycin (lane 3) or for 4 h at 42°C in complete medium (lane 4).

partially modified, possibly with the incomplete PG repeat units (Man α 1-PO₄ instead of Gal β 1,4-Man α 1-PO₄) as seen with LPG (Figure 1A). In both antigen capture and mobility assays, the properties of sAP were restored to that of WT upon re-expression of *LPG3* (Figure 8C, lane 3). Thus, while loss of *LPG3* has little effect on sAP secretion or activity, it is required for PG addition.

In addition to the GPI-anchored glycolipids LPG and GIPLs, the *Leishmania* surface contains abundant levels of glycosylated GPI-anchored proteins, including PPG, gp63 and gp46 (Olafson *et al.*, 1990; Schneider *et al.*, 1990; Rivas *et al.*, 1991). Unlike PPG or sAP, neither gp63 nor gp46 contains PG repeating units. Western blot analysis with appropriate sera showed that in contrast to the results obtained with sAP above, both gp63 and gp46 levels were significantly reduced in the *lpg3*⁻ null mutant, although the mobility of the residual protein was similar to that of WT (Figure 8D). Re-expression of *LPG3* expression restored gp63 and gp46 levels to that of WT (Figure 8D).

***LPG3* binds to itself and at least one other *Leishmania* protein, but not to LPG**

The relationship of *LPG3* to *GRP94* and the pleiotropic effects of *LPG3* mutations on PG-bearing molecules (LPG, sAP, PPGs) and GPI-anchored protein synthesis suggested that *LPG3* might function as a molecular chaperone, with specificity for protein or possibly nascent PGs. Several experiments were performed to search for binding of a protein with the predicted size of *LPG3* to LPG, using total labeled proteins from WT or OB1 cells, but we were unable to detect specific binding that differed between the two lines (data not shown). Thus we sought evidence for specific protein binding by immunoprecipita-

