Leishmania mexicana Mutants Lacking Glycosylphosphatidylinositol (GPI):Protein Transamidase Provide Insights into the Biosynthesis and Functions of GPI-anchored Proteins

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The major surface proteins of the parasitic protozoon *Leishmania mexicana* are anchored to the plasma membrane by glycosylphosphatidylinositol (GPI) anchors. We have cloned the *L. mexicana GPI8* gene that encodes the catalytic component of the GPI:protein transamidase complex that adds GPI anchors to nascent cell surface proteins in the endoplasmic reticulum. Mutants lacking *GPI8* (Δ*GPI8*) do not express detectable levels of GPI-anchored proteins and accumulate two putative protein–anchor precursors. However, the synthesis and cellular levels of other non–protein-linked GPIs, including lipophosphoglycan and a major class of free GPIs, are not affected in the Δ*GPI8* mutant. Significantly, the Δ*GPI8* mutant displays normal growth in liquid culture, is capable of differentiating into replicating amastigotes within macrophages in vitro, and is infective to mice. These data suggest that GPI-anchored surface proteins are not essential to *L. mexicana* for its entry into and survival within mammalian host cells in vitro or in vivo and provide further support for the notion that free GPIs are essential for parasite growth.

INTRODUCTION

Leishmania are protozoan parasites that cause a spectrum of diseases in humans. These parasites alternate between a flagellated promastigote stage that proliferates within the midgut of the sandfly vector and a nonmotile amastigote stage that invades mammalian macrophages, where they occupy the phagolysosome compartment. The cell surface of the promastigote stage is coated by a protective glycocalyx that comprises a number of glycosylphosphatidylinositol (GPI)-anchored glycoproteins, a complex GPI-anchored lipophosphoglycan (LPG), and a family of free GPIs (termed glycoinositolphospholipids [GIPLs]) (McConville and Ferguson, 1993; Beverley and Turco, 1998; Figure 1). Components in this glycoclayx are thought to be essential for parasite survival and infectivity in the diverse host (McConville and Ferguson, 1993; Beverley and Turco, 1998). The GPI-anchored glycoproteins include an abundant metalloproteinase, termed gp63 or leishmanolysin, and the promastigote surface antigen (PSA2)/gp46 family of glycoproteins

(Medina-Acosta *et al.*, 1989; Murray *et al.*, 1989; Frommel *et al.*, 1990; Lohman *et al.*, 1990). Gp63 has been shown to be proteolytically active against a wide variety of different peptide substrates and has been reported to act as a ligand for macrophage receptors, either directly or after opsonization with complement, to protect the parasites from complement-mediated lysis and also to contribute to the pathology of lesion development (see Alexander and Russell, 1992; Joshi *et al.*, 1998). However, the fact that these proteins are encoded by multicopy polymorphic genes (Button *et al.*, 1989; Lohman *et al.*, 1990; Symons *et al.*, 1994) has hindered elucidation of their function by genetic analysis (Joshi *et al.*, 1998). Moreover, surface expression of gp63 and PSA2 is dramatically down-regulated in the amastigote stage of some *Leishmania* species and variable within particular parasite populations of others (Bahr *et al.*, 1993; Handman *et al.*, 1995) such that the precise function of these parasite proteins in the mammalian host remains unclear.

Proteins that are destined to receive a GPI anchor are synthesized as precursor proteins with an N-terminal signal sequence that targets the protein to the endoplasmic reticulum (ER) and a C-terminal GPI attachment signal. The latter

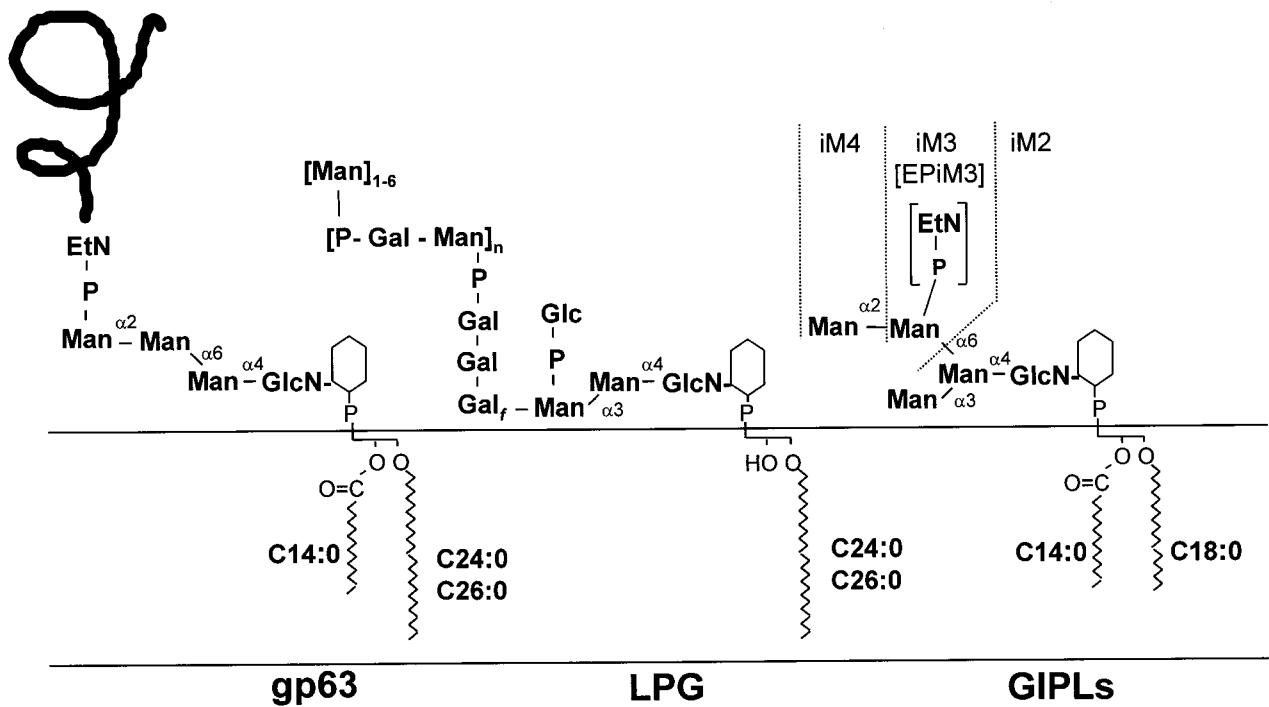§ Corresponding author. E-mail address: j.mottram@udcf.gla.ac.uk.

Figure 1. Structures of the three distinct classes of GPIs synthesized by *L. mexicana* promastigotes. These glycolipids act as membrane anchors for the major surface glycoprotein (gp63) and an abundant surface lipophosphoglycan (LPG) as well as being the major class of free glycolipids (GIPLs) (McConville *et al.*, 1993; Ralton and McConville, 1998). The nomenclature used for different GPI structures is as follows: M2–4 denotes GPIs with the structures $Man_{2-4}GlcN-PI$; EP denotes the presence of an EtN-P cap or side chain; and the prefix i denotes the presence of an $\alpha 1$–3-linked mannose in the core structure.

consists of three main domains: a C-terminal hydrophobic portion, a short hydrophilic spacer, and an attachment site (ω) (Caras *et al.*, 1989; Udenfriend and Kodukula, 1995). This signal is recognized by the GPI:protein transamidase complex, which proteolytically cleaves the precursor protein at the ω amino acid and links the newly generated C terminus to the terminal ethanolamine phosphate (EtN-P) of a preassembled GPI anchor precursor having the structure EtN-P-Man_3GlcN-phosphatidylinositol (PI) (Udenfriend and Kodukula, 1995). The transamidase is thought to form an intermediate with the precursor protein, resulting in an activated carbonyl group at the ω site (Maxwell *et al.*, 1995b; Sharma *et al.*, 1999). The nucleophilic ethanolamine component of the GPI anchor reacts with the active carbonyl, and the resultant GPI-anchored protein is then trafficked to the cell surface. This proposed reaction mechanism is supported by the finding that nucleophiles such as hydrazine can replace the GPI anchor in a cell-free assay system for GPI anchoring (Maxwell *et al.*, 1995b; Sharma *et al.*, 1999) and that the process is ATP and GTP independent (Mayor *et al.*, 1991). Interestingly, the GPI signal sequence differs between species, and that of *Trypanosoma brucei* variable surface glycoprotein functioned poorly when expressed in mammalian cells (Moran and Caras, 1994). Mutagenesis studies have suggested that the sizes of the peptide binding pocket in the trypanosome and mammalian GPI:protein transamidases differ (Moran and Caras, 1994). There are hopes that these differences may be exploited in the design of new antiparasite agents.

The GPI:protein transamidase complex contains at least two protein components, Gaa1p and Gpi8p, which have recently been identified by analysis of several temperature-sensitive yeast mutants defective in the GPI-anchoring of proteins. Gaa1p is a lumenally oriented ER glycoprotein with several transmembrane domains (Hamburger *et al.*, 1995), whereas Gpi8p is an ER–membrane glycoprotein with a large lumenal domain, a single C-terminal transmembrane domain, and a short cytoplasmic tail (Benghezal *et al.*, 1996). Yeast mutants defective in the functioning of these individual proteins accumulate preassembled GPI anchors and have no GPI-anchored proteins. Homologues of both of these proteins have now been identified in humans (Yu *et al.*, 1997; Hiroi *et al.*, 1998). It is believed that Gpi8p is the catalytic subunit (Yu *et al.*, 1997). This protein shares significant homology with a family of previously characterized cysteine proteinases, the asparaginyl endopeptidases known as legumains (Benghezal *et al.*, 1996), which were originally identified in the seeds of leguminous plants (Ishii, 1994). Together these proteins have been categorized as family C13 of cysteine peptidases (Riezman and Conzelmann, 1998).

