Identification and Characterization of *Leishmania major* UDP-sugar Pyrophosphorylase and Determination of its Impact on UDP-galactose Metabolism

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meinen Großeltern

Herbert & Lucie

„It is not the strongest species
that survives, nor the most intelligent,
but the one most responsive to change."

Charles Darwin, 1809-1882

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Zusammenfassung

Leishmanien sind obligat parasitäre Protozoen aus der Familie der *Trypanosomatidae*, die vor allem in tropischen und subtropischen Regionen, die weit verbreitete Krankheit Leishmaniose verursachen. Umhüllt und geschützt von einer dichten Glykokalyx, widerstehen sie extrem lebensfeindlichen Umwelteinflüssen. Die zelluläre Ummantelung des Parasiten besteht hauptsächlich aus den speziellen Lipophosphoglycanen (LPGs), den mucin-artigen Proteophosphoglycanen (PPGs) und den leichtkettigen Glycoinositolphospholipiden (GIPLs) und ist unentbehrlich für das Überleben im Darm seines Vektors, der Sandfliege, und für die Infektiosität im Säugetier, seines Reservoirs.

Vor allem die Phosphoglycanstructuren von *Leishmanien* sind extrem galactosehaltig und setzen eine spezialisierte, enzymatische Maschinerie voraus, um die hohen Ansprüche der UDP-Galactose-Biosynthese zu decken. Die vor kurzem biochemisch charakterisierte *L. major* UDP-Glucose-Pyrophosphorylase (UGP), die äußerst spezifisch Glucose-1-Phosphat mit UTP umsetzt und UDP-Glucose und Pyrophosphat erzeugt, schien ein Schlüsselenzym der UDP-Galactose-Biosynthese zu sein, da UDP-Galactose (UDP-Gal) entweder über Epimerisierung von UDP-Glucose (UDP-Glc) oder durch Uridylyltransfer von UDP-Glc zu Galactose-1-Phosphat entstehen sollte (Leloir Stoffwechselweg). Eine gezielte Gendeletion der UGP (Δ*ugp*) zeigte allerdings nur geringfügigen Einfluss auf die Phosphoglykanbiosynthese, wobei galactosehaltige GIPLs überhaupt nicht betroffen waren. Dementsprechend war die Virulenz der Δ*ugp* Mutante nur moderat herabgesetzt. In Anbetracht dieser Datenlage war anzunehmen, dass *Leishmanien* einen zweiten, UDP-Glucose unabhängigen Stoffwechselweg nutzen können, was mit dem offensichtlichen Fehlen des Leloir-Stoffwechselwegs in *Leishmanien* in Einklang war, da bisher kein verantwortliches Gen (von GALT) im *Leishmanien* Genom annotiert werden konnte.

Es konnte jedoch ein aus Pflanzen stammendes Homolog der UDP-Zucker Pyrophosphorylase (USP) in *Leishmanien* und einigen anderen Protisten identifiziert werden. Charakteristisch für diese neue Klasse von Enzymen (EC 2.7.7.64) war ihre Fähigkeit, eine Bandbreite an Monosaccharid-1-Phosphaten mit UTP zu aktivieren, wie Galactose-, Glucose-, Xylose-, L-Arabinose-, Galacturonsäure- und Glucuronsäure-1-Phosphat, um daraus das jeweilige UDP-Monosaccharid und Pyrophosphat zu formen. Es konnte gezeigt werden, dass dieses Enzym eine hohe Affinität zur Bindung von UTP zeigt und Galactose-1-Phosphat bevorzugt. Es zeigt einen gerichteten Bi-Bi Mechanismus, wobei zuerst UTP binden muss, gefolgt vom jeweiligen Monosaccharid-1-Phosphat, wodurch sehr wahrscheinlich die Erzeugung des UDP-Monosaccharids vorangetrieben wird. Somit stehen die *in vitro* gemessenen Eigenschaften der USP in Einklang mit der postulierten Funktion der Galactoseverwertung. Damit einhergehend ließ die erste Analyse einer *L. major* USP Gendeletionsmutante (Δ*ugp*) eine verminderte Galactosylierung der LPG Seitenketten vermuten.

Darüber hinaus konnte gezeigt werden, dass bereits die heterozygote Deletion der *USP* in der Δ*ugp* Mutante ausreichend war, um die restliche LPG Expression dieses Stammes einzudämmen, und

demzufolge die Rolle der USP im UDP-Galactose Stoffwechsel verdeutlicht. Interessanterweise war es trotz mehrmaliger Versuche nicht möglich eine Doppelmutante (Δ*ugp*/Δ*usp)* zu generieren, was darauf hindeutet, dass UDP-Gal und/oder UDP-Glc in *L. major* essentiell sind. Letztlich wurde die subzelluläre Lokalisation der USP und einiger anderer an der UDP-Glc/UDP-Gal Biosynthese beteiligter Enzyme aufgeklärt, wobei sich herausstellte, dass es bedeutende Unterschiede mit anderen Trypanosomatiden gibt, die, anders als *L. major*, ihre UDP-Zucker in spezialisierten Organellen, den Glycosomen, synthetisieren.

Schlagwörter: Galactose, UDP-Zucker Pyrophosphorylase, LPG, Leishmanien

Summary

The protozoan parasites *Leishmania spp.*, causing tropical and sub-tropical diseases called leishmaniases, are surrounded by a thick glycocalyx that protects them from the hostile environments in which they live. This cellular coat mainly consists of unique phosphoglycans, comprising the highly abundant lipophosphoglycan (LPG) and mucin-like proteophosphoglycans (PPGs), as well as low molecular weight glycoinositolphospholipids (GIPLs) and is indispensable for survival of the parasite in the insect vector and for establishment of infection in mammals.

Leishmania phosphoglycans are extremely rich in galactose and require thus a specialized enzymatic machinery to cover the high demand on UDP-galactose (UDP-Gal) for biosynthesis. The recently biochemically characterized *L. major* UDP-glucose pyrophosphorylase (UGP), very specifically utilizing glucose-1-phosphate and UTP to form UDP-Glucose (UDP-Glc) and pyrophosphate, was supposed to be the key enzyme in UDP-Gal biosynthesis, either via subsequent epimerization of UDP-Glc or by uridylyl transfer from UDP-Glc to galactose-1-phosphate. Targeted gene deletion of UGP (Δ*ugp*), however, only partially affected the synthesis of the galactose rich phosphoglycans, while no alteration in the abundant galactose-containing GIPLs was found. Consistent with these findings, Δ*ugp Leishmania* virulence was only modestly affected. These data implied that *Leishmania* elaborates a UDP-Glc independent salvage pathway for UDP-Gal biosynthesis and is consistent with the absence of GALT gene essential for the Leloir pathway in *Leishmania* genome. However, a homologue of the plant UDP-sugar pyrophosphorylase (USP) was found in *Leishmania* parasites and several other protists. Characteristic for this new class of enzyme (EC 2.7.7.64), *L. major* USP catalyzes the reaction of a broad pool of monosaccharide-1-phosphates, such as galactose-, glucose-, xylose-, L-arabinose-, galacturonic acid- or glucuronic acid-1-phosphate with UTP to form the respective UDP-monosaccharide and pyrophosphate. We have notably shown that this enzyme possesses a high affinity for UTP, favors Gal-1-P and proceeds via an ordered Bi-Bi substrate mechanism in which UTP binds first followed by the sugar monophosphate, and thus most likely promotes nucleotide sugar synthesis rather than their pyrophosphorolysis. The *in vitro* characteristics of USP are hence in perfect agreement with a postulated function of this enzyme in galactose salvage. In agreement with this role, first analyses of the *L. major* USP gene deletion mutant suggest a reduction of side chain galactosylation of the abundant cell surface polysaccharide LPG. Moreover the heterozygous deletion of *USP* in the Δ*ugp* mutant abolished the residual LPG expression that was still present in the Δ*ugp*, thus supporting a role of USP in the UDP-galactose pathway. Interestingly, a mutant deficient in both UGP and USP could not be obtained despite repeated attempts suggesting an essential role for UDP-Gal and/or UDP-Glc. Finally, the cytosolic localization of USP and several other enzymes involved in the UDP-Glc/UDP-Gal biosynthesis was established, highlighting an important difference with other trypanosomatids that seem to synthesize these nucleotide sugars in a specialized organelle called glycosomes.

Keywords: galactose, UDP-sugar pyrophosphorylase, LPG, *Leishmania*

CHAPTER 1 – General Introduction

1.1 *Leishmania*, Leishmaniasis and Leishmanicidals

Leishmania parasites are protozoan responsible for the disease leishmaniasis occurring in tropical regions of America and Africa and temperate regions of South America, South Europe and Asia. According to the World Health Organization, 12 million people are infected worldwide with an annual incidence of approximately 2 million new cases and 350 million people are threatened by these parasitic infections (*Farrel*, *2002*; *WHO*, *2009*).

Depending on *Leishmania* species and its host fitness the severity of symptoms range from disfiguring local or diffuse cutaneous lesions to mucocutaneous and lethal visceral appearances. Cutaneous leishmaniasis, with clinical manifestations of up to 200 skin ulcers and sore wounds of several centimeters, is the most frequent form and is transmitted by *L. major*, *L. tropica*, and *L. aetiopica*, as well as *L. braziliensis*. The latter also provokes mucocutaneous leishmaniasis, which can lead to necrosis of mucosa, nose, palate, tongue and lips. Lethal visceral leishmaniasis, caused by *L. donovani* and *L. infantum*, affects the reticulo-endothelial system and therein lymph nodes, spleen and liver. Uncured, this fatal form of leishmaniasis leads to death by a chance of 90 %, whereas the mortality rate decreases to 15 % after medical treatment (*WHO*, *2009*).

At present neither a vaccine nor specific drug without any drastic side-effect and low cost is available for prevention or therapy. It is alarming, that the few known chemotherapeutical drugs in use are compromised by a quick development of resistance. For example, a widespread resistance to the front line drugs pentavalent antimonials like sodium stibogluconate (PENTOSAM®) or meglumine antimoniate (GLUCANTIME®) has occurred in many countries while its biochemical mode of action is still under investigation (*Ashutosh et al.*, *2007*). Most of the currently used second-line drugs like Amphotericin B, Paromomycin (Aminosidine) and Miltefosine (IMPAVIDO®) arose from empirical testing or different therapeutic indications, being unaware of its exact molecular mechanism in the parasite's system much less the human ones.

Amphotericin B is believed to interact with membrane ergosterol (*Kshirsagar et al.*, *2005*) and displays severe side effects. A new formulation of a liposomal Amphotericin B (AMBISOME®) has much less adverse effects but its costs are high and unachievable for populations living in the endemic areas (*Croft and Coombs*, *2003*; *Sundar et al.*, *2003*).

The orphan drug paromomycin sulfate is an antibiotic aminoglycoside which inhibits protein synthesis by binding to 30S ribosomal RNA (*Kanyok et al.*, *1994*). Resistance could already be reported in *in vitro* studies (*Maarouf et al.*, *1998*). If resistance mutations are stable, transmission from such patients would lead to primary resistance in others (*Davidson et al.*, *2009*).

Recent discovery of miltefosine (IMPAVIDO®) as the first oral drug for treatment of cutaneous and visceral leishmaniasis gave new hope in treatment of Leishmaniasis. Cure rates are around 95% for

visceral and cutaneous infections (*Fischer et al.*, *2001*). It is a highly efficient and simple molecule, stable at room temperature and, compared to others, has tolerable side effects like nausea and diarrhea. Nevertheless its teratogenicity is one main disadvantage. Unfortunately, again, in vitro studies have shown miltefosine resistance developing quickly in Leishmania promastigotes (*Perez-Victoria et al.*, *2006*).

New therapeutical approaches based on the knowledge of *Leishmania* biology are thus needed.

1.2 Biology & Pathobiology

The protozoan *Leishmania* is a single-celled eukaryotic and obligate living endoparasite, unable to thrive without its two hosts, an insect vector and a mammalian reservoir. According to this biphasic lifestyle, *Leishmania* adapted a polymorphic phenotype, as there are the single anterior flagellated promastigotes with a long and slender body of about 20 x 2 µm, living intercellularly within the midgut of the insect vector, and the non-flagellated amastigotes having an ovoid body of about 4 µm in diameter, able to persist or proliferate intracellularly within macrophages. Both promastigotes and amastigotes house particular organelles like the kinetoplast, one big mitochondrium near the flagella rod, containing around 15% of the total DNA, which groups *Leishmania* into the order of kinetoplastida (**Figure 1**). Further taxonomical classification of kinetoplastida separates the two families of free living, double flagellated *Bodonida* from the usually parasitic, single flagellated *Trypanosomatida*. The latter can be sub grouped into nine distinct genders including the gender *Trypanosoma* and *Leishmania* responsible for human diseases (*Simpson et al.*, *2006*).

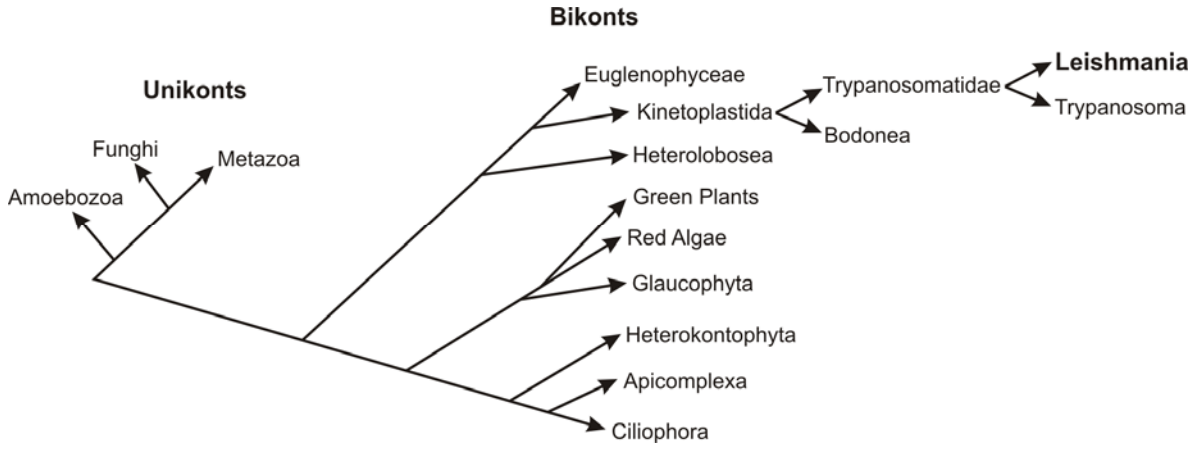

Figure 1. **Classification of the genus *Leishmania*** (based on Cavalier-Smith 2003 (*Cavalier-Smith and Chao*, *2003*)).

1.2.1 Hosts

In nature, *Leishmania* parasites are alternatively hosted by an insect vector or by mammalian reservoirs. Approximately 70 species, belonging to the genera *Phlebotomus* and *Lutzomyia*, are proven or suspected habitats for *Leishmania* parasites, which show specialization to different sand fly species (*Killick-Kendrick*, *1990*). The nocturnal insects feed on plant saps, but before they are able to lay their eggs in wet soil rich organic material the females need a blood meal (*Lane et al.*, *1985*). The sand fly feeding on a potential reservoir host, like human, dog or rodent, is one of the crucial events within *Leishmania* life cycle. *Leishmaniases* are zoonoses or zoonotic, meaning that the causative agent usually stems from animal reservoirs which are responsible for the long term maintenance of *Leishmania* in nature. Often parasites persist in these animals, displaying only mild symptoms while dogs commonly develop a fatal disease (*Sang et al.*, *1992*).

