Cloning, Functional Characterisation and Deletion of UDP-Galactopyranose-Mutase of *Leishmania major*

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Abstract

Leishmania are protozoan endoparasites belonging to the family of *Trypanosomatidae* and are responsible for a widespread group of diseases collectively known as leishmaniases. These diseases encountered in tropical, subtropical areas of the world and middle Asia range from self-healing cutaneous leishmaniasis to lethal visceral leishmaniasis. The variability of clinical manisfestations results from both the diversity of *Leishmania* species and the immune response of its host. Over 12 million of people are infected world wide. No effective vaccines, drugs or chemoprophylaxe exists to protect or heal leishmaniases.

The search of more effective and rational drug treatments led researchers to investigate the role of surface molecules in the parasite infectivity. *Leishmania* parasites are surrounded by a dense glycocalyx involved in parasite virulence. The cell surface glycoconjugates protect the parasites from hostile environments and are involved in subversion of the host immune response. Interestingly, *Leishmania* glycoconjugates contain galactofuranose (Gal*f*), an uncommon monosaccharide expressed at the cell surface of many human pathogens. Gal*f* is found in a variety of bacteria (e.g. *Escherichia coli, Klebsiella pneumoniae*, *Mycobacteria ssp*.), fungi (e.g. *Aspergillus* species) or protozoan parasites (*Leishmania* species, *Trypanosoma cruzi*). Because Gal*f* residues are often present in structures considered essential for virulence but are lacking in metazoans, the enzymes involved in its biosynthesis could be attractive targets for chemotherapy. In recent years, different enzymes involved in Gal*f* metabolism have been characterised in bacteria. Gal*f* was shown to arise from the action of the UDP-Galactopyranose mutase (UGM). This flavoprotein catalyses the interconversion of UDP-Galactopyranose (UDP-Gal*p*) into UDP-Galactofuranose (UDP-Gal*f*), which is the donor of galactofuranose to the various galactofuranosyltransferases involved in glycoconjugate and polysaccharide synthesis. Although the existence of such enzyme in eukaryotes was suspected, it had thus far not been identified. Moreover, with the exception of a putative galactofuranosyltransferase of *Leishmania*, all other enzymes involved in Gal*f* metabolism of eukaryotes are still unknown.

Here we report the characterisation of *Leishmania major* UGM and the determination of its role in glycoconjugate biosynthesis and parasite virulence by a targeted gene deletion approach. In *Leishmania major,* Gal*f* is present in the GPI-anchor of the abundant cell surface Lipophosphoglycan (LPG) and the Glycosylinositolphospholipids (GIPLs). Consequently, we observe the absence of LPG and the presence of truncated GIPLs lacking Gal*f* in our *Δglf* mutant. Other glycoconjugates were, however, unaffected. The

virulence of the Δ*glf* mutant was assessed by lesion formation of Balb/c mice after inoculation with the parasites. Remarkably mice inoculated with the Δ*glf* mutant did not develop any lesion, indicating the importance of this gene for the pathogen survival and proliferation in the mammalian host. This study represents the first investigation of the role of galactofuranose in eukaryotes.

Keywords: Galactofuranose, UDP-Galactopyranose mutase, *Leishmania*

Zusammenfassung

Leishmanien sind einzellige Endoparasiten der Familie *Trypanosomatidae* und verursachen die weit verbreitete Krankheit Leishmaniose. Das Hauptverbreitungsgebiet der Krankheit liegt in den Tropen, Subtropen und Zentral Asien. Das Krankheitsbild reicht von der selbst heilenden kutanen Leishmaniose bis hin zur tödlichen viszeralen Form. Die Variabilität in der klinischen Erscheinungsform hängt zum einen von der Artenvielfalt der *Leishmanien* und zum anderen von dem Immunstatus des Wirtes ab. Über zwölf Millionen Menschen sind weltweit infiziert. Derzeit existiert weder ein Impfstoff oder eine effektive medikamentöse Behandlungsmöglichkeit noch eine Chemoprophylaxe, die vor Leishmaniose schützt oder die Krankheit heilt. Die Suche nach effektiveren Behandlungsmöglichkeiten führte zu der Untersuchung von Oberflächenmolekülen und deren Beteiligung an der Infektiosität des Parasiten. Verantwortlich für die Virulenz der *Leishmanien* ist die Glykokalyx, die den Parasiten umgibt. Sie schützt den Parasiten im Sandfliegen-Vektor und vor der Immunantwort des Wirtes. Interessanterweise enthalten die Glykokonjugate der *Leishmanien* das seltene Monosaccharid Galactofuranose (Gal*f*), das auch auf Zelloberflächen von vielen Humanpathogenen exprimiert wird. So kommt Gal*f* z.B. in verschiedenen Bakterien (z.B. *Escherichia coli, Klebsiella pneumoniae*, *Mycobacteria ssp.)*, in Pilzen (z.B. *Aspergillus ssp.)* oder auch in einzelligen Parasiten (*Leishmania ssp.*, *Trypanosoma cruzi*) vor. Da Gal*f* in Strukturen vorhanden ist, die für die Virulenz von Humanpathogenen verantwortlich sind und die in den Metazoa vollständig fehlen, stellen Enzyme in der Biosynthese des Zuckers potenzielle Ansatzpunkte für die Entwicklung von Medikamenten dar. In den letzten Jahren wurden verschiedene Enzyme im Stoffwechselweg der Gal*f* in Bakterien charakterisiert. Es konnte gezeigt werden, dass Gal*f* von dem Enzym UDP-Galactopyranose mutase (UGM) gebildet wird. Dieses Flavoprotein katalysiert die reversible Umwandlung von UDP-Galactopyranose und UDP-Galactofuranose. Die UDP-Galactofuranose ist der Vorläufer der Galactofuranose, die von der Galactofuranosyltransferase zu den Glykokonjugaten und Polysacchariden transportiert wird. Die Existenz der UGM wurde schon lange auch in Eukaryoten vermutet, aber so weit noch nicht identifiziert. Eine Ausnahme bildet die Galactofuranosyltransferase in *Leishmanien*. Alle anderen Enzyme in der Biosynthese der Galactofuranose in Eukaryoten sind jedoch unbekannt.

In dieser Arbeit wurde sowohl die UGM aus dem Eukaryoten *Leishmania major* charakterisiert, als auch die Rolle des Enzyms in der Biosynthese der Glykokonjugate untersucht. Um die Bedeutung des Enzyms für die Virulenz des Parasiten zu ermitteln, wurden Deletionsmutanten (Δ*glf*) hergestellt.

In *Leishmania major* ist Gal*f* zentraler Bestandteil im Glycosyl-Phosphatidylinositol – Anker des prominent vertretenen Lipophosphoglykan (LPG), sowie der Glycoinositolphospholipide (GIPLs). Folglich enthielten die *Δglf* Mutanten kein LPG und exprimierten nur verkürzte GIPLs, während die anderen Glykokonjugate unverändert blieben. Die Virulenz der Δ*glf* Mutanten wurde durch Bildung von Läsionen in Balb/c Mäusen nach Inokulation des Parasiten untersucht. Bemerkenswerterweise entwickelten die Mäuse, die mit den Δ*glf* Mutanten infiziert worden waren, keine Läsionen, was die Wichtigkeit des Gens für das Überleben und die Vermehrung des Parasiten im Säugetier unterstreicht. Die Untersuchungen dieser Arbeit bieten erstmalig Daten über die Funktion der Gal*f* innerhalb der Eukaryoten.

Schlagwörter: Galactofuranose, UDP-Galactopyranose mutase, *Leishmanien*

1 Introduction

1.1 Leishmania and Leishmaniases

Leishmania (L.) are protozoan endoparasites belonging to the order of the *Kinetoplastida* and the family of *Trypanosomatidae* (Figure 1).

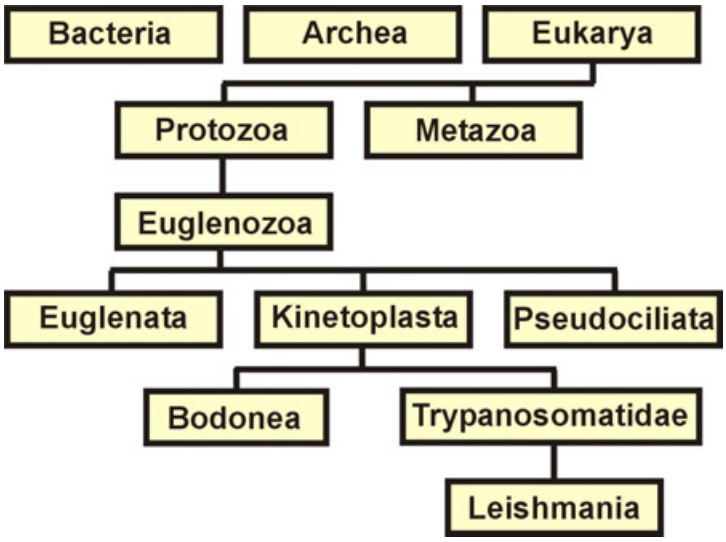


Figure 1: Classification of the genus *Leishmania*.

The genus *Leishmania* includes almost 30 different species, of which 21 are known to infect people, causing disease symptoms collectively known as leishmaniasis (Lane, 1993). They infect numerous mammalian species acting as reservoir, and are propagated through the bite of different species of sand fly belonging to the genus *Phlebotomus* or *Lutzomya*, depending on the region. *Leishmania* parasites present two distinct morphological stages: the intracellular amastigote and the extracellular promastigote stage (Figure 2). The amastigotes have a round or oval body of about 2-6 µm in diameter, containing a large nucleus, a kinetoplast and a residual flagellum attached to the blepharoblast. They multiply within the parasitophorus vacuoles of macrophages in the mammalian host. The promastigotes have a long and slender body (about 15-30 µm by 2-3 µm), with a central nucleus, a kinetoplast and a long anterior flagellum. They can be further classified as procyclic promastigotes, which multiply in the gut of the sand fly, or as the infective metacyclic promastigotes, which are found in the mouth part and anterior gut and do not divide (Farrel *et al*., 2002).

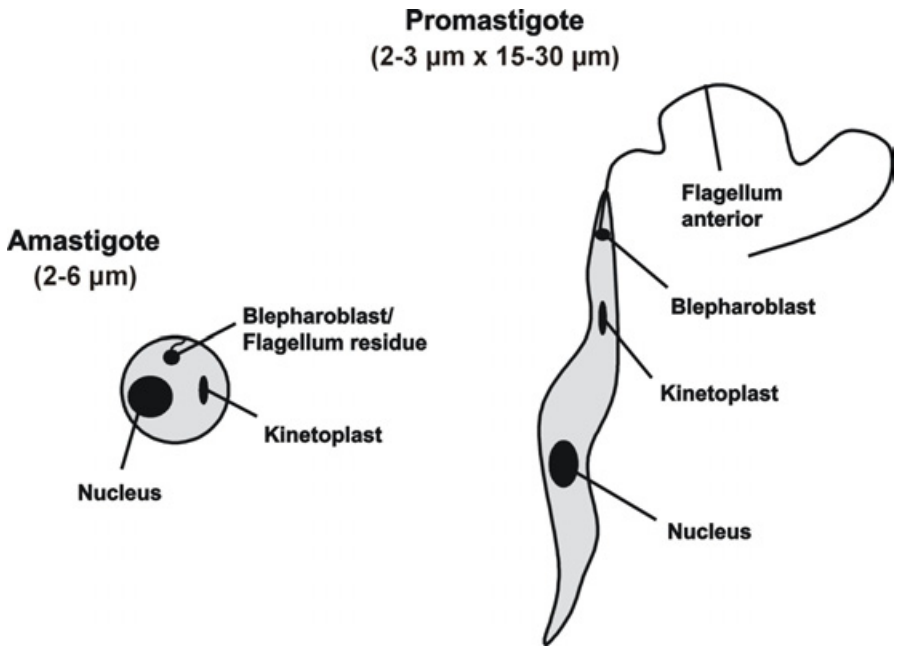


Figure 2: Morphology of *Leishmania*.

The life cycle of the parasites is shown in Figure 3. The infective metacyclic promastigotes are transmitted by the bite of an infected female sand fly to the mammalian host. The parasites produce a chemotactic factor (LCF) that recruits polymorphonuclear neutrophil granulocytes (PMN) (Laskay *et al*., 2003). The latter phagocyte *Leishmania* and release IL-8 that amplifies the migration of PMN to the site of infection, as well as the monocyte attractant MIP-1β. Recruited cells of the monocyte/macrophage lineage ingest apoptotic PMN harboring viable parasites. This infection strategy is one of the sophisticated mechanisms used by *Leishmania* to silence the antimicrobial functions of macrophages. Once inside the macrophage, the parasites transform into non motile amastigotes and proliferate despite the acidic pH until the macrophage burst. The released parasites infect surrounding macrophages, which results in disease development. During a blood meal on an infected person, a sand fly can ingest infected macrophages or free amastigotes. These parasites transform rapidly into motile procyclic promastigote that attach to the midgut of the sand fly vector to avoid excretion and proliferate. They undergo a process called metacyclogenesis during which they convert into non-dividing infective metacyclic promastigotes (Sacks, 1989), detach from the midgut of the sand fly vector and migrate to the pharynx and oesophagus. During another blood meal the metacyclic promastigotes are transferred with the saliva of the sand fly which supports the establishment of the parasites infection in the mammalian host most likely by inhibition of oxidative metabolic processes and antigen presentation (Lerner *et al*., 1991, Theodos *et al*., 1991).

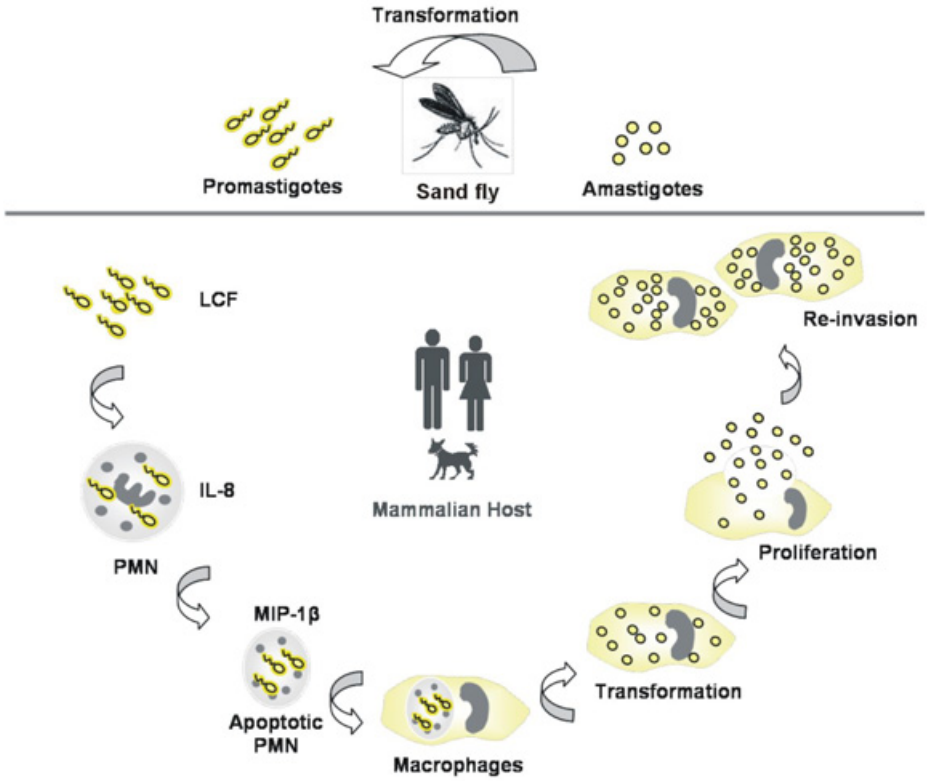


Figure 3: Bigenetic life cycle of *Leishmania* *Leishmania* are dimorphic parasites present in two morphological stages: the intracellular amastigotes that survive and proliferate inside mammalian macrophages, and the extracellular promastigotes that multiply in the sand fly vector.

Leishmania parasites are the etiologic agents of leishmaniases, a widespread group of diseases encountered in tropical, subtropical areas of the world and middle Asia. Old world species of *Leishmania*, such as *L. donovani* and *L. major* cause pathology from southern Europe to Africa, the Middle East, and throughout Asia, whereas New World species (e.g. *L. mexicana*, *L. amazonensis* and *L. chagasi*) are found throughout South America and Central America as far north as the United States. Leishmaniasis presents itself in four different clinical forms. The variability of the clinical manifestations results from both the diversity of *Leishmania* species and the immune response of its host. All forms can have devastating consequences. Visceral leishmaniasis (*kala azar*), caused by the species *L. donovani* or *L. chagasi,* is the most severe form of the disease. The parasites infect the liver, spleen and bone marrow leading to death if not treated. 90% of annual cases of visceral leishmaniasis are reported in Bangladesh, Brazil, India and Nepal. The cutaneous forms of leishmaniasis are the most common and represent 50 to 75% of all new cases (WHO), 90% of which occur in Afghanistan, Brazil, Iran, Peru,

Saudi Arabia and Syria and are caused by *L. major* and the *L. mexicana* subgenus. An ulcer forms at the site where a sand fly has bitten. The parasite is usually only found in the region of the ulcer but in some species, it also infects the lymphatic system, causing numerous skin wounds along the lymphatic ducts. The lesions are self-healing but leave the patients permanently scarred. The main causative species are *L. major, L. mexicana, L. tropica, L.aethiopica, L. guyanensis and L. panamensis*. The Viannia subgenus (e.g. *L. braziliensis, L. panamensis)* is encountered solely in the Americas and is responsible for the clinical form named mucocutaneous leishmaniasis (also called *espundia*) (Descoteaux and Turco, 1999) characterised by extensive and disfiguring destruction of mucous membranes of the mouth, nose and throat cavities. Healing is never spontaneous and untreated patients can die from secondary infections. The last form is called diffuse cutaneous leishmaniasis and is characterised by widespread papules or nodules in the skin all over the body. It never heals spontaneously and tends to relapse after treatment. In Africa (Kenya, Ethiopia) it is caused mainly by *L. aethiopica* and in Central America and northern South America it is caused mainly by *L. amazonensis*. In 2000, the World Health Organization estimated that over 12 million people are infected by the various *Leishmania* species in 88 countries and hundreds of million are at risk. There is no vaccine against leishmaniases. Available drugs are in most cases not efficient or reliable, very expensive and cause strong side effects. Another problem is the ever-increasing drug resistance. Standard therapy of leishmaniasis consists of repeated injections of pentavalent antimonials, associated with important side effects. Many parasites are resistant against this treatment. If these drugs are not effective, the second line drugs are pentamidine and amphotericin B. They are only successful when injected at high doses and are costly (Croft and Coombs, 2003). The recently introduced alkylphospholipid Miltefosin, originally developed as anticancer agent, is active orally against visceral leishmaniasis but its side effects do not allow application under all circumstances, e.g. in pregnancy or in severely malnourished patients. Vector and reservoir controls may be useful under certain conditions but are not applicable in every epidemiological setting and require infrastructure and vigilance beyond the capability of many endemic countries. There is thus a clear need for a vaccine or for more effective and rational drug treatments that are based on the fundamental knowledge of the pathogen biology.

1.2 Leishmania surface

1.2.1 Composition of *Leishmania* glycocalyx

The densely organised parasite surface is the primary interface with the insect and mammalian hosts and plays essential roles in the sophisticated mechanisms that *Leishmania* has evolved to survive and proliferate in the highly hostile environment associated with its life style. Procyclic promatigotes are covered by a 7 nm thick glycocalyx. This cell coat reaches at least 17 nm in metacyclic parasites but is almost completely absent from amastigotes. This jacket comprises several glycoconjugates such as the lipophosphoglycan (LPG), glycosylphosphatidylinositol (GPI)- anchored proteins and free glycosylinositolphospholipids (GIPLs) (Figure 4). Additionally, *Leishmania* secrete various molecules such as the proteophosphoglycans (PPGs) that have also directly been linked to parasite survival and pathogenicity.

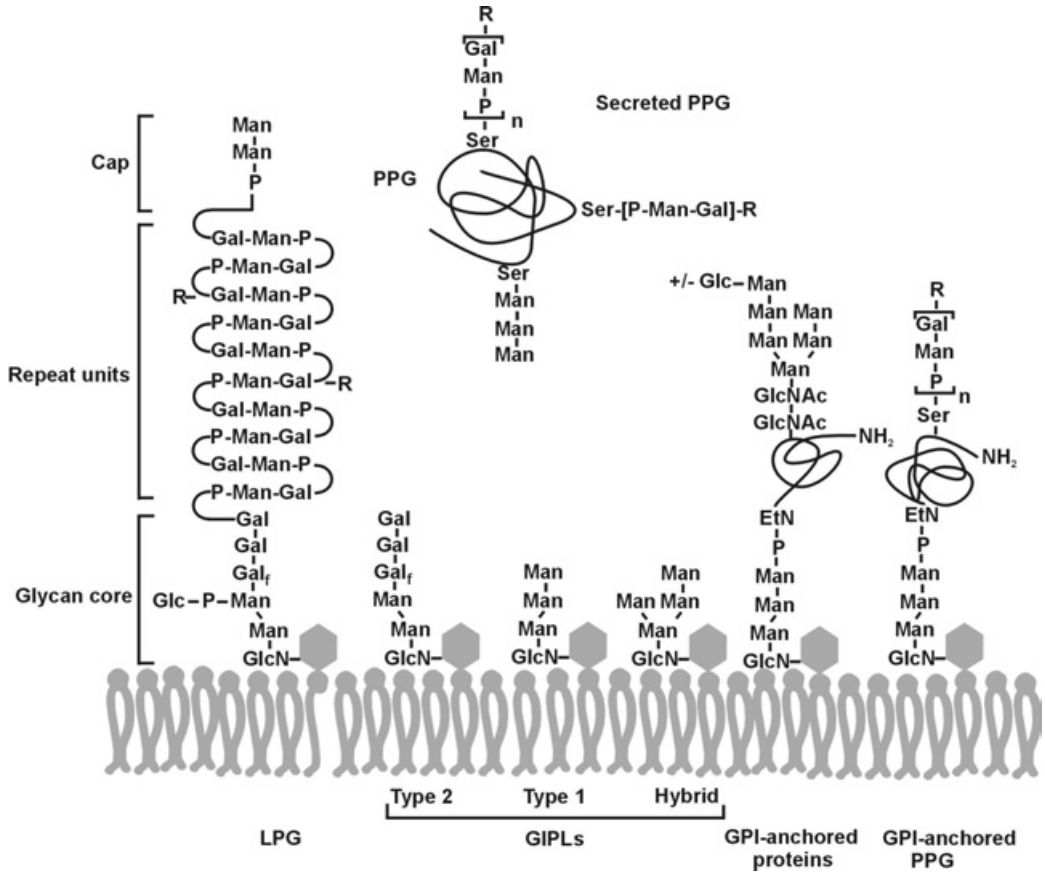


Figure 4: Surface glycoconjugates of *Leishmania* promastigotes: *Leishmania* promastigotes are coated with a thick glycocalyx comprised of LPG, GIPLs, GPI-anchored proteins and membrane bound PPGs. Additionally they secrete PPGs. Gal, galactose; Gal*f*, galactofuranose; Man, mannose; P, phosphate, PI, phosphatidylinositol, R, side chain modification; Glc, glucose; GlcN, glucosamine; EtN, ethanolamine.

1.2.1.1 The lipophosphoglycan

LPG is the predominant cell surface glycoconjugate of *Leishmania* promastigote. It is present all over the surface, including the flagellum. Found in all *Leishmania* species, LPG is composed of four domains, (1) a 1-O-alkyl-2-lyso-phosphatidyl-*myo*-inositol-anchor, (2) a conserved glycan core, (3) a backbone of 15 to 30 Galß1,4Manα1–phosphate repeat units and (4) an oligosaccharide cap structure (Figure 5) (Turco *et al.*, 1992, Descoteaux and Turco, 1999, McConville *et al.*, 1993, Guha-Niyogi *et al.*, 2001).

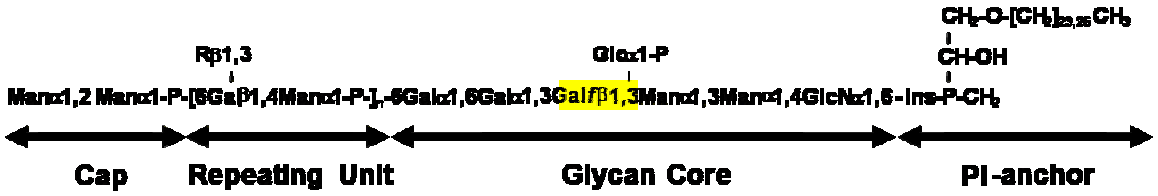


Figure 5: Structure of LPG from *L. major* promastigote highlighting the presence of a Gal*f* residue in the glycan core. R = H; Galβ(1-; Galβ(1-3)Galβ(1-; Araβ(1-2)Galβ1-(McConville *et al.,* 1990b, McConville *et al.,* 1992).

Leishmania species differ remarkable by the presence of glycan side chains as well as by their composition and positioning on the LPG core structure (McConville *et al.*, 1995). LPG of *L. major*, for example, is highly branched with oligosaccharides containing β-Gal, β-glucose and β-arabinose (Ara) residues attached to the C3 hydroxyl of the repeat unit Gal, whereas that of *L. donovani* does not possess any side chains. Furthermore, the structure of LPG differs between procyclic and metacyclic promastigotes, being significantly longer in the latter. The structural changes associated with the acquisition of infectivity during metacyclogenesis, have been particularly well described in *L. major*. In this species, the doubling of repeat units is associated with an increased frequency of terminal β-arabinose in LPG side chains. The thickening of glycocalyx reflects these changes. Similarly the differentiation of promastigotes into amastigotes is associated with a strong down regulation of LPG that is almost absent from amastigotes and is reflected by the lack of conspicuous surface glycocalyx. Comparable developmental changes in LPG structure also occur in *L. donovani* and *L. chagasi*. However, in *L. donovani,* changes in LPG conformation rather than side chain modifications are thought to regulate the stage specific expression of surface glycan epitopes. In contrast, recent

studies suggest that cellular levels of LPG decrease drastically in *L. mexicana* metacyclic promastigotes.