We report here the cloning of *GPI8* from the parasitic protozoon *Leishmania mexicana* and the production of *GPI8* null mutants by targeted gene replacement. Despite the finding that GPI-anchored proteins are no longer detectable on the cell surface of the null mutants, these parasites show normal growth in in vitro culture, are able to differentiate into amastigotes, and survive within macrophages in vitro and can establish an infection in mice. These results suggest

that GPI-anchored proteins are not essential for the parasite's infectivity and survival in a mammalian host. In addition, we show that some of the protein anchor precursors in *Leishmania* contain an acyl modification on the inositol head group. These data suggest that the leishmanial pathway of protein anchor biosynthesis is more similar to that of the intensively studied African trypanosomes than was previously thought and provide another example of how different classes of GPI intermediates are differentially processed in these parasites.

MATERIALS AND METHODS

Growth of Parasites

Wild-type *L. mexicana* promastigotes (MNYC/BZ/62/M379) were maintained at 25°C in either modified Eagle's medium (HOMEM) or RPMI-1640 supplemented with 2 mM L-glutamine (Life Technologies, Gaithersburg, MD) containing 10% (vol/vol) heat-inactivated fetal calf serum. The required antibiotics were added as follows: ClonSat (Hans Knoll Institute, Jena, Germany) at 25 μg/ml, puromycin (Calbiochem, La Jolla, CA) at 10 μg/ml, and neomycin (G418, Geneticin; Life Technologies) at 25 μg/ml. Parasites were grown as amastigotes axenically in vitro using the method described previously (Bates *et al.*, 1992).

Cloning of GPI8

Degenerate oligonucleotide primers (IUB group codes) were designed against the following three peptide sequences based on those of yeast, human, and *Caenorhabditis elegans* GPI8 proteins: 1) TNNWAVLV (OL76, 5'-ACS AAY AAY TGG GCN GTB CTY GT-3'; 2) RFWFNYRH (OL77, 5'-CGB TTY TGG TTY AAY TAY CGB CA-3'; and 3) (I/F)YMTGHGG (OL79, 5'-CCV CCR TGV CCS GTV AKR TAR CT-3' (antisense).

Nested PCR was carried out with *Taq* Polymerase (Applied Biosystems, Foster City, CA) in a 20-μl reaction volume. In the first round, primers OL76 and OL79 were used with 100 ng of procyclic promastigote, metacyclic promastigote, and amastigote *L. mexicana* first-strand cDNA and genomic DNA using the following conditions: 1 cycle of 94°C for 4 min, 25 cycles of 94°C for 1 min, 42°C for 1 min and 72°C for 1 min, and 1 cycle of 72°C for 4 min. Two microliters of first-round PCR were then reamplified with primers OL77 and OL79 using the same conditions as for the first round. Reactions were electrophoresed on a 1% agarose gel, and the 362-bp PCR products were gel purified (Qiaquick gel extraction kit; Qiagen, Hilden, Germany) and cloned into pTAG vector (R & D Systems, Minneapolis, MN) for sequencing.

The full-length *GPI8* was isolated by screening an *L. mexicana* λ DashII genomic library (Mottram *et al.*, 1997) using the PCR product, labeled with α-^{32}P, as a probe. Two λ clones were isolated, and three subclones containing the *GPI8* gene were isolated and cloned into pBluescript (Stratagene, La Jolla, CA); a 2.1-kb *Sal*I fragment (pGL185), a 2.4-kb *Xho*I fragment (pGL186), and a 4.5-kb *Xba*I fragment (pGL187). Portions of these subclones were sequenced to provide double-stranded sequence covering the *GPI8* gene and ~1 kb of 5' and 3' flanks. The nucleotide sequence of *L. mexicana GPI8* is available in the European Molecular Biology Laboratory database under accession number AJ242865. Multiple sequence files produced using the program Pileup were used with the Internet analysis tool Boxshade version 3.21 (http://www.isrec.isb-sib.ch/software/BOX_form.html) to highlight amino acid identities and similarities.

Disruption of GPI8

Sequential rounds of transfection with *SAT* and *PAC* resistance knockout constructs were used to disrupt the *GPI8* gene. These constructs were designed to replace precisely the *GPI8* with each antibiotic resistance gene so that the endogenous processing signals would allow expression of the resistance genes. A 1.2-kb DNA fragment containing the 5' flank of *GPI8* was amplified from plasmid pGL186 with primers OL83 and OL84 and cloned into pCR script. A 900-bp DNA fragment containing the 3' flank of *GPI8* was amplified from plasmid pGL185 with primers OL85 and OL86 and cloned into pCR script. OL83 and OL84 were engineered with *Hin*dIII and *Spe*I sites, respectively, whereas OL85 and OL86 were engineered with *Bam*HI and *Bgl*II sites, respectively. Primer sequences were as follows: OL83, 5'-GCCAAGCTTTGCTCAGAT-GACCGAGCCGGCGC-3'; OL84, 5'-GACTAGTAAACAGCCGGA-ACTGCACTAGCT-3'; OL85, 5'-CGCGGATCCCGTGTGGCATC-TACCTCCCTGCG-3'; and OL86, 5'GAAGATCTTTGCTCGTGAT-ACGACGGCGTGG-3'.

The *Bam*HI–*Bgl*II fragment containing the *GPI8* 5' flank was cloned into *Bam*HI–*Bgl*II-digested pGL207 and pGL51 (plasmids that contain the *PAC* and *SAT* genes, respectively (Brooks and J.C. Mottram, unpublished data). Subsequently the *GPI8* 3' flank was used to replace the *Hin*dIII–*Spe*I fragment to give pGL236 (PAC) and pGL237 (SAT). The integration cassette was released by digestion with *Hin*dIII and *Bgl*II. The complete open reading frame (ORF) of *GPI8* together with 1.1 kb of 5' and 1.0 kb of 3' flanking regions, respectively, is found in the λ clone on a 3.1-kb *Hin*cII fragment. This *Hin*cII fragment was cloned into the *Sma*I site of pXG (Ha *et al.*, 1996) to produce plasmid clone pGL269.

Transfection of *L. mexicana* promastigotes was as described previously (Mottram *et al.*, 1996). Briefly, for gene knockout experiments the cassettes were excised by digestion with *Hin*dIII and *Bgl*II, and the insert was gel purified using a Qiextract kit (Qiagen). Five to 10 μg of DNA were used for each transfection with 4×10^7 promastigotes. For introduction of the episome pGL269, DNA was prepared using a Qiagen Tip100 column as outlined by the manufacturer (Qiagen). Transfection used 10 μg of pGL269 DNA and 4×10^7 Δ*GPI8* promastigotes. After electroporation, cells were allowed to recover in 10 ml of HOMEM for 24 h at 25°C, and then transfectants were selected on 1% agar/HOMEM plates containing appropriate antibiotics.

DNA Isolation and Southern Blot Analysis

Southern blotting using the hybridization membrane Hybond-N (Amersham, Little Chalfont, United Kingdom) was carried out as previously described (Mottram *et al.*, 1993) with 5 μg of genomic DNA prepared by the method of Medina-Acosta and Cross (1993). Probes were labeled by a random priming using Prime It II (Stratagene) and purified using a NucTrap column (Stratagene).

Analysis of GPI-anchored Proteins

For Western blotting, 10^8 promastigotes were lysed on ice with 100 μl of 0.25% Triton X-100 before immediate addition of reducing Laemmli sample buffer. Samples were boiled for 5 min before fractionation by 10% SDS-PAGE. Proteins were electroblotted onto Hybond-C, and the membranes were incubated overnight with blocking buffer (10% horse serum and 5% nonfat milk powder in 20 mM Tris-HCl, pH 7.6, 300 mM NaCl, and 0.05% Tween 20) at 4°C. Anti-gp63 monoclonal antibodies were a gift from Dr. Robert McMaster (University of British Columbia, British Columbia, Canada). They were used at a 1:25 dilution in blocking buffer at room temperature for 90 min followed by washing in 20 mM Tris-HCl, pH 7.6, 300 mM NaCl, and 0.05% Tween 20. Affinity-purified anti-CRK1 antibodies (Mottram *et al.*, 1993) were used at a 1:100 dilution. Secondary antibody (either anti-mouse or anti-rabbit immunoglobulin-horseradish peroxidase conjugate; Promega, Madison, WI) was used at a 1:5000 dilution. Enhanced chemiluminescence (Pierce, Rockford, IL) was used to detect antibody binding.

GPI-anchored proteins were metabolically labeled with [^{3}H]EtN as described below. Proteins were extracted in boiling 1% SDS and

solvent precipitated for analysis by 10% SDS-PAGE. Gels were incubated in En³Hance (New England Nuclear, Boston, MA) for detection of labeled protein by fluorography.