1.2.3 Life Cycle

In the fly

Sand fly infection begins with ingestion of blood from a mammalian reservoir, containing amastigote infected macrophages or free floating parasites (**Figure 2**). Within the insect midgut digestion of the blood-meal is initiated with secretion of digestive enzymes and a peritrophic membrane, which completely covers the early blood-meal as sac of chitinous mucopolysaccharides. Here, amastigotes quickly start differentiating into small, sluggish procyclic promastigotes entering the first multiplication step in the sand fly vector, always facing onslaught of digestive proteinases and premature excretion. Accordingly, surviving procyclics develop into slender, agile and non-dividing promastigotes, called nectomonads, and exit the lethal casing into the interluminal space via the release of chitinases (*Schlein et al.*, *1991*; *Rogers et al.*, *2008*; *Sacks*, *2001*). *Leishmania* nectomonads first attach to the midgut thereby avoiding excretion and thereafter directly migrate by taxic responses (saliva- and sugar-taxis) to the anterior foregut (*Kamhawi et al.*, *2004*; *Barros et al.*, *2006*). Accordingly, nectomonads enter a new stage, which classifies them as leptomonad promastigotes, starting the second round of multiplication of an insect sugar-meal phase (*Gossage et al.*, *2003*). Finally, two additional stages are observed at the stomodeal valve, haptomonads and metacyclics. Haptomands are non-motile short flagellated and highly specialized promastigote forms building a parasite plug and are thought being responsible for the ongoing destruction of the constrictor by release of chitinases, leading to a successive parasite leakage (*Schlein et al.*, *1992*; *Volf et al.*, *2004*). The unattached, motile metacyclics display the infective stage behind the stomodeal valve and are highly adapted for an effective transmission, displaying a small cell body with elongated flagellum and a dense glycocalyx. A gel-like matrix secreted by *Leishmania*, termed promastigote secretory gel (PSG), is thought to contribute to a behavioural manipulation of the fly (*Kamhawi*, *2006*; *Bates and Rogers*, *2004*; *Rogers et al.*, *2004*). That is the so called ‘blocked fly hypothesis’ as a ‘blocked’ sand

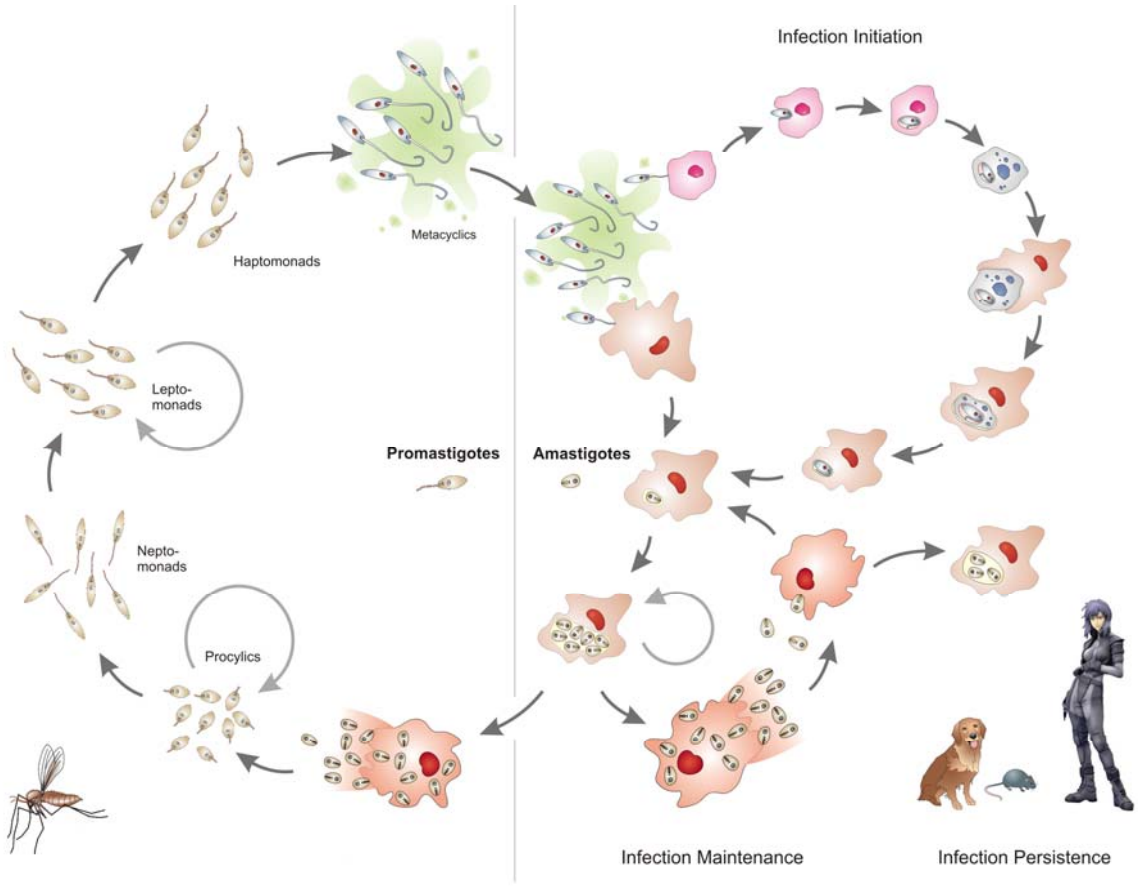

Figure 2. ***Leishmania* digenetic life cycle.** *Leishmania* parasites are transferred by an insect sand fly vector into mammals, their natural reservoir hosts. They are present in two main morphological forms: amastigotes that survive and proliferate inside mammalian macrophages, and the different stages of extracellular promastigotes that multiply in the sand fly vector.

fly attempts to feed multiple times, spreading its infectious load, whereby it regurgitates wide ranging doses of 10-10.000 metacyclics into the vertebrate host (*Rogers et al.*, *2004*; *Rogers et al.*, *2009*).

In mammalian

Upon dermal transmission *Leishmania* metacyclics rely on passive invasion and attraction and are confined to professional phagocytes like their main targets the macrophages, but infect also neutrophils and dendritic cells (*Rittig and Bogdan*, *2000*; *Sacks and Sher*, *2002*) (**Figure 2**).

Attracted by tissue damage neutrophils are the first leukocytes that migrate to the site of infection ingesting *Leishmania* parasites but without killing them. Accordingly, the parasite infected apoptotic neutrophils attract macrophages by chemokines. Their subsequent phagocytosis lessens inflammatory signalling which is beneficial for *Leishmania* survival, while allowing a "silent" entry of parasites into their final host cells, the macrophages, resembling a "Trojan horse" strategy (*van Zandbergen et al.*, *2007*; *Peters et al.*, *2008*).

Within macrophages *Leishmania* efficiently circumvents lysosomal disintegration, by preventing phagosome maturation, the oxidative burst, and altering interleukin signalling, like the inhibition of

IL-12 release, which is an essential cytokine for the development of acquired resistance to most intracellular pathogens (*Piedrafita et al.*, *1999*; *Sacks and Sher*, *2002*).
Upon replication the accumulating amastigotes are finally released by cell burst into the interstitial lumen and blood, where they encounter new target cells for re-infection.

Cultivation of this dimorphic protozon parasite is nowadays feasible. At least for the flagellated promastigotes this could fairly be accomplished. In culture media their growth resembles promastigote development within the fly, since promastigotes and metacyclics can be detected in the stationary phase. Keeping macrophage colonizing amastigotes alive *in vitro* as so called axenic amastigotes is discussed concerning their experimental significance (*Gupta et al.*, *2001*).

1.2.3 Genome

To date the genomes of three *Leishmania* species from *L. major*, *L. infantum* and *L. braziliensis (Ivens et al., 2005; Peacock et al., 2007)*, as well the ones from *Trypanosoma brucei* and *T.cruzi* have been sequenced (*Aslett et al.*, *2009*), allowing insights not only into the unique aspects of the biology of these parasites, but also eukaryote evolution, like their early divergence. The *L. major* Friedlin genome comprises 32.8 Mb in size, with a diploid karyotype of 36 chromosomes and an estimated number of 8311 genes and 900 RNA genes (*Dujardin*, *2009*). The key differences with other eukaryotes are an unusual genome organization into polycistronic gene clusters without gene fragmenting introns, simplified transcriptional machinery, and mRNA trans-splicing coupled with polyadenylation. The gene clusters can be divergently organized in head-to-head or in a convergent tail-to-tail fashion, whereas transcription is initiated bi-directionally in the divergent strand-switch regions between these clusters, terminating in the strand-switch region, which separates convergent clusters. Genes within these large (60 kb) transcripts of polycistronic mRNA are trans-spliced at their 5'-ends by addition of a 40 bp spliced-leader RNA (SL-RNA) and a cap structure. This so called trans-splicing is, compared to cis-splicing, a special form of RNA processing where exons from two different primary RNA transcripts are joined end to end and ligated. Polyadenylation occurs at the 3'-end, whereas the 3'-untranslated regions (3'UTR) drastically influence mRNA stability and translational efficiency (*Clayton et al.*, *2000*). A promoter based gene regulation is almost absent in *Trypanosomatids*, thus the main control of protein level or activity is governed post-transcriptionally by mRNA stability (*Clayton et al.*, *2000*), translationally or post-translationally via protein modification and half life. *Trypanosomatids* demonstrate extensive posttranslational protein modification, especially for surface and secreted proteins, and have a considerable species-specific repertoire of glycoconjugate biosynthetic enzymes (*Ivens et al.*, *2005*). Gene duplication and amplification are biological mechanisms to modulate gene expression and enabling a rapid adaptation to a changing environment. In *Leishmania* this process is well documented, as we can find tandem repetitions of housekeeping

genes required in high abundance like tubulin or rRNA (*Spithill and Samaras*, *1987*; *Inga et al.*, *1998*; *Kebede et al.*, *1999*), the amplification of short or long chromosome fragments in the form of linear or circular DNA (*Papadopoulou and Ouellette*, *1993*) and ploidy changes in the entire chromosome or genome (*Cruz et al.*, *1993*).

1.3 *Leishmania* cell surface and roles in pathogenicity

The *Leishmania* promastigote glycocalyx is composed of phosphoglycans (PGs) that comprise the highly abundant lipophosphoglycan (LPG) and mucin-like proteophosphoglycans (PPGs), glycoinositolphospholipids (GIPLs) and GPI-anchored proteins such as gp63, also referred as 'leishmanolysin'. All these molecules belong to the GPI-membrane anchor family by virtue of containing the conserved backbone structure $Man_{(\alpha 1\text{-}4)}GlcN_{(\alpha 1,6)}$-phosphatidylinositol-lipid. Beside, *Leishmania* secretes PPGs and a phosphoglycosylated acid phosphatase (sAP) (**Figure 3**).

Phosphoglycans are glycoconjugates that share a conserved backbone polymer of phosphorylated galactose-mannose disaccharide repeats ($-_6Gal_{(\beta 1\text{-}4)}Man_{(\alpha 1)}\text{-}PO_4-$) with a neutral oligosaccharide cap. Among *Leishmania* species, the phosphoglycan domain is polymorphic and is either unsubstituted as

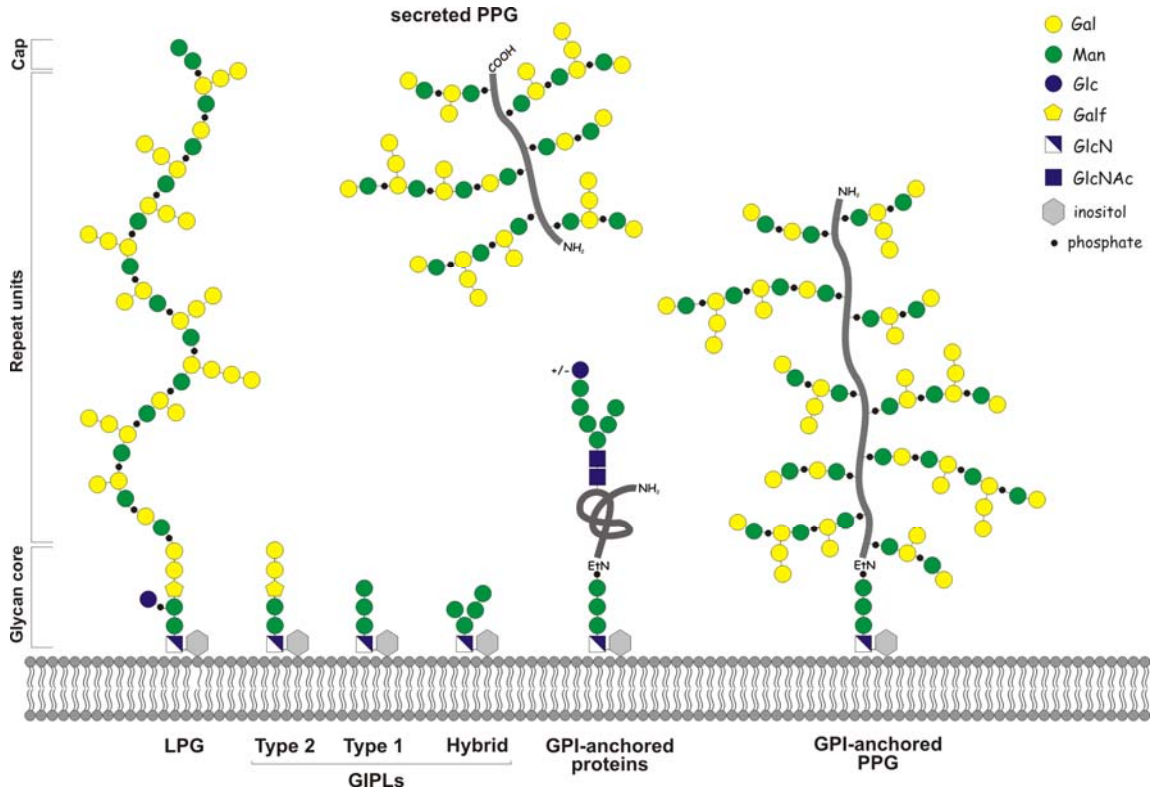

Figure 3. ***Leishmania major* surface glycoconjugate structures** (based on *McConville et al., 1990, Naderer et al., 2004* and *Ilg, 2000b*). No arabinose capping of galactose on phosphoglycan side chains is depicted, which would represent the situation in metacyclic promastigotes.

in *L. donovani* or variably substituted with oligosaccharide side chains (*L. major*, e.g.) *(Ferguson et al., 1994*; *Mcconville et al., 1995*; *Ilg, 2000b*).

These unique glycan structures represent the protecting interface between parasite and an always changing, hostile environment, providing survival and conferring virulence, and thus have drawn the attention of researchers in order to find attractive drug targets within their biosynthetic machinery. Several deletion mutants have been designed in order to dissect their biosynthesis in *Leishmania* and evaluate their importance for parasitic life.