1.2.1.2 GPI-anchors

Although some of the plasma membrane proteins of the parasitic protozoa use transmembrane polypeptide anchors, most of the major cell surface proteins of these organisms are GPI-anchored. These proteins are functionally diverse and include coat proteins, surface hydrolases and receptors. In all GPI-anchors characterised to date (from protozoa, yeast, fish, mammals, etc.) the C-terminus of the protein is linked via ethanolamine phosphate to a glycan with the conserved backbone sequence Manα1-2Manα1-6Manα1-4GlcN, which in turn is linked to the 6-position of the *myo*-inositol ring of phosphatidylinositol. The tetrasaccharide backbone may be substituted with other sugars in a species and stage specific manner. The GPI-anchored 58-65kDa metalloprotease termed GP63 or leishmanolysin is abundantly expressed by all *Leishmania* promastigotes and has thus been widely studied. Like LPG, GP63 shows marked heterogeneity among *Leishmania* species (Medina-Acosta *et al.*, 1993). The major promastigote isoform is modified with high mannose N-linked glycans and a GPI anchor and is proteolytically processed during transit to the cell surface. Like LPG, the expression of GPI-anchored proteins is mostly downregulated as promastigote differentiate to amastigotes suggesting that these proteins are primarily required for life in the sand fly vector (Naderer *et al*., 2004).

1.2.1.3 GIPLs

The glycosylinositolphospholipids (GIPLs) are the main class of glycolipids synthesised by all developmental stages of *Leishmania* parasites. They are members of the GPI family by virtue of containing the core sequence Manα1-6GlcNα1-6*myo*-inositol. Three different lineages of GIPLs have been distinguished, which are expressed in markedly different levels in different species or developmental stages. The type 1 and 2 GIPLs have glycan headgroups which are structurally related to GPI protein anchors (Manα1-2Manα1,6Manα1,4GlcNα1,6-PI) and the LPG anchor respectively (Galα1,6Galα1,3 Gal*f*α1,3Manα1,3Manα1,4GlcNα1,6-PI), while the hybrid type has branched glycan headgroups containing elements of both anchors Manα1,6(Manα1,3)Manα1,4GlcNα1,6-PI (McConville *et al.,* 1993). However they are far more abundant than GPI- and LPG-anchors, contain distinct lipid moieties and are clearly end products. These glycolipids cover a large proportion of the plasma membrane and are likely to be the major surface

components exposed on the amastigote stage. Unlike LPG which is continuously shed from the membrane, GIPLs have a long half life. Moreover the type-2 GIPLs present in *L. major* are highly immunogenic due to the presence of galactofuranose (Figure 6).

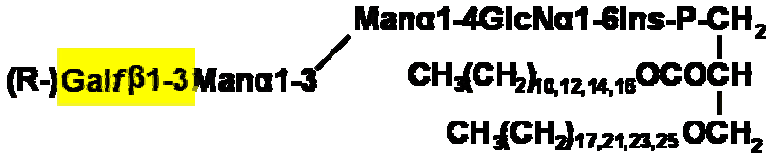


Figure 6: Structure of GIPLs from *L. major* highlighting the presence of a Gal*f* residue. R = H; Galα(1-3); Galα(1-6)Galα(1-3); Galβ(1-3)Galα(1-3); McConville *et al.,* (1990).

1.2.1.4 PPG

Cell surface and secreted proteophosphoglycans (PPGs) are an expanding family of highly glycosylated *Leishmania* proteins (Ilg, 2000) containing similar phosphoglycans chains to those found in LPG (Jaffe *et al.*, 1990, Bates *et al.*, 1990). Phosphoglycosylation is the most abundant type of protein glycosylation in these parasites and is initiated by the transfer of Manα1-phosphate to serine and threonine residues in the polypeptide backbone (Moss *et al.*, 1999). This mannose phosphate residue is then extended with mannose or with one or two Galß1,4Manα1–phosphate repeating units (Ilg *et al.*, 1994). PPG profiles of different *Leishmania* species and developmental stage can vary markedly (Ilg *et al.*, 1994 and 1996). For example, *L. mexicana* promastigotes secrete three distinct classes of PPG (two forms of secreted acid phosphatase and a filamentous PPG) while amastigotes only secretes one form of PPG that accumulates in the parasitophorus vacuole and extracellular milieu of the lesion. *L. major*, however, secretes fewer PPGs than *L. mexicana,* although some strains express a GPI-anchored form of PPG on their cell surface (Ilg *et al,* 1999).

1.2.2 Function of the surface glycoconjugates of *L. major* in the survival and proliferation

Surface glycoconjugates of *Leishmania* parasites are key determinants in parasite survival and virulence in both the insect vector and the mammalian host. The promastigote glycocalyx consists mainly of LPG, GPI-anchored glycoproteins and GIPLs (Pimenta *et al.*, 1991, McConville *et al.*, 2002, Descoteaux and Turco, 1999) and is

indispensable for the parasite survival in the sand fly vector and during the initial steps of macrophage infection (Naderer *et al*., 2004). In contrast the amastigotes lack a conspicuous surface glycocalyx. They are coated with a layer of GIPLs and host-derived glycosphingolipids (McConville, 1991b). The following paragraph outlines the role of promastigote and amastigote surface glycoconjugates.

1.2.2.1 Lipophosphoglycan

LPG is the predominant cell surface glycoconjugate of *Leishmania* promastigotes. It is clearly essential for parasite survival in the sand fly vector and at least for some species, for infectivity in the mammalian host (Sacks and Kamhawi, 2001, Ilg *et al*., 2001). Initially a large number of functions have been attributed to LPG. Recently *L. donovani, L. mexicana* and *L. major* LPG deficient mutants have been obtained by targeting gene deletion of the *LPG1* gene and have allowed to reassess many of these functions (Sacks *et al*., 2000, Ilg, 2000a, Späth *et al*., 2000). *LPG1* encodes a putative Golgi galactofuranosyltransferase that catalyzes an early step in the synthesis of the LPG anchor (Ryan *et al*., 1993). The targeted gene deletion of *LPG1* resulted in complete loss of LPG without affecting any other glycoconjugates including the structurally related PPGs and GIPLs. Thus these mutants provided excellent tools to rigorously test the roles of LPG. LPG roles that have been ascertained are presented below.

LPG promotes the attachment and detachment from the sand fly midgut. For example, in *Phlebotomus papatasi*, the natural vector of *L. major*, a lectin recognizing the terminal β-galactose residues of LPG side chains is responsible for the binding of parasites, preventing their excretion with the digested blood meal. The stage specific variations of LPG structures described previously are thus essential for the detachment of infectious metacyclic parasites and their migration toward the proboscis. In the mammalian host, LPG protects the parasite against complement lysis by preventing the insertion of the C5b-9 membrane attack complex into the promastigote membrane (Puentes *et al*., 1990, Guha-Niyogi *et al*., 2001). It can also scavenge oxygen radicals *in vitro* and seems to protect invading promastigotes from the oxidative burst of macrophage generated during phagocytosis. Moreover there is strong evidence that LPG inhibits the fusion between late endosomal compartments and the newly formed phagosome. Unlike amastigotes, promastigotes are vulnerable to degradation by the acidic and hydrolytic environment of the phagosome. The delay in phagosome maturation would thus provide a window during which promastigotes can differentiate into the more resistant amastigotes. However the importance of this delay in phagosome maturation for promastigote survival was recently called into question (Murray, 2005). On the other hand, LPG does not seem

to be the major surface ligand for macrophage attachment as previously thought. Indeed *L. major* Δ*lpg1* mutant was opsonised with C3b to the same extend and was equally effective at invading macrophages as wild type promastigotes, although their proliferation was clearly reduced *in vitro* (Späth *et al*., 2003a). Importantly, mice infection studies with *L. major* Δ*lpg1* mutant resulted in delayed lesion formation indicating the importance of LPG for the initial establishment of infection (Späth *et al*., 2000). However, as LPG is not expressed by amastigotes, its role is transient and confined to only the early stage of infection. The down regulation of LPG even seems indispensable as LPG would compromise parasite survival in the host cell (Proudfoot *et al.,* 1996). Interestingly *L. mexicana* LPG is not required for infection of macrophages (Ilg, 2000a). In contrast to what is observed in *L. major, L. mexicana* LPG is not modified nor rearranged during metacyclogenesis and LPG expression is dramatically downregulated in the infective metacyclic promastigotes. Thus *Leishmania* species differ dramatically in their reliance upon LPG for virulence (Turco *et al.,* 2001).

1.2.2.2 Proteophosphoglycans

The proteophosphoglycans represent a number of cell surface and secreted proteins, which are modified with similar phosphoglycan chains to those found in LPG (Ilg, 2000b). The filamentous form of the PPGs, secreted by all *Leishmania* species, has been shown to form a highly viscous mesh within which the parasites lay embedded (Ilg *et al.*, 1996). The PPGs block the cardia and the stomodeal valve of the sand fly (Lawyer *et al.*, 1990). It has been suggested that the blocking of the digestive tract play a role in the transmission of the metacyclic promastigotes (Warburg *et al.,* 1986). Using a *L. donovani* mutant lacking the GDP-mannose transporter gene (*LPG2*), Descoteaux *et al.* (1995) demonstrated that PPGs are responsible for the protection of the parasite from the complement system and proteases in the midgut. The Δ*lpg2* mutant that lacks both LPG and PPGs is indeed rapidly killed in the sand fly midgut while Δ*lpg1* mutant that lacks exclusively LPG remained viable during this initial period of infection. Furthermore *L. major* Δ*lpg2* is avirulent in a mouse- and macrophage infection model (Späth *et al.,* 2003b) while the Δ*lpg1* mutants lacking exclusively LPG (Sacks *et al.,* 2000) only showed a reduced virulence. These result suggested that the PPGs play an important role in parasite survival in the mammalian host. PPGs may contribute to the protection against complement lysis, to the attachment and invasion of the macrophages (Naderer *et al.,* 2004) and may modulate signalling events in the early stages of the infection (Guha-Niyogi *et al*., 2001). The *Δlpg2* mutant is rapidly killed by macrophages and does not established lesion in susceptible mice even with relatively high inoculums.

Remarkably some viable *L.major* Δ*lpg2* can be recovered from the site of infection months after injection. However, this persistence is not sufficient to maintain protective immunity (Kebaier *et al.,* 2006). Interestingly, *L. mexicana* differs again in the mechanism of infection, showing no reduced virulence while lacking LPG and PPG (Ilg *et al*., 2001).

1.2.2.3 GP63

The glycoprotein 63 (GP63) is a GPI-linked metalloproteinase that is abundantly expressed on cell surfaces by all *Leishmania* species. The protease is believed to facilitate survival in the mammalian host. GP63 has been reported to hydrolyse surface opsonised complement components (Brittingham *et al.,* 1999), act as a ligand for macrophage receptors, either directly or after opsonisation with complement (Alexander and Russell, 1992, Joshi *et al.,* 1998), and degrade the extracellular matrix of proteins (Joshi *et al.*, 2002). After deletion of the *L. major* GP63 genomic region (Hilley *et al.*, 2000), the mutants were 10-fold more sensitive to complement lysis in human serum and showed a delayed lesion development in susceptible mice. The amastigotes were as virulent as the wild type, indicating that GP63 is not essential for continued survival in the mammalian host. As proposed for LPG, there might be a strong selective pressure to down-regulate the surface expression of GP63 and other surface proteins with large ectopic domains in the amastigote stage as this might be detrimental for amastigote survival.

1.2.2.4 GIPLs

Free GPI or GIPLs are a family of low molecular weight glycolipids that are not attached to either proteins or polysaccharides. Because other glycoconjugates are dramatically downregulated in the amastigote stage of the parasites, the GIPLs are the predominant glycoconjugate in this developmental stage (Naderer *et al.*, 2004). Despite their abundance, little is known about the function of these glycolipids. The GIPLs are believed to play a role in parasite survival by modulating signaling events. GIPLs are notably able to inhibit protein kinase C (an enzyme involved in the first step of the macrophage oxidative burst) and nitric oxide production, a compound with leishmanicidal activity (Proudfoot *at al.,* 1995, Tachado *et al.,* 1997, McNeely *et al.,* 1989). Recently a *Leishmania major* mutant lacking detectable levels of GIPLs and LPG was generated by target gene deletion of a gene encoding the alkyldihydroxyacetonephosphate synthase (*ADS*), a key component in ether lipids biosynthesis (Zufferey *et al.,* 2003). Interestingly GPI-anchored proteins like GP63 were still synthesized by this mutant and are thought to

be anchored to the membrane via a diacylglycerol. Unexpectedly, the effect on virulence of this Δ*ads* mutant toward mice and macrophages was not stronger than the one observed with Δ*lpg1*. This observation suggests that the GIPLs are not essential for infectivity. However the Δ*ads* mutant expresses an unidentified lipid (Zufferey *et al.,* 2003) that could have compensated for the loss of these abundant glycolipids.

Throughout their life cycle, *Leishmania* survive and proliferate in very hostile enviroments and have evolved special mechanisms involving the cell surface glycocalyx and secreted glycoconjugates that enable them to endure these adverts conditions (Orlandi and Turco, 1987, McConville and Blackwell, 1991, Ilg *et al.*, 1996). Furthermore various mutants deficient in several glycoconjugates nicely demonstrated that the glycocalyx is indispensable for parasite virulence toward the insect vector and the mammal host (Guha-Niyogi *et al.*, 2001). For example, targeted deletion of the gene encoding dolichol-phosphate mannose synthase (DPMS) resulted in *L. mexicana* parasites that lacked all GPI-anchored molecules (LPG, GPI-anchored proteins and GIPLs) and had lost virulence (Garami and Ilg, 2000). Additionally, parasite surface molecules play an important role in the invasion of macrophages. *In vivo* opsonisation of metacyclic promastigotes by C3b and C3bi permits the interaction with macrophage complement receptor 1 (CR1) and CR3 respectively. However GP63 converts C3b into C3bi and thus favor internalisation via the macrophage complement receptor 3 (CR3), which is advantageous to parasite since it will not trigger the oxidative burst during phagocytosis. Moreover both GP63 and LPG interact with various macrophages receptors (mannose-cose receptor, C-reactive protein receptor, fibronectin receptor, CR4). More recently a number of surface molecules that play a role during the initial interaction with macrophage have been described, although the receptors for these molecules are not yet identified. For instance an antibody directed against the terminal β-D-Galactofuranose residue of GIPLs from either promastigotes or amastigotes inhibited internalisation of *L. major in vitro* (Suzuki *et al.,* 2002).

Although the initiation of infection is due to the promastigote, the maintenance of infection in the mammalian host relies on the amastigotes and their ability to replicate in macrophages, and to exit and re-infect new host cells. Less is known about sugars mediating interaction of the obligatory intracellular amastigote and its host cells in an already established infection (Handman, 2000). For all these reasons, the enzymes involved in the biosynthesis of *Leishmania* cell surface glycoconjugates attract the attention of researchers in the hope of defining potential targets that may be exploited against leishmaniasis.

1.3 Galactofuranose containing glycoconjugates

1.3.1 Occurrence of Galactofuranose

The monosaccharide D-galactose occurs naturally in a common pyranic form (a six-membered carbohydrate ring) and a more unusual furanic form (a five-membered carbohydrate ring). D-galactopyranose is widespread and is notably abundant in mammalian glycoconjugates. The occurrence of Galactofuranose (Gal*f*) is more restricted. It is notably present in structures considered to be essential for virulence of pathogenic bacteria, fungi and protozoa but is absent in mammals. In prokaryotes, Gal*f* constitutes an essential part of the mycobacterial cell wall (Pan *et al.*, 2001) and occurs in lipopolysaccharide O-antigens, extracellular and capsular polysaccharides (Nassau *et al.*, 1996, Köplin e*t al.*, 1997). In fungi, Gal*f* residues are often found within the cell wall polysaccharides and on glycoproteins; e.g., the cell wall galactomannan, some N- and O-glycans of *Aspergillus fumigatus* (Latge *et al.*, 1994). Finally, Gal*f* is a key component of the abundant protozoan surface GPI-anchored glycoconjugates such as the type 2 GIPLs and the LPG-anchor of *Leishmania* or the mucins, GPI-anchored proteins and lipids of *Trypanosoma cruzi* (McConville *et al.*,1990a, Naderer *et al.*, 2004). Importantly, Gal*f* is absent in human and thus the enzymes that are involved in its biosynthesis could be attractive targets for development of new antimicrobial drugs (Lederkremer and Colli, 1995, Pedersen and Turco, 2003).

1.3.2 Gal*f* biosynthetic pathway

The Gal*f* biosynthetic pathway is simple to describe but has been difficult to unravel. UDP- Gal*f* was first shown to be the activated precursor for the synthesis of bacterial and fungal polysaccharides (Trejo *et al.,* 1970; Sarvas and Nikaido, 1971) and to originate from UDP-galactopyranose (UDP-Gal*p*) (Trejo *et al.*, 1971). In free solution galactopyranose (Gal*p*, the common form of galactose) and Gal*f* can interconvert via the open chain form of galactose. However, once the reducing oxygen is covalently bonded as in UDP-Galactose, the ring is locked. The interconversion of UDP-Gal*p* into UDP- Gal*f* requires thus the action of the flavoprotein UDP-galactopyranose mutase (UGM) (Figure 7). Gal*f* is then transferred from UDP- Gal*f* onto various acceptors by specific galactofuranosyltransferases involved in the synthesis of glycoconjugates and polysaccharides. To date, very few galactofuranosyltransferases have been identified (Huang and Turco, 1993; Kremer *et al*., 2001; Guan *et al*., 2001) while UGM has been

extensively studied in bacteria because of its key role in Gal*f* metabolism. The gene encoding UGM (*GLF*) has been cloned from a variety of bacterial sources (Nassau *et al.*, 1996; Weston *et al.*, 1997; Koplin *et al*., 1997). The crystal structures of *E. coli* and *K. pneumoniae* UGMs have been solved (Sanders *et al.,* 2001b; Beis *et al.*, 2005) and different mechanisms of action proposed (Fullerton *et al.,* 2003; Soltero-Higgin *et al.,* 2004a). Importantly, targeted gene deletion of UGM affects the cell wall integrity of *Mycobacterium* and results in a lethal phenotype (Pan *et al.*, 2001). As this enzyme appears as an attractive target for the development of new antituberculosis drugs, assays for inhibitor screens have been developed, and compounds showing activity against UGM activity have been identified in prokaryotes (Scherman *et al.,* 2003, Soltero-Higgin *et al.*, 2004b).

Figure 7: UDP-galactopyranose-mutase (UGM) catalyzes the rearrangement of UDP-galactopyranose into UDP-galactofuranose.

1.4 Aim of this study

Does Gal*f* play a critical role in the viability or virulence of *Leishmania* parasites? *Leishmania* galactofuranosyltransferase LPG1 was the only enzyme of *Galf* metabolism described in eukaryotes (Späth *et al.,* 2000) until recently (Bakker *et al.,* 2005; Beverley *et al,* 2005). LPG1 was identified by functional complementation of the *Leishmania donovani* R2D2 mutant (Ryan *et al.*, 1993) and was later deleted in *L.donovani* (Sacks *et al.,* 2000), *L. mexicana* (Ilg, 2000b) and *L. major* (Späth *et al.*, 2000). These mutants were devoid of the abundant cell surface LPG and the remaining LPG core structure pointed out to the potential role of LPG1 in Gal*f* transfer. As mentioned previously, the targeted gene deletion of this putative galactofuranosyltransferase (LPG1) led to attenuated virulence in mice and macrophages and established the role of LPG, and therefore Gal*f* metabolism, in oxidant resistance, human complement resistance and parasite virulence (Späth *et al.*, 2000). Interestingly the expression of Gal*f* containing type 2 GIPLs was not influenced by this gene deletion (Zhang *et al.,* 2003) indicating the

existence of a GIPL-specific galactofuranosyltransferase. Because of their abundance in the intracellular amastigote stage and their ability to modulate key signaling pathways, GIPLs are believed to play important roles in survival of the parasite in and outside the macrophages, and are thus suggested to be essential for virulence (Naderer *et al.,* 2004). Some of the GIPLs functions seem to be associated with their lipid moiety (Proudfoot *et al.,* 1996), however, Gal*f* has also been directly implicated in macrophage-parasite interaction. Indeed an antibody directed against Gal*f* containing GIPLs has been shown to inhibit attachment of *L. major* to macrophages (Suzuki *et al.*, 2002).

Finally it should be mentioned that *Leishmania* genome encodes at least 6 putative galactofuranosyltransferases homologous to *LPG1* whose function are yet undefined (Zhang *et al.,* 2004). The gene deletions of three of these *LPG1* homologous genes showed that they are not involved in LPG or GIPLs synthesis. Thus other leishmanial molecules might also contain Gal*f*. Targeting a central enzyme of Gal*f* metabolism such as UGM rather than individual galactofuranosyltransferases could thus have a more pronounced effect on *Leishmania* pathogenesis. Our objective was to confirm the presumed importance of Gal*f* metabolism for *Leishmania major* pathogenesis. As Gal*f* is absent from human this would validate UGM as target for the development of new chemotherapeutic agents against cutaneous leishmaniasis. A mutant deficient in Gal*f* was thus generated by targeted gene deletion of *GLF*, the gene encoding UGM. Biochemical characterisation of the mutant confirmed the *in vivo* role of UGM. Finally the virulence of the generated parasites was tested in a mice infection model.

2 Materials and Methods

2.1 Materials

2.1.1 Laboratory animals

Balb/c mice	Female 8-10 weeks old Balb/c mice were obtained from Charles River laboratories.

2.1.2 Eukaryotic cell line

L. major MHOM/SU/73/5ASKH:	*Leishmania major* promastigotes cell line, kindly provided by Prof. Joachim Clos (BNI Hamburg)

2.1.3 Bacterial strains

E. coli CWG287:	Genotype: (K-12 *lacZ trp* ∆(*sbcB-rfb*)*upp rel rpsL* λDE3); Bacteria were kindly provided by Chris Whitfield (Canada)
E. coli CWG288:	Genotype: (K-12 *lacZ trp* ∆(*sbcB-rfb*)*upp rel rpsL* λDE3 *galE*:Tn*10*)); Bacteria were kindly provided by Chris Whitfield (Canada)
E. coli XL-1 blue:	Genotype: *recA1 endA1 gyr96 thi-1 hsdR17 supE44 relA1 lac* (Stratagene) [F'*proAB lacI*^{q}*ZΔM15 Tn 10* (Tet^{r})]
E. coli YZ 2000:	Genotype: *thr-1 leu-6 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 his-4 argE3 str-31 tsx-33 supE44 recB21, recC22, sbcA23, rpsL31, tsx-33, supE44, his-328, mcrA, mcrBC, mrr, hsdMRS* (Gene Bridges)

2.1.4 Phage

Ffm	The phage Ffm was kindly provided by Chris Whitfield (Canada)

2.1.5 Plasmids

pBluescript II SK(+):	High copy number prokaryotic expression vector with ampicillin resistance (Stratagene)
pCR2.1hyg:	Plasmid bearing the hygromycin phosphotransferase gene inserted between the *Bsp*HI and *Nhe*I restrictions sites of vector pCR2.1 (Invitrogen). This plasmid was supplied by Dr. Martin Wiese, BNI, Hamburg.
pCR2.1phleo:	Plasmid bearing the phleomycin binding protein gene inserted between the *Nco*I and AvrII restrictions sites of vector pCR2.1 (Invitrogen) This plasmid was supplied by Dr. Martin Wiese, BNI, Hamburg.
pCR4-Topo:	Cloning vector allowing the insertion of DNA fragments by TA cloning (Invitrogen)
pET 22 b:	Prokaryotic expression vector for the expression of proteins under the control of the T7/lac promoter (Novagen).
pET Leis-mut:	Plasmid for expression of *L. major* UDP-galactopyranose mutase in prokaryotes. UGM was amplified from genomic DNA using the primer pair HB1/HB2 and inserted between the restriction sites *Nde*I/*Xho*I of pET-22b (Novagen).

pWQ66:	Expression vector carrying the the glf_{KPO1} gene of *Klebsiella pneumoniae* provided by Chris Whitfield (Canada)
pWQ70:	Expression vector carrying the O-antigen cluster of *Klebsiella pneumoniae* and an in-frame deletion in the glf_{KPO1} gene (UGM), provided by Chris Whitfield (Canada)
pXG:	*Leishmania* expression vector with neomycin resistance was kindly provided by Stephan M. Beverley (St. Louis, USA). (Map as appendice)
pXG-GLF:	Construct for episomal expression of UGM in *Leishmania*. *GLF* was amplified with the primer pair BK22/BK18 and cloned via *SmaI/BamHI* in pXG.
pXG-LMSAP1:	Construct for episomal expression of *L. mexicana* secreted acid phosphatase that was kindly provided by Stephan M. Beverley (St. Louis, USA).
pXG-GFP:	*Leishmania* vector containing a modified GFP was kindly provided by Stephan M. Beverley (St. Louis, USA). (Map as appendice)
pXG-GFP+2:	*Leishmania* vector for N-terminal GFP labelling of proteins was kindly provided by Stephan M. Beverley (St. Louis, USA). (Map as appendice)
pXG-GFP+2UGM:	Construct for UGM localisation. *GLF* lacking the start codon was amplified with the primer pair BK17/BK18 and cloned in the sense

	orientation in the *BamHI* site of pXG-GFP+2.
P5'UTR-Topo:	Construct bearing the 5' flanking region of *L. major GLF.* The fragment was amplified from genomic DNA with the primer pair BK13/BK14 and inserted into pCR4-Topo.
3'pBSK +:	Construct bearing the 3' flanking region of *L. major GLF.* The fragment was amplified from genomic DNA using the primer pair FR3/FR4 and inserted via the *SacI* and *XbaI* restrictions sites of pBluescript II SK(+).
3'5'pBSK +:	Construct bearing 3' and 5´ flanking region of *L. major GLF* . The 5'UTR from genomic DNA was amplified with the primer pair FR1/FR2 and inserted into the *XhoI/BamHI* sites of 3'pBSK +.
P3'5'phleo:	Construct for targeted gene replacement of *GLF*. Plasmid bearing the phleomycin resistance marker flanked by the 5' and 3' untranslated regions of *L. major GLF*. The phleomycin resistant marker was amplified by the primer pair BK7/BK8 from pCR2.1phleo and inserted between 5' and 3' UTR of *GLF* in 3'5'pBSK + by homologous recombination in YZ 2000.
P3'5'hyg:	Construct for target gene replacement of *GLF*. Plasmid bearing the hygromycin resistance marker flanked by the 5' and 3' untranslated region of *L. major GLF*. The hygromycin resistant marker was amplified by the primer pair FR6/FR7 from pCR2.1hyg and inserted between 5' and 3' UTR of *GLF*

in 3'5'pBSK + by homologous recombination in YZ 2000.

2.1.6 Oligonucleotides

All oligonucleotides were purchased from MWG.