Characterization of EtN-P-containing GPIs

Wild-type and $\Delta GPI8$ promastigotes were metabolically labeled with [³H]EtN (20 μCi/ml) in RPMI-1640 with 10% fetal calf serum for 15 h at 27°C. Alternatively, promastigotes were pulse labeled with [³H]mannose for 30 min, as previously described (Ralton and McConville, 1998). Metabolically labeled promastigotes were extracted in chloroform/methanol/water (1:2:0.8 vol/vol), and GPI lipids were recovered by biphasic partitioning between 1-butanol (200 μl) and water (200 μl) (Ralton and McConville, 1998). [³H]EtN-labeled GPIs were purified by high-performance TLC (HPTLC) on aluminum-backed Silica Gel60 (Merck, Darmstadt, Germany), developed in chloroform/methanol/ammonium hydroxide/ammonium acetate/water (180:140:9:9:23 vol/vol) (Ralton and McConville, 1998) and then reanalyzed by HPTLC in chloroform/methanol/1 M ammonium hydroxide (10:10:3 vol/vol) before or after various treatments. Digestion with *Bacillus cereus* PI-specific phospholipase C (PI-PLC) was performed in 50 mM triethanolamine-HCl buffer, pH 7.5, 10 mM EDTA, and 0.16% sodium deoxycholate for 15 h at 37°C. Jack bean α-mannosidase digestion was performed in 0.1 M sodium acetate buffer, pH 5.0, for 15 h at 37°C. Base hydrolysis was performed in either 0.1 M methanolic NaOH for 2 h at 37°C (to cleave all ester-linked fatty acids) or a mixture of NH_4OH/methanol (1:1 vol/vol) for 2 h at 37°C (to preferentially cleave inositol acyl groups). The products of these treatments were recovered by passage down a column (0.5 ml) of octyl-Sepharose, equilibrated in 0.1 M sodium acetate and 5% 1-propanol, and eluted in 40% 1-propanol (Ralton and McConville, 1998). For characterization of [³H]Man-labeled Lipid X, this species was purified by HPTLC as described above and then treated with jack bean α-mannosidase in 0.1 M sodium acetate, pH 5.0, containing 0.2% taurodeoxycholic acid for 15 h at 37°C to hydrolyze comigrating GIPLs. The α-mannosidase-resistant Lipid X was then recovered by 1-butanol and water partitioning, and the glycan head group was obtained by hydrofluoric acid (HF) dephosphorylation (48% HF, 0°C, 48 h) followed by nitrous acid deamination and reduction with $NaBH_4$ as previously described (Ralton and McConville, 1998). Partial acetolysis of the released glycan head group was also performed as previously described (Zawadzki *et al.*, 1998).

Infections of Macrophages In Vitro and in Mice

Freshly culled BALB/c mice were injected intraperitoneally with 5 ml of ice-cold RPMI-1640 medium complete with 10 U/ml penicillin and 10 μg/ml streptomycin sulfate (Life Technologies). After 5 min of gentle massaging of the mouse abdomen, the RPMI-1640 that contained macrophages was harvested and kept on ice. Macrophages (2×10^5) were placed in each chamber of an eight-chamber Permanox slide (Labtek, Campbell, CA) and allowed to adhere overnight at 32°C under 5% CO_2/95% air. Chambers were washed with RPMI-1640 to remove nonadhered cells before 2×10^5 stationary phase promastigotes were added in RPMI-1640 with 10% fetal calf serum and incubated at 32°C under 5% CO_2/95% air. After 4 h, slides were again washed, and fresh RPMI-1640 with 10% fetal calf serum was added. Infection was allowed to proceed at 32°C under 5% CO_2/95% air. Individual chambers were fixed with 100% methanol for 5 min at 8 h and 1, 3, and 7 d after addition of promastigotes. Slides were stained with modified Giemsa stain (Sigma, St. Louis, MO), diluted 20-fold with distilled water for 30 min, and viewed by light microscopy using a Zeiss (Jena, Germany) Axioplan microscope. Infections of mice involved percutaneous inoculation of 5×10^6 stationary phase promastigotes into the shaven rumps of BALB/c mice. Amastigotes were isolated from lesions after 8 mo of infection and reinoculated into BALB/c mice (2.5×10^7 per mouse). The subcutaneous lesions produced were monitored over 4 mo.

RESULTS

Cloning and Characterization of the L. mexicana GPI8

Degenerate oligonucleotide primers, based on sequences of similarity between the *GPI8* sequences from *Saccharomyces cerevisiae*, human, and *C. elegans*, were used to amplify regions of *L. mexicana* cDNA or genomic DNA. A 362-bp PCR product was identified and cloned from cDNA produced from three life cycle stages of *L. mexicana*: procyclic promastigotes, metacyclic promastigotes, and amastigotes. Three identical PCR products were identified that showed encoded protein domains with significant sequence identity to human and yeast GPI8 proteins. The full-length *L. mexicana* GPI8 gene was subsequently isolated from a λ genomic library and sequenced. The *L. mexicana* GPI8 encodes a protein of 349 amino acids with a predicted size of 38 kDa. It has 31% sequence identity to yeast and human GPI8s and 19% identity to the plant asparaginyl endopeptidase legumain. A hydropathy analysis showed that the *L. mexicana* GPI8 has a similar overall profile to that of the yeast homologue, with an N-terminal hydrophobic domain that is likely to be a signal sequence directing entry into the ER. It lacks, however, a C-terminal hydrophobic domain found in yeast and human GPI8s that is predicted to be a transmembrane helix (Benghezal *et al.*, 1996). The GPI:protein transamidases belong to family C13 of the cysteine peptidases (Riezman and Conzelmann, 1998) and so are likely to possess the classical catalytic dyad residues, cysteine and histidine, that mediate activity. It has been shown that GPI:protein transamidase activity is susceptible to sulfydryl alkylating agents, implying that the protein has an essential cysteine (Sharma *et al.*, 1999). Two cysteine (Cys^{94} and Cys^{216}, *L. mexicana* GPI8 numbering) and two histidine (His^{63} and His^{174}) residues are conserved among leishmanial, yeast, and human GPI8 (Figure 2). Both histidine residues are also conserved in legumain, whereas only one of the cysteine residues is conserved across all C13 family members (Cys^{216}). Thus this residue is the prime candidate for the active site cysteine. Three serine residues are also conserved between the three GPI8 sequences and legumain (Ser^{55}, Ser^{236}, and Ser^{242}), and two more (Ser^{165} and Ser^{244}) are conserved between the three GPI8 proteins but are absent from legumain.

Genomic Organization of GPI8 and Production of GPI8 Null Mutants

Southern blot analysis revealed that *L. mexicana* GPI8 is a single-copy gene (Figure 3). A single hybridizing fragment was detected with *Hinc*II, *Hind*III, *Xba*I, and *Xho*I digests, whereas two DNA fragments were detected with a *Pst*I digest because of the presence of a *Pst*I site in the *GPI8* gene (Figure 4A). The sizes of the *Hinc*II (3.1 kb; Figure 3, lane 1) and *Xho*I (2.4 kb; Figure 3, lane 5) fragments correlate precisely with the 3.3 kb of sequence data obtained for the *GPI8* locus.

To investigate the role of GPI8 in *L. mexicana*, sequential rounds of targeted gene replacement were performed using *SAT* and *PAC* antibiotic resistance genes flanked by *GPI8* 5′ and 3′ sequence to replace *GPI8* (Figure 4A). A heterozygote clone (W1238, *GPI8::PAC*) in which one allele of *GPI8* had been removed by integration with *PAC* was subjected to a

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Lm   1    MRTTAYVMTSPTRCIATALIVFAFLVLTAAAAASAPLGATGKGQSNNWAVIV
Sc   1        MRIAMHLPLLLYIFLLPLSGANNTDAAHEVIATNTNNWAVLV
Hs   1    MAVTDSLSRAATVLATVLLLSFGSVAASHTEDQAEQFFRSGHTNNWAVLV
Leg  1       MVMMLVMLSLHGTAARLNRREWDSVIQLPTEPVDDEVGTRWAVLV

            2
      ─────────────
          ^           #                            *
Lm   53   SSSRYLFNYRHTANALIMYHLLRQHGIDDDHILLFLSDSFACDPRNVYPAEIFSQPPGAH
Sc   44   STSRFWFNYRHMANVLSMYRTVKRLGIPDSQIILMLSDDVACNSRNLFPGSVFNNK....
Hs   51   CTSRFWFNYRHVANTLSVYRSVKRLGIPDSHIVLMLADDMACNPRNPKPATVFSHK....
Leg  46   AGSNGYGNYRHQADVCHAYQLLIKGGVKEENIVVFMYDDIAYNAMNPRPGVIINHPQGP.