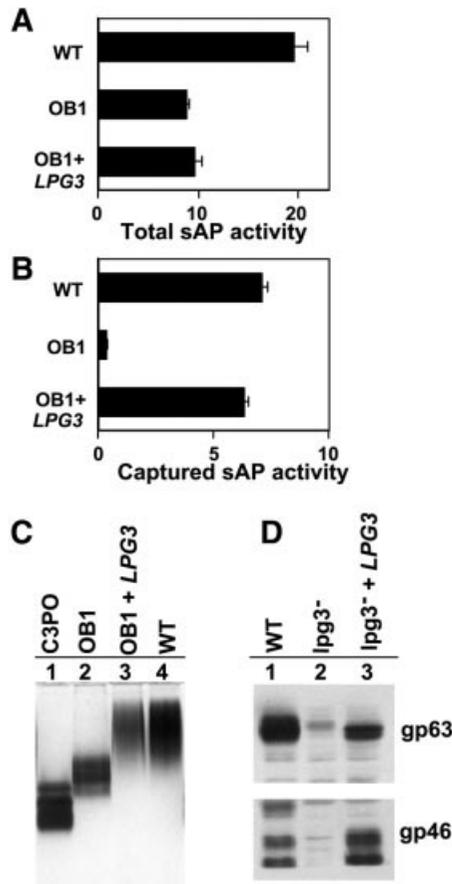


Fig. 8. Analysis of glycoconjugate expression in *lpg3*⁻ mutants. (A) Secretion of active acid phosphatase (sAP) is independent of *LPG3*. Culture supernatant (100 μ l) from log phase WT, OB1 or OB1 transfected with an *LPG3* expression construct (cosOB1- Δ AHindIII; see Figure 3) was assayed for acid phosphatase activity (Descoteaux *et al.*, 1994; Privé and Descoteaux, 2000). sAP activity is expressed as nmol/min/ml/10⁷ cells. (B) *LPG3* is required for PG addition to sAP. An antigen capture assay was performed with the CA7AE anti-PG repeating unit antibody (Tolson *et al.*, 1989) using 100 μ l of culture supernatants described in (A); sAP activity was assayed and quantitated as in (A). (C) sAP mobility in non-denaturing gel electrophoresis. Culture supernatants were concentrated (5.5-fold) from C3PO (lane 1), WT (lane 2), OB1 (lane 3) and OB1 transfected with an *LPG3* expression construct (lane 4; cosOB1- Δ AHindIII; see Figure 3) and separated by electrophoresis in 7% acrylamide gels in the absence of SDS. The gel was stained for acid phosphatase activity as described previously (Descoteaux *et al.*, 1995). (D) Effect of *LPG3* on gp63 and gp46 levels. Gp63 (A) and gp46 (B) levels were assessed by western blots using anti-gp63 or anti-gp46 antisera as described in the Materials and methods, using protein extracts from WT promastigotes (lane 1), *lpg3*⁻ promastigotes (lane 2) and *lpg3*⁻ promastigotes transfected with an *LPG3* expression construct (lane 3).

tion of *LPG3* protein complexes. We used the active C-terminal GFP-tagged *LPG3* protein described previously, and a second version bearing the OB1 mutant allele G159A. These proteins were expressed using the pXG expression vector in WT and OB1 parasites; the WT *LPG3*-GFP protein was functional as it was able to restore *LPG* expression in OB1 mutants, while the *LPG3*^{OB1}-GFP protein was inactive (data not shown).

Lysates were prepared from the four transfectants, immunoprecipitated with an anti-GFP antibody, and proteins were separated by SDS-PAGE. In transfectants

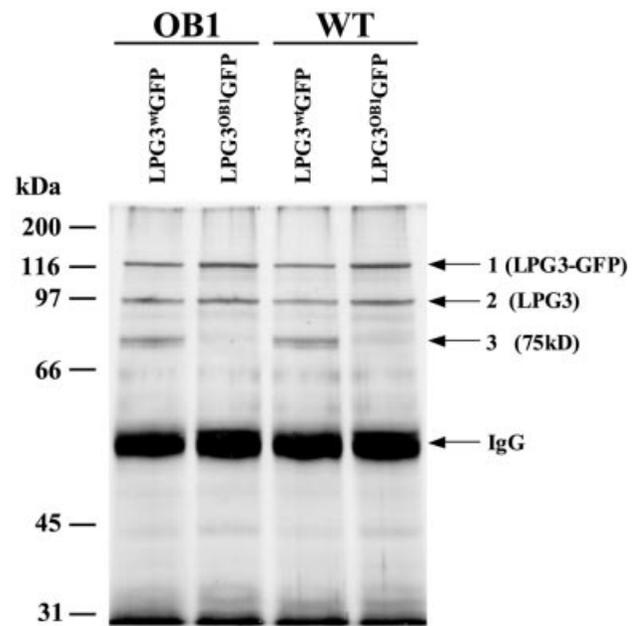


Fig. 9. Binding of *Leishmania* proteins to an *LPG3*-GFP fusion. Lysates from OB1 and WT *Leishmania* cells transfected with either pXG-*LPG3*^{wt}-GFP or pXG-*LPG3*^{OB1}-GFP were immunoprecipitated with anti-GFP antibodies (Clontech) and protein A-Sepharose as described in Materials and methods. Samples were subjected to SDS-PAGE (10% gel) under reducing conditions and silver-stained to visualize protein bands. Three distinct proteins are visible and labeled 1, 2 and 3. Band 1 corresponds to the *LPG3*-GFP fusion protein and band 2 has a size similar to the predicted *LPG3* protein as discussed in the text. The identity of the third band (75 kDa) is unknown.