Sequencing primers for *L. major GLF* (5' ➠ 3')

BK10	GCGTAGCGGGAGAAGATC
BK11	TGATCCCAGAGGCGCACA
BK26	TTCACCTTGGTTACGCGGCA
BK27	AGGCCGTGTGGAAGATGATC
BK28	GCCTCGACGCCCTGCATC
BK29	CTATTCACGCGGCCGCTTC

Sequencing primers for *L. major GLF* flanking regions (5' ➠ 3')

FR9	TCTCACGCCTCACTTCTAAG
FR10	CCATGGTGAAGGTCGTCGT
FR11	ACCATAGGCGTCGACGATGA
FR12	TGCTTCAATTTCGCACGTCT

Primers for homologous recombination control (5' ➠ 3')

ACM99	GACGTCGCGGTGAGTTCAGG
ACM100	GTCCGAGGGCAAAGGAATAG
ACM101	GAACGGCACTGGTCAACTTGG
ACM102	GTGGCCGAGGAGCAGGACTG
BK9	TGTGCAGGAGGGGTGGAA
BK12	ATCGGAGAACACGGCCTG

Sequencing primers for plasmids (5' ➠ 3')

ACM85	ATGAAAAAGCCTGAACTCACCG
ACM86	CTATTCCTTTGCCCTCGGAC
ACM87	ATGGCCAAGTTGACCAGTGC

ACM88	TCAGTCCTGCTCCTCGGCC
BK19	CTGCTGGAGTTCGTGACCG
BK30	AGGGCATGATAGTCCGCTCT
BK31	ATAGTCCGCTCTTGAGGGCA
T7	TAATACGACTCACTATA
T7term	GCTAGTTATTTGCTCAGCGG

PCR amplification primers (5' ➠ 3')

BK7	CATCAACTGACGCAACGCAGGCACACGCAGCA
	AATCCATCATGGCCAAGTTGACCAGTGCC
BK8	CTCCCTCGTCCTCCTCGTCCATGGCTGCCGACC
	TTGGCCTTCAGTCCTGCTCCTCGGCCAC
BK13	TTATCGTGTACTTCATTTGCTCG
BK14	TTCCACCCCTCCTGCACACT
BK17	GTCTGGATCCAGCGCTGACAAGGTGGTCATA
BK18	GCCTGGGATCCTACGAGGCCGTCGACGAC
BK25	GTCTGGATCCATGAGCGCTGACAAGGTGGT
FR1	CAAACTTCTTCGAGGCTATCAAG
FR2	GTCTGGATCCATGATGGATTTGCTGCGTGTG
FR3	CAAGTCTAGAGGCCAAGGTCGGCAGCCA
FR4	GCCTGGAGCTCAAACTCCGCCAAAAC
FR6	GACGCAACGCAGGCACACGCAGCAAATCCATC
	ATGAAAAAGCCTGAACTCAC
FR7	TCCTCCTCGTCCATGGCTGCCGACCTTGGCCTC
	TATTCCTTTGCCCTCGGAC
HB1	GCAAATCCATATGAGCGCTGACAAGGTG
HB2	TCGCTCGAGCATGTGCAGCGCATCTCACC

2.1.7 Antibodies

2.1.7.1 Primary antibodies

GP63-235: monoclonal antibody (mouse IgG1) directed against GP63 (hybridoma culture supernatant was generously provided by W. Robert McMaster ,Vancouver, Canada).

L-5-28: monoclonal antibody (mouse IgG1) reacting with GIPL2 and GIPL3 (purified antibody was generously provided by E. Handman, Victoria, Australia)

L-5-34: monoclonal antibody (mouse IgG1) reacting with GIPL2 and GIPL3 (purified antibody was generously provided by E. Handman, Victoria, Australia)

LT8.2: monoclonal antibody (mouse IgG1) directed against linear peptide epitope of *Leishmania mexicana* SAP (hybridoma culture supernatant was supplied by Martin Wiese)

MEST1: monoclonal antibody (mouse IgG1) directed against terminal galactofuranose of GIPL 1 (lyophilized hybridoma culture supernatant was generously provided by AH Straus, Sao Paulo, Brasil)

WIC79.3: monoclonal antibody (mouse IgG1) directed against galactose substituted repeating unit of LPG (ascites fluid supplied by Martin Wiese)

2.1.7.2 Secondary antibodies, sera and conjugates

Anti-mouse-IgG Cy3 conjugate	Sigma
Anti-Digoxigenin Fab AP conjugate	Roche
Anti-mouse-Ig AP conjugate	Dianova
Anti-mouse-Ig HRP conjugate	Dianova
DAPI	Sigma

2.1.8 Molecular weight markers

'1 kb DNA ladder'	Invitrogen
'SDS-PAGE molecular weight standards high range'	BioRad
'Prestained Precision Protein Standards'	BioRad

2.1.9 Enzymes

Alkaline calf intestine phosphatase	New England Biolabs
cloned Pfu-DNA-Polymerase	Stratagene
Lysozyme	Serva
Proteinase K	Boehringer- Mannheim
Restriction enzymes	New England Biolabs
T4-DNA-Ligase	New England Biolabs
Taq-DNA-Polymerase	Sigma

2.1.10 Culture media and additives

Adenin	Sigma
Ampicillin, sodium salt	Serva
Carbenicillin, disodium salt	Fluka
Fetal calf serum	Invitrogen
Hemin	Fluka
Hygromycin B	Sigma
Kanamycin	Sigma
LB-agar	Becton Dickinson
LB-medium	Becton Dickinson
M199 powder	GibcoBRL
Noble agar	Becton Dickinson
paraformaldehyde	Sigma
Penicillin/Streptomycin	Sigma
Phleomycin	Sigma
Poly (L)-lysine	Sigma
RPMI 1640 medium	Gibco
Terrific Broth medium	Sigma
Roti^{TM}Phenol	Roth

2.1.11 Kits and further materials

CarboPac PA- 10 colum, 2x 250mm	DIONEX
Cell culture bottles and dishes	Sarstedt
Cellulose acetate filter (0,22µm, 0,8 µm)	Sartorius
Electroporation cuvettes	BioRad
Filter paper	Whatman
Glas capillaries	Macherey-Nagel
Glas tubes with Teflon cap	DIONEX
Hyperfilm MP	Amersham Biosciences
HPTLC-plates Nano-DURASIL-20 size 10 x 10 cm	Macherey-Nagel
Microtiter plates 96-well polystyrol (U-bottom)	Greiner
Nitrocellulose membrane (0,45 µm)	Schleicher & Schuell
Nylon membrane (Hybond-N+)	Amersham Biosciences
PCR-tubes (0,2 ml)	Biozym
Plastic disposable pipettes (5 ml, 10 ml, 25 ml)	Sarstedt

Polypropylen tubes (14 ml, 50 ml)	Greiner
QIAGEN Plasmid Mini und Midi Kit	Qiagen
Qiaquick Gel Extraction Kit	Qiagen
Qiaquick PCR purification kit	Qiagen
Reaction tubes (0.5 ml, 1.5 ml)	Sarstedt
Reaction tubes safelock (1.5 ml, 2 ml)	Eppendorf
Sterile filters Millex GP (0,22 µM)	Milipore
Syringes (2 ml)	Braun

2.1.12 Standard buffer and media

20xSSC	3 M NaCl 0.3 M Sodium citrate pH 7.0
2xLaemmli	200 mM Tris-HCl pH 6.8 30% (v/v) glycerol 3% (w/v) SDS 0.1% (w/v) bromophenol Blue 5% (v/v) 2-mercaptoethanol
5xGEBS	20% glycerol 50 mM EDTA 0.05% bromophenol Blue 0.5% sarcosyl
5xLaemmli	600 mM Tris-HCl pH 6.8 40% (v/v) glycerol 10% (w/v) SDS 0.4% (w/v) bromophenol Blue 5% (v/v) 2-mercaptoethanol
AP-buffer	100 mM Tris-HCl pH 9.5 100 mM NaCl 5 mM $MgCl_2$
BCIP	25 mg/ml BCIP in 100% DMF
ECL-reagent	125 mM Luminol 45 mM p-Cumaric acid 1M Tris/HCl pH 8.5 15% H_2O_2
NBT	50 mg/ml NBT in 70% (v/v) DMF
PBS	10 mM sodium phosphate pH 7.4 150 mM NaCl
PBS/EDTA	10 mM sodium phosphate, pH 7.4 150 mM NaCl 2 mM EDTA
SOB (SOC)	2% bactotryptone 0.5% yeast extract 10 mM NaCl 2.5 mM KCl

	10 mM MgCl2 10 mM MgSO4 (2% glucose)
TAE	40 mM Tris-Acetate 2 mM EDTA pH 8.5
TBE	100 mM Tris-HCl, pH 8.0 100 mM Borate 2.5 mM EDTA
TELT	50 mM Tris-HCl, pH 8.0 62,5 mM EDTA pH 9.0 2,5 M LiCl 4% (v/v) Triton x-100
TBS	20 mM Tris-HCl, pH 7.4 150mM NaCL
TE	10 mM Tris-HCl, pH 8.0 1 mM EDTA

2.1.13 Chemicals

ABTS	Roche
Acetic acid (100 %)	Merck
Aceton	Baker
Acrylamide 40% 4 K-Mix (37.5:1)	Serva
AEC	Sigma
Agarose	Serva
Ammonium chloride	Merck
Ammonium persulfate (APS)	Serva
BCA *Protein Assay Reagent*	Pierce
BCIP (5-Bromo-4-chloro-3-indolyl-phosphate)	Fluka
Beta-Mercaptoethanol	Sigma
Borate, sodium salt	Merck
Bromophenol Blue, sodium salt	Applichem
BSA (Fraktion V)	Applichem
BSA protein standard	Pierce
Chloroform	Baker
Citric acid	Merck
CSPD	Roche
Dimethylsulfoxide (DMSO)	Merck
Dipotassium hydrogen phosphate	Merck
Disodium hydrogen phosphate	Merck
Disodium hydrogen phosphate	Merck
Dithiothreitol (DTT)	Sigma
dNTPs (100 mM each)	Pharmacia
Dry milk	Applichem

EDTA, Disodium salt (Titriplex III)	Merck
Ethanol, absolut	Baker
Ethidium Bromide	USB Corporation
Formaldehyde	Sigma
Glycerin (99%)	KMF
Glycine	Sigma
Hydrochloric acid (38%)	Baker
Hydrogen peroxide	Fluka
Imidazole	Fluka
Isopropanol (2-Propanol)	Merck
Korsolex plus	Roche
Methanol	Baker
Methanolic- HCL (0.5N)	Supelco
Moviol	Baker
NBT (Nitrotetrazolium bluechloride)	Fluka
Pepstatin	Roche
Phenylmethylsulfonyl fluorid (PMSF)	Sigma
Polyisobutylmethacrylat	Sigma
Ponceau S, sodium salt	Sigma
Potassium dihydrogen phosphate	Merck
Potassium chloride	Applichem
Roti-Blue Coomassie-stain (5x)	Roth
Saponin	Sigma
Sodium acetate	Merck
Sodium chloride	Merck
Sodium dihydrogen phosphate	Merck
Sodium dodecylsulphate (SDS)	Merck
Sodium hydroxide	Merck
Sodium periodate	Sigma
TEMED (N,N,N',N'-Tetramethyl-ethylendiamin)	Serva
TRIS (Tris(hydroxymethyl)-aminomethan)	Merck
Tween-20	Fluka

All chemicals not explicitly listed above were purchased in p.a. quality from either Merck or Sigma.

2.1.14 Laboratory Equipment

Blotting chamber	Fast-Blot B44	Biometra
Centrifuges :	- Biofuge fresco	Heraeus
	- Biofuge pico	Heraeus
	- Multifuge 3 S-R	Heraeus
	- Centrifuge 5415C	Eppendorf
	- Coulter Avanti J-30I	Beckman
Rotors:	- JA 25.50	Beckman
	- JLA 10.500	Beckman
	- JS-24.15	Beckman
Easy Enhanced Analysis System (E.A.S.Y RH-3, Videocamera 429K)		Herolab
Electroporator		BioRad

Electrophoresis chamber for agarose-gels	peqlab
Electrophoresis chamber for SDS-PAGE	BioMetra
ELISA-Reader: DigiScan	Asys Hitech
Heatingblock TB1	BioMetra
HeraSafe Hood	Heraeus
High pH Anion Exchange chromatograph	DIONEX BioLC
Incubators	Heraeus
Peristaltic pump P-1	Amersham Biosciences
Scales CP 224S (µg) / CP 3202 (g)	Sartorius
Sonifier 450	Branson
Spectrophotometer Ultrospec 2100 pro	Amersham Biosciences
Speedvac RVC 2-18	Christ
Standard Power Pack P25	Biometra
Stratalinker	Stratagene
Thermocycler T1 and T Gradient	Biometra
Thermomixer compact	Eppendorf
ULTRAFLEX™ MALDI-TOF/TOF spectrometer	Bruker

2.2 Cell biological approach

2.2.1 *Leishmania major* culture conditions

Promastigotes of *L. major* MHOM/SU/73/5ASKH were grown at 27°C in M199 medium supplemented with 10% fetal bovine serum, 40mM Hepes pH 7.5, 0.1mM adenine, 0.0005% hemin, 0.0002% biotin and 50 U/ml penicillin/streptomycin. As needed, G418, hygromycin B and phleomycin were added at 30, 50 and 5 µg/ml respectively. The 10 ml cultures were passaged every 3-4 days at a dilution from 1:50. The saturation density of this *L. major* strain ranged from 4-5 $x10^7$ cells/ml. Selection of individual clones after transfection was done on semi-solid M199 medium containing additionally 1% noble agar.

For long time storage, $4x10^7$ cells were pelleted, resuspended in 1,5 ml FCS supplemented with 10% DMSO and transferred into cryo-tubes. Cells were slowly frozen in the gas phase of the liquid nitrogen tank or in the -80°C fridge over night. The next day tubes were transferred into liquid nitrogen. To revive *L. major* culture, an aliquot was thawed in a water bath at 37°C and transferred to 10 ml fresh M199 media containing the appropriate antibiotics.

2.2.2 *In vitro* growth

For analyzation of the *in vitro* growth of the Leishmania parasites fresh 10 ml M199 media, containing the appropriate antibiotics, was inoculated with 5, 5 x 10^6 parasites. The cultures were counted every 24 h for 5 days in duplicate using a light microscope.

2.2.3 Electroporation conditions for *L. major*

The protocols for target gene replacement in *L.major* were kindly provided by Prof. Jon LeBowitz (Purdue University, West Lafayette, USA) and combined with an improved transfection protocol according to Robinson *et al*., (2003). Parasites were grown to a density of approximately 10^7 cells/ml and 1-2x10^8 parasites were harvested by centrifugation at 1300 xg for 10 min at 4°C. Parasites were washed once in electroporation buffer (21 mM Hepes, pH 7.5, 137 mM NaCl, 5 mM KCl, 0.7 mM $NaHPO_4$, 6 mM glucose), resuspended in 500 µl electroporation buffer and mix with 2 µg linearised targeted gene replacement construct bearing a resistant marker or 10 µg of the appropriate plasmid in a 4 mm cuvette. The cells were electroporated by two pulses at 25µF and 1.5 kV, incubated on ice for 10 min, transferred into 10 ml M199 media and left at 27 °C for 24 h. The culture was then centrifuged at 1300 xg for 10 min at 20°C, resuspended in 100 µl culture media and transferred on M199 agar plates (2x M199 culture media combined with an equal volume of 2% noble agar) containing the appropriate antibiotics. The M199 agar plates were protected from dehydration by wrapping them with parafilm and were incubated upright in the incubator at 27°C. After two weeks the colonies on the plates became visible and were transferred and expanded in 10 ml M199 media containing the appropriate antibiotics.

2.2.4 Fluorescence and Immunofluorescence microscopy

Leishmania parasites were analysed by immunofluorescence using the monoclonal antibodies WIC 79.3 or GP63-235. The cells were immobilised on 10-well glass coverslips coated with poly(L)-lysine (0.1 mg/ml in PBS). 500 µl parasites from a 2 x 10^7 cells/ml dense culture were harvested, washed once in PBS and resuspended in 500 µl PBS. 20 µl cells were transferred with the same amount of 4% PFA in each well and incubated for 20 min at room temperature. The cells were washed twice with PBS and permeabilized with 50 mM NH_4Cl for 15 min. Incubation with 2% BSA in PBS for at least 15 min was used to prevent unspecific absorption of antibodies. This solution was kept in subsequent incubation with the primary antibody (WIC 79.3 mAb ascites fluid 1:1000

or GP63-235 mAB hybdridoma culture supernatant 1:10). After 1 h, cells were washed 4 times with PBS and incubated for 30 min in the dark with a mixture of secondary antibody goat α–mouse Cy3 1:500 and 8µg/ml DAPI in 2% BSA/PBS. After five washing steps with PBS the slides were embedded in Mowiol and inspected with the 40 x and 100 x magnification of a fluorescence microscope.

Similar procedure was used for localisation experiments using a GFP tagged *GLF* construct. Parasites were immobilised to poly-L-lysine coated coverslips and fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. Additionally the cells were stained with 8µg/ml DAPI for 30 min in the dark before embedding in Mowiol and inspection by fluorescence microscopy at a 100 x magnification.

2.2.5 Mice infection model

Because *Leishmania* tend to lose virulence in culture the parasites were passaged once through mice. Parasites were grown to late logarithmic phase and 10^7 parasites per mouse were injected in the hind left footpad. After 10 days the parasites were re-extracted from lymph nodes and transferred to liquid media at 27 °C, to allow the transformation of the parasites from amastigote to promastigote. The reisolated parasites were expanded and DMSO stocks were prepared. These cultures were passaged less than three times. For the mice infection studies the parasites were grown to late logarithmic phase. 2 x 10^6 parasites were harvested at 1300 xg for 10 min at 4°C. The cell pellet was resuspended in 100 µl PBS and immediately injected into the left hind footpad of a Balb/c mouse (Charles River). The infection was controlled once a week by determining the infected and non infected footpad with a Vernier calliper. The median size difference of the infected and non infected footpad was monitored for 11 weeks after post infection.

2.3 Biochemical techniques

2.3.1 Assignation of protein concentration

Protein concentration was determined using the 'BCA Protein Assay Reagent' (Pierce) following the manufacturers instructions. Shortly the reagents A and B were mixed in a ratio of 50:1 before use. 200 µl of the mixture were mixed with 10 µl of sample in a 96-well microtiter plate. For reference a dilution series of 25-200 µg/ml BSA was included. The plate was incubated for 30 min at 55°C and absorbance was measured at 540 nm in

the ELISA-Reader 'DigiScan' (Asys Hitech). The protein concentration of the samples was calculated from the BSA standard curve.

2.3.2 Activity assays of UGM

2.3.2.1 *In vivo* activity assay of UGM

The activity of UGM of *Leishmania major* was analysed by an in vivo complementation of *E. coli* CWG287 lacking a functional UGM. The open reading frame of *Leishmania major* was amplified by PCR using the primer pairs HB1/HB2 and cloned via *NdeI/XhoI* into pET22b, resulting in pET-Leis-mut. The plasmid pWQ70 containing the O-antigen gene cluster from *Klebsiella pneumoniae* KPO1 with a deletion in the UDP-galactopyranose mutase gene (glf_{KPO1}) was transformed into *E.coli* CWG287. Chemically competent cells were prepared with these bacteria and transformed with a second plasmid containing either Glf_{KPO1} (pWQ66), the candidate gene of *Leishmania major* (pET Leis-mut) or empty pET22b vector. The transformants were grown in LB media with antibiotics for 10 hours. Antibiotics were added to 5 ml LB maintained at 50^{o}C and mixed with 1 ml fresh bacterial culture and immediately plated. Bacteria were allowed to grow for 2 H after which 5 µl drops containing 10^6 (top left), 10^5 (top right), 10^4 (bottom left) and 10^3 pfu/ml of phage Ffm were deposit on each plate. The plates were incubated at 37°C for 10h.

Protein-free LPS preparations were obtained by the SDS-proteinase K lysate method. Freshly transformed bacteria were harvested from an agar plate with PBS (pH 7,2) and the OD adjusted to 0.4. Bacteria from 1.5ml suspension were pelleted and solubilized in 50 µl of lysing buffer containing 2% SDS, 4% 2- mercaptoethanol, 10% glycerol, 0.1 M Tris-HCl (pH 6,8) and bromphenol blue. The lysated were heated at 100°C for 10 min.- For protein digestion, 25 µg of proteinase K was solubilized in 10 µl of lysing buffer was added to each boiled lysate an incubated at 60°C for 60 min. Protein-free LPS preparations obtained by the SDS-proteinase K lysate method were analyzed by SDS-PAGE (2.3.9) and silver-stained (2.3.11).

2.3.2.2 *In vitro* activity assay of UGM

Plasmid containing either glf_{KPO1}or the potential *GLF* gene of L. major (pET-Leis-mut) was transformed into *E.coli* CWG288. Transformants were then grown in terrific broth with antibiotic until OD_{600} 0.6 and induced with 0.4mM IPTG for 3H at 30^{o}C. Cells were collected by centrifugation, washed with ice cold buffer and resuspended at an OD_{600} 10 in 50mM Hepes pH 7.4, 1mM PMSF; 1mM DTT. Cells were then treated with lysozyme (100µg/ml) at room temperature for 30min and lysed by sonication using a Branson 450

sonifier. Cell debris was removed by centrifugation at 16000G for 30min at 4°C. Protein concentration of the supernatant was determined using the BCA assay (Pierce). The assay was based on the method developed by Scherman *et al*. (2003) using UDP-[6-^{3}H]Gal_p purchased from Amersham. Briefly, 0.5µg of crude protein extract were incubated with 100µM radioactive UDP-Gal_p (specific activity: 75 Bq/pmol) in 25µl buffer containing 25mM Hepes pH 7.2, 1mM $MgCl_2$ and 1mM NADH for 20min at 37°C. Nucleotides were then oxidized by $NaIO_4$ (200 mM) and the reaction was stopped by the addition of 3µl of ethylene glycol. After dilution with 100µl H_2O, products were loaded on a 300µl column of AG 1X8 (200-400 mesh; acetate form) (Biorad) and the column was washed with 100µl H_2O. After addition of 2ml of Lumasafe Plus scintillation cocktail (Lumac, The Netherlands) to the efluent, radioactivity was counted.

2.3.3 Preparation of total cell lysates for GP63 and LPG analysis

For analysis of GP63, LPG and PPG, 1x10^8 parasites were centrifuged at 1300 xg for 10 min and washed two times with cold PBS. The cells were resuspended in 100 µl lysis buffer (50 mM Tris/HCl pH 8,0, 0,1% Triton X-100, 1 mM PMSF, 2 µg/ml Leupep-tin, 5 µg/ml Pepstatin) and disrupted by sonification in a beaker resonator 4 times for 30 seconds (Branson, 100% Duty cycle, output control 5, 4°C). Disruption was checked under a light microscope. Cell debris were removed by centrifugation at 13000xg for 10 min at 4°C. 2 x Laemmli buffer was added to an aliquot of the lysate, and 12,5 µg protein per sample were loaded on a 8% ProSieve gel (2.3.9).

2.3.4 Preparation of lysates for GIPLs analysis

4 x 10^8 parasites were pelleted at 1300xg for 10 min and washed to times in cold PBS. The cell pellet was resuspended in 500 µl Chloroform/Methanol/Water ratio of 1:2:0,8. The cells were sonified in a beaker resonator 5 times for 30 seconds (Branson, 100% Duty cycle, output control 5, 4°C). Cell debris was removed by centrifugation at 13000 xg for 10 min at 4°C. The supernatant were kept and the cell pellet was reextracted with 500 µl Chloroform/Methanol/Water ratio of 1:2:0,8 following by sonification and centrifugation. The combined supernatants were evaporated to dryness under a stream of nitrogen, resuspended in water and purified over a C_{18}/SepPak® Plus column (Waters). GIPLs were eluted in methanol, dried under a stream of nitrogen, resuspended in Chloroform/ Methanol/ Water (30:60:8) and analysed by mass spectrometry.

2.3.5 HPTLC analysis

After extraction of GIPLs (2.3.4) the samples were resuspend in 15µl Chloroform: Methanol:0.2% KCL (10:10:3 v/v). 150ml Chloroform:Methanol:0,2% KCL (10:10:3 v/v) were added to a glass chromatophy tank (25cm x 27cm x 10cm) lined with filter paper (ca. 17cm x 19cm) and covered tightly. The vapors in the tank were equilibrated for at least 2h. The Glycolipids were spotted on the HPTLC plate using a glass capillary. The plate was placed in the tank with the samples just above the level of solvent. The tank was covered tightly and the plate was left until the ascending solvent line reached the top of the plate. The TLC plate was dried under the hood for 10 min. The plates were incubated 2 h with the primary antibodies (L-5-28 (1:40), L-5-34 (1:200) and MEST1(1:5)) in BSA/TBS. The plates were washed three times for 5 min with TBS and incubated with α-mouse Ig coupled to AP antibody (1:2000) in BSA/TBS. The plates were rinsed three times with TBS and once with AP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). The visualization of the glycolipids was achieved by incubation with the substrate BCIP/NBT (0.3 mg/ml NBT, 0.15 mg/ml BCIP in AP-buffer). The reaction was stopped by the addition of water.

2.3.6 Matrix assistance laser desorption (MALDI) mass spectrometry

The GIPLs were further analysed by mass spectrometry using 6-Aza-2-thiothymidine (5μg/μl) as matrix. 1 µl of the matrix was added and fixed on a high grade steel plate under a stream of hot air. 5 µl of the GIPLs extracts (2.3.4) was layered on the matrix by hot air drying. Finally 1 µl of the matrix was coated on top. Mass spectra were recorded on a Bruker ULTRAFLEX™ MALDI-TOF/TOF spectrometer in the negative mode and the instrument was used at a maximum accelerating potential of 20 kV. The data were analysed with the Bruker flexAnalysis 2.0 program.

2.3.7 HPAEC analysis

The GIPLs were investigated by High pH Anion Exchange chromatography (HPAEC). GIPLs from 4 x 10^8 *Leishmania* promastigotes were extracted (2.3.4) and evaporated to dryness in a glass tube with teflon cap. Methanolysis of glycosides was performed by addition of 150 µl 5mM methanolic-HCL (prepared by diluting 0.5N methanolic-HCL with anhydrous methanol) and incubation at 84°C for 3h. The samples were evaporated under a stream of nitrogen at room temperature. 50µl butanol were added and the samples dried again. This step was repeated 3 times. The samples were dissolved in

100 µl H_2O, injected on an analytical CarboPac PA-10 (2 x 250 mm) column and eluted isocratically in NaOH (18mM) at a flow rate of 0,25ml/min. The data were analysed with the DIONEX Chromeleon software.