                                                                      3
                                                                 ─────────────
Lm   113  DADGRASVNLYGCSAQVDYAGSDVDVRRFISVLQGRYDENTPPTRRLLSDN.TSNIIIYV
Sc   100  ....DHAIDLYGDSVEVDYRGYEVTVENFIRLLTDRWTEDHPKSKRLLTDE.NSNIFIYM
Hs   107  ....NMEINVYGDDVEVDYRSYEVTVENFLRVLTGRIPPSTPRSKRLLSDD.RSNILIYM
Leg  105  ........DVYA.GVPKDYTGEDVTPENLYAVILGDKSKVKGGSGKVINSNPEDRIFIFY

            #                                              *
Lm   172  AGHGAKSYFKFQDTEFLSSSDISETLTMMHQQRRYGRVVFLADTCHAIALCEHVEAP..N
Sc   155  TGHGGDDFLKFQDAEEIASEDIADAFQQMYEKKRYNEIEFMIDTCQANTMYSKFYSP..N
Hs   162  TGHGGNGFLKFQDSEEITNIELADAFEQMWQKRRYNELLFIIDTCQGASMYERFYSP..N
Leg  156  SDHGGPGVLGMPNAPFVYAMDFIDVLKKKHASGGYKEMVIYIEACESGSIFEGIMPKDLN

               ^         ^ ^
Lm   230  VVCLAASDAESESYSCQYDEQLGTHMVSFWMNEMYLLLNGTSCSNPLTRRIGDDAVSVLH
Sc   213  ILAVGSSEMDESSYSHHSDVEIGVAVIDRF...TYYCLDFLEQIDKNSTLTLQDLFDSFT
Hs   220  IMALASSQVGEDSLSHQPDPAIGVHLMDRY...TFYVLEFLEEINPASQTNMDLFQVCP
Leg  216  IYVTTASNAQENSEG.TYCPCMNPPPPEEYVTCLGDLYSVSWMEDSETHNLKRETVQQQY

Lm   290  QSWYNFNYHPYRVEASRNRSKPAHRDAVNDPTALREWIVADFVCGQVSAAVPVD..VRYD
Sc   270  FEKIHSHVGVRTDLFDRNPSEVLITDFFANVQNV...IPDDSKPLSVSHYHHYK..DHID
Hs   277  KSLCVSTPGHRTDLFQRDPKNVLITDFFGSVRKVE..ITTETIKLQQDSEIMES..SYKE
Leg  275  QSVRKRTSNSNSYRFGSHVMCYGDTNITAEKLYLYHGFDPATVNFPPHNGNLEAKMEVVN

Lm   348  LE
Sc   325  TA  +++  411
Hs   333  DQ  +++  395
Leg  335  QR  +++  475
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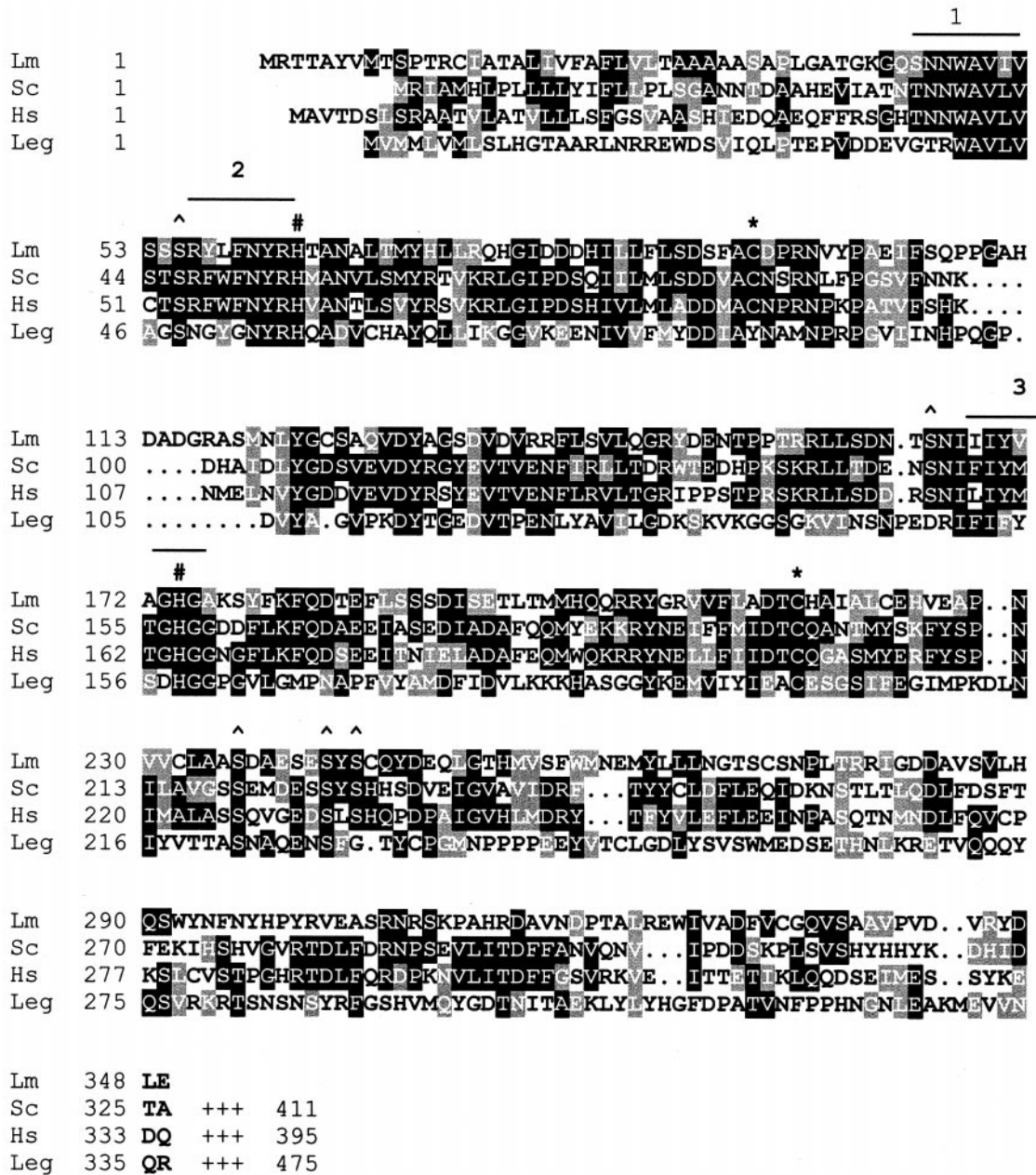
Figure 2. GPI8 protein sequence of *L. mexicana* (Lm; this study) aligned with *S. cerevisiae* (Sc; SwissProt, accession number P39012), human (Hs; Q92643), and *C. ensiformis* legumain (Leg; P49046). Gaps (.) were introduced to help the alignment; the C-terminal ends of Sc, Hs, and Leg have been omitted. Amino acid identities and similarities are highlighted. The highly conserved regions used to design degenerate oligonucleotide primers to isolate the *L. mexicana GPI8* are overlined and numbered. Conserved cysteine (∗), histidine (#) and serine (^) residues are indicated. *L. mexicana* GPI8 has three potential N-glycosylation sites at positions N^{163}, N^{268}, and N^{307}, none of which is conserved with yeast GPI8 that is heavily glycosylated (Benghezal *et al.*, 1996).

second round of transfection with the *SAT*-containing cassette, and clones resistant to both antibiotics ($\Delta GPI8$ clones W1234 and W1236) were analyzed by Southern blotting to confirm that they were indeed *GPI8* deficient and had undergone the predicted genomic rearrangements. *Xho*I-digested genomic DNA from wild-type *L. mexicana*, W1238 (*GPI8::PAC*, clone 1), W1234 ($\Delta GPI8$, clone 1), and W1236 ($\Delta GPI8$ clone 2) was probed with a 1-kb *Xba*I–*Xho*I fragment containing the *GPI8* ORF. A 2.4-kb *Xho*I fragment was detected in wild-type *L. mexicana* corresponding to the two *GPI8* alleles (Figure 4B, lane 1). One *GPI8* allele remained in the W1238 heterozygote (lane 2); however, the *GPI8* gene
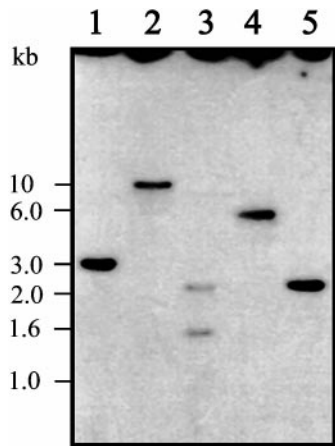
Figure 3. Southern blot. Restriction digests of *L. mexicana* wild-type genomic DNA (5 μg) using *Hinc*II (lane 1), *Hind*III (lane 2), *Pst*I (lane 3), *Xba*I (lane 4), and *Xho*I (lane 5) were electrophoresed on a 0.8% agarose gel and blotted onto a nylon membrane. The blot was probed with a radio-labeled 1.1-kb *Xba*I–*Xho*I fragment containing the *GPI8* ORF.

could not be detected in either of the Δ*GPI8* mutants (lanes 3 and 4). To confirm that *SAT* and *PAC* had integrated into the correct locus, the same blot was stripped and reprobed with the drug resistance genes (Figure 4, C and D). As

predicted, the *PAC* probe hybridized to a band of 3.7 kb with the W1238 heterozygote (Figure 4C, lane 2) and both Δ*GPI8* clones (lanes 3 and 4) but not wild-type parasites (lane 1). The *SAT* probe hybridized to a 3.6-kb band in the Δ*GPI8* clones (Figure 4C, lanes 3 and 4), which was absent from the wild-type and W1238 heterozygote (lanes 1 and 2). These results, together with further Southern blot analysis with a 5′ flank probe (our unpublished results), indicate that both alleles of *GPI8* had been replaced with *SAT* and *PAC* and that the mutants are *GPI8* deficient. *GPI8* was cloned into the pXG episomal expression vector and introduced into Δ*GPI8* clones 1 and 2 by electroporation to give G418-resistant cell lines W1235 Δ*GPI8*[pXG-*GPI8*] and W1237 Δ*GPI8*[pXG-*GPI8*].