1.3.1 GPI-anchored Proteins

In *trypanosomatids*, proteins are often attached to the outer cell surface via GPI-anchors. Usually an ethanolamine phosphate mediates attachment of the protein C-terminus to the glycan core ($Man_{(\alpha1-2)}Man_{(\alpha1-6)}Man_{(\alpha1-4)}GlcN_{(\alpha1-6)}PI$), which can be substituted in a protein, species and developmental stage specific manner (**Figure 4**). GPI-Proteins are functionally diverse including receptors, coat proteins and hydrolases. Amongst the latter, the major surface protease (MSP) gp63 has been widely studied, as it is abundant in all *Leishmania* species. Gp63 has been described to hydrolyze surface opsonized complement factors (*Brittingham et al.*, *1999*), and mediate ligand attachment to macrophage receptors (*Alexander*, *1992*; *Joshi et al.*, *2002*), thus promote phagocytosis and the save entry of the parasites. Gene deletion of this protein (*Hilley et al.*, *2000*) lead to a 10-fold increased sensitivity to complement lysis and displayed attenuated virulence in mice. This protease is however not essential for continued survival after infection establishment. Recent studies suggest that amastigote and promastigotes both express multiple MSP isoforms, differing biochemically and localizing differently between the parasite stages, and thus play diverse roles in the extracellular versus intracellular forms of *Leishmania* species (*Hsiao et al.*, *2008*).

1.3.2 LPG

The unique lipophosphoglycan is the dominant protruding structure all over the cell surface of *Leishmania* promastigotes, with an estimated number of approximately 3-5 million copies expressed per cell (Sacks DL 1992). Within all *Leishmania spp.* LPG is made of four domains, as there are the PI-anchor, a glycan core, a backbone of 15-30 $_6Gal_{(\beta1-4)}Man_{(\alpha1)}PO_4$ repeating units, and an oligosaccharide cap structure, mostly a mannose disaccharide (*Turco and Descoteaux*, *1992*; *Descoteaux and Turco*, *1999*; *Mcconville et al.*, *1993*; *Guha-Niyogi et al.*, *2001*) (**Figure 4**). Some of these elements are highly unusual for a eukaryotic glycoconjugate. This includes the presence of a galactofuranose in the glycan core and the repeating phosphorylated saccharide backbone containing a unique 4-O-linked mannose. While the GPI-lipid anchor of LPG is highly conserved, but glycosylated on the second mannose residue, the backbone varies stage specifically in its length. Most strikingly is the doubled increase in size as displayed by metacyclic stages of *L. major* (*Bogitsh et al.*, *2005*).

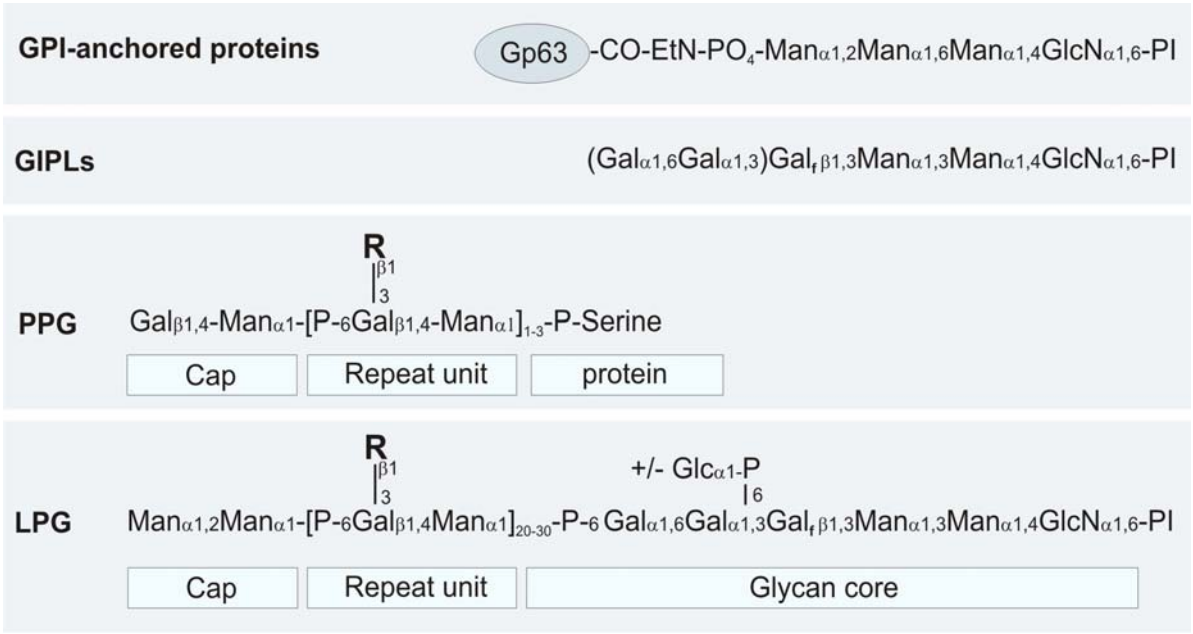

Figure 4. ***Leishmania major* surface determinant configurations** (based on *McConville et al., 1990, Naderer et al., 2004* and *Ilg, 2000b*). PPG and LPG are both composed of the same phosphoglycan repeat unit, which are additionally equipped, indicated by the residue (R). R = H-; Galβ1-; Galβ1-3Galβ1-; Araβ1-2Galβ1-; Araβ1-2Galβ1-3Galβ1-; Galβ1-3Galβ1-3Galβ1- .

Among *Leishmania* species a differing LPG backbone decoration is found. For example, *L.major* and *L.tropicana* LPG is additionally branched with galacto- or gluco-oligosaccharide side chains, respectively, which are optionally covered with additional arabinose caps. In contrast *L.donovani* LPG has an unmodified LPG backbone.

LPG is thought to shield the parasites by preventing insertion of the macrophage C5-9 membrane attack complex (MAC) into the membrane in order to avoid perforation. The very long LPG chain of metacyclics seems to be of advantage for this role (*Sacks and Sher*, *2002*). After phagocytosis LPG repeating units have been shown to transiently inhibit phagosome maturation. This delay may be pivotal, allowing sufficient time for promastigotes to differentiate into more hydrolase-resistant amastigotes (*Desjardins and Descoteaux*, *1997*; *Dermine et al.*, *2000*). Furthermore, Leishmania LPG protects invading promastigotes from the modest oxidative burst generated during phagocytosis consistent with its ability to scavenge oxygen radicals *in vitro* (*Chan et al.*, *1989*).

Besides innate microbicidal responses, macrophages can initiate the host activation cascade by presenting antigens and providing regulatory cytokines to T cells. Studies suggest intracellular protozoa interfering with the immune-initiation functions of macrophages. A striking dysfunction observed in macrophages and induced by *Leishmania* is their inability to produce IL-12, which is an essential cytokine for the development of acquired resistance to most intracellular pathogens, inducing interferon-γ and T helper type 1 (T_H1) cell differentiation. This was shown by inhibiting interleukin IL-12p40 expression with LPG (*Piedrafita et al.*, *1999*; *Sacks and Sher*, *2002*). During transformation from promastigotes to amastigote stage the major surface macromolecules like LPG are down-regulated, whereas GIPLs are expressed at near-constant levels in both developmental stages

(*Mcconville and Blackwell*, *1991*; *Winter et al.*, *1994*; *Bahr et al.*, *1993*; *Schneider et al.*, *1993*). Since LPG is not expressed by amastigotes, while only retaining a glycocalyx of GIPLs, its role is transient and confined to only the early stage of infection, and down regulation seems indispensable as LPG would compromise parasite survival in the host cell, as it is associated with activation of dendritic cells, natural killer (NK) cells and NK T cells (*Becker et al.*, *2003*; *Amprey et al.*, *2004*; *Aebischer et al.*, *2005*).

Several gene deletion mutants interfering with LPG synthesis have been generated during the last years, many of them also influencing other surface determinants. LPG1, a putative galactofuranosyltransferase, was the first and to date only mutant lacking LPG exclusively. This mutant revealed, LPG being of importance for the initial establishment of infection, displaying a typical attenuated lesion phenotype in mice footpad infection studies (*Spath et al.*, *2000*). Contradictory *L. mexicana* LPG is not required for infection of macrophages (*Ilg*, *2000a*). Furthermore its LPG is not modified nor rearranged during metacyclogenesis. Thus *Leishmania* species differ dramatically in their reliance upon LPG virulence (*Turco et al.*, *2001*).

1.3.3 GIPLs

Low molecular weight glycoinositolphospholipids (GIPLs) are the major class of glycolipids, with an estimated number of 10 million molecules per cell, synthesized by all *Leishmania* stages (*Mcconville et al.*, *1993*; *Bogitsh et al.*, *2005*). Depending on species or developmental stage three types of GIPLs have been described in *Leishmania*: type-1 containing the same glycan structure as protein anchors ($Man_{(\alpha1\text{-}2)}Man_{(\alpha1\text{-}6)}Man_{(\alpha1\text{-}4)}GlcN_{(\alpha1\text{-}6)}PI$), type 2 reflecting analogues of the LPG glycan core, which includes also the above mentioned galactofuranose ($Gal_{(\alpha1\text{-}6)}Gal_{(\alpha1\text{-}3)}Gal_{\mathbf{F}(\alpha1\text{-}3)}Man_{(\alpha1\text{-}3)}Man_{(\alpha1\text{-}4)}GlcN_{(\alpha1\text{-}6)}$ PI) but without any glucose-modification at the second mannose residue, and hybrid type GIPLs containing features of both the protein and the LPG anchor (*Guha-Niyogi et al.*, *2001*) (**Figure 4**). Studies of the functions of GIPLs are more limited than studies of LPG. Nevertheless the GIPLs are believed to play also a role in modifying signaling events within macrophages, by inhibiting PKC signaling and ROS production (*Proudfoot et al.*, *1995*; *Tachado et al.*, *1997*; *Mcneely et al.*, *1989*). The *Leishmania major* ADS (alkyldihydroxyacetonephosphate synthase) gene deletion mutant deficient in a key component in ether lipid biosynthesis, lacks GIPLs and LPG (*Zufferey et al.*, *2003*). Unexpectedly gp63 that is normally anchored by an ether lipid was still synthesized. The effect on virulence of the ADS mutant was not stronger than the one observed with LPG1 suggesting a minor role for GIPLs in the amastigote stage. Similarly, the UDP-galactofuranose mutase null mutant (GLF) represents one of the rare sugar-metabolic mutants defective in GIPLs synthesis, since the unique galactofuranosyl residue was missing for type-2 GIPL synthesis. Because the LPG core is composed of galactofuranose, the whole LPG synthesis was perturbed, leading to the typical LPG1 phenotype. These observations implied that GIPLs are not essential for infectivity (*Kleczka et al.*, *2007*).

1.3.4 PPGs

The proteophosphoglycans (PPG) constitute a family of molecules composed of the characteristic $_6Gal_{(\beta 1\text{-}4)}Man_{(\alpha 1)}PO_4$ phosphoglycan repeating units directly attached to serine residues of proteins (**Figure 4**). The fairly uncommon and heavily O-glycosylated proteins comprise membrane bound (mPPG), filamentous (fPPG) or specific amastigote (aPPG) and promastigote (pPPG) proteophosphoglycans. In addition, the majority of *Leishmania* species secretes phosphoglycosylated enzymes like non-specific acid phosphatases (sAP). The mucin-like filamentous PPGs are secreted by the flagellar pocket of pro- and amastigotes and are also referred as promastigote secretory gel (PSG) embedding metacyclic promastigotes in the stomodeal valve of the fly. These structures predominantly consist of phosphoglycans ($96\%_{w/w}$) and in small amounts of amino acids. It is estimated that every second amino acid carries a phosphoglycan chain, conferring proteinase resistance to the polypeptide, and inhibiting secondary structure formation. The membrane bound PPG is linked via GPI-anchorage and is also heavily glycosylated providing many more potential receptor- and complement-binding sites than LPG, displaying up to 800 serine-linked phosphoglycan chains clustered in a specific domain, whereas LPG carries one (*Ilg*, *2000b*).

The secreted PPGs partially block the stomodeal valve forcing the fly to feed more often and thus increase the chance of parasite transmission. Very recently it was demonstrated that regurgitated PPGs by *Leishmania* infected sand flies powerfully recruited macrophages, thereby increasing L-arginine catabolism and the synthesis of polyamines essential for intracellular parasite growth. Thus demonstrating that secreted PPGs are essential components of the infectious sand fly bite for the early establishment of infection (*Rogers et al.*, *2009*). An *L. donovani* GDP-mannose transporter null mutant (LPG2) lacking both LPG and PPG is indeed rapidly killed in the sandfly midgut, while LPG1 remained viable, thus implying hydrolytic protection (*Descoteaux et al.*, *1995*). Accordingly the LPG2 mutant is avirulent in mouse and macrophage infection studies (*Spath et al.*, *2003a*) while the LPG1 mutant only presents an attenuated virulence. These results suggest that PPGs play important roles in parasite survival in insect vector and mammalian host. Remarkably some viable *L. major* LPG2 revertants ($LPG2^{REV}$) could be recovered months after application from the site of infection. However, this persistence is not sufficient to maintain protective immunity. Intriguingly, *L. mexicana* differs again in the mechanism of infection, since the lack of LPG and PPG did not lead to reduced virulence (*Ilg et al.*, *2001*).

1.4 From Monosaccharide to Glycan

The glycocalyx and secreted phosphoglycans of *Leishmania* parasites are very rich in galactose. Taken into account that these structures provide essential functions for parasite survival within its hosts, the enzymes involved into phosphoglycan biosynthesis might constitute attractive drug targets.

1.4.1 Glycan assembly

Leishmania glycoconjugate core structures are synthesized within the endoplasmic reticulum (ER) and optionally processed in the Golgi apparatus. The similar ER derived core structures of LPG and type-2 GIPLs are galactofuranosylated within the Golgi at the terminal mannosyl residue, followed by addition of two galactopyranosyl units. Whereas type-2 GIPL assembly is then complete, synthesis of the core glycan is followed by addition of 15-30 phosphoglycan repeating units homogeneously expanding from the terminal galactosyl residue forming LPG and are generally capped by two mannose in *L. major* (*Turco and Descoteaux*, *1992*; *Mcconville et al.*, *1993*). Synthesis of hybrid and type-1 GIPLs, as well as N-glycosylated GPI-anchored proteins like gp63, only involves the ER and its set of attributed transferases, whereas O-glycosylation of secreted and GPI-anchored proteophosphoglycans (sPPGs/mPPGs) is taking place within the Golgi and is initated by the transfer of phosphomannose to serine rich sequences of the polyamine core, subsequently elongating the mannosyl residue with phosphomannogalactopyranosyl repeating units, similarly to LPG biosynthesis. The phosphoglycan portions of LPG and PPG are assembled by the sequential and alternating transfer of mannose-P and galactose from their respective nucleotide-sugar donors, GDP-Man and UDP-Gal. Depending on the species of *Leishmania*, additional branching sugars can be added, creating a remarkable array of side chains, indicative for a diverse subset of species dependent nucleotide sugar transferases. For example, in *L. major* the C3 hydroxyl group of galactose is galactosylated with one to four residues that may be capped by arabinose (*Mcconville et al.*, *1995*; *Varki et al.*, *2008*).

The maintenance of *Leishmania* glycan assembly drastically depends on nucleotide sugar availability (*Stewart et al.*, *2005*; *Spath et al.*, *2003b*; *Capul et al.*, *2007*). Consistent with the glycocalyx composition, the main nucleotide sugars detected in *L. major* promastigotes comprise a pool of UDP-Glc, followed by UDP-N-acetylglucosamine (UDP-GlcNAc), UDP-Gal, GDP-Man, completed by smaller amounts of UDP-galactofuranose (UDP-Gal_F), GDP-D-arabinose (GDP-Ara) and GDP-L-fucose (GDP-Fuc). The pool of UDP-Glc synthesized from the abundant metabolites UTP and glucose-1-phosphate by the UDP-glucose pyrophosphorylase (UGP) is relatively large and partially used to produce UDP-Gal by epimerization. The biosynthesis of these two nucleotide sugars is thus intimately linked.