2.3.8 Immunoprecipitation

For immunoprecipitations of the *L. mexicana* secreted acid phosphatase, 1 ml cell culture supernatant was incubated with purified LT8.2 monoclonal antibody for 1h at 4°C. 50 µl of a 50% slurry Protein-A sepharose beads (Sigma) were added and after 1h at 4°C, the samples were centrifuged at 500 x g for 30 seconds. The supernatant was removed and the beads washed four times with 1 ml 50 mM Tris/HCl, pH 8.0, 0.5% NP-40. The samples were resuspended in 20 µl 2x Laemmli buffer, boiled at 95°C for 5 min and separated on a SDS PAGE.

2.3.9 SDS-PAGE

The SDS-polyacrylamide gels were performed according to Laemmli (Laemmli, 1970). The protein samples were separated on SDS-polyacrylamide gels consisting of a 5 % stacking gel (125 mM Tris/HCl pH 6.8, 0.1 % SDS, 5 % polyacrylamide) and a 10 % separating gel (375 mM Tris/HCl pH 8.8, 0.1 % SDS, 7-14 % polyacrylamide) or 8% ProSieve® 50 (gel solution, Cambrex) gradient gel. For the gel preparation the buffer, SDS and the acrylamide stock solution were mixed (37.5 % acrylamide, 1 % bisacrylamide or ProSieve) and polymerisation was initiated by adding 0.1 % TEMED and 1 % ammonium persulfate. 2x or 5x Laemmli-buffer was added to the protein samples and heated to 95 °C for 5 min. Electrophoresis was performed in SDS-electrophoresis buffer (50 mM Tris, 350 mM glycine, 0.1 % SDS) at 15 mA (stacking gel) and 20 mA (separating gel) per gel.

2.3.10 Coomassie staining of SDS gels

The Roti®-Blue (Roth) staining solution was used for the protein gels staining. Immediately after electrophoresis (2.3.9) the gels were incubated in 20 ml 1xRoti®-Blue in 20 % methanol over night. The gels were destained in 20 % ethanol containing 10 % glycerol and dried.

2.3.11 LPS Silver staining

The SDS gels were soaked overnight in 40% isopropanol and 5% acetic acid in 200 ml of distilled water. The gels were incubated for 5 min in periodic acid solution (0.7% periodic acid 40% ethanol 5% acetic acid) to oxidise the LPS and rinsed in running destilled water for 2 h. The gel was stained for 10 min with freshly prepared staining solution which was prepared as follows. A 2 ml volume of concentrated ammonium hydroxide was added to 28 ml of 0.1 N sodium hydroxide. After the addition of about 115 ml of water, 5 ml of 20% silver nitrate was added in drops with stirring. The gel was washed three times in deionised water for 15 min and incubated afterwards in freshly prepared developer solution containing 10 mg citric acid and 0,1 ml 30% formaldehyde in 200 ml of water until bands were lighting up. The color reaction was stopped and fixed by exposure to 0.35% acetic acid for one hour. The gels were destained in drying buffer (20 % ethanol, 10 % glycerol) and dried after 3-4 hours.

2.3.12 Western Blot

After the electrophoresis the protein samples on the SDS page were transferred to Hybond nitrocellulose membranes (Amersham, Bioscience) using a *semidry* blotting chamber (Biometra) at 2 mA/cm^2 for 1 h. Gel and membrane were placed in between two layers of Whatman filter papers soaked in blotting buffer (48 mM Tris, 39 mM glycine). Transfer efficiency was checked by labeling with Ponceau S-solution (0.2 % (w/v) Ponceau S in 3 % TCA) and destained in deionised water and PBS.

2.3.13 Immunostaining of Western Blots

After western blotting (2.3.12) the membrane was blocked over night in blocking buffer consisting of 2% BSA/PBST (2% BSA, PBS, 0,1 % Tween). After three times washing of 10 min in PBST (PBS, 0,1% Tween), the membrane was incubated for at least 1 h with the first antibody in 2% BSA/PBST (WIC 79.3 mAb ascites fluid 1:4000; GP63-235 hybridoma supernatant 1:50). The blots were washed in PBST (PBS, 0,1% Tween) three times for 10 min and two times for 15 min. Finally the blots were incubated with α–mouse Ig coupled to HRP at 1:50 000 in 2% BSA/PBST, 0,1% Tween for 1 hour at room temperature. The membranes were washed 3 times for 10 min and two times for 15 min in TBST (0,1 % Tween). To detect the signals an ECL detection system was used. The ECL detection solution was prepared by mixing a Luminol solution (250 mM Luminol, 90 mM p-Cumaric acid, 1 M Tris/HCl, pH 8.5) with a hydrogen peroxide solution (1 M

Tris/HCl, pH 8.5, 30% H_2O_2) in a 1:1 ratio. After 15 min of incubation, the ECL solution was removed from the membrane. The blot was wrapped in a plastic sheet and the signals exposed for 1-10 min to a Hyperfilm MP.

2.4 Molecular biology techniques

2.4.1 Nucleic acids precipitation

The DNA was precipitated by adding 1/3 volume 7.5 M ammonium acetate (pH 5) and 2.5 volumes ethanol (100%) to the aqueous solutions for 1-2 hours at -80°C or over night at -20°C. Afterwards the samples were centrifuged at 13000xg for 30 min at 4°C and the pellet was washed in 500 µl 70% Ethanol. After another centrifugation step at 1300 xg for 10 min. the supernatant was removed completely. The pellets were air dried and dissolved in water or TE.

2.4.2 Phenol Chloroform extraction

For purification of the DNA the samples were extracted by phenol/chloroform following by precipitation (2.4.1). An equal volume of Roti^{TM}Phenol was added to the DNA sample and mixed. The samples were centrifuged at 1300 xg for 5 min at RT. The upper aqueous solution was removed and combined with an equal volume of chloroform, following by centrifugation (1300xg, 5 min, RT). The DNA of the aqueous solution was precipitated as mentioned before (2.4.1).

2.4.3 Determination of DNA concentrations

The DNA concentrations were determined photometrically and calculated from the absorbance measured at 260 nm: c(DNA) = absorbance(260 nm) x 50 µg/ml and c(RNA) = absorbance(260nm)x40 µg/ml.

If the DNA concentrations were to low, the concentrations were determined on ethidium bromide plates by comparing the signal intensity with serial dilutions of a DNA sample with known concentration. Therefore 100 ml of 0.8 % agarose in TAE buffer containing 1 µg/ml ethidium bromide was added and the solution was plated in five petri dishes. The standards were prepared by seven serial dilutions in 100 mM EDTA in a range of 10 ng/µl to 200 ng/µl and stored at -20°C. The same amount of 1 µl of the standards and the samples were spotted on the plates and incubated at RT for 15 min. The DNA was detected at 302 nm using the ‘Easy Enhanced Analysis System’ (Herolab).

2.4.4 DNA electrophoresis on agarose gels

The DNA was diluted in DNA sample buffer and separated on horizontal agarose gels (0.6 to 1.5 % agarose in TBE buffer). Agarose gels in TAE buffer containing ethidium bromide were used for extraction of DNA from the gels. Electrophoresis was performed at 5 V/cm in TBE buffer. The DNA was detected after staining in ethidium bromide (50 µg/ml) at 302 nm. For documentation the 'Easy Enhanced Analysis System' (Herolab) was used.

2.4.5 General cloning approaches

2.4.5.1 Digestion and dephosphorylation of DNA

Digests of DNA were performed using the restriction enzymes and reaction buffers following the manufacturers instructions (New England Biolabs). 2 µg of DNA were incubated with 5 U of enzyme in a total volume of 50 µl. The digests were incubated over night at temperatures indicated by the manufacturers. For analytical digests, 1 µg of plasmid DNA was incubated for 2 hours with 5 U of enzyme.

To avoid self ligation of vector fragments, the 5´-phosphate groups of vector DNA were removed by adding 1 U of calf intestine alkaline phosphatase (NEB) per µg of vector DNA and incubate at 37°C for 15 min.

2.4.5.2 DNA extraction from agarose gels

After restriciton enzyme digests the DNA was separated on an agarose gel and the desired fragments were excised from the gel. 'Qiaquick PCR Purification Kit' (Qiagen) was used for further purification following the manufacturers instructions. The excised gel fragments were solved in 300 µl buffer QG per 100 mg gel at 50°C and applied to a Qiaquick column. The column was centrifugated at 13000 xg for 1 min and the flow through was discarded. After one washing step with 500 µl buffer PE, bound DNA was eluted in 50 µl EBC (10 mM Tris-Cl pH 8.5).

2.4.5.3 DNA ligation

For ligation of DNA fragments 30 ng of digested (and if necessary dephosphorylated) vector DNA and a 3 molar excess of the respective insert were incubated with 1 U of T4-

DNA-Ligase (NEB) in 20 µl ligation buffer (50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 1 mM ATP, 10 mM DTT, 25 µg/ml BSA). After incubation over night at 16°C the ligation was transformed into competent *E.coli* cells.

The sequencing of constructs generated by PCR amplification steps was done by the companies MWG or GATC.

2.4.5.4 Analytical plasmid preparation

The 'Qiaprep spin Miniprep Kit' was used for analytical plasmid preparation following the manufacturers instructions. A single bacterial colony was transferred from a selective agar plate into 3 ml of LB medium containing the appropriate antibiotic. The bacteria were incubated for 12-16 h at 37°C and 250 rpm. For the plasmid preparation 2 ml of the bacteria culture were harvested for 1 min at 13000xg at RT and resuspended in 250 µl buffer P1 (50 mM Tris/HCl pH 8.0, 10 mM EDTA, 100 µg/ml RNaseA). Afterwards 250 µl of buffer P2 (1 % SDS, 0.2 M NaOH) and 350 µl of chilled buffer P3 (3.0 M potassium acetate, pH 5.5) were added, each buffer addition was followed by a cautions mixing step. After centrifugation (10 min, 13000xg, RT) the supernatant was applied on a Qiaprep spin column. The column was washed with 750 µl buffer PE and the DNA eluted with 50 µl TE buffer.

2.4.5.5 Preparative plasmid preparation

The 'Qiagen plasmid Kit Midi' was used for the preparative plasmid preparation following the manufacturers instructions. A single bacterial colony from a selective agar plate was inoculated in 100 ml LB containing the appropriate antibiotic. The culture was incubated for 12-16 h at 37°C and 200 rpm. The bacteria were harvested (15 min, 6000xg, 4°C) and resuspended in 4 ml buffer P1 (50 mM Tris/HCl pH 8.0, 10 mM EDTA, 100 µg/ml RnaseA). 4 ml buffer P2 (1 % SDS, 0.2 M NaOH) were added and after a cautious mixing bacteria were lysed for 5 min at room temperature. 4 ml of chilled buffer P3 (3.0 M potassium acetate, pH 5.5) were added, the sample was cautiously mixed again and incubated on ice for 15 min. The samples were centrifuged (30 min, 20000xg, 4°C) and the supernatant was poured over a filter and applied to a QIAGEN-Tip 100 column equilibrated in QBT buffer (750 mM NaCl, 50 mM MOPS pH 7.0, 15% isopropanol, 0.15% (v/v) TritonX-100). The columns were washed two times with 10 ml QC buffer (1 M NaCl, 50 mM MOPS pH 7.0, 15% isopropanol) and the bound plasmid DNA was eluted in 5 ml QF buffer (1.25 M NaCl, 50 mM Tris-HCl pH 8.5, 15% isopropanol). 3.5 ml isopropanol were added to the eluent and the samples were centrifuged (30 min,

20000xg, 4°C). The DNA pellet was washed with 2 ml ethanol (70 %), centrifuged again (15 min, 20000xg, 4°C), air dried, resuspended in 100 µl TE buffer and stored at -20°C. The DNA concentration was determined as described in 2.4.3.

2.4.5.6 Preparation of chemically competent *E.coli*

Chemically competent cells were produced following the protocol of Molthoff *et al.,* (1990). Briefly the *E. coli* were grown on selective LB agar plates containing the appropriate antibiotic. One colony was transferred to 2 ml SOB media and incubated at 37°C over night. 1ml of the culture was inoculated into 200 ml SOB medium and incubated for 1-2 days (200 rpm) at 18°C until an OD_{600nm} of 0.6 was reached. The culture was harvested (2000xg for 10 min at 4°C) and the pellet resuspended in 1/3 of the original volume cooled TB (250 mM KCl, 10 mM Hepes free acid, 15 mM $CaCl_2$, 55 mM $MgCl_2$). Another centrifugation step was performed (2000 xg for 10 min. at 4°C) and the pellet was dissolved in 1/12 of the original volume TB. After addition of DMSO to a final concentration of 7%, 100 µl cells aliquots were immediately frozen in liquid nitrogen and stored at -80°C.

2.4.5.7 Transformation

For the transformation of chemically competent E. coli, 100 µl of the bacteria were thawed on ice for 10 min. Afterwards 10 ng of plasmid DNA or 20 µl of ligation mix (2.4.5.3) were added and incubated on ice for 30 min. The cells were subjected to a heat shock at 42 °C for 50 s followed by incubation on ice for 2 min. After adding of 1 ml LB-medium the bacteria were incubated for 1 h at 37°C and plated on selective LB-agar plates containing the appropriate antibiotics.

2.4.5.8 Preparation of electrocompetent *YZ 2000*

The bacterial strain *YZ 2000* was purchased from Gene Bridges and the electrocompetent cells were prepared using a combined protocol of Zhang *et al.* (1998) and the manufacturer's instructions. *YZ 2000* were grown on LB agar plates at 37°C and one colony was inoculated in 10 ml LB medium and incubated over night at 37°C and 250 rpm. The culture was diluted 100-fold in LB medium and grown to an OD_{600nm} of 0.5. The cells were chilled on ice for 30 min and centrifuged at 5000 rpm for 15 min at -5°C. The pellet was washed three times in 10% ice-cold glycerol. Bacteria were harvested at

6000 rpm for 15 min at -5°C and resuspended in 600µl 10% ice-cold glycerol. 40 µl aliquots were immediately frozen in liquid nitrogen and stored at -80°C.

2.4.5.9 Homologous recombination in *YZ 2000*

The bacterial strain *YZ 2000* was electroporated according to the manufacturers instructions. 40 µl competent *YZ 2000* were thawed on ice and both 0,4 µg vector and 0,2 µg PCR-product were added and incubated on ice for 10 min. The mixtures were transferred into a 0.2 cm cuvette and placed into a Bio-Rad electroporator. The samples were pulsed once with 2.5 kV, 200 ohms and 25 µF. 1 ml of LB medium was immediately added and the cell suspension was transferred into a 1,5 ml eppendorf tube and incubated for 70 min at 37°C and 600 rpm. Samples were spread on selective LB-agar plates containing the appropriate antibiotics and incubated at 37°C for 12-16 h.

2.4.5.10 Preparation of *E.coli* DMSO stocks

For long time storage, 930 µl of an *E.coli* over night culture was mixed with 70 µl DMSO (final 7%) and frozen at -80°C.

2.4.5.11 Polymerase chain reaction (PCR)

2.4.5.11.1 DNA amplification for cloning

For cloning procedures the DNA was amplified by PCR using 10 ng of plasmid DNA or genomic DNA, sense and antisense primer (25 pmol each), 2,5 U *Pfu*-polymerase (Stratagene), dNTP´s (20 µM each) and 5% DMSO were mixed in a total volume of 50 µl 1xPCR buffer (Stratagene). The PCR-mix was initially denatured at 94°C for 5 min in a thermocycler followed by 30 consecutive cycles of: 45 s denaturing at 95°C, 45 s annealing of the oligonucleotide primers (5°C below their calculated melting point) and elongation at 72°C for 130 s with increasing the elongation time in each round for 5 sec. PCR reaction were either performed using the 'GeneAmp System 2400' (Perkin Elmer) or the 'T1-Thermocycler' (Biometra). The PCR products were cleaned up by using the 'Qiaquick PCR purification kit'. The samples were mixed with 5 volumes of buffer PB and applied on a Qiaquick column. After centrifugation for 1 min at 13000xg, the column was washed with 750 µl buffer PE and bound DNA fragments eluted in 50 µl TE. Afterwards the DNA concentration was determined on ethidium bromide agarose plates (2.4.3) and directly used for restriction digests (2.4.5.1).

2.4.5.11.2 Analysis of L. major GLF gene deletion mutants by PCR

To proof the correct insertion of the resistant marker cassettes into the *Leishmania* genome, the obtained clones were subjected to a 5' and 3' PCR reaction. In the 5' PCR reaction, a sense primer (BK9) was designed that bound 5' to the 5' flanking region used for homologous recombination. The antisense primer (ACM99: Hyg, ACM101: Phleo) was directed against the 5' end of the resistance marker to yield a PCR product of about 1.5 kb. For the 3' PCR reaction, the sense primer was directed against the 3' end of the resistant marker (ACM 100: Hyg, ACM102: Phleo), while the antisense primer (BK12) annealed to a sequence 3' of the 3' flanking region used for homologous recombination, The 3' end the PCR product comprehended 1700 nucleotides. For a 20 µl reaction, 10 ng genomic DNA, 4 pmol of sense and antisense primer, dNTP´s (20 µM each), 0.5 U *Taq* polymerase and 5% DMSO were mixed in 1x PCR buffer (Sigma). After 7 min of a initial denaturation at 94°C, the PCR-mix was incubated in a Thermocycler for 30 of the following cycles: 45 sec denaturation at 94°C, 45 sec annealing at 61°C, 1,30 min elongation at 72°C with increasing the elongation time in each round for 5 sec.. For the final step, a 10 min elongation at 72°C was applied. Afterwards the samples were loaded on a 1% agarose gel and visualized with ethidium bromide (2.4.4). Clones that were positives for both reactions were further analysed by Southern Blotting (2.4.15).

2.4.5.11.3 Digoxigenin (DIG) labelled DNA probes

A mix containing 2 mM dATP/dGTP/dCTP, 1.3 mM dTTP and 0.7 mM DIG-11-dUTP was used to prepared DIG-labelled DNA probes. For the amplification of the DNA probes, 10 ng of plasmid DNA (pCR2.1hyg, pCR2.1phleo, p5'UTR-Topo, pET Leis-mut) 4 pmol of sense and antisense primer (Hyg probe: ACM85/ACM86; Phleo probe: ACM87/ACM88; UGM probe: HB1/HB2, 5'UTR probe: BK13/BK14), 5 µl of dNTP mix and 1.25 U *Taq* polymerase in a total volume of 50 µl in 1x Taq PCR buffer were mixed. After 7 min of an initial denaturation step at 94°C, the PCR-mix was incubated in a Thermocycler for 30 of the following cycles: 45 s denaturation at 94°C, 45 s annealing at 58°C (Hyg and Phleo porbe) or 64°C (UGM and 5'UTR), 1 min elongation at 72°C. In a final step, a 10 min elongation at 72°C was applied. With the exception of the 5'UTR probe that was gel purified (2.4.5.2) after synthesis, the PCR products were directly used in Southern Blot analysis.

2.4.6 Extraction of genomic DNA from *L. major*

For the extraction of genomic DNA from *L. major*, $1x10^8$ cells were centrifuged (1300xg, 10 min 4°C) and resuspended in 400 µl TELT (50 mM Tris/HCl, pH 8.0, 62.5 mM EDTA, 2.5 M LiCl, 4% Triton X-100). After 5 min incubation at RT, 400 µl Roti^{TM}Phenol was added and mixed for 5 min at 4°C. Samples were centrifuged for 10 min at 14000xg and 4°C to separate the two phases. The upper phase was mixed with 400 µl chloroform/isoamylalcohol (24:1), incubated for 5 min at 4°C and centrifuged as before. DNA present in the aqueous phase was mixed with 1 ml ethanol (100%), incubated on ice for 5 min and centrifuged. The DNA pellet was washed with 1 ml ethanol (70%), centrifuged, dried, resuspended in 100 µl TE and stored at 4°C.

2.4.7 Southern Blotting

2.4.7.1 Preparation of the Southern Blots

2,5 µg DNA was digested with 5 U enzyme in a total volume of 25 µl at 37°C over night. The digested samples and 1ng of PCR product of the corresponding DNA probe were separated on a 0.7% agarose gel containing 5 µg/ml ethidium bromide. The gel was then incubated with 250 mM HCl for 15 min to depurinate the DNA and two times for 10 min in denaturing solution (0.5 M NaOH, 1.5 M NaCl). The DNA fragments were transferred to a nylon membrane by capillary forces in denaturing solution over night. The set up is described in the following:

1 glass plate
1 thick whatmann filter paper soaked in denaturing solution
1 thin whatmann filter paper soaked in denaturing solution
agarose gel up side down
nylon membrane, soaked in denaturing solution
3 thin whatmann filter paper, soaked in denaturing solution
6 layer dry cellulose filters
glass plate
0,5 kg weight

The following day the blot membrane was washed for 10 min in 50 mM sodium phosphate buffer pH 7.2 at RT and dried. UV crosslinking (Stratalinker, Stratagene) was used to fix the DNA to the membrane. The membrane was hybridised and developed as described in 2.4.7.2 and 2.4.7.3.

2.4.7.2 Hybridisation of Southern Blots

The membrane was prehybridised in high SDS (7% SDS, 50% formamid, 5x SSC, 50 mM NaPO4, pH 7.0, 0.1% N-lauroyl sarcosine, 2% blocking solution (Roche) for 1 hour at 42°C. The DIG-labelled probe was mixed with an equal volume of TE, denaturated at 95°C and transferred on ice to cool down. The solution was incubated with the membrane in high SDS (7% SDS, 50% formamid, 5x SSC, 50 mM NaPO4, pH 7.0, 0.1% N-lauroylsarcosine, 2% blocking solution (Roche)) for 1 hour at 42°C. The membrane was incubated shortly with 1 x SSC (0.1% SDS), following by two washing steps with 2xSSC (0.1% SDS) for 15 min at RT. The membrane was washed another two times with 0.1x SSC (0.1% SDS) for 30 min at 65°C. The bound probe was detected with CSPD (2.4.7.3).

2.4.7.3 Detection of Southern Blots by chemiluminescence

The membrane was equilibrate in buffer 1 (0.1 M maleic acid, 0.15M NaCl, pH 7.5) and blocked with buffer 2 (10x blocking solution (Roche) diluted 1:10 in buffer 1) for 30 min. Afterwards the membrane was incubated with AP-coupled α-DIG Fab fragment (1:10000 in buffer 2) for 30 min at RT. The membrane was washed three times with buffer 1 containing 0.3% Tween, equilibrate in buffer 3 (100 mM Tris/HCl pH 9.5, 100 mM NaCl) and incubated with 10-12 drops of CSPD (Disodium 3-(4-methoxyspiro {1.2-dioxetan-3.2'-85'-chloro} –tricyclo decan} -4-yl)phenylphosphate, Roche) for 15 min at 37°C. The CSPD was removed and the membrane sealed into plastic foil. Due to dephosphorylation of the CSPD by the AP activity, light was emitted at 477 nm. The signals were visualized on Hyperfilms MP.

3 Results

3.1 Identification and Characterisation of the UDP-galactopyranose mutase from Leishmania major

3.1.1 Analysis of the amino acid sequence

The UDP-galactopyranose mutase (UGM), encoded by the *GLF* gene, is a flavoprotein that catalyzes the rearrangement of UDP-galactopyranose into UDP-galactofuranose (Figure 7). As the latter is the donor for all galactofuranosyltransferases, UGM represents a central enzyme in Gal*f* metabolism. Although the first prokaryotic *GLF* was identified in 1996, previous effort to detect eukaryotic *GLF*s had not been successful. So not surprisingly, no sequence highly homologous to bacterial UGMs could be found in *Leishmania major* genome. However as the flavin cofactor is indispensable for catalytic activity of bacterial UGM (Nassau *et al.,* 1996, Köplin *et al.,* 1997, Soltero-Higgin *et al*., 2004a), we hypothesized that eukaryotic UGMs are also flavoproteins. Several sequences with conserved sequence motifs for FAD binding were identified in blast searches. Sequences exhibiting the dinucleotide binding motif xhxhGxGxxGxxxhxxh$(x)_8$hxhE(D) where h is a hydrophobic residue (Wierenga *et al*., 1983) were closely examined. This motif located at the N- terminus is characteristic of proteins of the glutathione reductase structural family (GR) including *E.coli* UGM (Sanders *et al.*, 2001b). Amongst protein sequences containing this motif, a candidate gene showing an overall identity of 18% with *E.coli* UGM was selected. The putative enzyme contained most of the amino acid residues involved in substrate binding, residues that are highly conserved within the bacterial UGMs (Figure 8). Additionally, a gene showing 51% identity to the *Leishmania* candidate was present in the genome of *Aspergillus fumigatus*, an organism known to synthesise Gal*f*, while no homologous genes were found in other completely sequenced eukaryotic genomes like *Drosophila melanogaster*, *Arabidopsis thaliana*, yeast or mammal, organisms in which Gal*f* has never been found.

Figure 8: Alignment of key eukaryotic and prokaryotic *GLF* proteins. Conserved residues of *L. major, A. fumigatus, K. pneumoniae* and *E. coli K12* UDP-galactopyranose mutase are boxed. The asterisks indicate the *E. coli* substrate binding site. The dinucleotide binding motif is underlined. Numbers refer to the *E. coli* sequence.

3.1.2 *In vivo* activity assay

The activity of the *L. major* UGM was tested in an *in vivo* complementation system, adapted from the assay described by Köplin *et al*. (1997) for the characterisation of *Klebsiella pneumoniae* UGM. In gram-negative bacteria the O-antigen of the lipopolysaccharide (LPS) is encoded by a gene cluster called *rfb*. The *rfb* gene cluster of *K. pneumoniae* O1 containing an in frame deletion in glf_{KPO1} gene, was introduced into the plasmid pWQ70 (Köplin *et al.,* 1997). After introduction into *E. coli* CWG287, a strain deleted for the endogenous *rfb* cluster, no LPS biosynthesis could occur. However when an additional plasmid containing the glf_{KPO1}gene was transformed, LPS biosynthesis was restored (Köplin *et al.,* 1997). To analyse the activity of the putative *L. major* UGM, the candidate gene was amplified by PCR from genomic DNA digested with NdeI and XhoI and cloned into the vector pET22b. The resulting plasmid was then transformed with the plasmid pWQ70 into *E. coli* CWG287. The LPS phenotype conferred by these plasmids was first analysed by susceptibility to the phage Ffm (Wilkinson *et al.,* 1972). The candidate gene of *L. major* like the characterised *K. pneumoniae* glf_{KPO1} conferred resistance to the phage (Figure 9), suggesting that the LPS biosynthesis was restored. In contrast bacteria containing pWQ70 and an empty vector were susceptible to the phage Ffm, resulting in phage plaque formation. The LPS biosynthesis was further analysed by SDS-PAGE and silver staining. If the CWG287 bacteria contained pWQ70 and the characterised K. pneumoniae glf_{KPO1} gene or the candidate gene from *L. major*, a ladder of smooth LPS could be detected, indicating that the gene transformed are functional. As expected no LPS biosynthesis was observed with an empty vector control (Figure 10).