Δ*GPI8* Mutants Lack GPI-anchored Proteins

The major GPI-anchored surface protein of *Leishmania* promastigotes is the metalloproteinase gp63 (Schneider *et al.*, 1990). Immunofluorescence was carried out with a monoclonal antibody directed against *L. mexicana* gp63 on promastigotes of wild-type *L. mexicana*, Δ*GPI8*, and Δ*GPI8*[pXG-*GPI8*]. A strong signal was detected with wild-type *L. mexicana*, but no gp63 was detected with Δ*GPI8* (our unpublished results). Levels of surface expression of gp63 were restored to wild-type levels in the cell line expressing an episomal copy of *GPI8*. The presence of gp63 in cell lysates from these cell lines was investigated by Western blotting
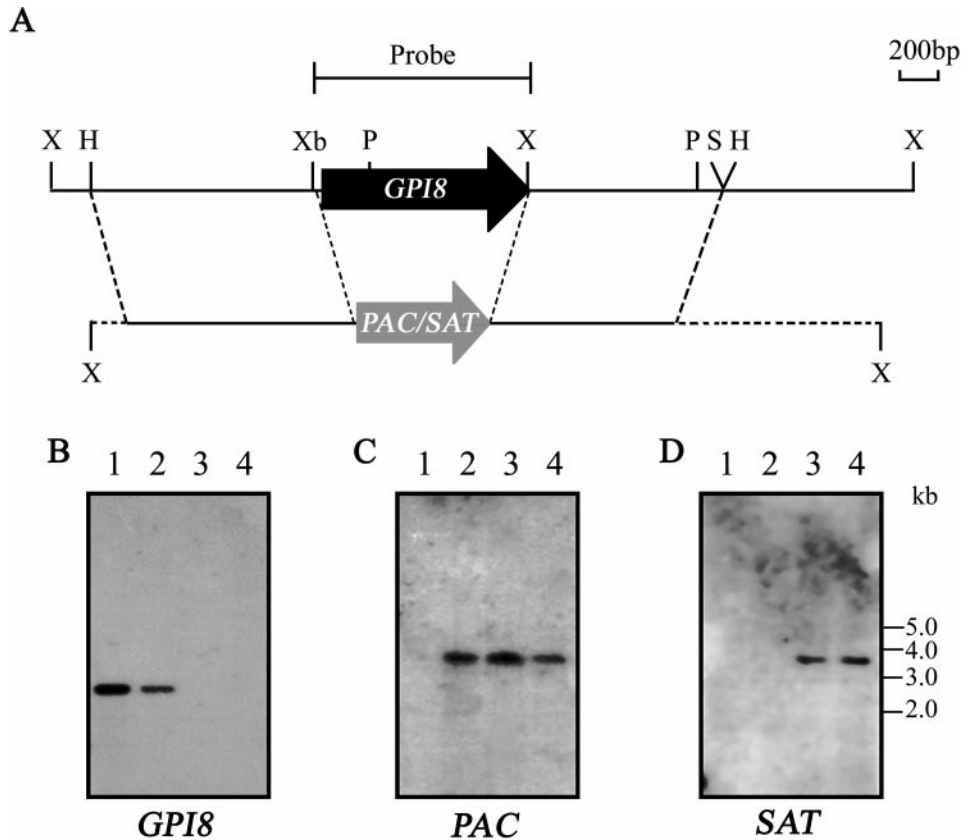

Figure 4. Southern blot analysis of *GPI8* mutants. (A) Map of *GPI8* locus and predicted recombination events. X, *Xho*I; H, *Hinc*II; Xb, *Xba*I; P, *Pst*I; S/H, *Sal*I and *Hinc*II. Note the loss of the *Xho*I site within *GPI8* after recombination. (B) Five micrograms of genomic DNA from wild-type *L. mexicana* (lane 1), W1238 heterozygote mutant (lane 2), W1234 Δ*GPI8* clone 1 (lane 3), and W1236 Δ*GPI8* clone 2 (lane 4) were digested with *Xho*I and fixed to a nylon membrane. The blot was probed with a radiolabeled 1.1-kb *Xba*I–*Xho*I fragment containing the *GPI8* ORF. The same blot was stripped and reprobed with radiolabeled *PAC* (C) and *SAT* (D) probes.
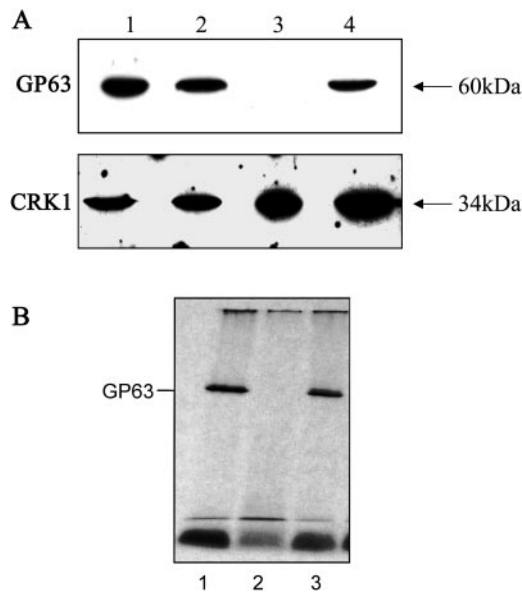
Figure 5. Analysis of gp63 expression. (A) Western blotting. Lysates of wild-type (lane 1), W1238 *GPI8* heterozygote (lane 2), W1234 Δ*GPI8* (lane 3), and W1235 Δ*GPI8*[pXG-*GPI8*] (lane 4) promastigotes were probed with an anti-gp63 mouse monoclonal antibody or anti-CRK1 kinase antibodies as a control for protein loading. (B) Metabolic labeling. Promastigotes were metabolically labeled to steady state with [³H]EtN, and labeled GPI:proteins were detected after separation by SDS-PAGE. Lysates of wild-type (lane 1), W1234 Δ*GPI8* (lane 2), and W1235 Δ*GPI8*[pXG-*GPI8*] (lane 3) promastigotes are shown.

with anti-gp63 antiserum (Figure 5A). An ∼60-kDa protein was detected by immunoblotting with wild-type *L. mexicana* (lane 1) and the heterozygote (lane 2) but was absent from Δ*GPI8* (lane 3). gp63 was restored in the Δ*GPI8*[pXG-*GPI8*] cell line (lane 4). Equal loading of cell lysates was confirmed with an antiserum against the CRK1 protein kinase (Mottram *et al.*, 1993). Long-term exposure of the blot revealed that Δ*GPI8* contained a small amount of protein recognized by the anti-gp63 antibody (our unpublished results). It had a similar mass to gp63 itself and could either have been undegraded precursor protein or possibly the isoenzyme characteristically present in the lysosomes of amastigotes, which is not GPI anchored (Medina-Acosta *et al.*, 1989; Ilg *et al.*, 1993). The residual gp63 protein detected was not GPI anchored, because no [³H]ethanolamine-labeled gp63 could be detected in the Δ*GPI8* mutant (Figure 5B). Interestingly, even after a very-long-term (3 mo) exposure of the autoradiograph no other GPI-anchored proteins could be detected in this strain of *L. mexicana*.

Δ*GPI8* Mutants Accumulate a Novel Inositol-acylated GPI Species

Wild-type parasites and Δ*GPI8* and Δ*GPI8*[pXG-*GPI8*] mutants were metabolically labeled to steady state with [³H]EtN to investigate whether disruption of protein anchoring resulted in the accumulation of any EtN-P-containing protein–anchor precursors. Two [³H]EtN-labeled species

were present in fivefold higher levels in Δ*GPI8* compared with wild-type cells (Figure 6A, compare lanes 1 and 2). We have previously shown that one of these bands, EPM3$_{VLAC}$, is likely to be the major protein GPI anchor precursor in *L. mexicana* and has the structure EtN-P-Man$_3$GlcN-PI (Ralton and McConville, 1998). The second species, termed Lipid X, represents a novel species that has not been previously characterized. Interestingly, levels of both EPM3$_{VLAC}$ and Lipid X were decreased in Δ*GPI8*[pXG-*GPI8*] compared with the wild-type parasites, suggesting that both species may be used at a higher rate in cells overexpressing this component of the transamidase (Figure 6A, lane 3). The cellular levels of other EtN-labeled phospholipids, as well as EPiM3, the major EtN-P-containing free GPI (see Figure 1 for structure), were essentially unchanged in all three cell lines (Figure 6A, lanes 1–3).

To characterize Lipid X further, the [³H]EtN-labeled species was purified by HPTLC and subjected to a series of enzymatic and chemical treatments. Lipid X was resistant to both PI-specific phospholipase C and jack bean α-mannosidase digestions (Figure 6B, lanes 6–8). In contrast, EPiM3 was completely digested by PI-PLC and was partially converted to a faster HPTLC migrating species after jack bean α-mannosidase digestion, consistent with the removal of the α1–3-linked mannose side chain (Figures 1 and 6B, lanes 1–3). Treatment of Lipid-X with methanolic-NaOH using conditions that should remove all ester-linked acyl groups generated a new species with a slower HPTLC mobility (species γ; Figure 6B, lane 11) that was sensitive to PI-PLC digestion (Figure 6B, lane 12). This species migrated with a slightly faster HPTLC mobility than the base-generated *lyso*-EPiM3, which will retain a single C18:0 alkyl chain (Figures 1 and 6B, lanes 4 and 5). When Lipid X was subjected to milder base hydrolysis, using conditions that preferentially cleave the inositol-linked acyl chains rather than glycerolipids, two new species with intermediate HPTLC mobilities were generated (species α and β; Figure 6B, lane 9). Species α had a slightly slower HPTLC mobility than Lipid X and remained resistant to PI-PLC, whereas species β migrated with the same HPTLC mobility as the putative protein anchor precursor EPM3$_{VLAC}$ and was sensitive to PI-PLC digestion (Figure 6B, lane 10). Collectively these results suggested that Lipid X corresponds to a GPI with two base-sensitive acyl chains and a single base-resistant (alkyl) chain. The resistance of Lipid X and species α to PI-PLC suggested that both of these lipids retained a base-labile constituent on the inositol head group. Species α (which is present in small amounts in untreated samples) may be generated by base-catalyzed migration of an acyl group around the inositol ring. Species β most likely corresponded to Lipid X minus the inositol acyl modification (rendering it susceptible to PI-PLC), whereas species γ corresponded to Lipid X minus both the inositol acyl modification and the *sn*-2 fatty acyl chain in the PI glycerolipid.