bearing *LPG3*^{wt}-GFP, three major proteins with estimated sizes of 125, 92 and 75 kDa were identified by silver staining (Figure 9; similar results were obtained with [³⁵S]Met/Cys *in vivo*-labeled cell lysates; data not shown). Notably, protein bands comparable in size to the GFP reporter or other degradation products were not observed in immunoprecipitation or western blot studies with the anti-GFP antisera, suggesting that the *LPG3*-GFP fusion protein was largely intact (data not shown). The 125 kDa band corresponded to the predicted 112 kDa *LPG3*-GFP fusion protein, as shown by western blot analysis with anti-GFP antibody (data not shown). The apparent size of the 92 kDa protein was similar to that of *L. infantum* GRP94 (Larreta *et al.*, 2000) and that expected for mature *LPG3* (calculated to be 85 kDa). Immunoprecipitation of native *LPG3* by the *LPG3*-GFP fusion protein suggests that *LPG3* exists in oligomeric complexes, like mammalian GRP94, which is known to exist as a dimer (Lewis *et al.*, 1985; Qu *et al.*, 1994). Both the *LPG3*-GFP and *LPG3* proteins were immunoprecipitated from both OB1 and WT lysates, with either the *LPG3*^{wt}-GFP and *LPG3*^{OB1}-GFP fusion proteins. This is consistent with the location of the *LPG3*-OB1 mutation in the nucleotide-binding region, which is distinct from the GRP94 dimerization domain (Figure 4).

Significantly, a 75 kDa protein band was immunoprecipitated only by the *LPG3*^{wt}-GFP protein, but not by the *LPG3*^{OB1}-GFP protein, in both WT and OB1 transfectants. Thus, the *LPG3*^{OB1} nucleotide binding domain mutation

appears to preclude the interaction between LPG3 and this protein. The identity of this protein remains to be determined; its mobility suggests that it is unlikely to be gp46, gp63 or other known or abundant *Leishmania* proteins, and potentially it is involved in one or more steps of glycoconjugate synthesis defective in OB1.

Discussion

In this report we have characterized the biochemical defect associated with LPG assembly in the *L.donovani* mutant OB1, and shown that it affects the addition of the galactosyl residue within the first PG repeat added to the nascent LPG chain (Figure 1). We used a functional complementation and gene knockout approach to identify *LPG3* as the gene responsible for this defect. Remarkably, rather than encoding a PG-specific GalT as we had supposed, *LPG3* encodes a member of the GRP94/HSP90 protein family, which are chaperones whose multiple functions include protein assembly and secretion, and antigen presentation (Melnick and Argon, 1995; Nicchitta, 1998; Argon and Simen, 1999). The homology to mammalian GRP94 spans the entire molecule and includes domains implicated in secretion, nucleotide binding and dimerization (Figure 4). Our data, as well as searches of the *Leishmania* genome databases, suggest that the *L.infantum* *GRP94* gene, identified previously on the basis of its homology to mammalian GRP94, is orthologous to *L.donovani* *LPG3*.

Like other GRP94s, *LPG3* is targeted to the parasite ER, although our data suggest that the mechanism by which this occurs may use mechanisms in addition to the canonical KDEL ER retrieval signal characterized previously for other ER resident proteins of trypanosomes and other eukaryotes (Munro and Pelham, 1987; Bangs *et al.*, 1996). In addition, *LPG3* shares many functional properties with GRP94, as *lpg3*⁻ mutants show pleiotropic defects, including down-regulation of surface GPI-anchored glycoproteins and mild effects on *N*-glycosylation and glycolipid synthesis (Table I; Figures 2 and 8). While many chaperones show a broad ability to bind to many nascent proteins, mammalian GRP94s bind to a more limited set of proteins (Argon and Simen, 1999). Similarly, *Leishmania* *LPG3* can form complexes with a small number of proteins, including itself and at least one unidentified 75 kDa protein (Figure 9). It is possible that this protein may be involved in one of the defects evident in the *lpg3*⁻ mutants, such as GPI protein or PG galactosyltransferase activity, and we are investigating this question. Since PG biosynthesis occurs in the *Leishmania* Golgi apparatus (Bates *et al.*, 1990; Descoteaux *et al.*, 1995; Ha *et al.*, 1996), the role of *LPG3* in PG synthesis probably occurs by ensuring that at least one of the LPG biosynthetic enzymes is properly folded in the ER prior to transit. Although the GalT responsible for PG repeat synthesis has not been identified, searches of *Leishmania* databases of putative galactosyltransferase genes, as well as other likely glycosyltransferase genes, predict rather large proteins (90–160 kDa) compared with typical 40–50 kDa glycosyltransferases size found in other species (Field and Wainwright, 1995 and references therein). If relevant to the PG GalT,

perhaps the larger size places more emphasis on *LPG3*/GRP94-dependent folding.