Figure 9: Resistance of *E. coli* CWG287 (pWQ70) to phage Ffm after transformation with a functional *GLF* gene. *E. coli* CWG 287 was transformed with pWQ70 (A) and the gene encoding *K. pneumoniae* (B) or *Leishmania major* (C) UDP-galactopyranose mutase.

Figure 10: Silver staining of the LPS of *E. coli* CWG 287 after transformation with *GLF* from *K. pneumoniae* or *L. major*.

3.1.3 *In vitro* testing of *L. major* UGM

The activity of *L. major* UGM was analysed using an *in vitro* assay. The assay (Figure 11) is based on the release of tritiated formaldehyde by periodate oxidation from the product UDP-[6-^{3}H]Gal*f* but not from its precursor UDP-[6-^{3}H]Gal*p* (Scherman *et al.,* 2003). Radioactive product and precursor can then be easily separated by anion exchange chromatography. *E. coli* CWG288, a derivate from CWG287 was used to express the different UGMs. This strain is deficient in endogenous UGM and in UDP-glucose-4-epimerase, an enzyme that converts UDP-Gal*p* in UDP-Glucose. Consequently crude protein extract can be used for this assay. The reaction catalysed by the mutase is a reversible reaction in which the production of UDP-Gal*f* is disfavoured. Only 5 to 7% of UDP-Gal*f* would be present at the equilibrium. The activity of the different enzyme tested was comparable. The average values from three independent experiments using *L. major and K. pneumoniae* enzymes were 4.3% and 3.6%, respectively, of total UDP-Gal*p* used in the experiment. A background of 2.1% was observed with bacteria containing only an empty vector. These data suggested that the *L. major GLF* gene encodes a protein with UGM activity that promotes the formation of UDP-Gal*f* from UDP-Gal*p*.

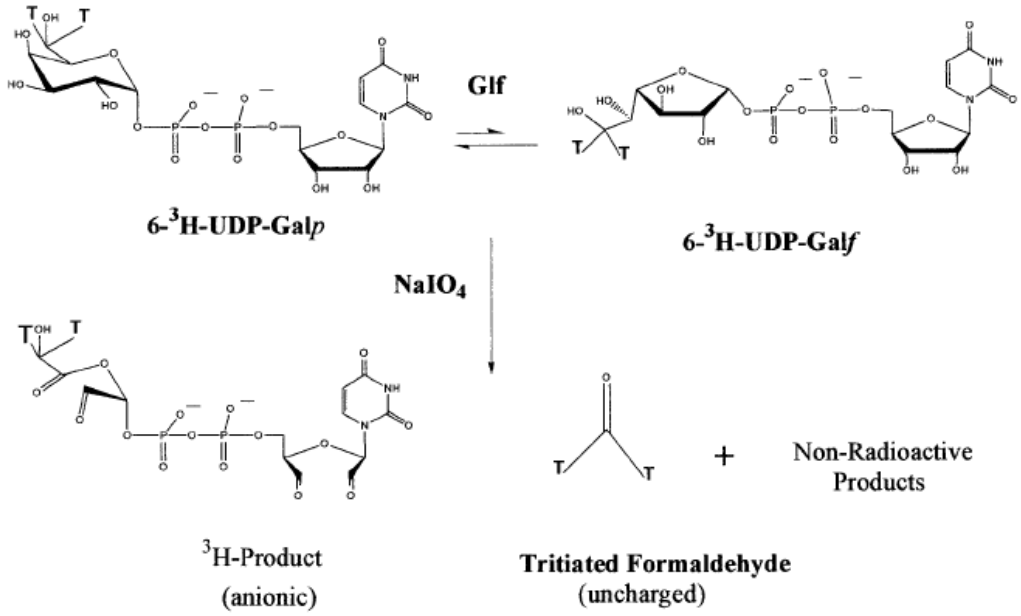


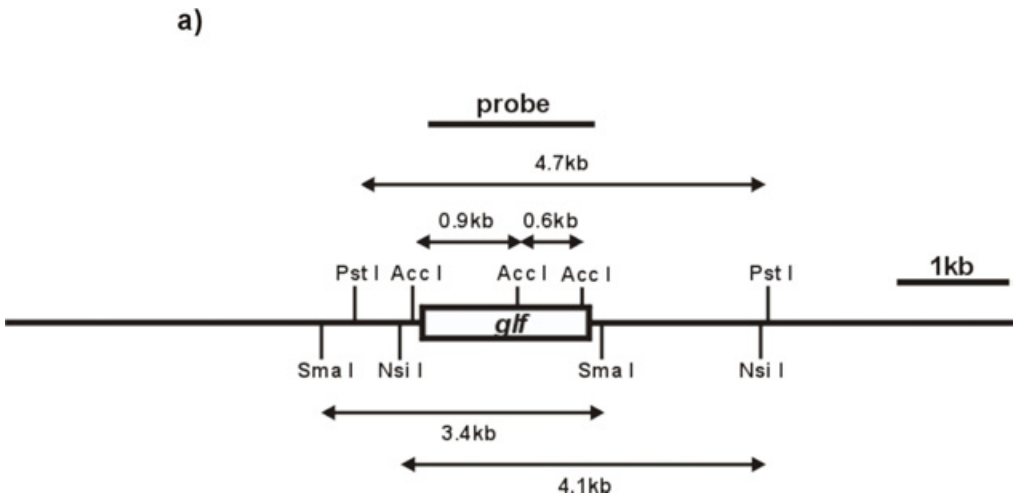
Figure 11: Assay for UDP-galactopyranose mutase. UDP-Gal*f* but not UDP-Gal*p* forms neutral tritiated formaldehyde when treated with sodium periodate (from Scherman *et al.,* 2003).

3.2 Generation of a L. major GLF gene deletion mutant

In parasites of the *Trypanosomatidae* family, gene regulation and expression are characterised by a combination of atypical mechanisms. The genes in *Leishmania* do not contain any intron like in other eukaryotes and are separated by short intergenic regions. Promoters are absent and the transcription takes place in a polycistronic fashion (Borst *et al*., 1986, Kapler *et al*., 1990). The long polycistronic pre-mRNAs are then processed by coupled trans-splicing and polyadenylation reactions to generate mature monocistronic mRNAs, each of which bears at its 5´end a common 39-nucleotide mini-exon. Sequences within the intergenic regions provide the signals for these processes as well as regulation of gene expression (Curotto de Lafaille *et al*., 1992, Stiles *et al*., 1999, Liang *et al*., 2003). Furthermore, *Leishmania* are diploid asexual parasites thus deletion of any gene requires two rounds of targeted replacement to substitute each allele by a dominant drug-resistance marker. Our strategy for the obtaining of a targeted gene deletion mutant was influenced by the peculiarities in the mechanisms of gene expression used by trypanosomatids. Notably, the targeting constructs were prepared by Red/ET recombination in bacteria in order to leave the 5´and 3´ untranslated regions (UTRs) completely unaltered and allow correct expression of the dominant markers in *Leishmania*.

3.2.1 *GLF* is a single copy gene in *L. major* 5ASKH

Another particularity of *Leishmania* and other kinetoplastida organisms is that many genes are present in the genome as multiple copies. In *L. major*, most of these multiple copies genes occur in 2 or more separate loci containing single genes and/or tandem arrays (Ivens *et al.,* 2005). When we initiated this project, *Leishmania* genome was incomplete and copy number of the *GLF* gene in *L. major* 5ASKH genomic DNA was thus investigated by Southern Blot. Sequence information could however be retrieved from the *L.major* genome sequencing project (The Wellcome Trust Sanger Institute) to reconstitute *GLF* locus. Genomic DNA was digested with *Acc I, Nsi I, Pst I* or *Sma I* in order to generate different diagnostic fragments as indicated in Figure 12a. Digests were then separated on a 0.7 % agarose gel, transferred to nitrocellulose and hybridised with a digoxigenin (DIG) labelled *GLF* probe consisting of the whole open reading frame. As expected fragments of approximately 4.1 kb, 4.7 kb and 3.4 kb were observed after digestion with *Nsi I*, *Pst I* and *Sma I,* respectively. Digest with *Acc I* gave rise to two signals of 0.9 kb and 0.6 kb as it cuts once in the *GLF* sequence. No additional bands could be observed indicating the presence of a single copy gene (Figure 12b). This result is consistent with the presence of a single homologous of *GLF* present on chromosome 18 as revealed after completion of *Leishmania major* genome (Ivens *et al.,* 2005). Moreover the presence of a single copy gene was later confirmed by targeted gene replacement of *GLF*.



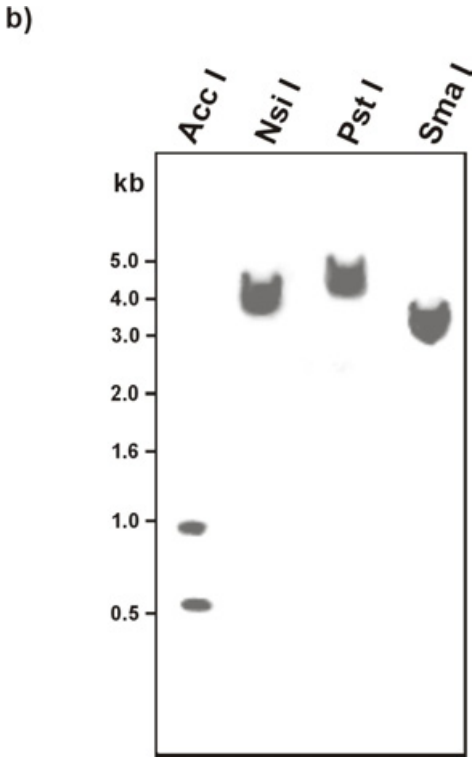


Figure 12: Investigation of the number of *GLF* gene copies

a) Schematic representation of *L. major GLF* locus indicating *Acc I, Nsi I, Pst I* or *Sma I* fragments expected after hybridisation with a *GLF* probe.

b) Southern blot analysis of genomic *L. major* 5 ASKH DNA. Genomic DNA was digested with *Acc I, Nsi I, Pst I* or *Sma I*. After separation on a 0.7% agarose gel, the fragments were transferred to a nitrocellulose membrane and hybridised with a DIG-labelled *glf* probe.

3.2.2 Cloning of the targeting constructs

As we have already emphasized it, two successive rounds of targeted gene replacement are required to inactivate any leishmanial gene because of the diploidy of this parasite. Two different targeting constructs in which the regions flanking the *GLF* gene are separated by an antibiotic resistance gene were thus necessary. For the generation of these constructs, a 1.5kb region directly upstream of the start codon and a 1.5kb region directly downstream of the stop codon were amplified by PCR from *L .major* genomic DNA and successively cloned into the vector pBSK+ (Stratagene) resulting in a plasmid called 5'3'pBSK+. Sequence information was available from *L.major* genome sequencing project (Welcome Trust Sanger Institute). The genes encoding hygromycin phosphotransferase (*hyg*) and phleomycin binding protein (*phleo*) that give resistance against the antibiotics hygromycin and phleomycin respectively were chosen amongst

the suitable selection markers and inserted between the 5'- and 3'-UTR of *GLF* gene by Red/ET recombination in bacteria. For this purpose, the open reading frames of the antibiotic resistance genes were amplified by PCR from plasmid pCR2.1hyg and pCR2.1phleo using primers having a homologous overhang of approximately 35bp to the end of the 5'-UTR or to the start of the 3'-UTR of *GLF.* These PCR products were then separately electroporated in *E. coli* YZ2000 (Gene Bridges, Germany) with the digested plasmid 5'3'pBSK+ to promote the insertion of the selection marker between the 5'- and 3'-UTR of *GLF* by homologous recombination. This procedure yielded two targeting plasmids called 5'3'Hyg pBSK+ and 5'3'Phleo pBSK+ in which the antibiotic resistance gene replaces precisely *GLF* gene. This particular point is important for correct expression of the selection markers once inserted in *Leishmania* genome.

3.2.3 Targeted gene deletion of *L.major GLF*

Most trypanosomatid species grow rapidly in standard culture media with doubling time of 6 to 10 hours. Moreover colonies can be obtained by plating on semi-solid media, so several rounds of genetic manipulation can be carried out in a reasonable amount of time. The targeting constructs, carrying the hygromycin and phleomycin resistance genes were first excised from plasmids 5'3'Hyg pBSK+ and 5'3'Phleo pBSK+ by digestion with *Xho I* and *Sac I* yielding fragments of 4.2 kb and 3.6 kb respectively. The later were purified by agarose gel electrophoresis, gel extraction and ethanol precipitation. In the first round, 2 µg of each fragment were separately electroporated in 1x 10^8 *L. major* wild type 5ASKH. To isolate clonal lines, the parasites were grown on agar plates containing the appropriate selective drug. Visible colonies of parasites were observed after 10 days at 27°C. More hygromycin (*GLF*/Δ*glf*::*HYG*) than phleomycin (*GLF*/Δ*glf*::*PHLEO*) resistant clones were obtained in the first electroporation round. This reproducible result suggests that the integration of the phleomycin resistance gene was more difficult than the hygromycin resistance gene. In this regard, it is worth mentioning that the *phleo* gene is a 373 bp gene while *hyg* contains 1026 bp and has thus a size comparable to *Leishmania GLF*. After selection on agar plates, different clones were transferred to liquid media in order to prepare genomic DNA and DMSO stocks. The clones were first screened for correct insertion of the antibiotic resistant markers by PCR and positives clones were further analysed by Southern Blotting. Two representative phleomycin resistant clones that were subsequently used for the obtaining of homozygous mutants are shown in the Figures 13 and 14. Genomic DNA from wild type *Leishmania* and from different mutant clones was digested with *Sac I* or *Pst I* and analysed by Southern Blotting using a DIG labelled probe consisting of the whole coding sequence of

phleomycin to exclude random integration. The signals of 6.5 kb and 3.6 kb confirmed single integration into the genome at the *GLF* locus (Figure 13). No unspecific hybridisation in wild type parasites could be detected.

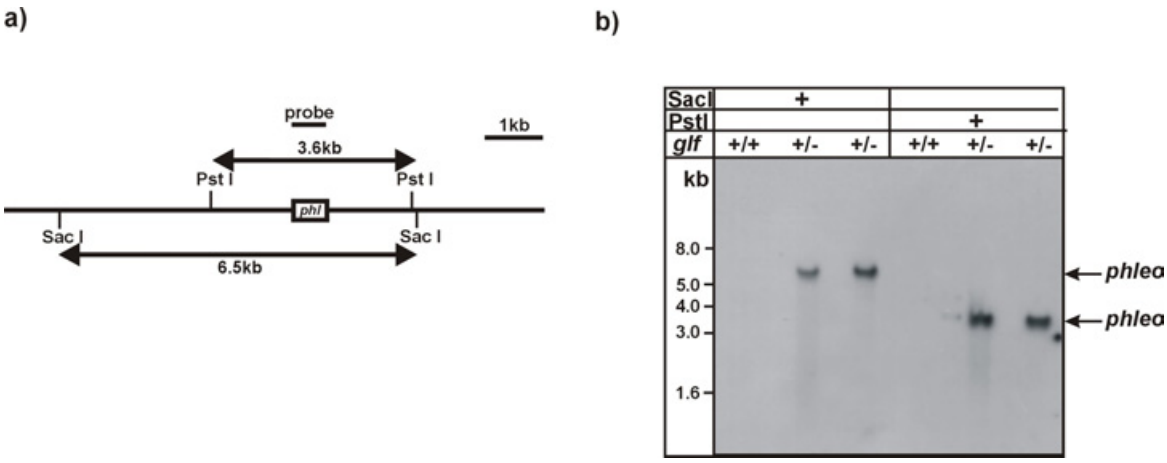


Figure 13: Determination of random integration of the selection marker in phleomycin resistant clones. Genomic DNA from *L. major* phleomycin resistant clones and parental strain was digested with *Sac I* or *Pst I* and analysed by Southern Blotting (b). The blots were hybridised with DIG labelled phleomycin. The sizes of the expected fragments are shown in a).

To confirm the integration of phleomycin at the right locus, the heterozygous mutants were further analysed by Southern Blotting using a 5'-UTR probe. A sequence lying outside the region previously used for homologous recombination was cloned into the vector pcR4-TOPO and the resulting plasmid used to generate the DIG-labelled 5'-UTR probe. Genomic DNA obtained from various heterozygous mutants and the parental strain was digested with *Apa I* or *Sac I*, separated on agarose gel, transferred to a nitrocellulose membrane and hybridised with this 5'-UTR probe. In all samples, presence of a wild type *GLF* allele was demonstrated by the fragments at either 5.4 kb or 4.3 kb when DNA is digested with *Apa I* or *Sac I* respectively (Figure 14). Additionally, the heterozygous mutants exhibit signals at 6.5 kb or 6.4 kb according to the restriction enzyme used, that confirm successful replacement of one *GLF* allele by the phleomycin resistant marker. The analyses described above allowed the isolation of *L. major* heterozygous mutants *GLF*/Δ*glf*::*HYG* or *GLF*/Δ*glf*::*PHLEO* and the confirmation of their identity.

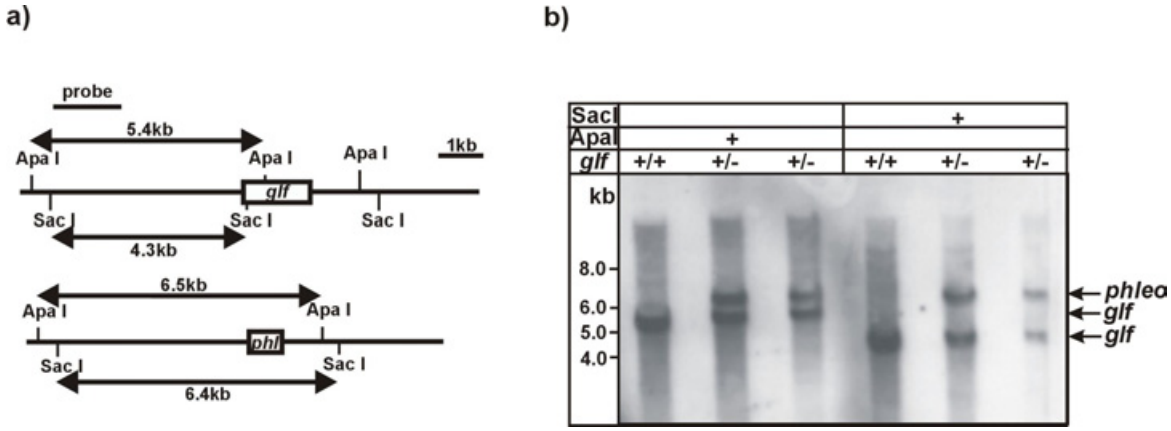


Figure 14: Southern Blot analysis of the heterozygous mutants. Genomic DNA was digested either with *Sac I* or *Apa I*, transferred to nitrocellulose and hybridised with a 5'-UTR probe (b). The sizes of the expected fragments are shown in a).

As mentioned before integration of the hygromycin resistance gene in *L. major* 5 ASKH genome seemed to be preferred over integration of the phleomycin resistance gene. Thus to ease the obtaining of homozygous mutants, *GLF*/Δ*glf*::*PHLEO* clones were selected and electroporated with the purified Xho I – Sac I fragment obtained from the hygromycin targeting construct 5'3'Hyg pBSK+. Transfectants were selected on media containing both phleomycin and hygromycin and expanded in liquid culture for the preparation of genomic DNA and DMSO stocks. Like for the heterozygous mutants, the integration of the selection marker at the right locus was first investigated by PCR and later confirmed by Southern Blotting using different restriction enzymes and probes. At least two clones resistant to phleomycin and hygromycin obtained from each heterozygous *GLF*/Δ*glf*::*PHLEO* clone were inspected. Genomic DNA of all clones as well as the parental strain were first digested with *Nsp I,* transferred to a nitrocellulose membrane and hybridised with a probe covering the whole coding sequence of hygromycin. As expected, a 1.9 kb fragment (Figure 15) was detected in the homozygous mutants only indicating the replacement of the second *GLF* allele. Since no other signal could be detected with this probe, random integration of the hygromycin resistant marker in the genome was excluded.

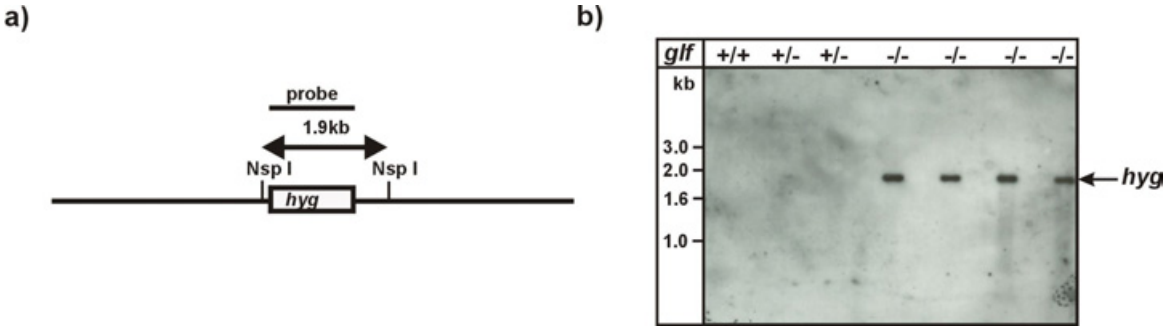
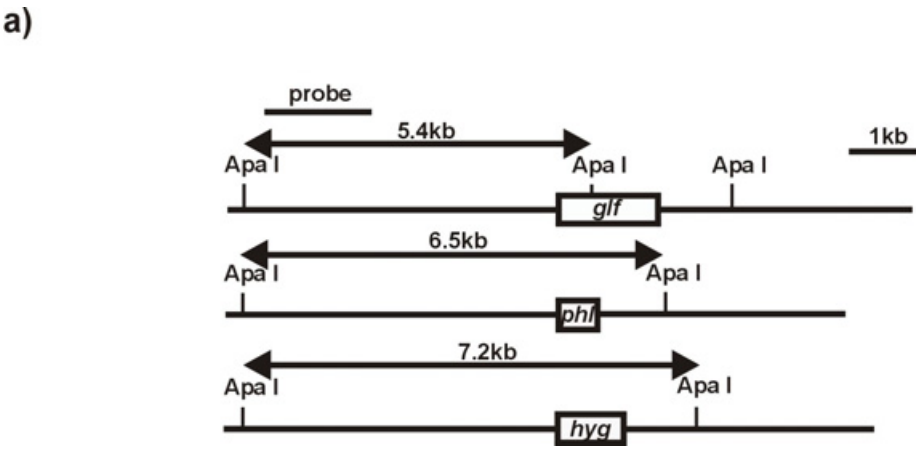


Figure 15: Determination of random integration of the hygromycin marker in homozygous mutants. Genomic DNA of wild type, heterozygous and homozygous mutants was digested with *Nsp I* and analysed by Southern Blotting (b). The blots were hybridised with DIG labelled hygromycin. The size of the expected fragment is shown in a).

As previously, the result was confirmed with a DIG-labelled 5'-UTR probe (Figure 16). In the later case, genomic DNA was digested with *Apa I*. The signals at 6.5 kb and 7.2 kb observed in the homozygous parasites correspond to the predicted fragment obtained after integration of phleomycin and hygromycin respectively at the *GLF* locus. On the other hand, no signal at 5.4 kb indicative of a *GLF* allele is observed in these parasites while it is present in the heterozygous mutant and wild type strain. These data are consistent with the desired recombination events.



b)

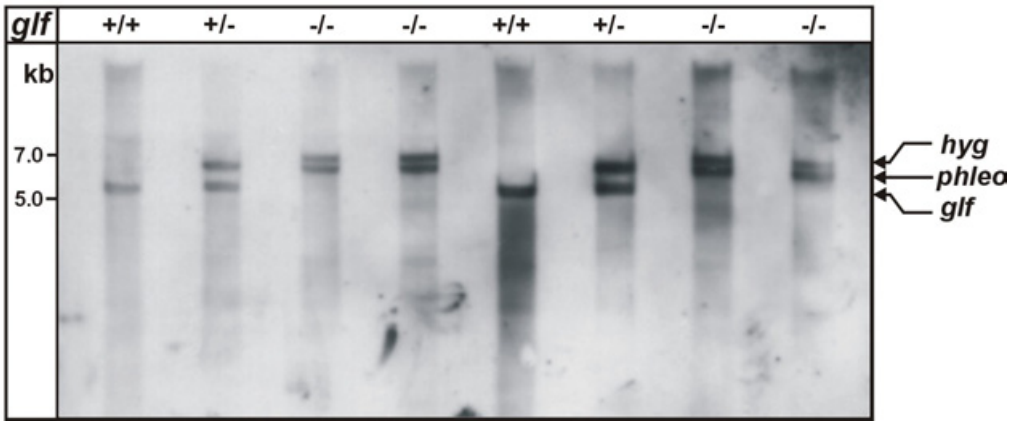


Figure 16: Southern Blot analysis of the homozygous mutants. Genomic DNA from wild type, heterozygous mutants and resulting homozygous mutants was digested with *Apa I*, transferred to a nitrocellulose membrane and hybridised with a 5'UTR probe (b). The scheme of the resulting fragments is depicted in a).

The absence of the two *GLF* alleles in the homozygous mutant is clearly illustrated in Figure 17 presenting a Southern blot hybridised with the whole coding sequence of *GLF*. As expected, two fragments of 0.91 kb and 0.56 kb are observed after digestion of genomic DNA from wild type and heterozygous mutants with *Acc I*, whereas no signal is visible in the homozygous mutants. All these analyses demonstrate unambiguously the replacement of both *GLF* alleles in *Leishmania major* 5 ASKH. We were thus able to generate Δ*glf*::*HYG*/Δ*glf*::*PHLEO* parasites further referred to as Δ*glf* mutant indicating that in contrast to mycobacteria, *GLF* is not essential for *Leishmania major* survival.