To define further the structure of the glycan head group of Lipid X, Δ*GPI8* was metabolically labeled with [³H]mannose, and a species with the same HPTLC mobility as Lipid X was purified by HPTLC and exhaustive α-mannosidase digestion to remove comigrating free GPIs (the latter species are not modified with an EtN-P and are therefore digested by α-mannosidase [Ralton and McConville, 1998]). The neutral glycan head group of Lipid X obtained by hydrogen
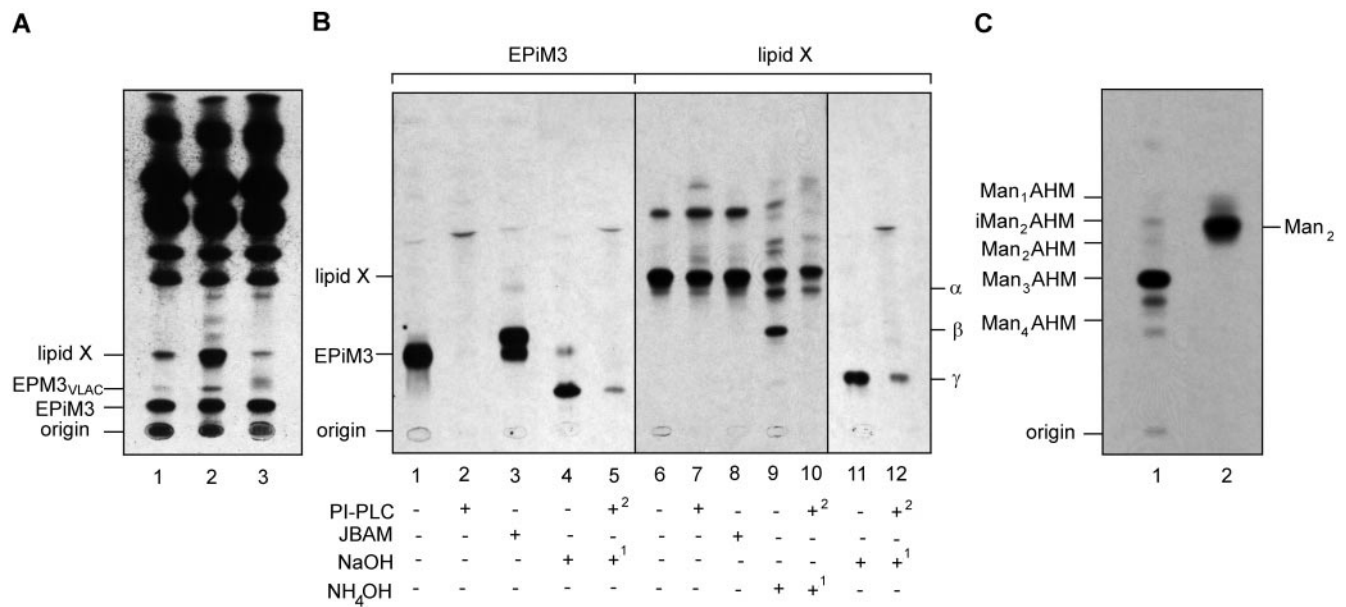
Figure 6. Accumulation of protein–anchor intermediates in *GPI8* mutants. (A) HPTLC analysis of [³H]EtN-labeled lipids in wild-type (lane 1), W1234 Δ*GPI8* (lane 2), and W1235 Δ*GPI8*[pXG-*GPI8*] (lane 3) promastigotes. The elution positions of the major EtN-P-modified GIPL, EPiM3, and a previously characterized protein anchor precursor, EPM3$_{VLAC}$, are indicated ($_{VLAC}$ refers to GPIs with very long [C24:0/C26:0] alkyl chains in the lipid moiety). Lipid X represents a second putative protein anchor precursor that accumulates in Δ*GPI8*. (B) [³H]EtN-labeled EPiM3 and Lipid X were purified by HPTLC and treated with PI-PLC, jack bean α-mannosidase (JBAM), and either strong (NaOH) or mild base (NH₄OH), as indicated. Numbers refer to the order in which each treatment was performed. The products were recovered by 1-butanol partitioning and analyzed by HPTLC. The base treatments generated several novel species (α, β, and γ) that may represent the products of acyl migration or deacylation (discussed in RESULTS). (C) HPTLC analysis of the neutral glycan head group of Lipid X. Lipid X was metabolically labeled with [³H]mannose and purified by HPTLC. The neutral glycan obtained after HF hydrolysis and nitrous acid deamination and reduction was analyzed by HPTLC before (lane 1) or after (lane 2) acetolysis. The migration positions of standard glycans, Man$_{1–4}$AHM (glycan head groups derived from Man$_{1–4}$GlcN-PI), and Manα1–2Man (Man$_2$ derived from [³H]mannose-labeled Man$_4$AHM) are indicated.

fluoride dephosphorylation, nitrous acid deamination, and reduction (which cleaves the PI lipid moiety and converts the terminal GlcN to 2,5-anhydromannitol [AHM]) comigrated with authentic Man$_3$AHM on HPTLC (Figure 6C, lane 1). Partial acetolysis of this glycan, which is expected to cleave the 1–6 linkage in the conserved Man$_3$GlcN backbone of the gp63 anchor (Schneider *et al.*, 1990), generated a faster migrating species, which comigrated with the disaccharide Manα1–2Man (Figure 6C, lane 2). This is the expected product if most of the [³H]mannose label was incorporated into the two outer mannose residues. These data suggested that Lipid X contained the same Man$_3$GlcN glycan core as the gp63 GPI anchor (Figure 1). Moreover, based on the HPTLC mobilities of the deacylated Lipid-X species it is likely that this species had the same 1-*O*-alkyl(C24:0/C26:0)-2-*O*-acyl-PI lipid moiety as EPM3$_{VLAC}$ and the mature protein-linked GPIs (Schneider *et al.*, 1990; Ralton and McConville, 1998). However, this species is unusual in containing an inositol acyl modification that has not been identified on any other leishmanial GPIs.

Cellular Levels of Non–Protein-linked GPIs Are Unchanged in the Δ*GPI8* Mutant

To determine whether the cellular levels of non–protein-linked GPIs were altered in the Δ*GPI8* mutants, cells were labeled to steady state with [³H]inositol, and total inositol lipids were analyzed by HPTLC. [³H]Inositol is incorporated into the major phospholipids PI and inositolphosphoceramide, as well as into the predominant free GPI species iM2, iM3, and iM4 (see Figure 1 for structures). The levels of these free GPIs, relative to the levels of PI, were essentially the same in wild-type parasites, Δ*GPI8,* and Δ*GPI8*[pXG-*GPI8*] (Figure 7A). Deletion of GPI8 also had no effect on the levels of the major GPI-anchored macromolecule LPG (Figure 7B). Thus an accumulation of protein–anchor precursors does not reduce the rate of synthesis of non–protein linked GPIs.