To date the Golgi import of GDP-Man, GDP-Ara, GDP-Fuc and UDP-Gal was experimentally confirmed. The generation of *Leishmania major* gene deletion mutants of the GDP-Man (GDP-Ara/GDP-Fuc) transporter or two UDP-Gal transporters referred as LPG2 and $LPG5_{a/b}$ respectively facilitated the dissection of glycoconjugate synthesis (*Hong et al.*, *2000*; *Capul et al.*, *2007*; *Spath et al.*, *2003b*). It has in particular been demonstrated that LPG2 leads to the concomitant deletion of LPG and PPGs and abolishes virulence. Contradictory the deletion of the $LPG5_{a/b}$ in the same species resulted only in attenuated virulence, although both LPG and PPGs are absent. To date this discrepancy remains unexplained.

1.4.2 UDP-Galactose metabolism

The main route of UDP-galactose synthesis within *Trypanosomatidae* engages four enzymes converting glucose into glucose-6-phosphate (1), glucose-6-phosphate into glucose-1-phosphate (2), glucose-1-phosphate into UDP-glucose (3) and UDP-glucose into UDP-galactose (4). The enzymes involved are glucokinase$^{Lmaj/Tcru}$/ hexokinase^{Lmex} (1: GlcK/ HK) (*Caceres et al.*, *2007*; *Pabon et al.*, *2007*), phosphogluco(manno)mutase$^{Tcru/Lmaj}$ (2: PGM/PMM) (*Penha et al.*, *2009*; *Garami et al.*, *2001*), UDP-glucose pyrophosphorylase^{Lmaj} (3: UGP) (*Lamerz et al.*, *2006*) and UDP-glucose 4-epimerase$^{Tbru/Tcru}$ (4: GALE) (*Roper et al.*, *2000*; *Macrae et al.*, *2006*) (**Figure 5**). These enzymes, although not all characterized yet in *L. major*, could be annotated within its sequenced genome, validating the existence of this pathway in *Leishmania*. Furthermore, galactokinase-like genes were found in *L. major* and *T. cruzi* genome, implicating that incorporated galactose can be transformed into galactose-1-phosphate (Gal-1P). Within most organisms, like mammals, plant, fungi, or bacteria, the so called Leloir pathway makes Gal-1P available for energy metabolism, that is glycolysis. The inability of Gal-1P depletion is reflected pathobiologically with the human disease galactosemia, lacking either the already mentioned UDP-glucose 4-epimerase (GALE) or an enzyme called UDP-glucose:galactose-1-phosphate uridyltransferase (GALT). The latter consumes Gal-1P which is activated by a UDP-moiety switch from UDP-glucose, generating UDP-galactose and Glc-1P. Accordingly, GALE converts the C4 hydroxyl group of UDP-galactose into UDP-glucose, which in turn can be re-used for Gal-1P activation. Only catalytical amounts of UDP-glucose are enough to convert Gal-1P into Glc-1P, which is able to enter glycolysis after reconfiguration into glucose-6-phosphate by the above mentioned phosphoglucomutase (PGM) that plays the role of a gatekeeper enzyme between catabolism and anabolism. Hence, in theory high levels of Glc-6P should fuel the anabolic pathway that is UDP-Gal synthesis.

It is likely that UDP-glucose production via activation of Glc-1P with UTP by the UGP enzyme and subsequent epimerization (GALE), is the main route to UDP-galactose in *L. major*, nevertheless it was demonstrated that *L. major* is able to take up and incorporate [^{3}H]-labelled galactose into its glycoconjugates, demonstrating that the existence of a salvage pathway for UDP-galactose in *L.major* (*Turco et al.*, *1984*) is possibly mediated by GALT as in many other organisms.

Thus, due to the fact that both GALT and GALE rely on UDP-Glc abundance within *Leishmania*, UGP can be ascribed as a key enzyme in UDP-galactose formation.

1.4.3 UDP-glucose pyrophosphorylase

The UTP:glucose-1-phosphate uridylyltransferase (EC 2.7.7.9) commonly termed UDP-glucose pyrophosphorylase catalyzes the reaction of Glc-1P and UTP to form UDP-glucose and inorganic pyrophosphate (PP_i). In *L. major* this enzyme is highly specific for Glc-1P or UDP-glucose. Like other pyrophosphorylases UGP follows an ordered bi-bi binding mechanism, with

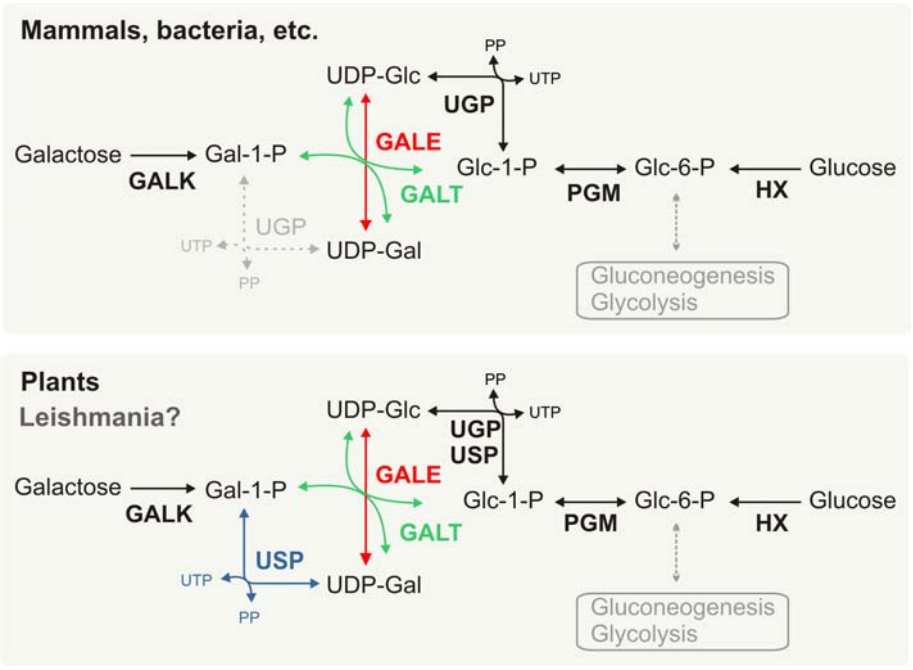

Figure 5. **Biosynthesis of UDP-galactose in various organisms.** UDP-galactose (UDP-Gal) is synthesized *de novo* by epimerization of UDP-glucose (UDP-Glc) by UDP-glucose 4-epimerase (GALT, EC: 5.1.3.2). In addition, galactose-1-phosphate (Gal-1-P) produced from galactose by galactokinase (GALK, EC: 2.7.1.6) is activated by the UDP-glucose:galactose-1-phosphate uridylyltransferase (GALT, EC: 2.7.7.12). These reactions rely on UDP-Glc production from glucose-1-phosphate (Glc-1-P) by the UTP:glucose-1-phosphate uridylyltransferase also named UDP-glucose pyrophosphorylase (UGP, EC: 2.7.7.9). The phosphoglucomutase (PGM, EC: 5.4.2.2) mediating the interconversion of Glc-1-P and glucose-6-P (Glc-6-P) connects the galactose metabolism to gluconeogenesis and glycolysis. Glc-6-P may also originate from phosphorylation of free glucose by glucokinase (EC: 2.7.1.1) or hexokinase (HX, EC: 2.7.1.2). The conversion of Gal-1-P into UDP-Gal described in mammals by Isselbacher is thought to be due to a weak UTP:galactose-1-phosphate uridylyltransferase activity (EC:2.7.7.10) of UGP. In plants, a third pathway for UDP-Gal biosynthesis is mediated by an unspecific UDP-sugar pyrophosphorylase (USP, EC: 2.7.7.64).

binding of UTP preceding entry of Glc-1P by inducing a conformational change from "closed" to "open", whereas for the reverse reaction UDP-glucose binds without the presence of PP_i. Another common feature is the need for a divalent metal ion like Mg^{2+}, which is presumably necessary to stabilize the negative charge of anhydrous phosphate-groups, e.g. UTP. Furthermore it is known from plant UGPs that activity is sequestrated by oligomerization, but the *L. major* UGP was shown to be active as a monomer. Moreover, the recently solved 3D-structure allows insights into substrate-protein interactions and amino acids involved into binding (*Steiner et al.*, *2007*; *Lamerz et al.*, *2006*).

1.4.4 UDP-galactose pyrophosphorylase

More than 50 years ago Luis Federico Leloir determined human GALT activity, and some years later Kurt Isselbacher found an impaired GALT being responsible for congenital galactosemia accompanied by his proposal of a direct activation of Gal-1P by UTP, due to a residual galactose metabolism (*LELOIR*, *1951*; *Isselbacher*, *1958*). To date both pathways are entitled after them, the Leloir pathway using UDP-glucose and the Isselbacher pathway using UTP for direct Gal-1P activation. Some studies elucidated UDP-gal pyrophosphorylase (UDP-gal PPase) activities in different human tissues and

developmental stages, whereas one referred about different splice variants of UGP1 and UGP2 showing that UGP2 displays residual activity to Gal-1P but with a very slow turnover rate compared to GALT, which explains a compensatory and approximately 1000 times higher expression pattern in GALT blocked cells (*Lai et al.*, *2003*; *Leslie et al.*, *2005*; *Wehrli et al.*, *2007*). This finding clarifies that UDP-gal PPase activity can be assigned to the UGP. No specialized and highly active UDP-gal PPase (EC 2.7.7.10) could be identified and biochemically characterized so far.

1.4.5 UDP-sugar pyrophosphorylase

At the beginning of the century, a new class of enzyme termed UDP-sugar pyrophosphorylase (USP) entered the stage, especially drawing attention to plant biologists. Its name is reflecting the broad substrate activity, not only activating hexose-1-phosphates but also pentose-1-phosphates with a stringent nucleotide donor, UTP (**Figure 6**). Several publications today characterized USPs in pea, melon fruit and *Arabidopsis thaliana (Kotake et al., 2004; Dai et al., 2006; Litterer et al., 2006b)*. Interestingly, in 1983 Patricia Lobelle-Rich and Richard Reeves already identified two UTP-utilizing enzymes in *Entamoeba histolytica*, the first was classified as UTP:glucose-1-phosphate uridylyltransferase (UGP, see above) and the second as a new defined UTP:hexose-1-phosphate uridylyltransferase (EC 2.7.7.10, as noted above) (*Lobelle-Rich and Reeves*, *1983*). Whereas the analyzed UGP exhibited activities as expected nowadays as a Glc-1P specific enzyme with a 20-fold greater velocity compared to Gal-1P, the latter enzyme in all likelihood reflected the activities of a USP. Although they did not measure more sugar-1-phosphates, they found a characteristic 1.35 times greater maximum velocity with Gal-1P than Glc-1P. At present the USP was assigned to EC 2.7.7.64 on all proteomic and enzyme databases and is referred under its accepted name UTP:monosaccharide-1-phosphate uridylyltransferase. Completing the above denoted substrate spectrum this class of enzyme utilizes galactose-1P, glucose-1P, glucoronic acid-1P, arabinose-1P and xylose-1P *(Kotake et al., 2004; Dai et al., 2006; Litterer et al., 2006b)*. Especially in plants, more precisely in *Arabidopsis thaliana*, it was shown by deletion mutants that USP is a prerequisite for pollen development (*Kotake et al.*, *2007*; *Schnurr et al.*, *2006*). Overall this enzyme was expected to play a role in salvage pathways. It is retooling hydrolysed polymer-sugars by activation and generating new building blocks for transferases.

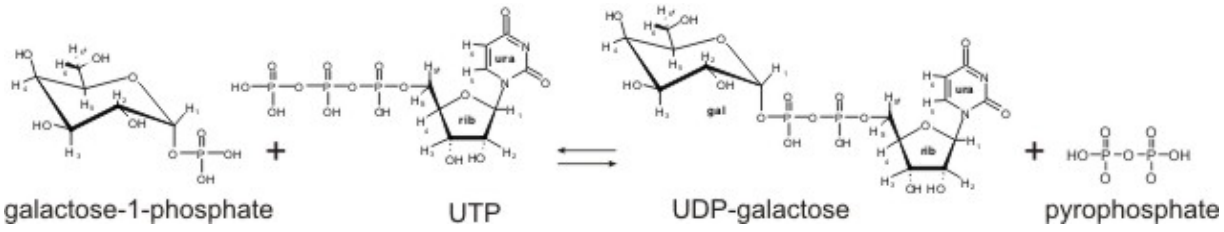

Figure 6. **Exemplified reaction of a UDP-sugar pyrophosphorylase.**

1.4.6 Glycosomes

While speaking of sugars fate within a *trypanosomatid* organism one must not forget mentioning their unique and special microsomal compartments termed glycosomes. They are related organelles to peroxisomes and glyoxysomes lacking a genome and are enclosed by a single, protein dense membrane. Peroxysomes contain redox-enzymes, like catalase, for β-oxidation and other oxidative reactions. In contrast, the specialized plant glyoxysomes are also able to generate glucose from fatty acids, via the eponymous glyoxylate cycle. From early studies it could be shown that at least some pathways or enzymes commonly found in peroxisomes are also present in glycosomes of some *trypanosomatid* species (*Hart and Opperdoes*, *1984*; *Wiemer et al.*, *1996*). Glycosomes and peroxisomes display close evolutionary relationships, like conserved (peroxisomal) targeting sequences (PTS) and homology in peroxin (PEX) proteins required for matrix protein import from *trypanosomatids*, yeast and humans. Targeting sequences are either localized C-terminal (PTS1: -SKL, or conservative variant) or N-terminal (PTS2: [RK]-[LVI]-x5-[HQ]-LA]), but also transport by "piggybacking" on other PTS-proteins is reported (*Gould et al.*, *1989*; *Subramani et al.*, *2000*; *Swinkels et al.*, *1992*). However, the finding that the first seven glycolytic enzymes are localized to the *trypanosomatid* peroxisome-like organelles is unique, hence the name glycosome (**Figure 7**). Beside glycolysis and β-oxidation pathways, also ether lipid biosynthesis, pyrimidine metabolism and purine salvage have been identified (*Parsons*, *2004*; *Opperdoes and Szikora*, *2006*). Interestingly, phylogenetic studies suggest that glycosomal proteins were recruited from a photosynthetic organism through lateral gene transfer, whether by endosymbiosis (*Hannaert et al.*, *2003*) or phagocytosis (*Waller et al.*, *2004*) remains unanswered. Nevertheless, the signature of the genetic donor can be traced in *trypanosomatid* enzymes and pathways, that are related to those of green algae, plants and chloroplasts, implying that the genetic partner was a green algae (*Hannaert et al.*, *2003*). Within *trypanosomatid* parasites the glycosomal compartmentation is thought to prevent toxic accumulation of phosphorylated intermediates and therefore functioning as an alternative to allosteric regulation. This finding was supported by computational metabolic models and a peroxin PEX14 knock-down which compromised glycosomal import of matrix proteins (*Haanstra et al.*, *2008*; *Furuya et al.*, *2002*). Supporting this theory, most of the glycosomal enzymes which are artificially mislocated to the cytosol display cytotoxic effects, like the phosphoglycerate kinase or phosphomannomutase (PMM) from *T.brucei* or *L.major*, respectively (*Blattner et al.*, *1998*; *Opperdoes and Szikora*, *2006*). Enzymes involved in nucleotide-sugar metabolism are speculated to reside within the glycosome, in this regard all *trypanosomatid* hexokinases are glycosomal as well as the putative galactokinases housing equal targeting sequences. Furthermore the already mentioned *L. major* phosphomannomutase (PMM), which also possesses a strong phosphoglucomutase (PGM) activity (*Garami et al.*, *2001*) and the *T.brucei* and *T.cruzi* UDP-glucose 4-epimerases (GALE) are located to glycosomes. Nevertheless, enzymes, like e.g. the transketolase in the pentose phosphate pathway, may also be located in both glycosomes and cytoplasma (*Veitch et al.*, *2004*).