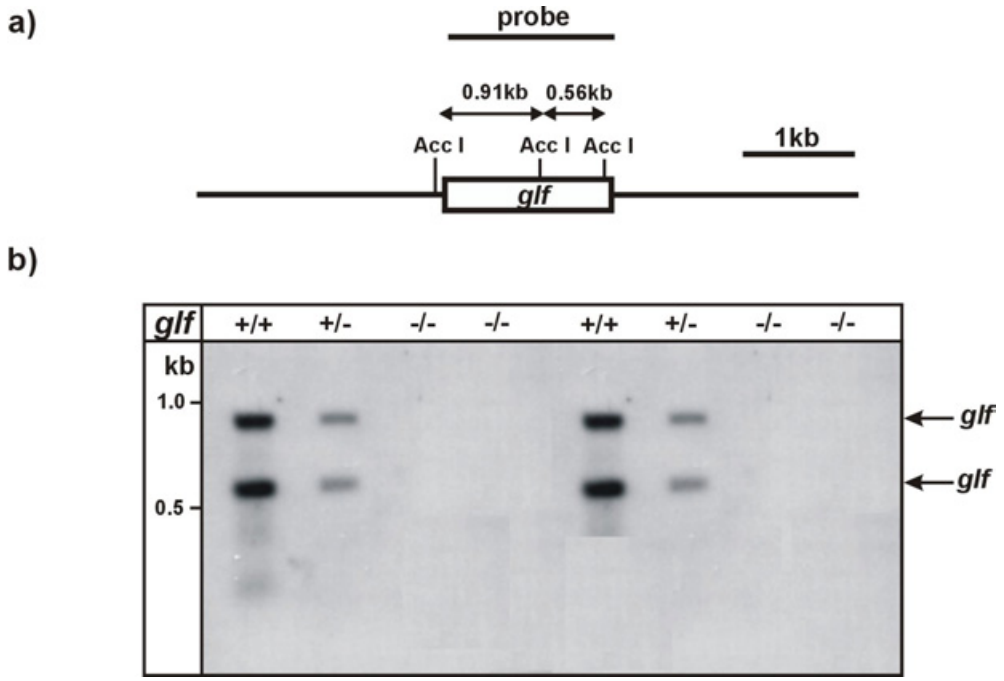


Figure 17: Confirmation of *GLF* deletion. Wild type, heterozygous and corresponding homozygous mutants were analysed by Southern blotting after *Acc I* digest using the complete coding sequence of the *GLF* as a probe.

3.2.4 Re-expression of UDP-galactopyranose mutase in *L. major* Δ*glf* mutant

As a control for subsequent experiments, the expression of the UDP-galactopyranose mutase (UGM) was restored in the deficient mutants in order to assign an observed phenotype to *GLF*. The complete coding region of *GLF* was PCR amplified and cloned into the *Leishmania* pXG(NEO) expression vector using *Sma I* and *BamH I* restriction sites respectively, yielding pXG-*GLF*. The cloning site of this episomal vector is flanked by the intergenic regions of *Leishmania* dihydrofolate reductase thymidylate synthase gene that provide signals for trans-splicing and polyadenylation and drive the expression of gene inserted between them. PXG gives high level of expression, fairly constant trough the parasite life cycle (Chakkalath *et al.,* 2000). Additionally, it carries a neomycin resistance marker (neo) for selection of transfectants. To restore the expression of UGM, 2x 10^8 *L. major* homozygous parasites were thus transfected with 10 µg of pXG-*GLF*. The resulting Δ*glf*::*HYG*/Δ*glf*::*PHLEO* [pX-*GLF*] parasites further referred to as Δ*glf*/+*GLF* or “addback” were isolated on M199 agar plates containing phleomycin, hygromycin and neomycin. After 10 days of incubation at 27°C the colonies were visible and transferred

to liquid M199 media for preparation of cell pellets and DMSO stocks. In all further analyses, sibling clonal lines behaved similarly and thus results from a single Δ*glf* and Δ*glf* /+*GLF* mutant will be shown in illustrations.

3.3 *Characterisation of* L. major Δglf *mutant*

In order to understand the effect of *GLF* deletion and thus of the galactofuranose metabolism on important biological processes related with the pathogenesis of *Leishmania* and link these effects to specific molecules, the generated mutants need to be biochemically defined. As galactofuranose is present in the cell surface glycoconjugates of *Leishmania*, particular attention was paid to these molecules.

3.3.1 *In vitro* growth of Δ*glf* mutant

The *in vitro* growth behaviour of several Δ*glf* mutant and the corresponding *GLF*/Δ*glf*::*PHLEO* heterozygous mutant was first analysed. As shown in Figure 18 the heterozygous and homozygous mutants grew in culture as well as the parental wild type strain indicating that *GLF* is not critical for *in vitro* growth of *Leishmania* parasites. All parasites appeared morphologically normal under a light microscope. The growth of the Δ*glf*/+*GLF* mutant was also comparable to wild type *Leishmania*.

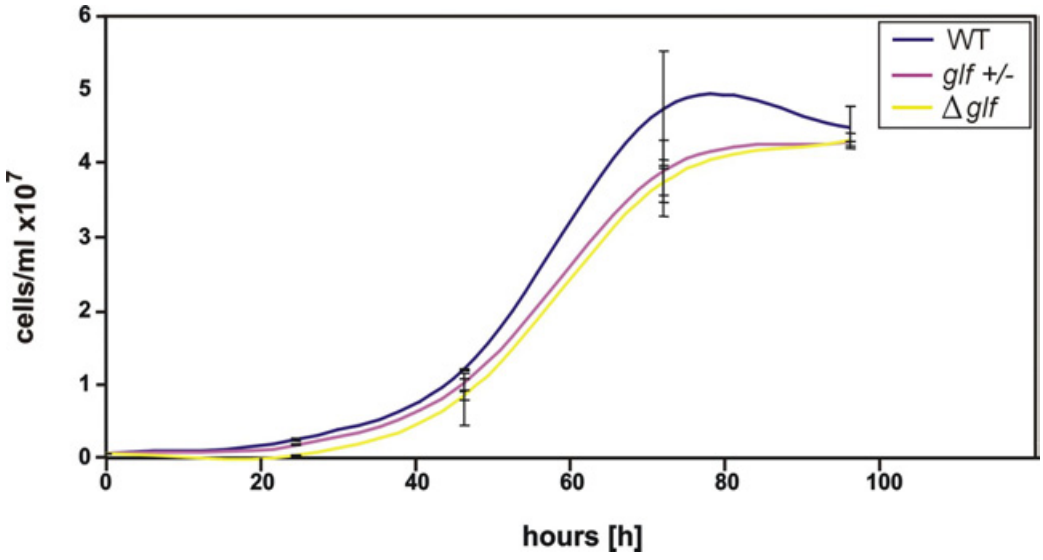


Figure 18: In vitro growth of Δ*glf* mutants. The cell count of wild type, heterozygous and the corresponding homozygous mutant were determined over 4 days using a light microscope. Results obtained from a representative heterozygous and homozygous mutant are presented.

3.3.2 Characterisation of *L. major Δglf* cell surface glycoconjugates

The cell surface glycoconjugate structures of *Leishmania major* have previously been characterised in detail. Gal*f* is present in the anchor of the abundant polysaccharide LPG and in GIPLs, the major glycolipids of the plasma membrane. Thus deletion of *GLF* is expected to affect the biosynthesis of these molecules. Furthermore *Leishmania* present structurally related molecules such as the GPI-anchor of proteins or PPGs, which share some steps of the LPG and GIPLs biosynthetic pathways. Therefore the effect of a *GLF* targeted gene deletion on the expression of all these related cell surface glycoconjugates was investigated.

3.3.2.1 Analysis of Lipophosphoglycan expression

LPG is the major glycoconjugate structure on the cell surface of *L.major* promastigote parasites. It is distributed over the complete cell surface including the flagellum. Moreover LPG plays an important role in the pathogenesis of *Leishmania*. This polysaccharide constituted of -6Galβ1-4Manα1-P- repeat units is attached to the plasma membrane via a GPI anchor (McConville and Ferguson, 1993). Non infectious *L. major* procyclic promastigotes contain in average 14 repeat units while the infectious metacyclic promastigotes enriched in stationary phase culture bear 30 repeat units in average. Since the GPI-anchor connecting LPG to the membrane contains a β-D-Gal*f* residue, LPG expression should be affected in Δ*glf* mutant due to absence of UGM.

3.3.2.2 Western Blot analysis of LPG synthesis

Synthesis of LPG in wild type, Δ*glf* and 'add back' (Δ*glf* /+*GLF)* parasites was investigated by Western blotting. An equal amount of each cell lysate was separated on a ProSieve SDS PAGE and the Western blot was analysed with the monoclonal antibody (mAb) WIC79.3 recognising galactose substituted repeat units specific for *L. major* LPG and PPGs (Greenblatt *et al.*, 1983). Signals were detected with an enhanced chemiluminescence (ECL) system. Equal loading of the cell lysates was confirmed by Commassie-Blue staining of a second gel. Due to microheterogeneity of the chain length (variation of number of repeat units), LPG appears as a diffuse distending signal between 30 and 100 kDa (Figure 19). LPG expression was observed in the parental wild type strain while no signal was discernible in the homozygous mutants confirming absence of

LPG backbone. When UGM expression was restored, LPG biosynthesis was resumed as shown by the strong signal present in the Δ*glf*/+*GLF.* Hence as expected, absence of UGM leads to the complete absence of LPG backbone in the null mutant.

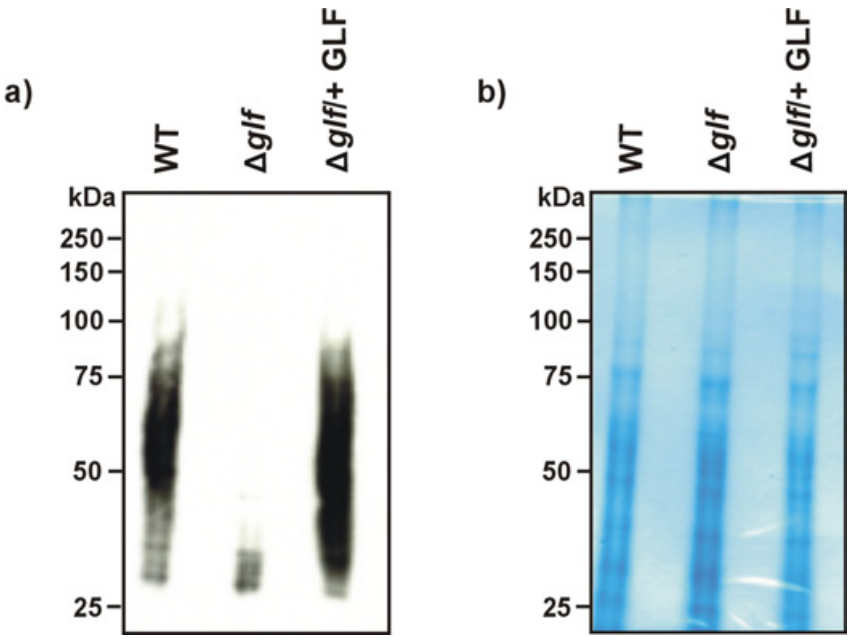


Figure 19: Analysis of LPG by Western Blotting using the monoclonal antibody WIC79.3. a) Lysates of wild type, homozygous and corresponding add back mutant were separated on 10 % SDS ProSieve gel and analysed with WIC79.3 and ECL detection. b) To confirm, equal loading in each lane, a second gel was stained with Commassie-Blue.

3.3.2.3 Analysis of phosphoglycans by immunofluorescence

LPG expression of wild type, and Δ*glf*/+*GLF* was also analysed by immunofluorescence microscopy. *Leishmania* promastigotes were stained with the mAb WIC79.3 and a Cy3-coupled secondary antibody and visualised by both immunofluorescence microscopy and transmitted light using the 40 x magnification. Wild type *Leishmania* show an intense staining revealing the abundance of LPG on the entire cell surface. In contrast no staining was detectable in the Δ*glf* mutant indicating the total absence of LPG (Figure 20). LPG expression could be restored in the 'add back' mutant (Δ*glf*/+*GLF*) confirming that the observed phenotype is specific for *GLF* deletion. Since UGM is overexpressed in the 'add back' mutant, a slight increase in fluorescence intensity is visible compared to wild type parasites.

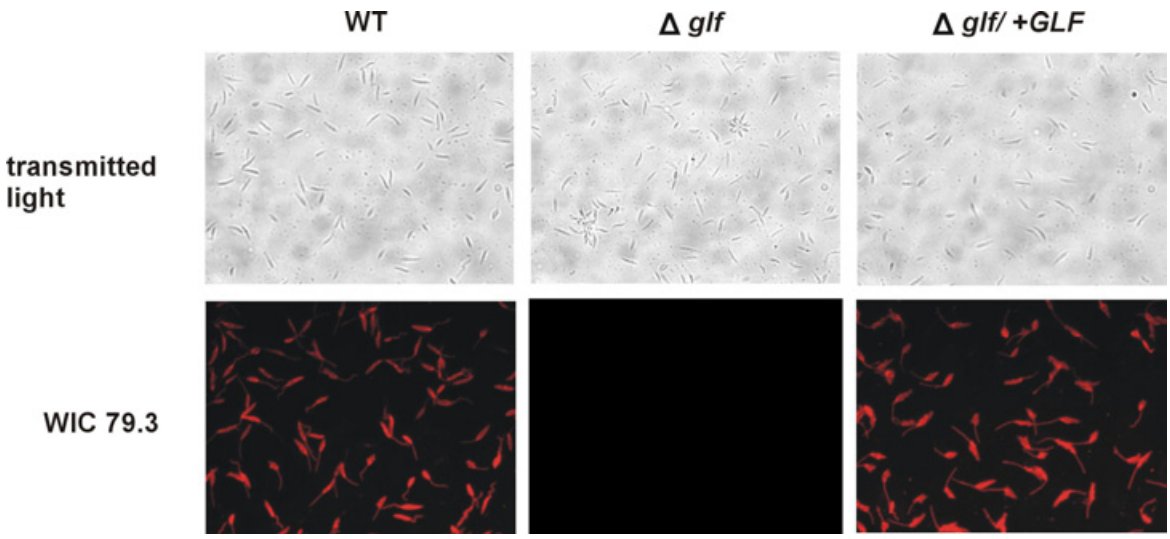


Figure 20: LPG cell surface expression of *L. major* wild type, Δ*glf* and Δ*glf*/+*GLF* mutants. Immunofluorescence with mAb WIC79.3. *Leishmania* promastigotes were fixed and LPG stained with WIC79.3 (1:1000). Cells were visualised by transmitted light.

3.3.2.4 Characterisation of PPGs in Δ*glf* mutants

The family of proteophosphoglycans is constituted of secreted and membrane bound proteins that are expressed in a stage and species specific manner. For example, *L. mexicana* promastigotes secrete two different acid phosphatases and a filamentous PPG. In contrast, *L. major* synthesise fewer PPGs and does not secrete any acid phosphatase (SAP, secreted acid phosphatase).

PPGs, like LPG, contain galactose substituted repeat units that can be recognised by the mAb WIC79.3. Thus membrane bound PPGs should be visible in the *Δglf* mutant by immunofluorescence using WIC79.3 antibody (Figure 20) while PPG expression should not be decreased by *glf* deletion. However, as mentioned above, no signal representing the corresponding epitope was visible on the *Δglf* mutant. This indicates the absence of membrane bound PPGs in *L. major* MHOM/SU/73/5ASKH. Since PPGs are high molecular weight structures they remain in the stacking gel and thus can be separated from LPG molecules. By western blotting of the stacking gel using WIC79.3 , membrane bound PPGs were analysed. However, no signals could be detected in wild type or mutant parasites confirming the lack of membrane bound PPGs. More surprisingly, the PPGs secreted in the media were hardly discernable after Triton X-114 partial purification and western blotting with WIC79.3 suggesting the low abundance of these molecules. Therefore the influence of *GLF* deletion on phosphogylcosylation of the PPGs was

analysed by heterologous expression of *L. mexicana* secreted acid phosphate (SAP) in *L. major* wild type and *Δglf* . SAP was immunoprecipitated from cell culture supernatant with the specific monoclonal antibody LT8.2 and the samples were analysed by Western blotting using the mAb WIC 79.3 (Figure 21, a). Equal protein loading was confirmed by staining with the mAb LT8.2 (Figure 21, b). As expected, no signal was observed for untransfected wild type parasites (Figure 21, lane 1) confirming absence of SAP in *L. major*. On the other hand the slower electrophoretic mobility of SAP in *Δglf* relative to wild type suggests that the degree of phosphoglycosylation is elevated in *Δglf* .

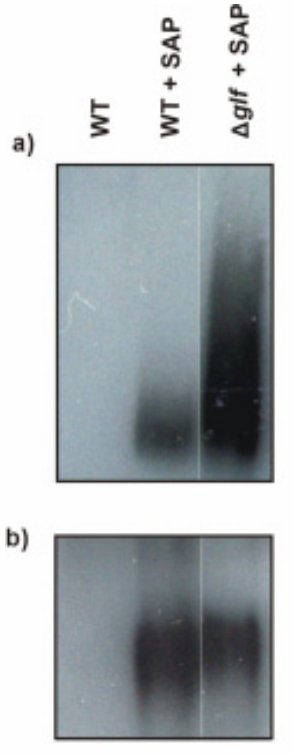


Figure 21: Proteophosphoglycan analysis. WT, WT+SAP and Δ*glf*+SAP were grown for two days in serum free M199 media. The SAP protein was immunoprecipitated with mAb LT8.2 and analysed by Western blotting using mAb WIC 79.3 (a). Equal loading was confirmed by staining with the mAb LT8.2 (b).

3.3.2.6 Characterisation of GIPLs in Δ*glf* null mutant

Leishmania major GIPLs are important constituents of the plasma membrane containing one residue of galactofuranose. Sometimes also called free GPI, these molecules are not attached to either proteins or polysaccharides and are expressed at very high copy numbers on both promastigotes and amastigotes surfaces. *Leishmania major* synthesises different type 2 GIPLs consisting of Gal*f*(β1-3)Man(α1-3)Man(α1-4)$GlcNH_2$-phosphatidylinositol; Gal(α1-3)Gal*f*(β1-3)Man(α1-3)Man(α1-4)$GlcNH_2$-phosphatidyl-inositol and Gal(α1-6)Gal(α1-3)Gal*f*(β1-3)Man(α1-3)Man(α1-4)$GlcNH_2$-phosphatidyl-

inositol. These are by convention termed GIPL-1, GIPL-2 and GIPL-3 according to the number of galactose residues that they contain. The lipid part generally consists of a *sn*1-alkyl-2-acyl-phosphatidylinositol. They were obtained by solvent extractions, purified by reverse phase chromatography and analysed by different techniques.

The GIPLs extracted from wild type *Leishmania* and Δ*glf* mutant were first analysed by High Performance Thin Layer Chromatography (HPTLC). After separation on a HPTLC-plate the glycolipids were stained either with the monoclonal antibody MEST-1, an antibody directed against terminal Gal*f* of GIPL-1 (Suzuki *et al.,* 2002) or with the antibodies L-5-28 and L-5-34 staining GIPL-2 and GIPL-3 (McConville and Bacic, 1989). Unfortunately no differences were observed between wild type and mutant. Furthermore the staining did not seem to depend on the primary antibodies since incubation of the HPTLC plates with only the secondary antibody gave the same pattern. Attempt to adjust the concentration of primary and secondary antibodies did not improve the result. Moreover as the specificity of L-5-28 and L-5-34 was not clearly defined, these analyses were not further pursued.

High performance anion exchange chromatography with pulsed amperometric detection (HPAEC) is a simple and convenient technique for monosaccharide analysis. The technique relies on the release of monosaccharide from oligosaccharides or glycoconjugates by acid hydrolysis and separation at high pH using hydroxide eluents. This technique is very sensitive and no sample derivation is needed. However when galactofuranose is liberated as a free monosaccharide, it is rapidly converted into the thermodynamically favoured galactopyranose form via the open chain of galactose. Moreover galactofuranose is acid labile. These problems can be circumvented by the use of mild acid methanolysis. This method releases terminal galactofuranose residues in the form of methylglycosides in which the reducing oxygen of galactofuranose is covalently bonded. Therefore the ring of galactofuranose is locked. Moreover the mild acidic condition used for the release preserve the integrity of the monosaccharide. Purified GIPLs from Leishmania major wild type and Δ*glf* mutant were thus subjected to mild acid methanolysis and analysed by HPAEC-PAD (Figure 22). The chromatogram obtained from wild type shows a small peak eluting at 5.6 min corresponding to methyl-α-galactofuranose. As methanolysis leads to an inversion of the anomerisation, this result is consistent with the presence of terminal β-linked galactofuranose in *Leishmania major* GIPLs. In contrast no peak was noticeable when Δ*glf* GIPLs were submitted to the same analysis indicative of the absence of terminal galactofuranose.

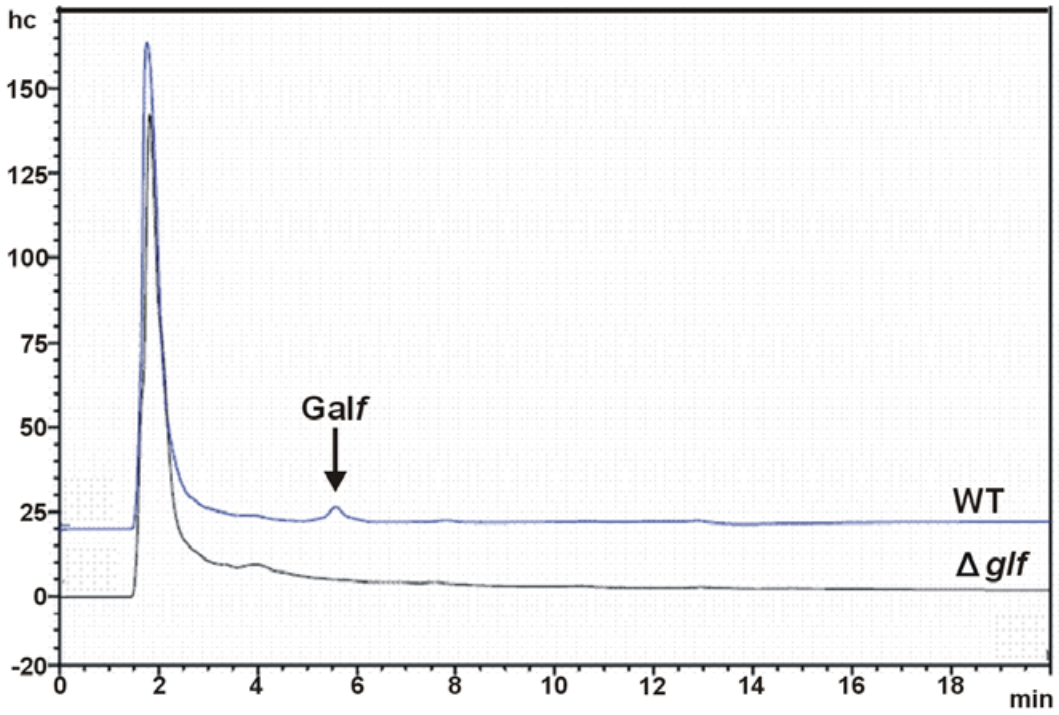


Figure 22: Analysis of terminal Gal*f* presence in GIPLs from *L. major* WT and Δ*glf* mutant. Gal*f* was released from purified GIPLs by mild acid methanolysis and analysed by High pH Anion Exchange Chromatography.

Finally GIPLs were analysed by matrix assisted laser desorption ionisation mass spectrometry (MALDI-MS) in the negative mode. As previously mentioned, a *sn*1-alkyl-2-acyl-phosphatidylinositol commonly links GIPL-1, GIPL-2 and GIPL-3 to the plasma membrane. Because of heterogeneity in the length of the acyl and alkyl chain, 10 distinct glycolipids ions can be observed in the negative ion spectrum of *Leishmania major* wild type GIPLs (Figure 23). The ions at m/z 1498.8, 1526.9, 1660.9, 1688.9, 1822.9 and 1851.0 represent GIPL-1, GIPL-2 and GIPL-3 with C24:0 alkyl and C12:0 or C14:0 acyl chains. Additionally, the spectrum presents ions at m/z 1414.7, 1442.8 and 1470.8 corresponding to GIPL-1 with C18:0 alkyl and C12:0, C14:0 or C16:0 acyl chains respectively. These assignments are in agreement with the structures previously described in *L. major* V121 (McConville *et al.,* 1990, Schneider *et al.,* 1994). Interestingly GIPL-1 containing a sn1-alkyl-2-lyso-phosphatidylinositol with C24:0 alkyl chain (1316.7) is also present in the wild type spectrum. The latter was not reported in *L. major* V121 but seems to be present in *L. major* Friedlin V1 (Zufferey *et al*., 2003).

The negative ion spectrum of GIPLs extracted from *GLF*/Δ*glf*::*PHLEO* heterozygous mutant was identical to the wild type spectrum (data not shown). In contrast, analysis of Δ*glf* mutant revealed the presence of truncated GIPLs. As expected the biosynthesis of GIPLs seems to stop after the addition of the second mannose giving rise to GIPLs

having the basic structure Man(α1-3)Man(α1-4)GlcNH_2-phosphatidylinositol known as iM2. The same lipid heterogeneity as seen in wild type GIPLs leads to the observation of 6 different iM2 peaks at m/z 1154.6, 1252.7, 1280.7, 1308.7, 1336.8 and 1364.8. As a result of UDP-Gal*f* absence, LPG biosynthesis is also prematurely stopped leading to the accumulation of a residual LPG anchor with the structure Glcα1-PO_4-6-Man(α1-3)Man(α1-4)GlcNH_2-sn1-alkyl-2-lyso-phosphatidylinositol. The spectrum is therefore dominated by an ion at m/z 1418.6 corresponding to a sodium adduct ($[M-2H]^{2-}$ + Na^+) of this residual LPG anchor with a C24:0 alkyl chain. The formation of a sodium adduct is enabled by the presence of two phosphate groups in this glycolipid. These groups are indeed easily ionisable. The molecular ion $[M-H]^-$ at m/z 1396.6 corresponds to the same residual LPG molecule. Additionally a small amount of truncated LPG anchor with a C26:0 alkyl chain can be observed (sodium adduct at m/z 1446.6). The presence of these glycolipids indicates that the enzyme involved in glucose phosphate addition does not require the prior addition of Gal*f* by LPG1.