Δ*GPI8* Infect and Replicate in Macrophages and Mice

Promastigotes of the two Δ*GPI8* clones were found to grow at the same rate in liquid culture as wild-type *L. mexicana* promastigotes and could differentiate into amastigote-like forms in vitro. To test whether the mutants were capable of surviving in mammalian cells, invasion experiments were performed with macrophages in vitro (Figure 8). The mouse macrophages were allowed to adhere to slides before a 1:1 exposure to stationary-phase promastigotes of wild-type *L. mexicana*, Δ*GPI8*, and Δ*GPI8*[pXG-*GPI8*]. Samples were taken at 8 h and 1, 3, and 7 d after infection. Δ*GPI8* and Δ*GPI8*[pXG-*GPI8*] mutants were found to infect macro-
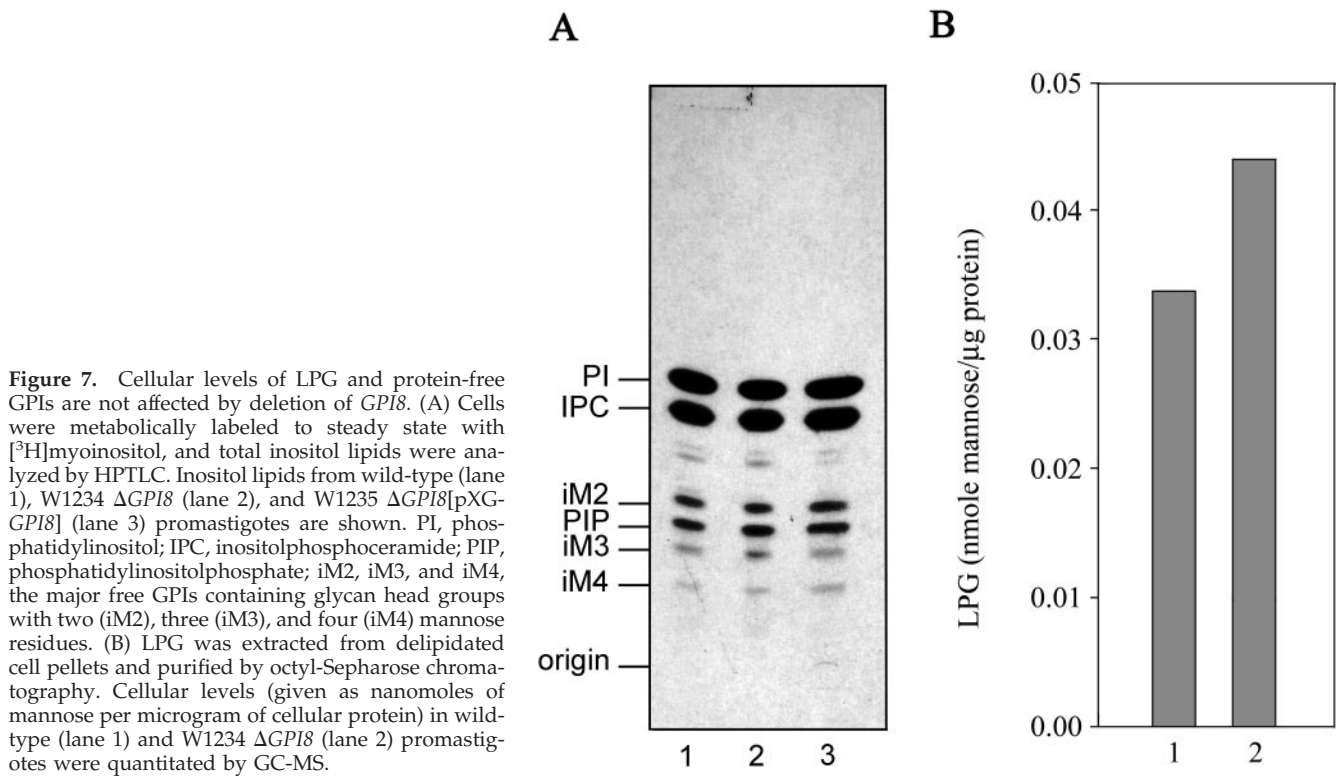
A

B

Figure 7. Cellular levels of LPG and protein-free GPIs are not affected by deletion of *GPI8*. (A) Cells were metabolically labeled to steady state with [³H]myoinositol, and total inositol lipids were analyzed by HPTLC. Inositol lipids from wild-type (lane 1), W1234 Δ*GPI8* (lane 2), and W1235 Δ*GPI8*[pXG-*GPI8*] (lane 3) promastigotes are shown. PI, phosphatidylinositol; IPC, inositolphosphoceramide; PIP, phosphatidylinositolphosphate; iM2, iM3, and iM4, the major free GPIs containing glycan head groups with two (iM2), three (iM3), and four (iM4) mannose residues. (B) LPG was extracted from delipidated cell pellets and purified by octyl-Sepharose chromatography. Cellular levels (given as nanomoles of mannose per microgram of cellular protein) in wild-type (lane 1) and W1234 Δ*GPI8* (lane 2) promastigotes were quantitated by GC-MS.

phages in vitro to wild-type levels (Figure 8A). In addition, the numbers of amastigotes per infected macrophage increased over the 7-d period to a similar extent for each of the three cell lines (Figure 8B). This shows that macrophage entry by promastigotes, differentiation into amastigotes, and survival and replication within the macrophage phagolysosome is not dependent on GPI-anchored proteins. Δ*GPI8* and Δ*GPI8*[pXG-*GPI8*] mutants were inoculated into BALB/c mice, and after 8 mo amastigotes were isolated from lesions for inoculation into six BALB/c mice. The size of the subsequent lesions was monitored over a 4-mo period (Figure 9). All animals infected with Δ*GPI8* and Δ*GPI8*[pXG-*GPI8*] produced large cutaneous lesions ranging in volume from 50 to 350 mm³ after 22 wk. In general, wild-type *L. mexicana* amastigotes produced larger lesions (100–800 mm³ after 22 wk) that formed slightly more rapidly than those caused by the mutant parasites. These data show that amastigote replication and survival in mice is not dependent on GPI-anchored proteins.

DISCUSSION

Several lines of evidence point to the *L. mexicana* GPI8 being a functional homologue of yeast Gpi8p and a component of the GPI:protein transamidase complex. First, there is the high level of sequence identity among the predicted yeast, human, and *L. mexicana* GPI8 proteins, including the conservation of cysteine and histidine residues that potentially have key involvement in the catalytic mechanism. Second, deletion of *GPI8* from *L. mexicana* led to the accumulation of the putative protein–anchor precursor EPM3$_{VLAC}$ (Ralton

and McConville, 1998). Third, Δ*GPI8* mutants are deficient in GPI-anchored gp63, the major surface protein of *L. mexicana*. Interestingly, [³H]ethanolamine labeling experiments (Figure 5B) confirmed the presence of GPI-anchored gp63 in wild-type *L. mexicana* but failed to detect other GPI-anchored proteins that have been reported to be present at low abundance on the surface of other *Leishmania* species (Murray *et al.*, 1989; Lohman *et al.*, 1990). It remains a formal, albeit unlikely, possibility that a transamidase structurally unrelated to GPI8 carries out the GPI:protein transamidase reaction for low-abundance GPI-anchored proteins. However, the buildup of the protein:anchor precursor and the loss of GPI-anchored gp63 in the Δ*GPI8* mutant, together with the finding that *GPI8* is a single-copy gene (as found in yeast), argues strongly that GPI8 is an essential component of the single GPI:protein transamidase in *Leishmania*.

Sequence comparisons suggest that the *L. mexicana* GPI8 protein is smaller than the *S. cerevisiae* and human homologues (38 vs. 46 kDa) and that it lacks the C-terminal hydrophobic domain that is thought to be involved in anchoring these other GPI8 proteins to the lumenal leaflet of the ER (Benghezal *et al.*, 1996; Yu *et al.*, 1997). Attachment of *L. mexicana* GPI8 to the ER membrane, where the protein will be able to interact with the preformed GPI anchor, may thus require one or more other integral membrane proteins. A possible candidate is Gaa1p, an integral membrane protein that appears to an essential component of the GPI:transamidation complex of yeast (Hamburger *et al.*, 1995). This type of variation between ER isoenzymes involved in GPI biosynthesis is not unprecedented. Two classes of dolichol-phosphate-mannose synthase have been identified in differ-
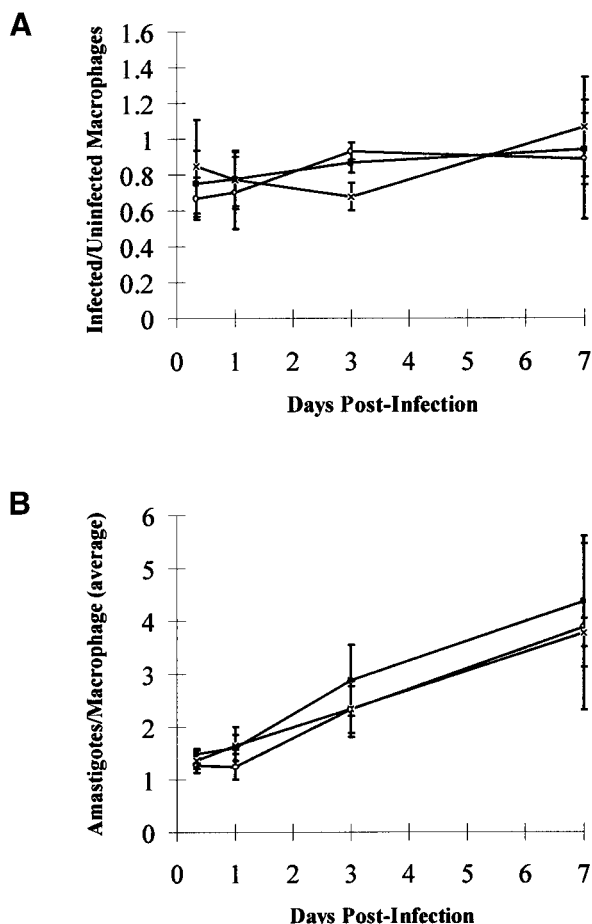
A

B

Figure 8. Macrophage infectivity assays. Peritoneal exudate cells from BALB/c mice were infected 1:1 with stationary phase promastigotes of wild-type *L. mexicana* (×), W1234 Δ*GPI8* (■), and W1235 Δ*GPI8*[pXG-*GPI8*] (○). Samples were taken over a 7-d period, and the number of intracellular parasites was counted after fixation and Giemsa staining. More than 100 macrophages were counted for each time point, and the results are expressed in terms of the ratio of infected to uninfected macrophages (A) and the number of amastigotes per infected macrophage (B). The data are means and SDs from several experiments.

Figure 9. Infectivity to mice. BALB/c mice were inoculated with lesion amastigotes of wild-type *L. mexicana*, W1236 Δ*GPI8,* and W1237 Δ*GPI8*[pXG-*GPI8*], and the subsequent lesion sizes were monitored over 22 wk. Data from individual mice in each group are shown to highlight the variation in lesion size between them.

ent species (Colussi *et al.*, 1997). The human and fission yeast enzymes lack a C-terminal hydrophobic domain, whereas the trypanosome and budding yeast enzymes contain one. The two classes of this enzyme, however, are functionally equivalent, because both the human and *S. cerevisiae* genes can complement a lethal null mutant in fission yeast (Colussi *et al.*, 1997).