Recently, a GRP94 homolog from the related species *L.infantum* was identified and shown to be highly immunogenic, potentially implicating this protein in interactions involving the host immune system known to play important roles in parasite virulence (Locksley *et al.*, 1999; Larreta *et al.*, 2000, 2002). Indeed, reactivity to other molecular chaperones and HSPs has been implicated in the pathology of a number of microbial infections, including *Leishmania* (Requena *et al.*, 2000; Srivastava, 2002). Interestingly, mammalian GRP94–peptide complexes have been shown to elicit cellular immune responses, an effect which can also be attained by exogenous administration of GRP94–peptide complexes (Nicchitta, 1998; Argon and Simen, 1999). This raises the possibility that complexes of *Leishmania* peptides with *LPG3*/GRP94 could interact with the host immune system and modulate immune responses, and this will be studied in the future.

Our data suggest that *Leishmania* *LPG3*/GRP94 shares many of the activities of GRP94s of other eukaryotes. However, despite these similarities, our most striking findings point to significant differences in the role of *LPG3* in parasite metabolism. First, gene knockout studies show that *LPG3* is not essential for parasite growth, whereas in mammalian cells or *Dictyostelium*, GRP94 is essential (Little and Lee, 1995; Morita *et al.*, 2000). Secondly, the effects on protein secretion and glycosylation are considerably less profound. Thirdly, *lpg3*⁻ mutants show severe defects in phosphoglycosylation, resulting in a complete lack of PG assembly on LPG and proteins. Fourthly, regulation of *LPG3* mRNA expression differs greatly from that seen in mammalian cells, where the *GRP94* mRNA typically is up-regulated in response to stress. In *Leishmania*, *LPG3* mRNA expression appears to be regulated primarily in the context of development, with levels declining in stationary phase promastigotes where differentiation to the infectious metacyclic promastigote stage occurs, and increasing in amastigotes that reside within the macrophage phagolysosome and cause disease. Unlike mammalian GRP94 mRNA or HSP mRNAs in *Leishmania* and other organisms, the *Leishmania* *LPG3* transcript is specifically not induced by canonical GRP94-inducing stresses such as heat, glucose deprivation or the glycosylation inhibitor tunicamycin (Figure 7B). Lastly, the LPG defect of the OB1 mutant could not be rescued by expression of mammalian GRP94.

Together, these data suggest that the role of *LPG3* in *Leishmania* metabolism differs greatly from its homolog GRP94 in mammalian cells. Effectively, the critical role(s) of *LPG3* is restricted to the synthesis of glycoconjugates implicated in parasite virulence, including LPG and other PG-bearing molecules, and GPI-anchored proteins. It is noteworthy that similar findings have emerged in studies of a number of *Leishmania* proteins associated typically with essential, ‘housekeeping’ functions of the secretory pathway in other eukaryotes. For example, Ilg and colleagues have shown that disruption of several genes of the mannose biosynthetic pathway required for protein glycosylation, such as phosphomannomutase, phosphomannoisomerase, GDP-mannose pyrophosphorylase and dolichol-phosphate mannose synthase, have surprisingly

little effect on parasite viability, yet have strong effects on the synthesis of glycoconjugates and virulence (Garami and Ilg, 2001a,b; Garami *et al.*, 2001). Similarly, the Golgi GDP-mannose transporter is essential in *Saccharomyces cerevisiae* (Poster and Dean, 1996), yet the *Leishmania LPG2* gene encoding this transporter is dispensable, with adverse effects only upon PG biosynthesis and parasite virulence (Descoteaux *et al.*, 1995; Ma *et al.*, 1997; Ilg *et al.*, 2001; Turco *et al.*, 2001). Notably, the viability of parasite mutants in these genes provides opportunities for their study not possible in other eukaryotes where they are essential.