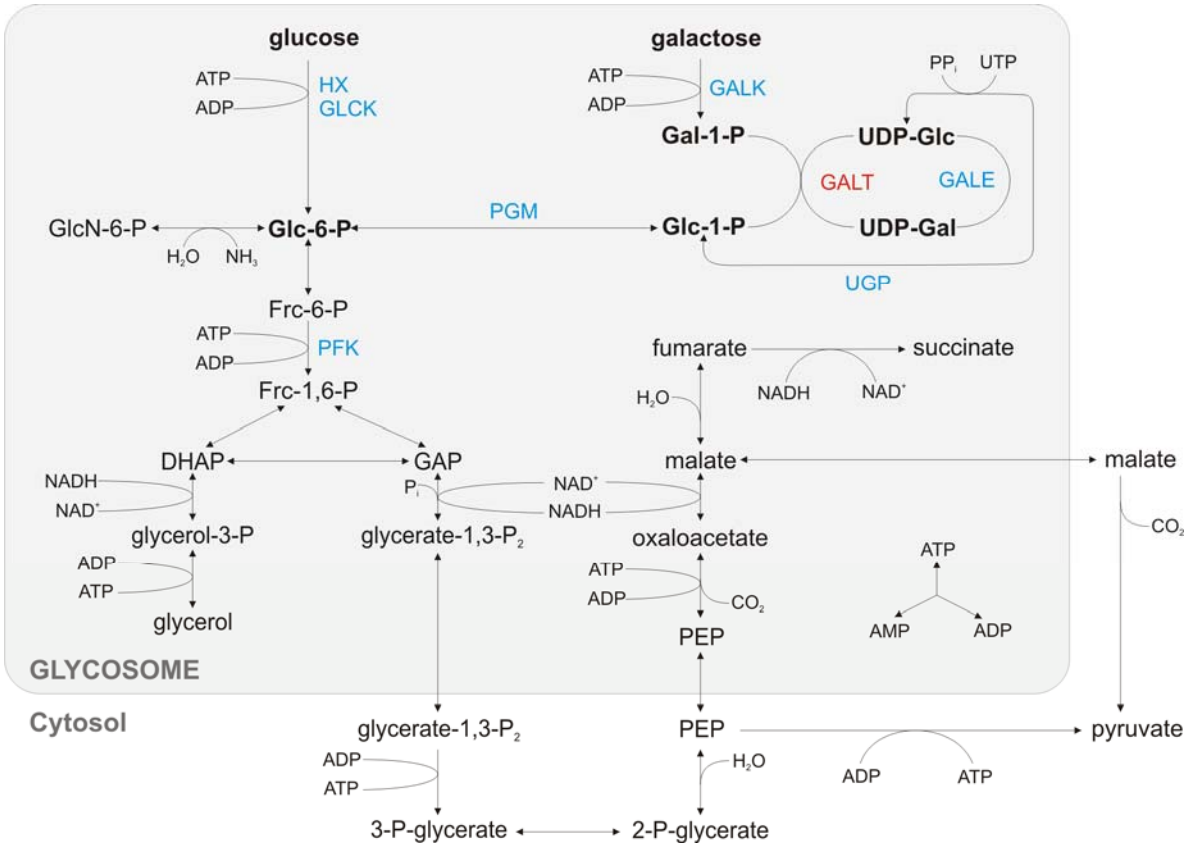

Figure 7. **Glycosomal pathways of carbohydrate metabolism in *Leishmania*** (modified from Opperdoes & Szikora 2006, "*In silico* prediction of the glycosomal enzymes of *Leishmania major* and *trypanosomes*."). Substrates and enzymes representative for this study are highlighted: bold, substrates; blue, the glycosomal assigned enzymes; red, enzymes without any sequence identified in *L. major*, *T. cruzi* or *T. brucei* gene database. HX: hexokinase, GLCK: glucokinase, PGM: phosphoglucomutase, UGP: UDP-glucose pyrophosphorylase, GALE: UDP-galactose 4-epimerase, GALT: UDP-glucose:galactose-1-phosphate uridylyltransferase.

1.5 Objectives

The development of novel and specific therapeutic strategies for treatment of leishmaniasis is desirable, but not achievable without fundamental knowledge of *Leishmania* biology. The glycocalyx of *Leishmania* parasites is known to be essential for parasite virulence, both in the insect and the mammalian hosts. However, the precise nature of the molecules involved is more difficult to determine due to the structural similarities between the glycocalyx components and sharing of several biosynthetic steps. Although a study involving deletion of the GDP-Man transporter clearly indicates that the concomitant deletion of LPG and PPGs is essential for parasitic virulence, a more recent mutant deficient in UDP-Gal transport questioned this result. Since to date this discrepancy remains inexplicable, our goal was to reinvestigate the role of these molecules rich in galactose.

In *Leishmania*, UDP-Gal arises *de novo* by epimerization of UDP-glucose via UDP-glucose 4-epimerase (GALE) or by a salvage pathway thought to involve phosphorylation of galactose by the putative galactokinase and activation into UDP-Gal via the action of UDP-glucose:α-D-galactose-1-phosphate uridylyltransferase (GALT) as described by Leloir. However, the genome of *Leishmania* does not contain any clear homologue of the last enzyme and our first study indicated that *Leishmania* galactose salvage pathway is indeed independent from UDP-glucose production.

Our aim was thus to first delineate the UDP-Gal biosynthetic pathways in *Leishmania* in order to subsequently obtain mutant deficient in UDP-Gal and evaluate the importance of this nucleotide sugar for parasite virulence. In this thesis, we report the identification and detailed biochemical characterization of an unusual enzyme potentially involved in galactose salvage, and present the first evidences of its *in vivo* function.

CHAPTER 2 – Deletion of UDP-glucose Pyrophosphorylase Reveals a UDP-glucose Independent UDP-galactose Salvage Pathway in *Leishmania major*

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Preface – About this manuscript

Prior to this study the *ugp* gene encoding a UDP-glucose pyrophosphorylase (UGP) was identified and biochemically characterized in *L. major* by our research group (Lamerz 2006). The following part of this manuscript aimed at generating a *L. major ugp* deletion mutant (Δ*ugp*) to determine the consequences of the loss of UDP-Glc for viability and virulence of this parasite.

For this purpose, a *L. major* mutant strain was generated by targeted gene deletion, using two selection marker cassettes in a homologous recombination approach, in which the *ugp* alleles were replaced by a resistance gene, respectively. To validate the results, an episomal expression vector ('add-back') was introduced into the Δ*ugp* mutant (Δ*ugp*/+*UGP*), in order to reconstitute the wild type situation. Subsequently, the mutant and add-back strain were analyzed for the presence of UDP-Glc and cell surface determinants of the glycocalyx. Therefore a combination of enzymatic, immunochemical, electrophoretic and mass-spectrometric techniques was employed. Finally, the virulence of the Δ*ugp* and Δ*ugp*/+*UGP* mutant strain was determined in an experimental mouse infection model and by *in vitro* macrophage infection studies.

My contribution to this manuscript comprised the detailed immunochemical analysis of surface lipophosphoglycan and proteophosphoglycan glycoconjugate structures.

The nucleotide sugar UDP-galactose is essential for the biosynthesis of several abundant glycoconjugates forming the surface glycocalyx of the protozoan parasite *Leishmania major*. Current data suggest that UDP-galactose could arise *de novo* by epimerization of UDP-glucose or by a salvage pathway involving phosphorylation of galactose and the action of UDP-glucose:α-D-galactose-1-phosphate uridylyltransferase as described by Leloir. Since both pathways require UDP-Glucose, inactivation of the UDP-glucose pyrophosphorylase (UGP) catalyzing activation of glucose-1 phosphate to UDP-glucose was expected to deprive parasites of UDP-galactose required for *Leishmania* glycocalyx formation. Targeted deletion of the gene encoding UGP, however, only partially affected the synthesis of the galactose rich phosphoglycans. Moreover, no alteration in the abundant galactose-containing glycoinositolphospholipids was found in the deletion mutant. Consistent with these findings, the virulence of the UGP deficient mutant was only modestly affected. These data suggest that *Leishmania* elaborates a UDP-glucose independent salvage pathway for UDP-galactose biosynthesis.

Introduction

Leishmania parasites are responsible for a group of diseases collectively known as Leishmaniases ranging from self-healing ulcerative skin lesions to lethal visceral infections. They alternate between flagellated procyclic promastigotes colonizing the midgut of the sandfly vector, metacylic promastigotes residing in the foregut and transmitted to the mammalian host via a bite, and non-flagellated amastigotes proliferating in the macrophage of the mammalian host. The promastigotes are coated with a thick glycocalyx rich in molecules of the glycosylphosphatidylinositol (GPI) family (Fig. S1). GPIs are based on the conserved backbone structure Manα1,4GlcNα1,6-phosphatidylinositol and, in *Leishmania*, anchor proteins such as the proteophosphoglycans (PPGs) or a polysaccharide called lipophosphoglycan (LPG). They can also be free and are then termed glycoinositolphospholipids (GIPLs) (Ferguson 1999; Guha-Niyogi et al 2001; McConville and Ferguson 1993; Mendonca-Previato et al 2005). *Leishmania* glycocalyx is particularly rich in galactose (Gal) since LPG, the most abundant glycoconjugate of promastigotes, and protein linked phosphoglycans (PGs) are comprised of linear chains of 6Galβ1,4Manα1-P repeating units (Fig. S1) (Ilg 2000; Turco and Descoteaux 1992). Moreover, in *L. major*, Gal residues substitute the backbone structure of LPG, PPGs and GIPLs (Ilg 2000; McConville et al 1990; Turco and Descoteaux 1992). While in protein linked PGs only the pyranic form of galactose exists, LPG and GIPLs contain in addition galactofuranose, an unusual conformer absent from vertebrate species but commonly expressed in eukaryotic and prokaryotic pathogens (Bakker et al 2005; Beverley et al 2005; Ilg 2000; McConville et al 1990; Turco and Descoteaux 1992).

Consistent with the glycocalyx composition, the main nucleotide sugars detected in *L. major* promastigotes are UDP-glucose (UDP-Glc), UDP-Gal, UDP-N-acetylglucosamine and GDP-mannose (Turnock and Ferguson 2007). The pool of UDP-Glc synthesized from the abundant metabolites UTP and glucose-1-phosphate by the UDP-glucose pyrophosphorylase (UGP) (also designated UTP:α-D-glucose-1-phosphate uridylyltransferase) is relatively large and is used to produce UDP-Gal by epimerization (Lamerz et al 2006; Turnock and Ferguson 2007) (Fig. 1). Besides this *de novo* synthesis of UDP-Gal mediated by the UDP-Glc 4-epimerase, the latter nucleotide sugar may be generated by direct activation of galactose-1-phosphate. *Leishmania* parasites, in contrast to the trypanosomatids *Trypanosoma cruzi* and *Trypanosoma brucei*, indeed can take up galactose from the environment and utilize it for the biosynthesis of glycoconjugates, although the enzymes involved in Gal activation are still unknown (Turco et al 1984). The UDP-glucose:α-D-galactose-1-phosphate uridylyltransferase that catalyzes synthesis of UDP-Gal from galactose-1-phosphate and UDP-Glc is classically involved in salvage of galactose (Leloir 1951) (Fig. 1). This enzyme delineates the Leloir pathway which like the *de novo* pathway for UDP-Gal biosynthesis depends on UDP-Glc biosynthesis (Fig.1). UDP-Glc is thus expected to be an important metabolite for the biosynthesis of *Leishmania* glycocalyx.

The relevance of the glycocalyx for *Leishmania* survival and infectivity was demonstrated by targeted deletion of individual genes involved in the biosynthesis of surface glycoconjugates (Naderer et al 2004). In particular, the contribution of LPG was unambiguously determined with a mutant exclusively deficient in this polysaccharide generated by targeted gene replacement of the putative galactofuranosyltransferase LPG1 (Spath et al 2000). In *L. major*, LPG is clearly essential for survival in the

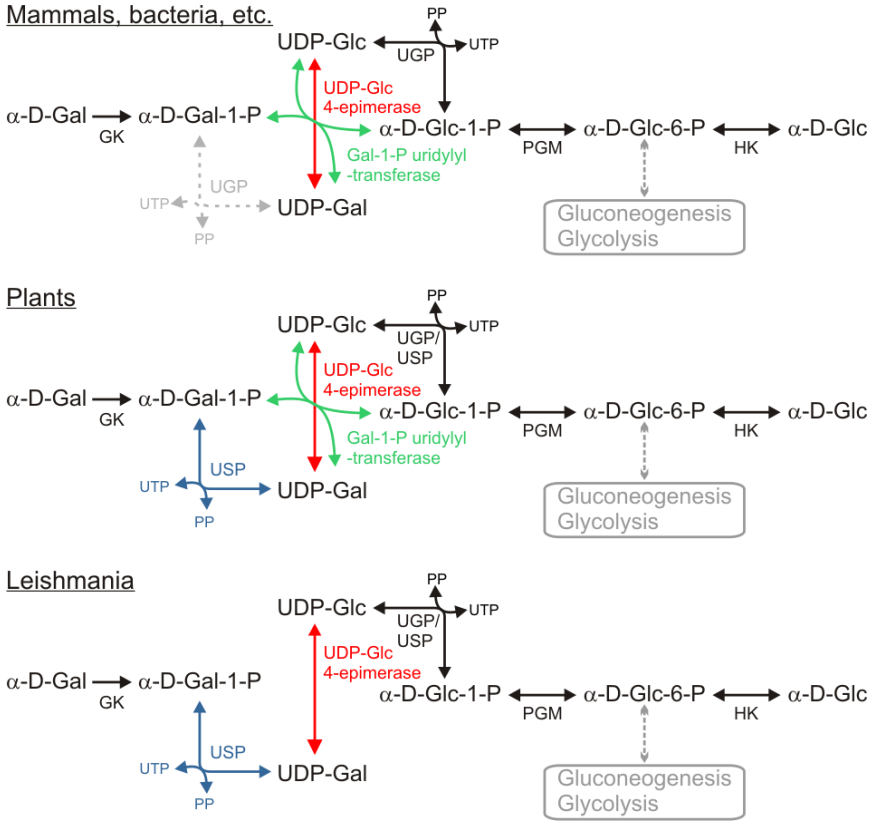

Figure 1. **Biosynthesis of UDP-α-D-galactose in various organisms.** UDP-α-D-galactose (UDP-Gal) is synthesized *de novo* by epimerization of UDP-α-D-glucose (UDP-Glc) by the UDP-glucose 4-epimerase (UDP-Glc 4-epimerase, EC:5.1.3.2). In addition, α-D-galactose-1-phosphate (α-D-Gal-1-P) produced from α-D-galactose (α-D-Gal) by the galactokinase (GK, EC:2.7.1.6) is activated by the UDP-glucose:α-D-galactose-1-phosphate uridylyltransferase (Gal-1-P uridylyltransferase, EC:2.7.7.12). These reactions depend on UDP-Glc production from α-D-glucose-1-phosphate (α-D-Glc-1-P) by the UTP: α-D-glucose-1-phosphate uridylyltransferase also named UDP-glucose pyrophosphorylase (UGP, EC:2.7.7.9). The phosphoglucomutase (PGM, EC:5.4.2.2) mediating the interconvertion of α-D-Glc-1-P and α-D-glucose-6-P (α-D-Glc-6-P) connects the galactose metabolism to gluconeogenesis and glycolysis. α-D-Glc-6-P may also originate from phosphorylation of free glucose (α-D-Glc) by the glucokinase (EC:2.7.1.1) or hexokinase (HK, EC:2.7.1.2) The conversion of α-D-Gal-1-P into UDP-Gal described in mammals by Isselbacher is thought to be due to a weak UTP:α-D-galactose-1-phosphate uridylyltransferase activity (EC:2.7.7.10) of UGP. In plants, a third pathway for UDP-Gal biosynthesis is mediated by an unspecific UDP-sugar pyrophosphorylase (USP, EC:2.7.7.64). The pathways proposed for *Leishmania* parasites are based on analysis of the genome and the existence of a UDP-glucose independent pathway for UDP-Gal biosynthesis demonstrated in this work. Activation of α-D-Glc-1-P and α-D-Gal-1-P by USP would explain the production of UDP-Glc and UDP-Gal in the *L. major ugp*$^-$ mutant.