Leishmania promastigotes synthesize a number of GPI-anchored proteins that are minor, but significant, components in the well-defined glycocalyx that covers the promastigote surface. The other major components in this glycocalyx are LPG and the family of free GPIs (GIPLs) that are likely to form a densely packed layer immediately above the plasma membrane (McConville and Ferguson, 1993). Although a variety of in vitro studies have suggested that these GPI-anchored proteins fulfill a number of functions in
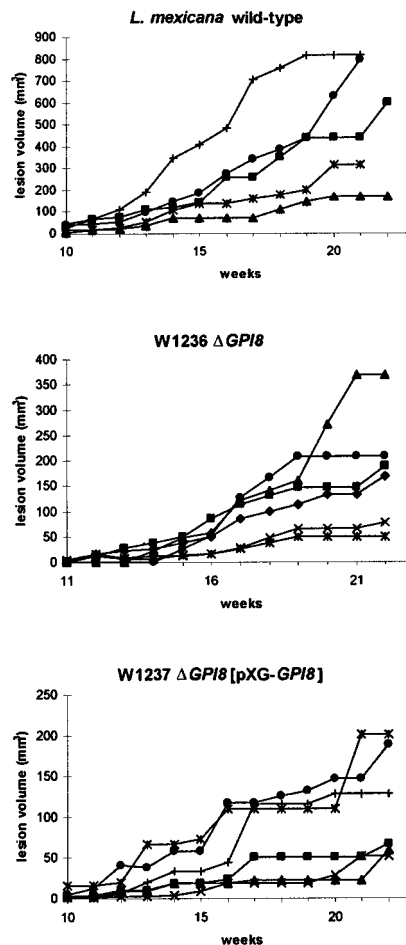
different developmental stages of the parasite, the relative significance of these proteins for parasite infectivity and survival in the insect or mammalian hosts has been unclear. Previous attempts to obtain parasites that are defective in one or more of these proteins by targeted gene deletion have been hampered by the fact that most of the proteins are encoded by multigene families. Interestingly, targeted deletion of six of the seven *L. major* gp63 genes did not affect growth in vitro or prevent the formation of disease in mice (Joshi *et al.*, 1998). An alternative approach to obtaining *Leishmania* parasites lacking GPI-anchored proteins exploited the finding that ectopic expression of *T. brucei* GPI-PLC in *Leishmania* results in the selective degradation of protein anchor GPI precursors (Mensa-Wilmot *et al.*, 1994; Ilgoutz *et al.*, 1999b; Mensa-Wilmot *et al.*, 1999). However, this approach does not result in the complete removal of all GPI-anchored proteins and may also lead to the partial degradation of non–protein-linked GPIs (Mensa-Wilmot *et*

al., 1999) and, therefore, is unable to provide definitive data on the specific functions of GPI-anchored proteins. In our study, we have eliminated the expression on the parasite surface of the major GPI-anchored glycoprotein gp63. Although expression of the much less abundant PSA2/gp46 could not be detected even in wild-type *L. mexicana*, it is likely that the GPI-anchoring and therefore surface expression of these glycoproteins are also prevented in the Δ*GPI8* mutant.

The absence of GPI-anchored proteins had no effect on the growth of the parasite in liquid culture or its ability to infect and survive in macrophages in vitro. Importantly, the Δ*GPI8* mutant was also able to establish an infection in mice. These data suggest that GPI-anchored proteins are not essential for growth or infectivity to mammals. It remains to be determined whether they play a key role for the parasite in the insect host. In contrast to our findings with the Δ*GPI8* mutant, we have recently shown that the gene encoding dolichol-phosphate mannose synthase, a key enzyme in GPI biosynthesis, is essential for growth of *L. mexicana* promastigotes (Ilgoutz *et al.*, 1999b). Taken together with the results described here, it is likely that this enzyme is essential because of a requirement for continued synthesis of non–protein-linked free GPIs in the promastigote stage.

Although all three classes of GPI synthesized by *Leishmania* contain the same core, they each contain distinct glycan head group modifications and lipid compositions (Figure 1). Moreover, the synthesis of the protein and LPG anchors, but not the GIPLs, is developmentally regulated (McConville and Blackwell, 1991). These findings suggest that the regulation of GPI biosynthesis in *Leishmania* is complex and may require the presence of distinct enzyme complexes and/or some degree of compartmentalization of these pathways (Ilgoutz *et al.*, 1999a). The finding in this study that a subpool of mature protein anchor precursors becomes inositol acylated provides another example of how this class of GPI intermediates is processed differently from the GIPL or LPG anchor intermediates. The inositol acylated GPI intermediate (Lipid X) has been overlooked in previous analyses (McConville *et al.*, 1993; Ralton and McConville, 1998), because it is present in low levels in wild-type cells and comigrates in HPTLC analysis with one of the major GIPL species. However, the involvement of Lipid X in the GPI protein anchor pathway is now strongly suggested by the structural characterization of this species and the finding that both Lipid X and the protein anchor precursor $EPM3_{VLAC}$ are specifically accumulated in Δ*GPI8* mutant cells. Inositol acylation of GPI anchor intermediates usually involves the addition of a saturated fatty acid to the 2-hydroxyl of the inositol residue and appears to occur in all eukaryotes (Ferguson 1999; Tiede *et al.*, 1999; McConville and Menon, 2000). However, the timing, nature of the acyl donor, and function of this modification may vary between higher and lower eukaryotes (Güther and Ferguson, 1995; Smith *et al.*, 1997). For example, in animal cells and yeast, inositol acylation occurs at an early stage in GPI biosynthesis (after de-N-acetylation of GlcN-PI), whereas in African trypanosomes it occurs later in the pathway (Güther and Ferguson, 1995). Inositol acylation in *Leishmania* appears to be analogous to the situation in African trypanosomes, because we have found no evidence for the inositol acy-

lation of early GPI intermediates. Moreover, as in the trypanosomes, there is no evidence that these inositol acylated GPI species are added to protein (Schneider *et al.*, 1990). In trypanosomes inositol acylation of $Man_3GlcN-PI$ is thought 1) to be a prerequisite for addition of the EtN-P head group and for subsequent fatty acid remodeling reactions of latter intermediates and 2) to play a role in regulating the catabolism of excess GPI intermediates (Güther and Ferguson, 1995; Milne *et al.*, 1999). Pulse–chase labeling experiments suggest that Lipid X may also be part of a catabolic pathway in *Leishmania* (J.L. Zawadzki and M.J. McConville, unpublished data). Whatever its function, this type of modification appears to be highly selective for protein anchor precursors, because it is not found on the more abundant and structurally similar $Man_3GlcN-PI$ intermediates in GIPL biosynthesis (Ralton and McConville, 1998). It is possible that the GPI inositol acyltransferase only recognizes GPI molecular species with very long alkyl chains (C24:0/C26:0) that are selectively incorporated into the protein and LPG anchors (Ralton and McConville, 1998). Alternatively, the acyltransferase may only have access to this pool of GPIs if intermediates from different pathways are present in different membranes or have different membrane topologies. Given the distinct properties and probable function of inositol acylation in trypanosomes compared with animal cells, it has been proposed that the inositol acyltransferase may be a target for new anti-trypanosome therapeutics (Smith *et al.*, 1999). However, the fact that this modification is restricted to nonessential protein–anchor precursors in *Leishmania* suggests that therapeutics such as these would not be useful against these parasites.

The lack of detectable gp63 in the Δ*GPI8* mutants suggests that the newly synthesized gp63 is either rapidly degraded or secreted. We have been unable to detect any secretion of gp63 from Δ*GPI8*, suggesting that this protein is being degraded intracellularly. In contrast, depletion of intracellular levels of GPI protein anchor precursors by ectopic expression of *T. brucei* GPI-PLC in *L. major* resulted in the secretion of a hydrophilic form of gp63 (Mensa-Wilmot *et al.*, 1994). Similarly, cleavage of the signal peptide without anchor addition has been reported in both mammalian and trypanosomal in vitro systems and resulted in the release of processed, but GPI-free, protein (Maxwell *et al.*, 1995a; Ramalingam *et al.*, 1996; Sharma *et al.*, 1999). However, in the absence of the proteolytic processing event (as would be the situation in Δ*GPI8*), the protein is targeted for degradation. Interestingly, mutation of the putative ω residue of gp63 (Asn^{577}) resulted in the secretion of the protein (McGwire and Chang, 1996). It now seems probable that this mutated protein was proteolytically processed but not modified with a GPI anchor.

The GPI:protein transamidase of yeast is essential (Benghezal *et al.*, 1996). It is also likely to be crucial for growth of parasitic protozoa that synthesize GPI:glycoprotein coats, such as the African trypanosomes and *Plasmodium falciparum* (McConville and Ferguson, 1993; Ferguson, 1999). As such, selective inhibitors of fungal or protozoan GPI8 may have therapeutic potential against a variety of pathogens. Despite the fact that some caution is required in interpreting the results from genetic manipulation to validate drug targets in parasitic protozoa (Barrett *et al.*, 1999), the finding that

ΔGPI8 mutants infected mice provides convincing evidence that the GPI:protein transamidase is not essential for *Leishmania* proliferation in mammals; therefore, inhibitors of the protein will not have clinically useful antileishmanial activity. However, the viability of the *GPI8* null mutants of *Leishmania*, together with the ability to reexpress mutated forms of *GPI8* in these parasites, makes *Leishmania* an excellent model system for analyzing the structure and function of the transamidase complex.

ACKNOWLEDGMENTS

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