Prior to knowledge of the biochemical role of *LPG2* in LPG and glycoconjugate synthesis, we had proposed that *LPG2* is part of a specialized pathway leading to parasite virulence (Descoteaux *et al.*, 1995). In light of recent knowledge of the role of *LPG3* and many genes affecting glycoconjugate synthesis cited above, this hypothesis must be updated. Indeed, given the limited consequences in *Leishmania* of deletion of a broad spectrum of typically 'essential' eukaryotic genes affecting glycoconjugate structure and synthesis, it would appear that the specialization may involve the entire parasite secretory pathway, towards molecules important to parasite virulence, and away from molecules essential for viability as in most other eukaryotes. This does not imply that the basic function or role of the secretory pathway differs; instead, its focus seems to have shifted greatly, towards 'cargo' implicated in parasite survival within the host.

Materials and methods

Parasites and mutagenesis

Leishmania donovani 1S2D promastigotes were grown in M199 medium supplemented with 10% fetal calf serum (Descoteaux *et al.*, 1994). Mutagenesis of promastigotes with 3 µg/ml of *N*-methyl-*N*-nitroso-*N*-nitroguanidine and selection of LPG-defective mutants based on resistance to agglutination by the lectin ricin agglutinin were performed as described previously (King and Turco, 1988; Descoteaux *et al.*, 1994).

Metabolic labeling and analysis of the truncated form of LPG from OB1

OB1 cells (1×10^9) were metabolically labeled with [2-³H]mannose (100 µCi) for 16 h in 2 ml of medium. [³H]GIPLs (some of which are the precursors to LPG) were extracted as described previously (McConville and Bacic, 1989). The GIPL extract was evaporated to dryness under N₂, further purified by chromatography on phenyl-Sepharose, and delipidated by nitrous acid deamination (Huang and Turco, 1993). The deaminated glycan portion was collected by chromatography on phenyl-Sepharose, desalted on a column of Sephadex G25 equilibrated in water, resuspended in 1.0 mM Tris-HCl, pH 8.0, and applied to a column of DE52 cellulose (1 × 2 ml) equilibrated in 1.0 mM Tris-HCl, pH 8.0. After the fifth fraction was collected, the column was eluted with a gradient of NaCl (0–0.1 M) in 1.0 mM Tris-HCl, pH 8.0. Fractions of 0.6 ml were collected and assayed for radioactivity by liquid scintillation counting. Following DEAE chromatography of the nitrous acid deaminated [³H]GIPLs, the [³H]mannose-labeled (–2) charged glycan was pooled and desalted. The [³H]glycan was subjected to mild acid hydrolysis in 0.02 N HCl for 5 min at 100°C to cleave the labile glycosylphosphate bonds, yielding neutral and (–4) charged fragments. The neutral fragment was subjected to Dionex HPLC. The (–4) charged fragment was further treated with alkaline phosphatase (0.3–0.5 U in 1 mM Tris) and then analyzed by Dionex HPLC as described previously (Mahoney *et al.*, 1999).

Microsomal membrane preparation and GalT assays

Parasites were harvested at a density of $2\text{--}4 \times 10^7$ cells/ml and microsomal membranes were prepared (Carver and Turco, 1991). Incubation mixtures (400 µl) contained 0.4 mg of membrane protein, 50 mM HEPES (pH 7.4), 25 mM KCl, 5 mM MnCl₂, 5 mM MgCl₂,

0.1 mM *N*α-*p*-tosyl-L-lysine chloromethyl ketone, 1 µg/ml leupeptin, 1 mM ATP, 0.5 mM dithiothreitol and 4 µCi UDP-[³H]Gal (specific activity, 15.3 Ci/mmol). In some reactions, Triton X-100 was added to 0.1% (v/v). The mixtures were incubated at 26°C for 1 h and the [³H-Gal]-LPG product was extracted, purified, and quantitated as described previously (Carver and Turco, 1991).