insect vector and promastigote infectivity in the mammalian host but is not required for amastigote survival (Naderer et al 2004; Spath et al 2000). In a mouse model of cutaneous leishmaniasis, the LPG1 deficient mutant induces lesion formation after a pronounced delay in the establishment of infection (Späth et al 2000). Similar delayed lesion appearance was observed with several other LPG deficient mutants lacking LPG obtained by genetic deletion of, for instance, the UDP-galactopyranose mutase or alkyldihydroxyacetonephosphate synthase involved in UDP-galactofuranose or ether phospholipid biosynthesis respectively (Kleczka et al 2007; Zufferey et al 2003). Besides corroborating the role of LPG in infectivity, the study of these mutants suggested that despite their abundance in amastigotes, GIPLs are not crucial for survival of this parasitic stage (Kleczka et al 2007; Zufferey et al 2003). Intriguingly, absence of LPG and other phosphoglycans induced by replacement of the *LPG2* gene encoding the Golgi GDP-Man transporter resulted in avirulence, whereas a mutant defective in UDP-Gal transport across the Golgi and essentially devoid of phosphoglycans only caused a modest delay in lesion appearance (Capul et al 2007; Späth et al 2003). One hypothesis advanced for these findings was the possibility of an undiscovered molecule requiring the LPG2 GDP-Man transporter for its biosynthesis (Capul et al 2007).

To interfere with the biosynthesis of galactosylated molecules and eventually shed light on their role in parasite virulence, we targeted UGP in the hope of

blocking not only the *de novo* synthesis of UDP-Gal but also its salvage pathway. Our data demonstrate, however, that the UDP-Gal salvage pathway is independent from UDP-Glc biosynthesis and able to sustain the biosynthesis of most of the glycocalyx.

Results

Targeted replacement of L. major UGP – The full length *L. major UGP* has been cloned previously and the enzyme partially characterized (Lamerz et al 2006). *L. major* genome (Ivens et al 2005) exhibits a single copy of *UGP* gene located on chromosome 18 (*LmjF*18.0990) and does not display any highly homologous gene. Prior to the generation of a null mutant, that was achieved by consecutive replacement of the two *UGP* alleles with genes encoding the selection markers hygromycin phosphotransferase

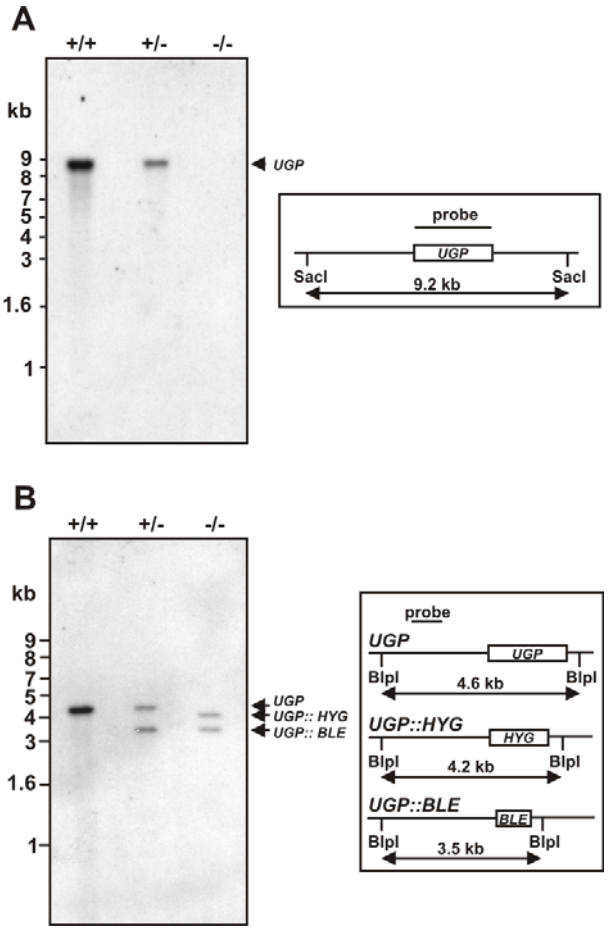

Figure 2. **Targeted gene replacements of *UGP* alleles.** Southern Blot analysis of genomic DNA from wild type (+/+), heterozygous *UGP*/Δ*ugp*::*BLE* (+/-) and homozygous *ugp*$^-$ mutant (-/-). DNA digested by SacI(A) or BlpI (B) was separated on agarose gel, transferred to nylon membrane and hybridized with a digoxigenin-labelled *UGP* probe or a digoxigenin-labelled 5'- flanking probe, respectively. The size of expected fragments is outlined in the right panel.

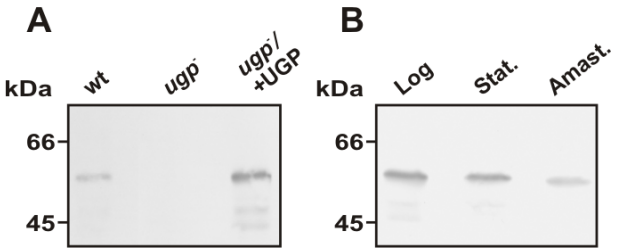

Figure 3. ***L. major* UGP is expressed through life stages and absent from the *ugp*$^-$ mutant** Whole cell lysates (12 µg/lane) of logarithmic and stationary phase wild type promastigotes, wild type amastigotes isolated from mice lesions and *ugp*$^-$ and *ugp*$^-$*/+UGP* promastigotes were subjected to SDS/PAGE and Western Blotting with anti UGP serum.

(*HYG*) and phleomycin binding protein (*BLE*), the gene copy number was confirmed by Southern blot analysis of genomic DNA (Fig. S2). The successful generation of *ugp*$^-$ mutant, was confirmed by Southern blotting (Fig. 2). After SacI digest, the *UGP* gene could be detected in wild type and in the heterozygous mutant but no signal was obtained in the *ugp*$^-$ mutant (Fig. 2A). Moreover, integration of the resistance markers into the correct gene locus was demonstrated with a probe hybridizing outside the region used for homologous recombination after BlpI digest (Fig. 2B). Multiple and/or random insertions of the resistant markers were excluded by additional Southern blots using probes specific for *HYG* or *BLE* (data not shown). Mutant parasites were morphologically identical to the parental strain and grew at similar rates and density under standard culture conditions. The absence of the UGP enzyme in the *ugp*$^-$ mutant and its re-expression in *ugp*$^-$*/+UGP* was ascertained by Western blotting of total cell lysates detected with the anti-UGP serum (Lamerz et al 2006) (Fig. 3A). Equal protein loading and transfer efficiency was assessed by reversible staining in Ponceau S-solution (data not shown). In addition, figure 3B demonstrates the expression of UGP in the logarithmic and stationary growth phase of promastigotes as well as in amastigotes. This expression through the parasite life cycle is consistent with the need of activated nucleotide sugars for glycoconjugate biosynthesis in both parasitic life stages. The lower amount detected in amastigotes is in agreement with previous reports indicating lower expression of enzymes involved in the glycolytic pathway at this parasitic stage (McConville et al 2007; Naderer and McConville 2008; Rosenzweig et al 2008).

Despite the complete absence of UGP (Fig. 3A), a residual UDP-Glc pyrophosphorylase activity was detected in the deletion mutant. UDP-glucose formation from glucose-1-phosphate was measured in

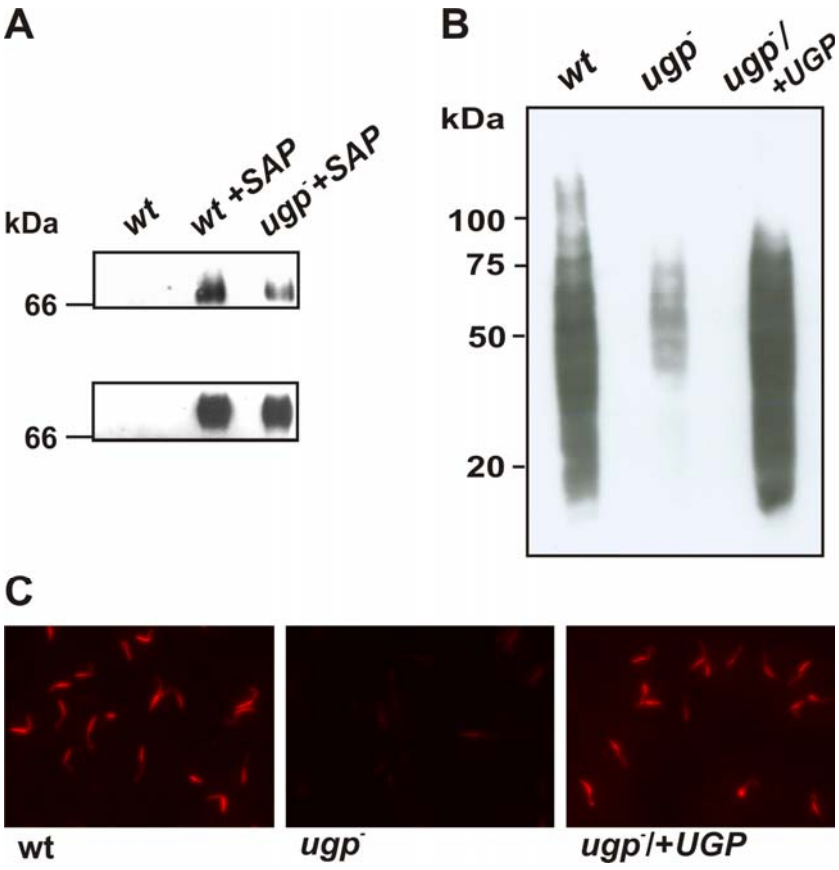

Figure 4. **Phosphoglycosylation of reporter secreted acid phosphatase and LPG in the *ugp*⁻ mutant.** A, SAP was expressed in wild type and *ugp*⁻ mutant, immunoprecipitated with mAb LT8.2 and subjected to Western Blot analysis with mAb WIC 79.3 (top panel). Loading was checked using mAb LT8.2 (lower panel). Untransfected wild type cells served as negative control. B, Cell extracts of wild type, *ugp*- and *ugp*-/+*UGP* parasites were analyzed by western blotting with mAb WIC79.3. C, LPG expression of wild type, *ugp*- and *ugp*-/+*UGP* parasites was analyzed by indirect immunofluorescence microscopy. Promastigotes were fixed, permeabilized and stained with mAb WIC79.3.

total cell lysates by a coupled enzymatic assay measuring reduction of NAD+ in presence of UDP-Glc dehydrogenase. In wild type cells an activity of 0.41 U/mg was measured whereas the *ugp*⁻ mutant showed a weak activity of 0.04 U/mg. These data suggest the existence of a second UDP-glucose forming activity in *L. major*.

Galactosylation is reduced in the ugp⁻ *mutant – L. major* mutant LPG and protein linked PGs are made up of linear chains of 6Galβ1,4Manα1-P repeating units, where the 3 position of the galactose may be substituted by side chains rich in galactose and arabinose (Ilg 2000; Turco and Descoteaux 1992). Their synthesis requires thus the availability of UDP-galactose.

The effect of *UGP* deletion on protein linked PGs was addressed first. We used a convenient PG reporter developed previously, expressing a secretory acid phosphatase (SAP) which is entensively phosphoglycosylated (Späth et al 2000; Wiese et al 1999). *L. mexicana* SAP1 was heterologously expressed (Wiese et al 1999) in wild type and the *ugp*⁻ mutant. After immunoprecipitation and Western blotting with the anti SAP mAb LT8.2 (Ilg et al 1993), a specific signal of about 70 kDa could be detected in the stacking gel area indicating phosphoglycosylation of the Ser/Thr-rich repetitive motifs of the protein (Fig. 4 A, lower panel) (Wiese et al 1995). The similar size of SAP expressed either in wild type or in the *ugp*⁻ mutant suggests that the protein is properly phosphoglycosylated in the mutant (Fig. 4 A, lower panel). Immunoblotting with mAb WIC79.3, recognizing the galactosylated side chains decorating the phosphoglycan backbone revealed however a decrease of Gal modified repeating units in the *ugp*⁻ mutant (Fig. 4A, top panel). These Gal modified repeating units were estimated to 70% from the intensity of the signal (Fig. 4A, upper panel) after correction for loading (Fig. 4A, lower panel). These results suggest that against expectations the *ugp*⁻ mutant is still able to produce substantial amounts of UDP-galactose.

This conclusion was supported by analysis of LPG (Fig. 4). Analysis of LPG in whole cell lysates using the monoclonal antibody WIC79.3 for detection revealed a strong decrease of Gal modified repeating units in the *ugp*⁻ mutant (Fig. 4B). Gal modified repeating units were estimated to 15% from the signal intensity and was hardly discernable by immunofluorescence microscopy (Fig. 4C). Together these results demonstrate significant, albeit reduced, production of UDP-Gal in the *ugp*⁻ mutant.