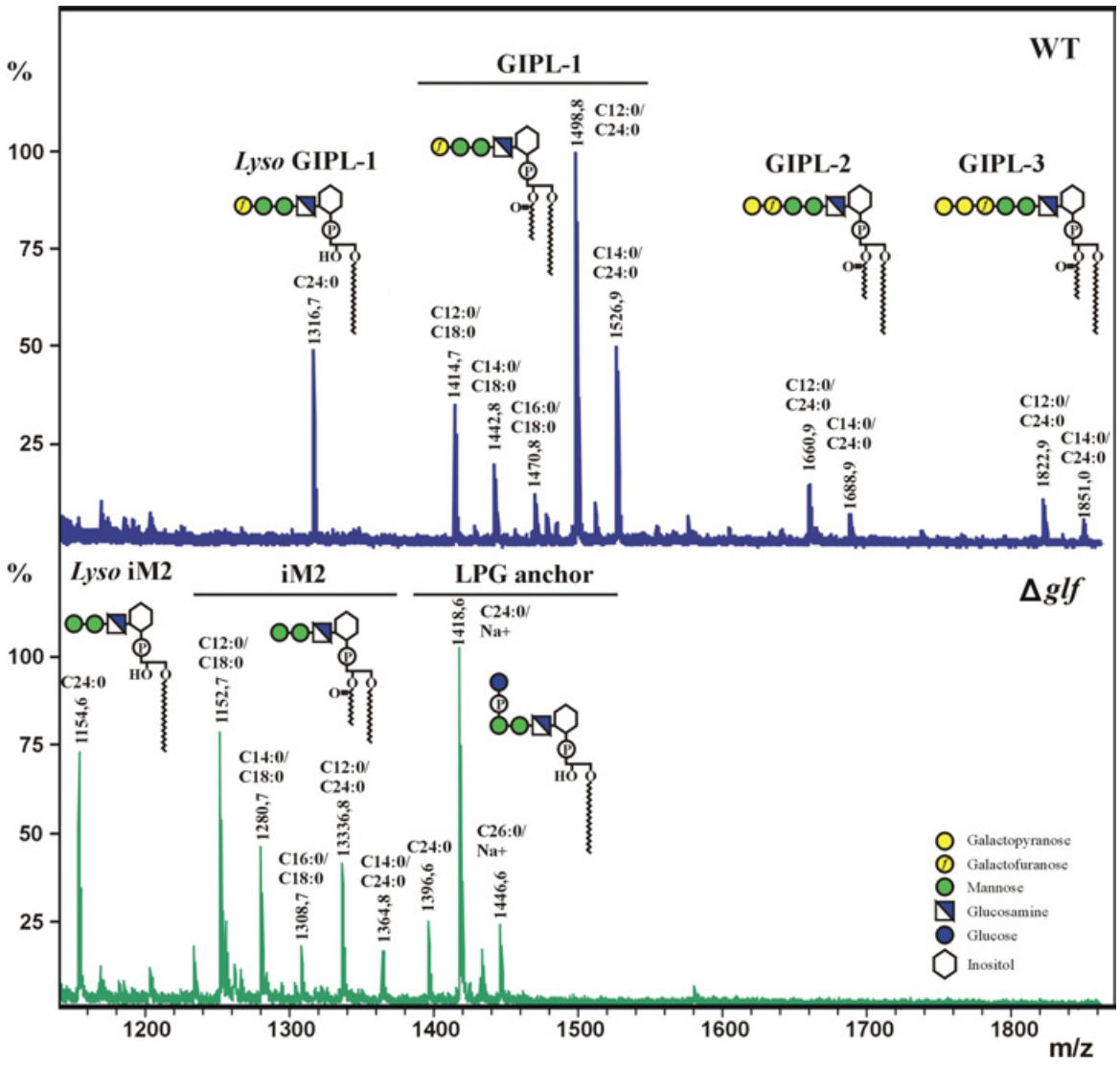


Figure 23: MALDI analysis of GIPLs. The GIPLs from WT and Δ*glf* mutants were extracted, analysed on a MALDI-TOF/TOF spectrometer in the negative mode and the instrument was used at a maximum accelerating potential of 20 kV.

All assignments were confirmed by tandem mass spectrometry. Tandem mass spectrometry allows indeed the selective determination of individual components within a complex mixture. After being selected in the first module of the tandem MS/MS, a specific glycolipid is dissociated into fragments that the second module of the apparatus can analyse. Some fragmentation pathways are favoured and lead to the obtaining of structurally diagnostic ions. A nomenclature system for the designation of fragments ions in mass spectra and tandem mass spectra of glycolipids has been introduced and will be used here. For glycoconjugates and oligosaccharides, A_n, B_n, C_n are used for fragments that retain the charge on the non reducing end and X_n, Y_n , Z_n are used for fragments that retain the charge on the reducing (lipid) end (Domon and Costello, 1988). Ions related to

phosphoric acid (m/z 97, $H_2PO_4^-$ and m/z 79, PO_3^-) and the (B_n+80), where n is equal to the number of sugar rings, dominate the negative ion MS/MS spectra of phosphoglycolipids. Moreover cleavage adjacent to the phosphate at the glycosidic bond results in an abundant Z_0 ion. Additionally, cleavage of the acyl moiety from the molecular ion $[M-H]^-$ can be observed. The absence of the latter is indicative of a *sn*1-alkyl-2-lyso-phosphatidylinositol. All ions observed retain the phosphate group. As illustrations, the daughter ions spectra of the molecular ion $[M-H]^-$ at 1154.6 m/z and m/z at 1418.6 are presented in Figures 24 and 25.

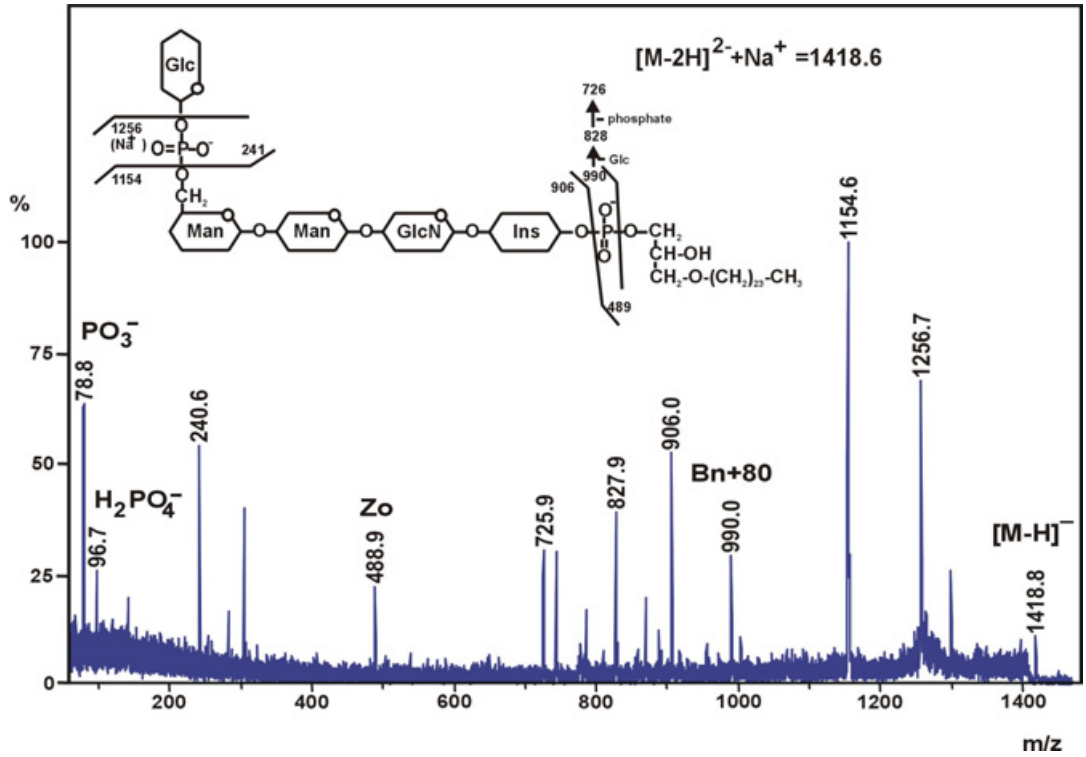


Figure 24: Fragmentation spectra of the molecular ion at m/z 1418.6. GlcN (Glucosamine), Glc (Glucose), Ins (Inositol) and Man (Mannose). For clarity the masses have been rounded to their nominal values.

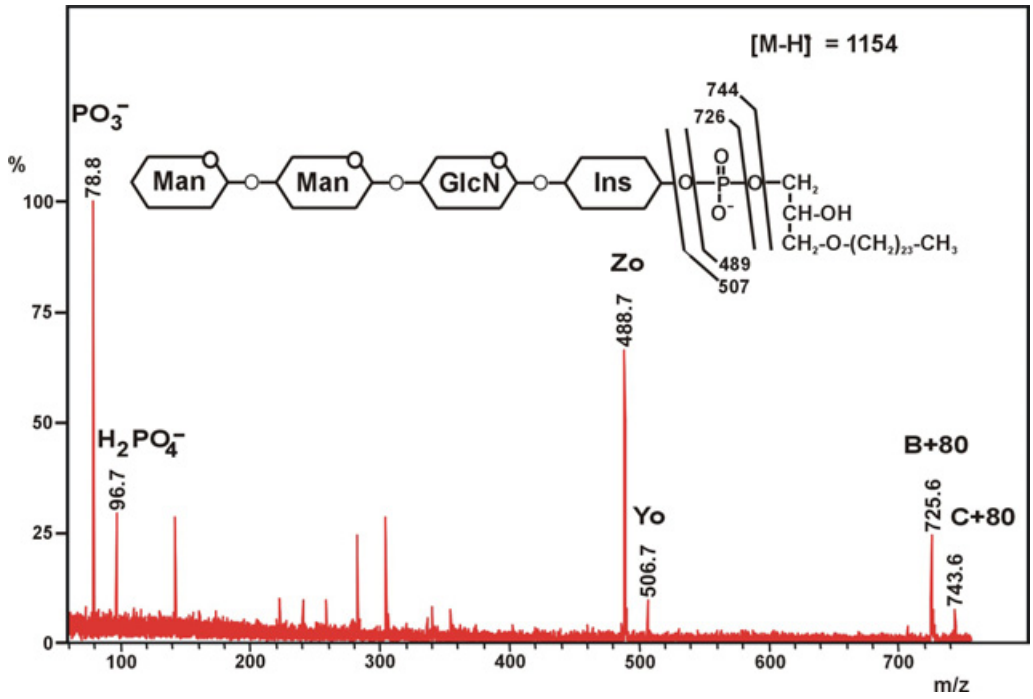


Figure 25: Fragmentation spectra of the molecular ion [M-H]$^-$ at m/z 1154.6. GlcN (Glucosamine), Glc (Glucose), Ins (Inositol) and Man (Mannose). For clarity the masses have been rounded to their nominal values.

3.3.2.6 Analysis of cell surface expressions of GP63 in Δ*glf* mutants

GP63 is a 63 kDa zinc metalloprotease bound by a myristic acid containing GPI-anchor to the plasma membrane. It is well expressed on the promastigote stage of *Leishmania* parasites, accounting for 1% of all cellular protein, but is down-regulated in amastigotes. As no galactofuranose is present in the GPI-anchor of GP63, the interruption of the galactofuranose biosynthesis by *GLF* gene deletion should not affect the expression of this glycoprotein. However GPI-anchors, LPG and GIPLs share some steps of their biosynthetic pathway and upregulation of GPI-anchored proteins was already observed in some LPG deficient mutants. We thus analysed GP63 expression of *Leishmania* wild-type parasites, Δ*glf* and Δ*glf* /+*GLF* mutants by immunofluorescence microscopy and western blotting using the monoclonal antibody GP63-235, that recognises specifically *L. major* surface protease GP63 (a generous gift of W. Robert McMaster, Vancouver, Canada). For immunofluorescence microscopy, *Leishmania* promastigotes of wild type, homozygous mutants (*Δglf/*) and add back (*Δglf/* + *GLF*) were immobilised on cover slides and stained with GP63-235 antibody and a Cy3-coupled secondary antibody. Western blot analysis was performed as described previously for LPG analysis. The

immunoblot was stained with a 1:50 dilution of a hybridoma culture supernatant of GP63-235 antibody and signals detected by an enhanced chemiluminescence (ECL) system. No differences of GP63 expression on *Leishmania* wild type, *Δglf* and *Δglf* / + *GLF* mutants could be detected neither in the immunofluorescence nor in the western blot analysis (Figure 26 and 27).

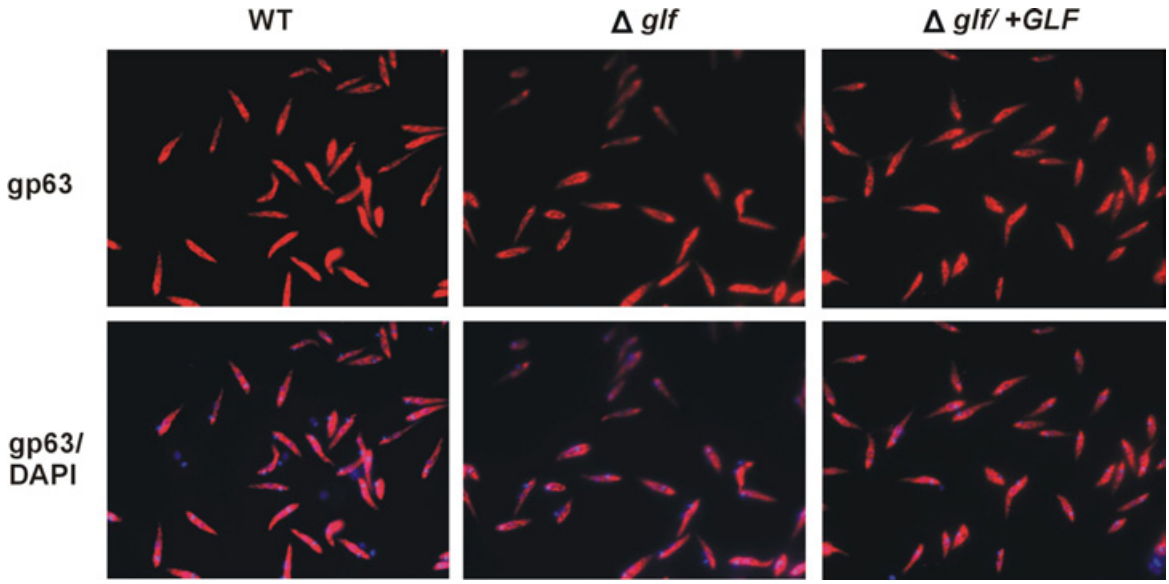


Figure 26: Detection of GP63 on the cell surface of *L.major* WT, Δ*glf* and *Δglf/+GLF*. The parasites were fixed on poly (L) lysine coated coverslips and incubated with monoclonal antibody GP63-235 and Cy3 coupled secondary antibody. Nuclei and kinetoplast were stained with DAPI.

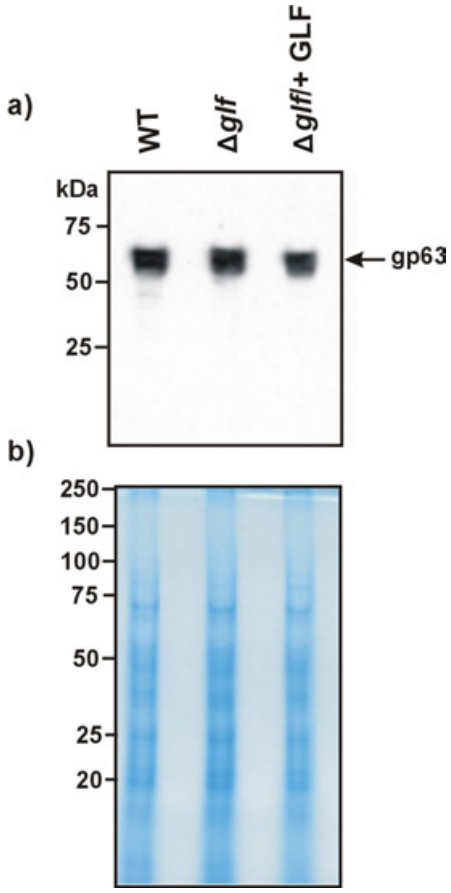


Figure 27: Deletion of *GLF* does not affect GP63 expression. a) Western Blot analysis. Parasites from *L. major* WT, Δ*glf* and *Δglf /+GLF* were extracted and separated on a ProSieve SDS Page and Western Blotting with anti – GP63 antibody. b) To confirm equal loading a second gel was stained with Commassie Blue.

3.4 Cellular localisation of UGM

The analysis of the UGM amino acid sequence revealed no known signal sequences for membrane association or anchorage suggesting that the UGM is soluble. To confirm this finding, the cellular localisation of UGM was examined using episomal expression of N-terminal GFP-tagged protein (*GFP*::*GLF*) in wild type *L. major* or in the Δ*glf* null mutant. The green fluorescent protein (GFP) of *Aequorea victoria* has been introduced as a convenient marker in eukaryotic organisms including *L. major* for localisation of engineered fusion proteins (Chalfie *et al.*, 1994; Ha *et al.,* 1996). To generate the N-terminal GFP tagged UGM, the full length open reading frame lacking start codon was PCR amplified and cloned in the sense orientation into the *BamH I* site of pXG-GFP+2. 10 µg of the resulting plasmid pXG-GFP+2-*GLF* were transfected in the Δ*glf* mutant. As a control *L. major* wild type cells were transfected with a plasmid expressing GFP (pXG-GFP+). LPG that is absent from Δ*glf* mutant was re-expressed after transfection with a

plasmid containing *GFP*::*GLF*, demonstrating that the GFP tagged protein is enzymatically active (data not shown). Fluorescence microscopy showed that the GFP tagged UGM is distributed throughout the whole cell within *L. major* wild-type (Figure 28) and Δ*glf* mutant supporting the cytoplasmic location of UGM.

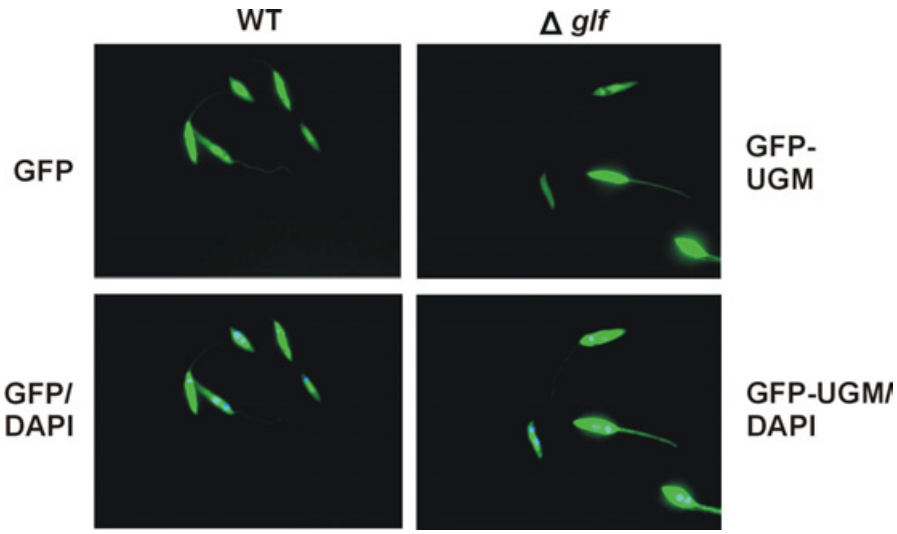


Figure 28: Expression of GFP tagged UGM in *L. major*. A N-terminal GFP tagged UGM (pXG-GFP+2-UGM) was transfected in the Δ*glf* mutants. *L. major* wild-type was electroporated with pXG-GFP+ (B2863). The cells were fixed with 4% paraformaldehyde on a poly-L-lysine coated coverslips. An additionally DAPI staining was performed.

*3.5 Experimental mice infection study with Δ*glf *mutant*

To investigate the participitation of the *GLF* gene in infectivity, a mouse infection model was used. First, as *Leishmania* loose virulence in culture, the parental wild type strain, the homozygous (*Δglf*) and the corresponding add back mutant (*Δglf/+GLF)* were passed once through mice to optimise the virulence of the parasites. For this purpose, all parasites were grown to late logarithmic phase to enrich the population of infectious metacyclic promastigotes and 1 x 10^7 parasites per mouse were injected in the hind left footpad. After 10 days, amastigotes were re-extracted from lymph nodes, transferred to liquid media at 27°C and cultured to allow re-differentiation into promastigotes. Importantly all re-isolated parasites were kept in culture for less than three passages before inoculation into mice. These freshly isolated parasites were grown to late logarithmic phase and 2 x 10^6 parasites per mouse were injected in the hind left footpad. The size of the lesion was measured weekly over a 10 weeks period by comparing of the

thickness of the infected and non infected hind footpad using a Vernier calliper (Figure 29). After four weeks, lesion were clearly visible in all mice infected with the parental wild type strain, while lesion formation was slightly delayed in mice injected with the add backs mutant. In contrast no lesion development was observed in mice that were inoculated with the *Δglf* mutant (Figure 30) showing that the UGM is an important virulence factor.

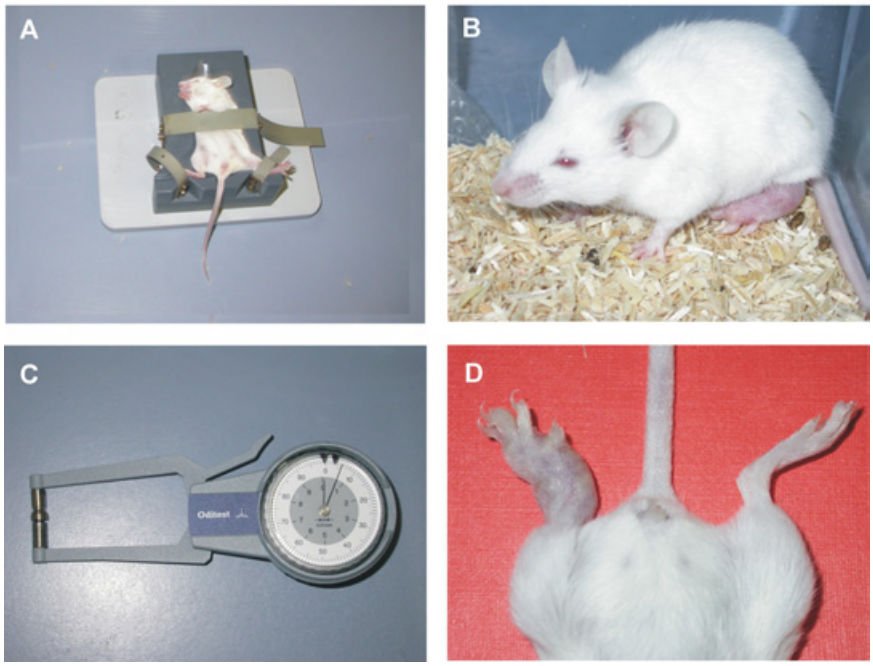


Figure 29: Infection of Balb/c mice with wild type and *Δglf* mutant. Balb/c mice were fixed (A) and infected with 2 x 10^6 wild type parasites, homozygous mutant and add back. (B) Lesion formation of *Leishmania* wild type parasites 11 weeks post infection. (C) The size of the lesion was determined using a Vernier calliper (C) comparing infected and non infected hind footpad (D).

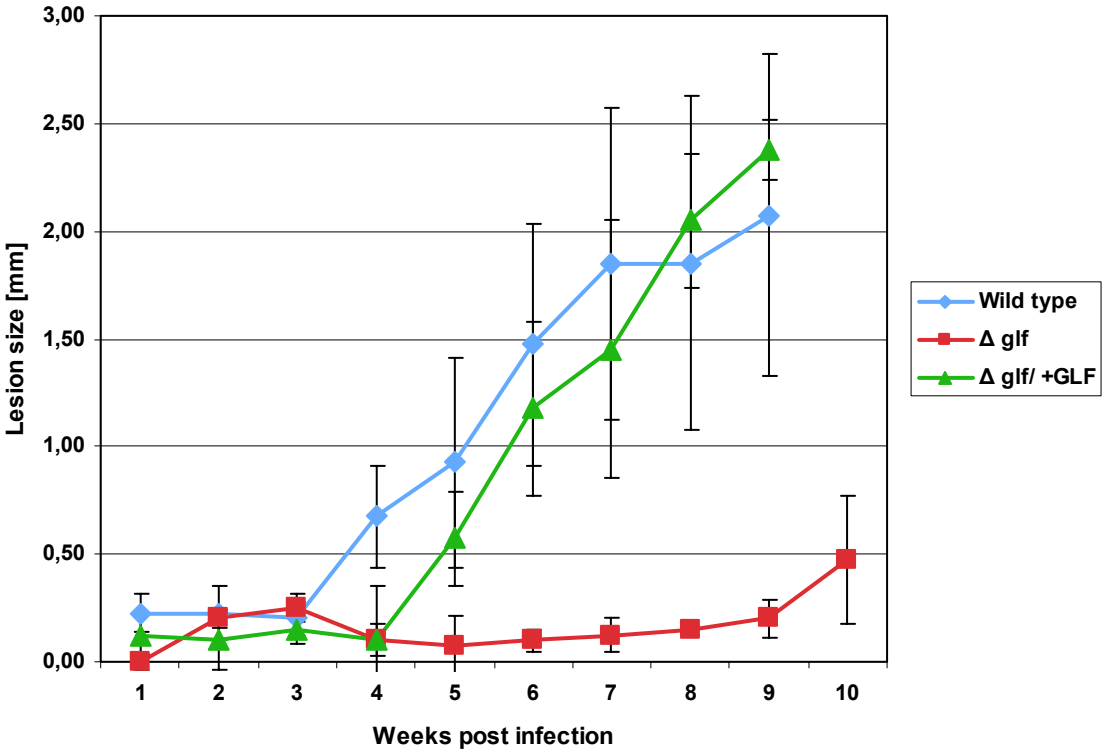


Figure 30: *In vivo* testing of virulence. The time course of lesion formation after infection of Balb/c mice with L. major wild type (blue line), homozygous mutant (red line) and the corresponding add back (green line) mutant strains were monitored by measuring the swelling footpad. The median size differences of the infected and the non infected hind footpad are plotted against the weeks post infection. For each clone, four animals were infected.

4 Discussion

The surface glycoconjugates, that forms a dense cell surface glycocalyx (Pimenta *et al.,* 1991, Garami and Ilg, 2001), comprised of GPI-anchored glycoproteins, lipophosphoglycan (LPG), free GPI glycolipids (GIPLs) and proteophosphoglycans (PPGs) are essential for the survival and virulence of *Leishmania* parasites (McConville, 2002, Descoteaux *et al.,* 1999). Hence some enzymes involved in their biosynthetic pathways might provide interesting drug targets. Because of its absence in human, the UDP-galactopyranose mutase (UGM) represents an attractive candidate. This enzyme, encoded by the *GLF* gene, is responsible for the interconversion of the activated UDP-galactopyranose and UDP-galactofuranose, and represents thus a central enzyme in galactofuranose biosynthesis. Moreover it is essential for the survival or virulence of various pathogenic bacteria. Although in eukaryotes the existence of such an enzyme was since long suspected, it had so far not been identified. Here we report the characterisation of *Leishmania* UGM and determine its role in glycoconjugate biosynthesis and importance for parasite virulence, by a targeted gene deletion approach.