Western blotting

Log-phase parasites were washed twice in phosphate-buffered saline (PBS). For analysis of gp46 and gp63, parasites were suspended in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 150 mM NaCl) containing protease inhibitors (Protease Inhibitor Cocktail, Roche Diagnostic). The extracts were incubated on ice for 15 min, vortexed and nuclei removed by centrifugation. Fifteen micrograms of protein/well were separated by SDS-PAGE on 8% polyacrylamide gels. For LPG, parasites were resuspended in SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 5% 2-mercaptoethanol) at 5×10^7 cells/ml. After incubation at 100°C for 5 min, lysates corresponding to 10⁶ cells/lane were separated by SDS-PAGE on 10% polyacrylamide gels. Samples were electroblotted onto Hybond-ECL membranes (Amersham Pharmacia Biotech), and detected using an enhanced chemiluminescence method (Amersham Pharmacia Biotech). PG-containing glycoconjugates were detected with the anti-PG monoclonal antibody CA7AE which recognizes the Gal(β1–4)Manα1-PO₄ repeating units (Tolson *et al.*, 1989). gp63 was detected using the mouse monoclonal anti-gp63 (mAb 235; Button *et al.*, 1991) and gp46 was detected using a rabbit antiserum to deglycosylated *Leishmania amazonensis* gp46 prepared and provided by D.McMahon-Pratt, Yale University Medical School (Champs and McMahon-Pratt, 1988). Peroxidase-conjugated anti-rabbit and anti-mouse antisera were from Amersham Pharmacia Biotech.

Secreted acid phosphatase analysis

Supernatants from promastigote cultures (ranging from 8 to 10×10^6 cells/ml) were concentrated 5.5-fold using Centricon concentrators. Twenty-five microliters of each concentrated supernatant was electrophoresed in non-denaturing conditions in 7% acrylamide gels, which were subsequently stained for acid phosphatase activity (Katakura and Kobayashi, 1988). A PG repeat antigen capture assay was performed on promastigotes culture supernatants as described previously (Descoteaux *et al.*, 1995) using the CA7AE antibody.

Fluorescence and immunofluorescence microscopy

WT *L. donovani* transfected with pXG-LPG3-GFP were grown in medium containing 50 µg/ml G418. Log phase cells were attached to poly-lysine coated cover slips and fixed in 3.5% formaldehyde (1 min at room temperature) followed by ethanol (15 min on ice). A rabbit anti-BIP polyclonal antibody (provided by E.Handman, Australia) was used to reveal the ER (1:1000, 20 min at room temperature), followed by goat anti-rabbit IgG-Texas Red labeled secondary antibody (1:2000).

Immunoprecipitation

Parasites (8×10^9 cells) were pelleted by centrifugation and washed once in ice-cold PBS. The cells were resuspended in 2.5 ml of prechilled lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM CaCl₂, 5 µg/ml leupeptin, 2 µg/ml pepstatin A, 0.5% Triton X-100), and incubated for 30 min on ice. Lysates were clarified by centrifugation at 1000 *g* for 10 min and then 10 000 *g* for 10 min at 4°C. Immunoprecipitations were performed by adding 1–2 µl of rabbit polyclonal anti-GFP antibodies (Clontech) for 1 h at 4°C with shaking. Immune complexes were precipitated by the addition of 20 µl of protein A-Sepharose and 40 µl of Sepharose (Pharmacia) for 2 h followed by centrifugation for 20 s at 12 000 *g*. Immunoprecipitated samples were washed three to four times with lysis buffer, boiled in sample buffer (2% SDS, 60 mM Tris pH 6.8, 2.5% β-mercaptoethanol) and then centrifuged for 20 s at 12 000 *g*. The supernatants were subjected to SDS-PAGE (10% polyacrylamide containing 1% SDS) in the presence of β-mercaptoethanol, and the gels were silver-stained to visualize protein bands, or analyzed by western blotting as described above with anti-GFP antibodies (1:1000).