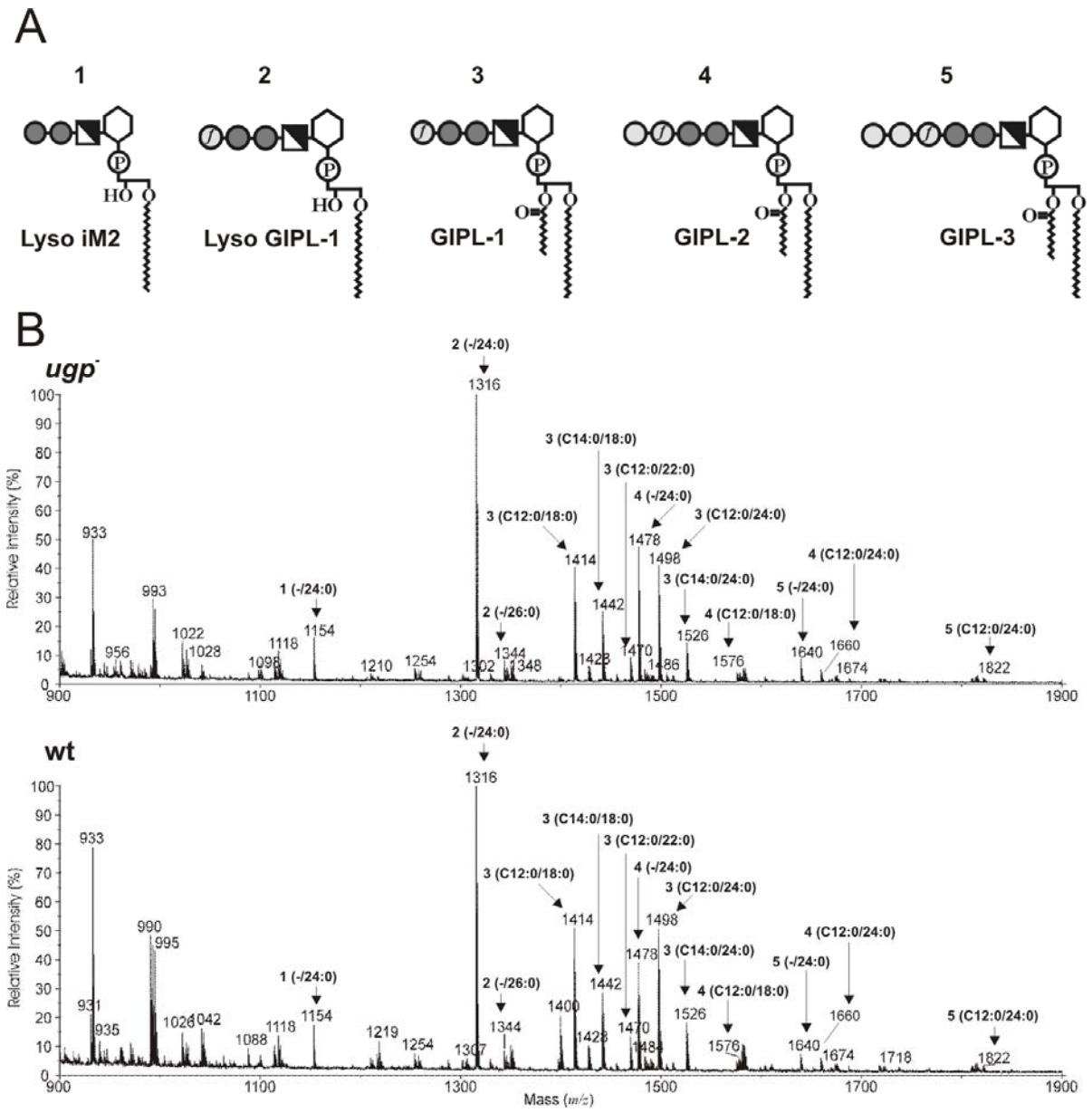

Figure 5. **GIPLs structures are unaffected in the *ugp*⁻ mutant.** A, Schematic representation of *Leishmania* GIPLs. Light shaded circles, Gal_p; light shaded circles with f, Gal*f*; dark shaded circles, Man; half shaded squares, GlcN; hexagons, myoinositol; and P, phosphate.
B, negative ion MALDI spectra of GIPLs isolated from *ugp*⁻ mutant (top panel) and wild-type (lower panel) parasites. The identities of the major ions are indicated by the schematics in A and can be inferred from the structure of GIPL3, which is: Galα1-6Galα1-3Gal_fβ1-3Manα1-3Manα1-4GlcNα1-6*myo*-inositol-1-HPO4-3(sn-1-alkyl-2-acylglycerol). The numbers of C atoms and of C=C double bonds in the acyl and alkyl chains, respectively, are indicated in brackets above each peak.

The structural composition of GIPL is not affected in the ugp⁻ *mutant – L. major* synthesizes three different type-2 GIPLs containing the common glycan core Gal*f*α1-3Manα1-3Manα1-4GlcN-phosphatidyl-inositol termed GIPL-1 that can be elongated by one (GIPL-2) or two (GIPL-3) terminal Gal residues (Galα1-3Gal*f*α1-3Manα1-3Manα1-4GlcN-phos-phatidylinositol and Galα1-6Galα1-3Gal*f*α1-3Manα1-3Manα1-4GlcN-phosphatidylinositol, respectively) (McConville et al 1990). To highlight eventual structural differences between wild type and *ugp*⁻ GIPLs, the glycolipids were extracted, purified and analyzed by negative ion matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) (Fig. 5). The ions at *m/z* 1414, 1498 and 1576, 1660 represent GIPL-1 and GIPL-2 species containing C12:0 acyl and C18:0 or C24 alkyl chains, respectively (Kleczka et al 2007). Ions of *m/z* 1316, 1478, and 1640 (Fig. 5.) represent lyso-structures with C24:0 alkyl chains corresponding to each of the three GIPL types. Furthermore, the ions at

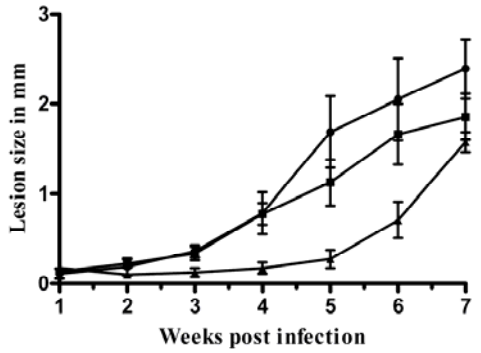

Figure 6. **Delayed lesion formation of mice infected with the *ugp*⁻ mutant**. Female Balb/c mice were inoculated in the footpad with 2 x 10^6 wild type (square), *ugp*⁻ mutant (triangle) or *ugp*⁻/+UGP (circle) and lesion formation was measured once a week .

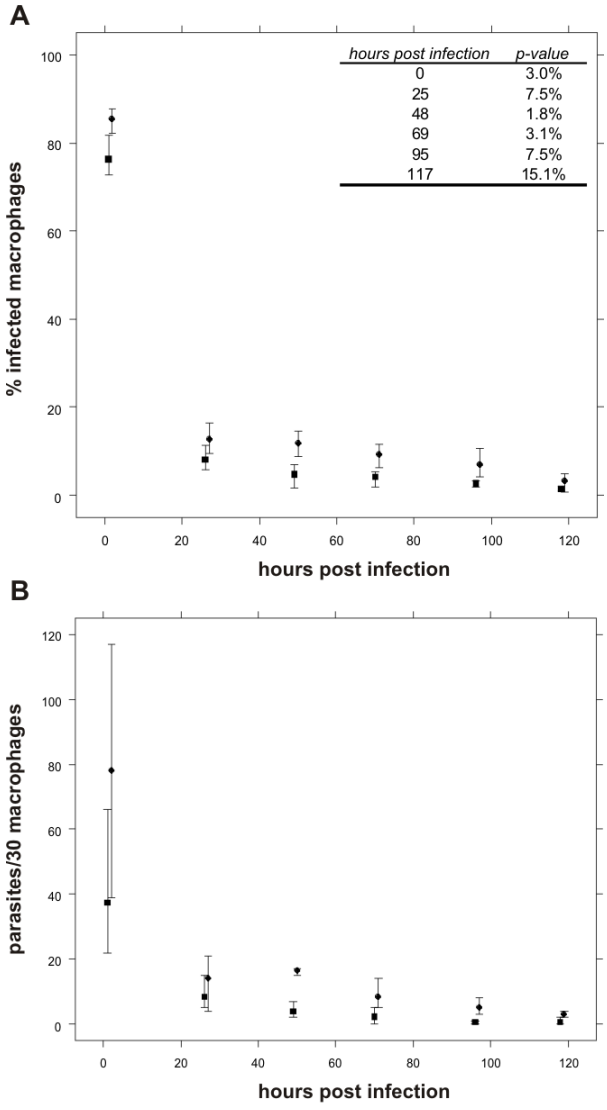

Figure 7. ***In vitro* invasion of human peritoneal macrophages with the *ugp*- mutant.** *In vitro* macrophage infection with C3 opsonized stationary phase wild type (circle) and *ugp*⁻ (square) promastigotes. The percentage of infected macrophages and the survival of parasite/30 macrophages as a function of time are shown in A and B, respectively. The data represent the mean, minimum and maximum from three independent experiments. The inlay in A displays the p-value of the Welch Two Sample t-test for each time point.

m/z 442, 1470 and 1526 represent GIPL-1 structures with C12:0 or C14:0 acyl and C20:0/C22:0/C24:0 alkyl chains, respectively. A very similar spectrum was obtained with the GIPL fraction from the *ugp*⁻ mutant (Fig. 5B) which indicates that the absence of UGP did not result in an increase of truncated GIPLs or precursor structures. Interestingly, the GIPL spectrum from wild type parasites contained an ion of *m/z* 1400 that is not present in the spectrum from *ugp*⁻ mutants (Fig. 5). Preliminary data suggest that this peak represents a GIPL-1 species with C17:0 alkyl and C12:0 acyl chains.

Delayed lesion formation in mice infected with ugp⁻ *mutant* – The effect of *UGP* deletion on infectivity was determined by infection with stationary phase promastigotes of susceptible Balb/c mice. Lesion formation of the wild type strain occurred three weeks after inoculation and progressed steadily (Fig. 6). In contrast, the *ugp*⁻ mutant showed a slight delay in lesion formation and swelling occurred five weeks after inoculation. Thereafter, the lesions developed as progressively as in mice infected with wild type cells and the lesions size correlated with parasite burden. As expected, the *ugp*⁻/+UGP cell line induced infections similar to wild type. The slight difference observed might be due to overexpression of UGP in the *ugp*⁻/+UGP (Fig. 3B). To exclude contaminants, amastigotes were recovered from infected animals and differentiated back into promastigotes. The identity of the re-isolated cell lines was confirmed by Western blotting using the anti UGP serum (data not shown).

Human peritoneal macrophage infections were performed with stationary phase promastigote parasites opsonized with C5 deficient mouse serum. The time course of the infection rate for the wild type and knock out groups was observed in three independent double blind experiments (Fig. 7A). The initial infection rate with wild type parasites (85%) is slightly higher than the one with *ugp*⁻ mutant (76%). After 25 h, the mean infection rate falls to 12% for wild type and 8% for the knock out and stays significantly lower with *ugp*⁻ parasites. After 117 h the difference in the mean infection rate between the two groups is too small to be significant. Additionally, the number of parasites per 30 macrophages was determined (Fig. 7B). The initial uptake into macrophages is approximately two times higher with wild type than *ugp*⁻ parasites. Within two days of infection about 80% and 90% of wild type and *ugp*⁻ parasites perished, respectively. The data indicate a similar clearance of wild type and *ugp*⁻ parasites and suggest that the delayed lesion formation observed in

mice infected with the *ugp*$^-$ mutant might be due to a lower initial uptake of parasites into macrophages.

Discussion

In *Leishmania* parasites, epimerization of UDP-Glc by the UDP-Glc 4-epimerase is likely the primary route of UDP-Gal formation (Turnock and Ferguson 2007) although a salvage pathway for UDP-Gal synthesis is also known to occur (Turco et al 1984). Gal is generally phosphorylated by a galactokinase before being converted to UDP-Gal by the UDP-glucose:α-D-galactose-1-phosphate uridylyltransferase (encoded by *GALT*) as described by Leloir (Leloir 1951). If the genome of *L. major* contains a putative galactokinase, no obvious *GALT* homologue was found. An alternative pathway, initially described by Isselbacher in mammals (Isselbacher 1958; Leslie et al 2005) has been associated with the weak UDP-galactose pyrophosphorylase activity of UGP (Knop and Hansen 1970; Lai and Elsas 2000). UGP occupies thus a central position in galactose metabolism and was expected to control *L. major* cell surface molecules biosynthesis and affect virulence as previously observed in several gram-negative and gram-positive bacteria (Chang et al 1996; Mollerach et al 1998; Vilches et al 2007).

Surprisingly, targeted gene replacement of *UGP* in *L. major* showed only modest effects on the synthesis of several key molecules of the glycocalyx. Whereas the biosynthesis of LPG seems to be markedly reduced, the influence on protein linked PGs is limited, and remarkably, the structure of GIPLs present in the *ugp*$^-$ mutant was totally unaffected. This potentially could arise through a requirement for UDP-Glc in the synthesis of the Glc-P modification found in the LPG anchor but not protein linked PGs or GIPLs. Consistent with the limited alteration of its surface glycocalyx components, the *L. major ugp*$^-$ mutant only induced a modest delay in lesion formation in susceptible Balb/c mice. Such delay in lesion emergence was previously observed with various LPG deficient mutants (Capul et al 2007; Kleczka et al 2007; Späth et al 2000; Zufferey et al 2003).

The characterization of the *ugp*$^-$ mutant thus suggests synthesis of a substantial, albeit reduced, UDP-Gal pool in the absence of UGP. Consistent with the observation made in this study, MacRae and collaborators showed that in presence of reduced amount of UDP-Gal due to deletion of one allele of the UDP-Glc 4-epimerase (*TcGALE*$^{+/-}$ mutant), *T. cruzi* preserved its galactofuranose-containing GIPLs, while the galactopyranose-rich mucins were more severely affected (Macrae et al 2006). These data led to the assumption that GIPLs are of major importance for basic parasite survival in culture. In *Leishmania* parasites, however, a mutant expressing agalactosylated GIPLs was generated by targeting galactofuranose metabolism and did not display any *in vitro* growth or morphological anomalies (Kleczka et al 2007). Similarly, deletion of *UGP* in *L. major* did not induce morphological abnormality or growth defect, whereas the *T. cruzi* UDP-Glc 4-epimerase heterozygote mutant exhibited severe changes in cell surface molecular architecture and aberrant morphology (Macrae et al 2006). This suggests that the UDP-Gal pool is larger in the *L. major ugp*$^-$ mutant than in the so called *TcGALE*$^{+/-}$. It should be mentioned that in *T. cruzi* and *T. brucei,* epimerization of UDP-glucose seems to be the exclusive path for UDP-Gal synthesis since the hexose transporters of these two parasites are unable to transport Gal (Barrett et al 1998; Tetaud et al 1997). Consequently, deletion of the UDP-Glc 4-epimerase is lethal in these two trypanosomatids (Macrae et al 2006; Urbaniak et al 2006b), which makes the enzyme an attractive drug target (Urbaniak et al 2006a). While we had expected in these studies to be a similar test of the importance of UDP-Gal in *Leishmania*, our data showed surprisingly that *Leishmania* has another pathway of UDP-Gal synthesis bypassing the *de novo* and Leloir salvage pathways.

A remaining question is how is UDP-Gal synthesized in the *ugp*$^-$ mutant? Intriguingly, the deletion mutant still exhibited a 10% UGP residual activity. In yeast, a greater than 95 % reduction in UGP activity obtained in a UGP antisense mutant did not lead to any obvious phenotype (Daran et al 1997), although UGP activity is essential for survival of this organism (Daran et al 1995). The 10% residual enzymatic activity for UDP-Glc synthesis detected in the *ugp*$^-$ mutant might thus be sufficient to maintain the biosynthesis of most surface glycoconjugates. Since absence of the *UGP* gene and protein were confirmed in the *ugp*$^-$ mutant, however, this residual activity can be clearly attributed to a different enzyme. Recently, it has become apparent that several organisms may contain isozymes of UGP encoded by different genes. For instance, deletion of a gene encoding UGP (*udpgp*1) in *Dictyostelium discodeum* pointed toward the importance of a second UGP involved in differentiation and development of the slime mold (Bishop et al 2002). Plants also often express different UGPs (Chen et al 2007; Meng et al

2007; Meng et al 2008). *Arabidopsis*, for instance, contains homologous *UGP* genes encoding two enzymes located in the cytoplasm and a chloroplastic UGP involved in sulfolipid biosynthesis (Meng et al 2008; Okazaki et al 2009). However, the genome of *Leishmania major* does not contain any close UGP homologues. Conversely, a leishmanial homologue of the recently described plant UDP-sugar pyrophosphorylase (USP) was found. USP is an enzyme that can non-specifically utilize UTP and glucose-1-phosphate or galactose-1-phosphate to produce UDP-glucose or UDP-Gal and pyrophosphate (Kotake et al 2004; Kotake et al 2007; Litterer et al 2006). Such an enzyme would be able to fuel the UDP-Gal pool by direct activation of galactose-1-phosphate and be responsible for the limited UDP-Glc production that takes place in the *ugp*$^-$ mutant. Like in the *L. major ugp*$^-$ mutant, deletion of the two *Arabidopsis* cytoplasmic UGPs had no effect on cell wall composition and resulted in a 15 to 25% residual activity. This outcome was at least partially due to USP overexpression (Meng et al 2009). A similar compensation mechanism might take place in *Leishmania major* and contribute to the mild phenotype obtained by UGP deletion. Altogether, this work demonstrates that the UDP-Gal salvage pathway of *Leishmania* does not proceed via the Leloir pathway and is able to contribute significantly to the biosynthesis of the glycocalyx.