4.1 Identification and partial characterisation of L. major UDP-galactopyranose mutase

The existence of an enzyme converting UDP-Gal*p* into UDP-Gal*f* was suggested by the early work of Trejo and colleagues in fungi (Trejo *et al.*, 1970). Nevertheless the genes encoding this enzyme (*GLFs*) were the only enzymes involved in the Gal*f* metabolism identified in bacteria (Nassau *et al.,* 1996; Weston *et al.*, 1997; Koplin *et al.,* 1997). In order to identify *GLFs* from eukaryotic pathogens, blast searches of *Leishmania major* genome with prokaryotic *GLFs* were conducted. Particular attention was paid to the presence of a flavin binding domain in the candidates, since flavin is essential for the catalytic activity of prokaryotic UGMs (Sanders *et al.,* 2001). Moreover the phylogenetic distribution of the gene, specifically its occurrence in fungi and protozoa known to synthesise Galf in comparison to taxa lacking Gal*f,* was used as additional selection criterion. A single *L. major* candidate gene encoding a 491 amino-acid protein with low homology to prokaryote UGMs was identified. Importantly, this protein was conserved in various eukaryotes known to express Gal*f* such as *Aspergillus fumigatus* and *Trypanosoma cruzi* but was absent from *Drosophila melanogaster, Arabidopsis thaliana*, yeast and mammals, where Gal*f* has never been found.

Direct evidence that the selected genes encoded active UGMs was provided by an *in vivo* complementation assay for UDP- Gal*f* synthesis in *E.coli*. This system was based on the expression of *Klebsiella pneumoniae* LPS O-antigen (that contains Gal*f*) in a strain of *E. coli* deleted for the endogenous LPS *rfb* locus. Inactivation of *Klebsiella GLF* abrogated LPS biosynthesis, but could be rescued by episomal expression of our candidate genes. Activity of the enzymes was then confirmed by an *in vitro* enzymatic assay. The same approach was used by Beverley and co-workers who independently reported the characterisation of some eukaryotic UGMs at the same time that we did (Beverley *et al.*, 2005; Bakker *et al.*, 2005).

Despite the extensive divergence, eukaryotic UGMs share key properties with those of prokaryotes. The eukaryotic UGMs show the characteristic dinucleotide binding motif of the glutathione reductase structural family (Dym and Eisenberg, 2001; Wierenga et al., 1983) and importantly most of the residues involved in UDP Galp binding were conserved. Considering the low homology between bacterial and eukaryotic UGMs, the conservation of many residues of the binding site is intriguing and the crystal structure should be solved to determine if the shape of the UDP-Gal binding site is conserved. However preliminary tests of a panel of prospective prokaryotic UGM inhibitors suggest that they fail to inhibit eukaryotic UGMs (Beverley et al., 2005). Thus it might be necessary to develop eukaryotic specific UGM inhibitors in the future.

As in prokaryotes, eukaryotic UGMs lack signals typical for membrane association or anchorage and are likely to be cytoplasmic. However, there is precedent in other systems for the compartmentalisation of proteins involved in nucleotide sugar interconvertion (Griffith *et al.*, 2004; Pattathil *et al*., 2005). Thus cellular localisation of UGM was examined using episomal expression of N-terminal GFP-tagged protein (*GFP*::*GLF*) in *Leishmania major*. The GFP tagged UGM is distributed throughout the whole cell supporting the cytoplasmic localisation of UGM. This implies that UDP- Gal*f* is synthesised in the cytoplasm, like most nucleotide sugars, and strongly suggests the existence of an UDP-Gal*f* transporter able to provide substrate to the galactofuranosyltransferases located within the Golgi apparatus (Späth *et al.,* 2000).

Interestingly a number of genes with strong homology to *GLF*s were found in lower eukaryotes not known to synthesise Gal*f*. The predicted proteins show also conservation of key residues involved in cofactor and substrate binding. Most of them have probably UGM activity as well. Thus Gal*f* synthesis seems to occur more widely than previously thought and would be found in various pathogens of humans, animals and plants. Amongst organisms that presumably synthesise Gal*f*, we could cite the human nematodes parasites Brugia and Onchorcerca.

4.2 Generation and characterisation of a L. major GLF *gene deletion mutant*

The targeted gene deletion strategy was determined by the genetic organisation of the parasite. The genome of *Leishmania* does not contain any introns and the transcription takes place in a polycistronic fashion. The polycistronic pre-mRNAs are further processed by coupled trans-splicing and polyadenylation reactions to generate mature monocistronic mRNAs. (Stiles *et al.,* 1999, Stein *et al.,* 1990). Gene expression is not directed by promoters (or at least these have not yet been identified) but by sequences present in the 3´and 5´flanking region. Thus each of the two *GLF* alleles was precisely replaced by the coding sequence of an antibiotic resistant gene, leaving the 3' and the 5' flanking regions completely unaltered to ensure correct expression of the markers. For this purpose targeting constructs were prepared by homologous recombination in bacteria (Zhang *et al*., 2000). Interestingly, the integration of the hygromycin resistance gene was preferred over the phleomycin resistance gene. The latter is a small gene encoded by a 373 bp region, while hygromycin and *GLF* are encoded by 1026 and 1473 bp respectively. The size of the DNA might influence the homologous recombination or the polycistronic gene transcription. In *Leishmania* parasites, targeted gene replacement is extremely influenced by the amount and quality of DNA. High amounts of the targeted gene deletion constructs resulted in multiple false insertions into the genome. This phenomenon was previously reported by Cruz *et al.* (1990 and 1991). *GLF* gene deletion and the correct insertion of the antibiotic resistant markers were confirmed by southern blot. For comparison of the results, the parental wild type strain was treated and cultivated in the same way as the mutants. Moreover several clones were analysed in all experiments.

4.2.1 Characterisation of *L. major GLF* gene deletion mutants

As no other *GLF* homologous was found in the *L. major* genome, no alternative pathway for galactofuranose should exist and the mutants were expected to lack any Gal*f*.

The clones obtained after two rounds of targeting gene deletion were viable, demonstrating that *GLF* is not essential for the survival of *Leishmania* parasites under *in vitro* conditions. Moreover all mutants analysed in this study grew as well as the parental strain of *Leishmania major*. In *Mycobacteria*, *GLF* deletion is lethal. However, the role of Gal*f* in the survival or virulence of bacteria depends greatly of its abundance and position

in the glycoconjugates. Galactofuranose is indeed an inner component of the mycobacterial cell wall and is hence necessary for its integrity. In *Leishmania* promastigotes (insect stage), Gal*f* occupies a similar position in the anchor of the abundant polysaccharide LPG. Consequently *GLF* deletion is expected to affect profoundly the glycocalyx. However, in the amastigotes (stage of the parasite in the mammalian host), LPG is down regulated and the glycocalyx almost absent. Thus these changes were not expected to influence *Leishmania* survival *in vitro*. Moreover various mutants deficient in LPG and/or other glycoconjugates have been described and are perfectly viable (Späth *et al.*, 2000 and 2003, Zufferey *et al.*, 2003).
In *Leishmania major*, galactofuranose is not only present in the GPI-anchor of LPG, but also in the main surface Glycosylinositolphospholipids (GIPLs). Thus in addition to the loss of LPG, deletion of *GLF* should result in truncated GIPLs because Gal*f* occupies the terminal, penultimate or antepenultimate position in these glycoconjugates. On the other hand, the synthesis of other molecules should not been altered. In order to confirm these predictions, the expression and structure of cell surface glycoconjugates was carefully analysed. The complete absence of LPG was observed by Western blotting using the monoclonal antibody WIC 79.3 directed against the galactose substituted repeating unit characteristic of *L. major* LPG and PPGs. Although the membrane bound PPGs and the newly synthesised PPGs accumulating in the flagellar pocket before secretion should weakly stain the parasite, no signal could be detected. Further experiments confirmed that unexpectedly *L. major* MHOM/SU/73/5ASKH expresses low level of PPGs. The absence of membrane bound PPGs was already reported in *Leishmania major* LV39 (Späth *et al.,* 2000) but the quasi absence of mucin-like PPGs is more surprising. The PPGs profiles are known to vary markedly in different *Leishmania* species and developmental stages. *L. major* secretes much fewer PPGs than *Leishmania mexicana*. Given the paucity of information on PPGs expression in different strains of *Leishmania,* it can only be speculated that different *Leishmania* strains vary in their glycoconjugates expression. By heterologous expression of secreted acid phosphatase, it was however demonstrated that the PPG biosynthetic machinery is intact in the Δ*glf* mutant. In these conditions, an increased phosphoglycosylation is even observed in the mutant. Such increase is a logic consequence of the absence of LPG backbone synthesis and was previously reported in another LPG deficient mutant (Zufferey *et al*., 2003).

In order to further characterise the Δ*glf* mutants, the structure of the GIPLs was intensively investigated. The GIPLs were extracted, purified by reverse phase chromatography and analysed by Matrix Assisted Laser Desorption Ionisation Mass spectrometry (MALDI-MS) and tandem mass spectrometry (MALDI-MS/MS). Like other

strains of *L. major* analysed to date, *L. major* MHOM/SU/73/5ASKH expresses only type 2 GIPLs. The structures of the glycan and lipid moieties deducted from our analyses are in perfect agreement with previous reports (McConville *et al.*, 1990, Zuffery *et al.,* 2003). As expected, the biosynthesis of GIPLs is interrupted after the addition of the second mannose in the *GLF* null mutant resulting in truncated GIPLs deficient in Gal*f*. In addition accumulation of the residual LPG anchor was observed. This residual anchor can be distinguished from GIPLs by the presence of a glucose phosphate residue located on the terminal mannose and a sn1- alkyl-2-lyso-phosphatidylinositol.

The early steps in GIPLs and LPG anchor biosynthesis are the same as those involved in protein anchor biosynthesis involving the transfer of N-acetylglucosamine to *sn*1-alkyl *sn*-2-acyl phosphatidylinositol, followed by de-N-acetylation and transfer of a mannose residue to form Manα1-4GlcN-PI. The first committed step for the GIPLs type 2 as for the LPG is the addition of the mannose residue in a α 1-3 linkage to this intermediate. Galactofuranose is added to this α 1-3 linked mannose residue. The existence of two different galactofuranosyltransferases involved either in the LPG anchor or in the GIPLs biosynthetic pathways was suggested by deletion of *LPG1* gene encoding the putative galactofuranosyltransferase that abolished Gal*f* addition to the LPG anchor but not to the GIPLs. The remaining steps in LPG anchor include the addition of two more galactose residues, one glucose phosphate residue and deacylation of the lipid moiety. However the ordering and enzymology of these reactions has not been elucidated. The presence of glucose phosphate in the residual LPG core of the Δ*glf* mutant indicates however that addition of this residue does not require the prior addition of Gal*f* by LPG1. Interestingly the amount of truncated GIPLs with a C24:0 alkyl and a C12:0 or C14: acyl chain is not abundant in the Δ*glf* mutant, although these two phosphatidylinositols are the main lipids found in wild type GIPLs. GIPLs biosynthesis requires remodelling of the *sn*-2 fatty acid before transport to the plasma membrane but remodelling of the alkyl chain has never been described. Therefore in absence of galactofuranose addition, GIPLs precursors with long alkyl chains seem to be directed towards LPG biosynthesis. This suggests a competition between the GIPLs specific galactofuranosyltransferase and the enzymes specifically involved in LPG synthesis (eg. glucose phosphate transferase, deacylase, LPG1). The differential expression of these enzymes could thus account for the selective down regulation of LPG in the amastigote stage which is a crucial step for survival of *Leishmania* in the mammal host.

As just mentioned the biosynthetic pathway of protein anchors is closely linked to those of LPG and GIPLs. Therefore although GPI-anchors of proteins do not contain any Gal*f*, their expression was investigated by indirect immunofluorescence and Western blotting

using the antibody GP63-235 directed against the metalloprotease GP63. Expression of GP63 was not affected in the Δ*glf* mutant. Thus as predicted the Δ*glf* mutant lacks LPG and expresses truncated GIPLs whereas the biosynthetic machinery of other glycoconjugates is unaffected.

4.2.2 Virulence

The characterised Δ*glf* mutant was used to elucidate the role of Gal*f* in parasite virulence and therefore assess if UGM is a potential drug target. The virulence of the Δ*glf* mutant was assessed by lesion formation of Balb/c mice after inoculation with the parasites. Because *Leishmania* parasites loose virulence in culture, all parasite lines were passed through mouse at high inoculating dose, re-isolated and maintained for less than 3 passages *in vitro* prior to virulence testing. Remarkably mice inoculated with the Δ*glf* mutant did not develop any lesion while in contrast mice infected with the wild type strain and "addback" line showed rapid disease progression. As Gal*f* is present in the anchor of LPG and this molecule is a known virulence factor in *L. major*, a decrease of virulence was expected. However, in contrast to *Leishmania major* Δ*lpg1* mutant that lacks exclusively LPG and shows a delayed disease progression, the Δ*glf* mutant seems completely avirulent. This virulence phenotype could be attributed to the simultaneous loss of LPG and Gal*f* in GIPLs. However it is difficult to determine the role of GIPLs in pathogenesis. Indeed the structural homology and the sharing of biosynthetic pathways with LPG and GPI-anchored proteins (McConville and Ferguson 1993, McConville *et al*., 2002) preclude the obtaining of mutant exclusively deficient in GIPLs. Thus genetic studies that attempted to assign a specific role to GIPLs yielded contradictory results (Ilgoutz *et al.,* 1999; Mensa- Wilmot *et al.,* 1999, Garami and Ilg 2001). Nevertheless the ability of purified GIPLs to inhibit protein kinase C activity and nitric oxide production implicated them in parasite survival in macrophages. Though, these properties are associated with the lipid part of GIPLs and should not be affected in the Δ*glf* mutant. Additionally GIPLs containing a terminal Gal*f* residue have been implicated in the interaction of *L. major* with macrophage (Suzuki *et al*., 2002). However the *L. major* Δ*ads1* mutant that lacks all ether lipids including LPG and GIPLs recently counteracted all these proposals. Indeed this mutant only exhibits a reduction of virulence similar to *lpg1*$^{-}$ and is able to enter macrophages as well as wild type parasites. Preliminary experiments indicate that promastigotes of the Δ*glf* mutant are able to enter macrophages as efficiently as the parental strain and in this respect behave similarly as the *ads1*$^{-}$ mutant. However the interaction promastigote-macrophage might not be relevant as the first cell interacting with *Leishmania* promastigotes seems to be

polymorphonuclear neutrophils (PMN). Interestingly the infectivity of Δ*glf* mutant toward PMN seems reduced. Additional experiments to study the infectivity of Δ*glf* promastigotes and amastigotes toward PMN and macrophages are required to determine the role of galactofuranose in macrophage invasion and parasite replication inside macrophages. It should be emphasised that LPG is absent from the intracellular amastigote stage of the parasite and thus in contrast to all other existing mutants, Δ*glf* amastigotes have an exclusive defect in GIPLs.

Unexpectedly MHOM/SU/73/5ASKH expresses low level of PPGs and thus the Δ*glf* mutant resembles the Δ*lpg2* mutant, an avirulent mutant deficient in LPG and PPGs. The loss of virulence observed after deletion of *GLF* could thus be due to a fortuitous choice of *L. major* strain and be attributed to the concomitant absence of LPG and PPGs. Over the last decade, researchers tried to determine the glycoconjugate responsible for virulence of *Leishmania*. However, there seems to be a certain level of functional redundancy between the different surface glycoconjugates of *Leishmania* and thus loss of virulence is generally associated with the absence of several glycoconjugates. Moreover, it is now acknowledged that different species of *Leishmania* rely differently on their surface glycoconjugates for virulence (Turco *et al*., 2001). Differences between strains have also been suggested in the case of *L. mexicana*. Indeed the deletion of dolichol-phosphate-mannose synthase gene resulted in a mutant that lack all GPI-anchored molecules while attempts to delete the same gene in another strain of *L. mexicana* were unsuccessful. In *L. major,* however, most studies highlighting the roles of glycoconjugates have been conducted with the same strain. The present work shows that differences in glycoconjugates expression are found between different strains. These might be reflected by the degree of virulence of each strain and account for some of the inconsistency found in literature.

The study presented in this thesis highlights the essential role of galactofuranose for *L. major* virulence and provides encouraging results for the chemotherapeutical potential of Gal*f* metabolism in *Leishmania major*. Although this role cannot be extrapolated to other species of *Leishmania*, it might not be restricted to *L. major*. Indeed a recent study suggested that galactofuranose containing GIPLs are important for basic survival of *Trypanosoma cruzi* (MacRae *et al*., 2006). In this respect, the identification of eukaryotic UGMs represents an important step that allows studying the requirement of Gal*f* for the viability or pathogenicity of different organisms. Such study with the opportunistic fungus *Aspergillus fumigatus* indicates an important contribution of Gal*f* to the virulence of this pathogen. An inhibitor of eukaryotic UGM might thus be active against a wide range of pathogens.

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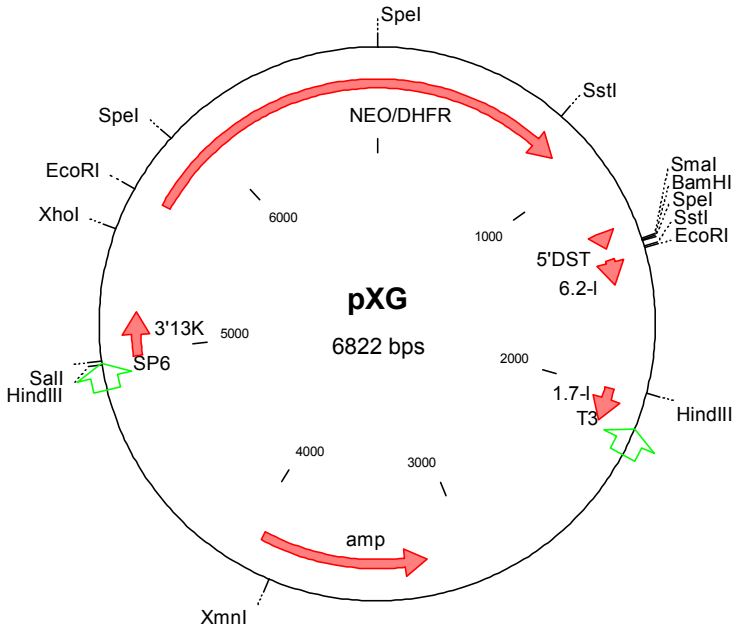
6 Appendix

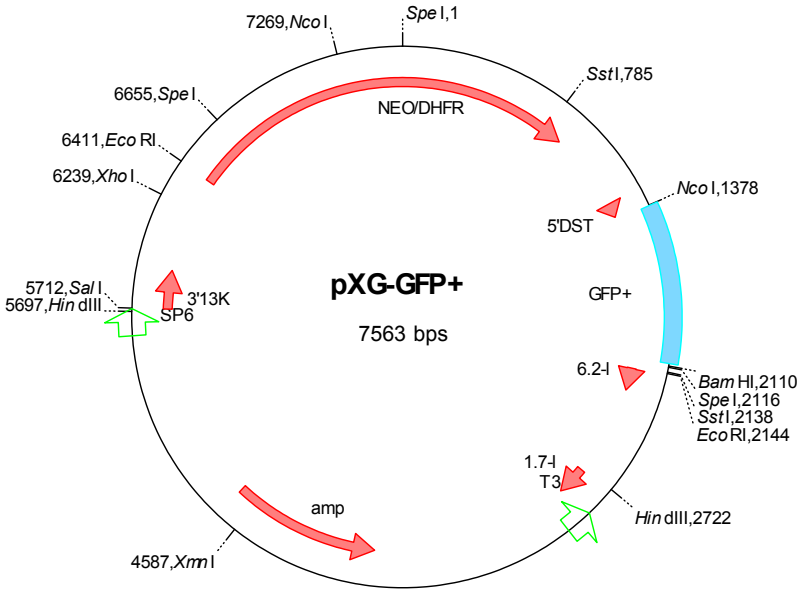
6.1 Map of pXG: Leishmania *expression vector with NEO marker*



In the vectors of pXG-serie, the expression of the gene is driven by a *Leishmania* intergenic region providing signals for trans-splicing and polyadenylation. This intergenic region flanks the unique polylinker *Sma*I or *Bam*HI sites. Insertion of ORF into these sites gives high level of expression, fairly constant through parasite life cycle (log, stationary phase promastigotes; lesion amastigotes) (Ha *et al.*, 1996; Chakkalath *et al.* 2000). This vector contains all genetic information required for expression and replication in *Leishmania*. It is originated from a circular 30Kb region bearing the dihydrofolate reductase thymidylate synthase gene amplified in certain methotrexate resistant *Leishmania.*

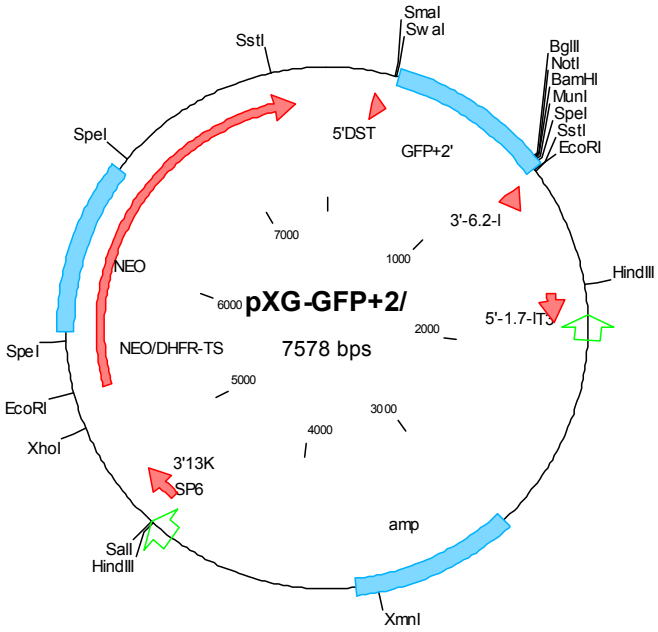
Any site in the vector (compatible with marker function) can be used for shuttle vector purposes. The *NEO* gene is originally from pR-NEO (Kapler *et al.* 1990)

6.2 Map of pXG –GFP+: Leishmania *GFP vector*



The pXG-GFP+ vector contains a modified GFP called “GFP+” (GC rich; S56T mutation) inserted into expression site of pXG (Ha *et al*., 1996).

6.3 Map of pXG –GFP+2: Leishmania vector for making N- terminal GFP fusions to protein



Gene fusions may be made conveniently by inserting a gene in the appropriate reading frame in the polylinker located 3'-ward of the GFP+2 gene. The GFP+2 gene is a GFP variant similar to GFP+; it contains GC-rich codons as well as mutations that improve the fluorescence signal (L64T65), making it suitable for 'fluorescein'-oriented fluorescence optics (Ha *et al.*, 1996).

In the map the blue bars represent ORFs, red arrows indicated mRNAs known or predicted from the *L. major* intergenic regions. All derived from regions surrounding the *DHFR-TS* gene.

7 Abbreviations

°C	degree Celsius
µm	micrometer
µl	microliter
α–	Anti-
ABTS	2,2'-Azino-di-(3-ethylenbethiazolin-sulphate)
A. fumigatus	*Aspergillus fumigatus*
AP	Alkaline phosphatase
APS	Ammonium peroxidisulphate
Ara	arabinose
ATP	Adenosine triphosphate
BCA	Bichionic acid
BCIP	5-Brom-4-chlor-3-indolyl-phosphate
bp	Base pairs
BSA	Bovine serum albumin
CMP	Cytidine 5'-monophosphat
Da	Dalton
DAPI	4',6-Diamedino-2-phenylindol
DMEM	'Dulbecco's Modified Eagle's medium'
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
DTT	Dithiothreito
E. coli	*Escherichia coli*
EDTA	Ethylendiamine-N,N,N',N'-tetraacetic acid
e.g.	*exempli gratia*
ELISA	Enzyme linked immono sorbant assay
et al.	*et alii*
EtN	ethanolamine
Fig.	figure
Gal	galactose
Gal*f*	galactofuranose
Gal*p*	galactopyranose
GFP	Green fluorescent protein
GIPLs	Glycosylinositolphospholipids
GlcN	Glucosamine
Glc	Glucose
GP63	glycoprotein 63
GPI	Glycosylphosphatidylinositol
h	hours
hyg	hygomycin
Ins	Inositol
kb	kilobase
kDa	kilodalton
K. pneumoniae	*Klebsiella pneumoniae*
L.	*Leishmania*
L.major/mexicana/donovani	*Leishmania major/mexicana/donovani*
LB	Luria-Bertani
LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
M	Molar
mM	millimolar
mAb	Monoclonal antibody

MALDI-MS	Matrix Assisted Laser Desorption Ionisation Mass spectrometry
Man	mannose
min	minutes
ml	millilitre
mm	millimeter
NBT	Nitrotetrazolium-blue chloride
NCAM	Neural cell adhesion molelcule
neo	neomycin
OD	Optical density
P	phosphate
PAGE	Polyacrylamide gelelectrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
phleo	phleomycin
PI	phosphatidylinositol
PMSF	Phenylmethansulfonylflouride
PPG	Proteophosphoglycan
RT	Room temperature
SAP	secreted acid phosphatase
SDS	Sodium dodecyl-sulphate
sec	seconds
TEMED	N,N,N',N'-Tetramethyl-ethylendiamin
TRIS	Tris(hydroxymethyl)-aminomethan)
UDP	Uridin 5'-diphosphate
UGM	UDP-galactopyranose mutase
UTR	untranslated region
wpi	weeks post infection
z.B.	zum Beispiel

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Promotion

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04.09.2005- 09.09.2005
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Topic:' Identification and functional characterisation of a UDP-Glucose Pyrophosphorylase from *Leishmania major*', Lamerz A.C., **Kleczka B.**, Wiese M., Routier F.H., Gerardy-Schahn R.

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Topic: 'Leishmania major UDP-Glucose pyrophosphorylase: identification, characterisation and analysis of a gene deletion mutant' Lamerz A.C., **Kleczka B.**, Wiese M., Routier F.H., Gerardy-Schahn R.

9 Erklärung

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbständig und nur unter Verwendung der angegebenen Quellen und Hilfsmittel angefertigt habe. Außerdem versichere ich an Eides statt, dass die zugrunde liegenden Ergebnisse nicht für eine andere Prüfungsarbeit verwendet worden sind.

Hannover, 31.05.2006

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