Parasite transfections and LPG⁺ selections

OB1 cells were transfected with WT *L. donovani* cosmid library DNA (Ryan *et al.*, 1993a) and HYG-resistant transfectants were selected as described previously (Ryan *et al.*, 1993b; Descoteaux *et al.*, 1994). LPG⁺ OB1 transfectants were selected by two rounds of LPG panning using 10 µg/ml of ricin agglutinin (Descoteaux *et al.*, 1994). An OB1 genomic DNA cosmid library was constructed in cLHYG as described previously

(Descoteaux *et al.*, 1994). The library was probed with the 0.85 kb *EcoRV*-*Bam*HI fragment from *LPG3* (Figure 3) to identify cosmids containing the OB1 *LPG3* gene.

LPG3 targeting construct and generation of *lpg3*⁻ null mutants

The *LPG3* deletion targeting construct (pLPG3-KO; strain D133) consisted of a 3.8 kb *Apal*-*Hpa*I fragment in which the first 1.3 kb of *LPG3* coding sequence was replaced by a *NEO* cassette followed by the 3' untranslated region of *DHFR/TS* (Figure 6A). This fragment was transfected into the WT line to generate G418-resistant clones with heterozygous *LPG3* replacements (*LPG3*/Δ*lpg3*::*NEO*). To obtain homozygous replacements, we used a loss-of-heterozygosity protocol (Descoteaux *et al.*, 1995; Gueiros-Filho and Beverley, 1996). Heterozygote clones were grown in 20 μg/ml G418 and were submitted to five successive rounds of agglutination with the CA7AE monoclonal antibody followed by plating to obtain a clonal *lpg3*⁻ line (Δ*lpg3*::*NEO*/Δ*lpg3*::*NEO*). *LPG3* expression was restored in the *lpg3*⁻ null mutant by transfection of several constructs including cosOB1-C (B1230), pXG-LPG3-GFP+ (below) or pXGSAT-LPG3 (B3366). This construct was made by ligation of the 2.3 kb *LPG3* *Nco*I-*Not*I fragment from pXG-LPG3-GFP+ into *Sma*I-*Bam*HI-digested pXGSAT (B2352), after all termini had been made blunt by the activity of the Klenow fragment of DNA polymerase I.

Expression of murine GRP94 in Leishmania

The mouse *GRP94* ORF, contained on a 2.7 kb *Bam*HI fragment, was excised from pGEM99.2 (Mazzarella and Green, 1987) and inserted into the *Bam*HI site of pXG (Ha *et al.*, 1996), to create pXG1-mGRP94 (B2283). This construct was transfected into OB1 and tested for *LPG* expression. Expression of mouse *GRP94* in *Leishmania* cells was assessed by western blotting with a rabbit polyclonal antiserum raised against the 16 C-terminal amino acids of murine *GRP94* (provided by M.Green, St Louis University, MO).

LPG3-GFP construct

An *LPG3*-GFP protein fusion expression vector (pXG-LPG3GFP; B-2396) was constructed using the pXG-GFP vector (Ha *et al.*, 1996; B-2295). The pXG-LPG3^{OB1}GFP construct (D106) was obtained by replacing the 1.7 kb *EcoRV*-*Asc*I fragment from pXG-LPG3GFP with the 1.7 kb *EcoRV*-*Asc*I fragment from p8kbNsiI(OB1) (B-2193), which contains a blunted 8.0 kb *Nsi*I fragment (containing the *LPG3*^{OB1} allele) from cos*LPG3*^{OB1} inserted into pUC π digested with *Eco*RI and *Hind*III.

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