Experimental procedures

Parasite culture and transfection – L. major MHOM/SU/73/5ASKH was grown at 27°C in M199 media (Invitrogen) containing 10% fetal calf serum, 40 mM Hepes pH 7.5, 0.1 mM adenine, 0.0005% hemin, 0.0002 % biotin and 50 U/mL penicillin/streptomycin. Parasites were transfected by electroporation (Robinson and Beverley 2003) and allowed to grow in 1x M199 medium for 24 hours before transfer to semi solid media containing 1% Noble agar (Becton Dickinson) and appropriate antibiotics. Individual colonies were picked and grown in selective M199 liquid media. The antibiotics phleomycin, hygromycin B, and puromycin were obtained from InvivoGen and G418 from Sigma.

Generation of L. major *UGP deletion mutants and add back lines* – For gene replacement by homologous recombination the resistance markers hygromycin B phosphotransferase (HYG) and the phleomycin binding protein (PHLEO) were cloned between the 5' and 3' regions directly flanking the UGP-gene. Therefore, sequences 1.5 kb upstream and downstream of the *ugp* locus were amplified by PCR from genomic DNA using the primers CTG ATC TAG AAA CGA AGA CGA GCT ACA GCG CAT G / TAA AGG ATC CCC ATG GCT TCA CCT CCG TGA CAG C and GAA AGG ATC CGC TAG CTA GGG GTC ACA AGC TGC TGA / ATA CGG TAC CCC GCC GTC ATC TGT CGA TTG CAC AC, respectively and ligated into the XbaI, BamHI and BamHI, KpnI restriction sites of the pcDNA3 vector respectively. The above primers contained additional BspHI and NheI restriction sites at the 3' and 5' ends of the 5' and 3' flanking region, respectively that allowed cloning of the selection markers amplified from the vectors pCR2.1hyg and pCR2.1phleo (M. Wiese, unpublished data) between the flanking regions. The resulting UGP::HYG and UGP::PHLEO targeting constructs were digested with BsaAI and the corresponding fragments purified by gel extraction and subsequent ethanol precipitation. The deletion mutant was generated by two consecutive rounds of homologous recombination using the UGP::PHLEO fragment in the first and the UGP::HYG in the second round. Southern blotting techniques were used to confirm the precise gene replacement. The obtained homozygous mutant was named *ugp*$^-$.

For episomal expression of UGP in the *ugp*$^-$ background, the construct pXG-UGP was transfected into several clones, referred to as *ugp*$^-$/+UGP. The plasmid was generated by PCR amplification of the UGP open reading frame with the primer pair AGT ACC CGG GAT GGA AAA CGA CAT GAA GTC C / AGT AGG ATC CCT ACT TGT TGG TCG ACT GCT G. After XmaI/BamHI restriction digest, the fragment was ligated into pXG-PAC (Freedman and Beverley 1993).

Western Blot Analysis – Whole cell lysates from exponentially growing and stationary phase *L. major* promastigote cultures as well as from amastigotes isolated from mice were separated by SDS/PAGE and transferred onto nitrocellulose membranes (Whatmann Schleicher & Schüll). Protein concentration was measured in triplicate by Bradford protein assay (Biorad) to ensure equal loading. Enhanced chemiluminescence detection (Pierce) was used after incubation with mAb WIC79.3 (ascites fluid; diluted at 1:2000) (de Ibarra et al 1982) whereas the Super Signal West Femto ECL substrate (Pierce) was required after incubation with mAb CA7AE (diluted at 1:1000) (Tolson et al 1989). In both case, goat anti mouse mAb coupled to peroxidase (Dianova) at a

dilution of 1:25000 was used as secondary antibody. *L. major* UGP was detected using a 1:60000 dilution of a recently prepared antiserum (Lamerz et al 2006) and AP-conjugated goat-anti-rabbit antibody (1:2000, Dianova).

Expression and analysis of L. mexicana *secreted acid phosphatase* – Proteophosphoglycosylation was analyzed by heterologous expression of the *L. mexicana* secreted acid phosphatase (SAP1) (Wiese et al 1999). Therefore, the plasmid pXG-*Lmex*-SAP1 (Späth et al 2000) was transfected into wild type and *ugp- L. major* cells. Recombinant proteins were immunoprecipitated from the cell culture supernatants with the anti-SAP mAb LT8.2 (Ilg et al 1993). Both expression of SAP and phosphoglycosylation were monitored by Western blotting using the mAbs LT8.2 and WIC79.3 and displayed with the Super Signal West Femto ECL substrate (Pierce).

GIPL analysis – GIPLs were extracted in chloroform/methanol/water (1:2:0.8), purified over a C18/SepPak® Plus column (Waters) and dried under a stream of nitrogen as described previously (16). -MALDI-TOF-MS analyses of lipid extracts were performed in the negative-ion mode with delayed extraction on a Voyager DE STR time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a 337 nm nitrogen laser. Analyses were performed in reflector mode over the *m/z* range 800–3000 with an accelerating voltage of 20 kV and a delay of 300 ns. The instrument was externally calibrated. A low-mass gate value of *m/z* 500 was selected to avoid saturation of the detector. α-Cyano-4-hydroxycinnamic acid (10 μg μl^{-1} in 60% ACN−0.1% TFA) was used as a matrix. Final mass spectra represented an average of 5–10 spectra, each of which is acquired from 200 laser shots. For structural assignment of GIPLs, linear ion trap (LIT) MS^n spectra (n = 2, 3, 4) were obtained as previously described (Althea A. Capul, F-F. Hsu and SMB unpublished; Althea A. Capul, Ph.D. Thesis, Washington University 2005). Briefly, $[M -H]^-$ ions were generated by electrospray ionization (ESI) and subjected to low energy CAD on a Thermo Finnigan (San Jose, CA) LTQ LIT mass spectrometer (MS) operated with Xcalibur software. Methanolic GIPL solutions were continuously infused into the ESI source with a syringe pump at a flow rate of 2 uL/min. The automatic gain control of the ion trap was set to $5x10^4$ and the maximum injection time 100 ms. Helium was used as buffer and collision gas at a pressure of $1x10^{-3}$ mbar (0.75 mTorr). The relative collision energy ranged from 20-30%, and an activation time of 30 ms and an activation q value of 0.25 were used, which resulted in a residual precursor ion abundance of about 20%. The mass resolution of the instrument was tuned to 0.6 Da at half peak height.

Infection of human peritoneal macrophages – Human peritoneal monocytes were isolated as previously described (van Zandbergen et al 2002). Briefly, freshly isolated human buffy coats were diluted and monocytes were isolated by Histopaque 1077 (Sigma) gradient centrifugation. Collected cells were subjected to magnetic cell sorting using CD14 microbeads (Miltenyi Biotec, Germany). Isolated monocytes cultured in RPMI 1660 media supplemented with 10% FCS, L-Glutamin and human macrophage colony stimulating factor (Tebu) for 7 days. Before use in infection studies parasites were 30 min incubated with 4% complement factor 5-deficient human serum (C5-deficient serum; Sigma) in RPMI. Parasites were then allowed to invade macrophages for 2 hours at 37°C in a parasite to macrophage ratio of 10:1. Measured infection rates were normalized to values obtained with wild type *L. major* after a 2 hours infection step.

Mouse infection – Promastigotes passed through BALB/c mice (Charles River) were grown to stationary phase and $2x10^6$ parasites were injected subcutaneously into the footpad of female Balb/c mice (Charles River). Each experimental group consisted of five individuals. Lesion formation was monitored once a week by measuring the infected and the non-infected footpad using a Vernier calliper. The median size difference (+/- MAD) of the infected and non-infected footpad was plotted against the weeks post infection. Mice were sacrificed when necrosis appeared in the group and lesion derived parasites were enumerated in limiting dilution assays. In addition, amastigotes were isolated from lesions and either used directly or after differentiation into promastigotes used for further analyses.

In vitro determination of UGP activity – For the *in vitro* testing of UGP activity, lysates obtained from promastigotes were assessed by a coupled enzymatic assay. The assay which measures the forward reaction of the enzyme has been previously described in detail (Lamerz et al 2006). Whole cell lysates were used as enzyme source.

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Abbreviations

UGP, UDP-glucose pyrophosphorylase; Gal, galactose; Gal*f*, galactofuranose; Glc, glucose; GlcN, glucosamine; Man, Mannose; GPI, glycosylphosphatidylinositol; GIPL, glycoinositolphospholipid; LPG, lipophosphoglycan; PPG, proteophosphoglycan; SAP, secreted acid phosphatase;

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Supplementary Material

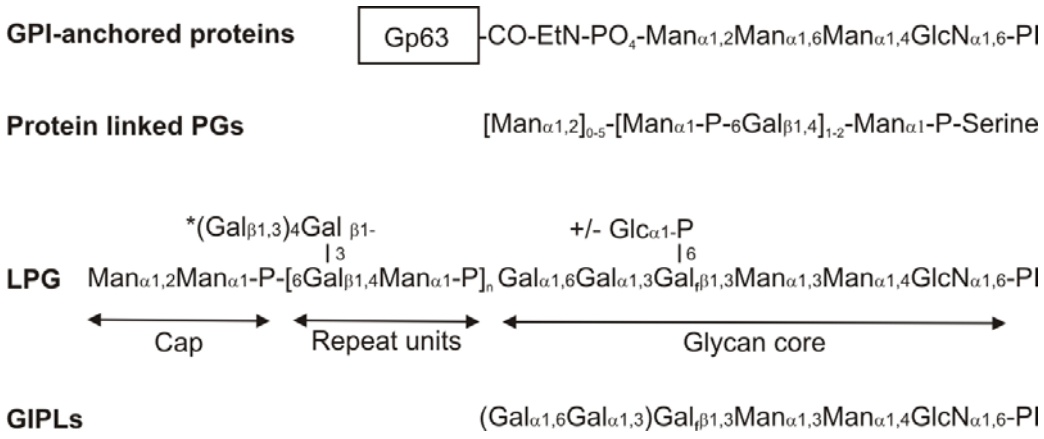

Figure S1. **Molecules of the GPI family dominating *Leishmania major* surface.** GPI-anchored proteins, phosphoglycosylated proteins (protein linked PGs), lipophosphoglycan (LPG) and glycoinositolphospholipids (GIPLs) are the main molecules constituting *Leishmania* glycocalix. Man, mannose; Gal, galactose, Glc, glucose, P, phosphate, GlcN, glucosamine, PI, phosphatidylinositol. *The side chains substituting LPG vary in the different developmental stages. The side chains depicted here are the main chains present in procyclic promastigotes. For more details, the reader is referred to McConville, M. J. and Ferguson, M. A. (1993) *Biochem.J.* 294 , 305-324.

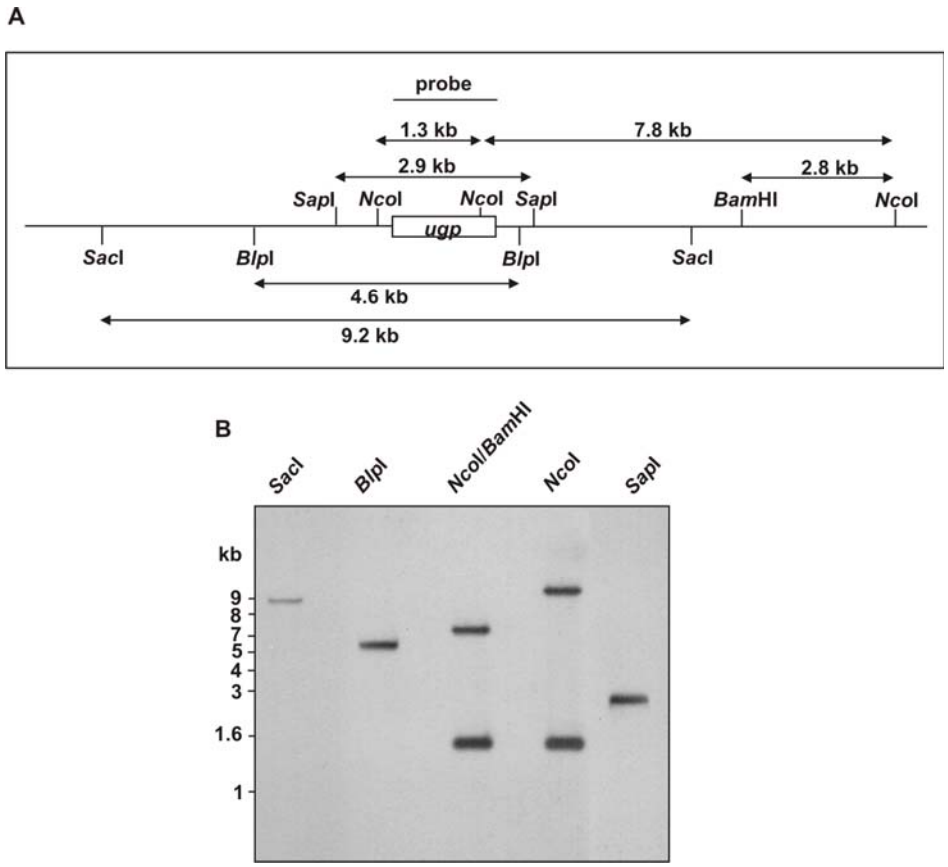

Figure S2. ***L. major UPG* is a single copy gene.** A, Schematic display of *L. major UGP* locus indicating the size of fragments expected after digestion with the indicated restriction enzymes. B, Southern Blot analysis of genomic DNA digested with either BlpI, SacI, NcoI, NcoI/BamHI or SapI. Fragments were separated on agarose gel, transferred to a nylon membrane and hybridized with a digoxigenin-labelled *UGP* probe.

Chapter 3 – *Leishmania major* UDP-Sugar Pyrophosphorylase: the Missing Link in Galactose Salvage?

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Preface – About this manuscript

This part of my work aimed at the identification and biochemical characterization of the assumed UDP-sugar pyrophosphorylase (USP) in *Leishmania major*. For this purpose the *L. major* Friedlin (*LmjF*) genome was searched using the BLAST algorithm with the known USP gene from *Pisum sativum*. The candidate gene *LmjF*17.1160 was identified, cloned, the protein overexpressed in *E. coli* and purified by chelating and affinity chromatography. Using purified protein for immunization trials in rabbits, USP anti-serum could be obtained. The *L. major* USP characteristics such as oligomerization status, kinetic parameters, substrate epitope binding and binding mode were investigated using an array of techniques comprising enzymatic, electrophoretic, immunochemical and NMR-spectrometric techniques. In particular, a novel versatile enzyme coupled *in vitro* assay system was established for detection of USP reactivity.

My contribution to this manuscript encompassed the purification, immunization and whole characterization of the *L. major* USP including the establishment of new techniques. Prof. Routier and I wrote the paper.

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The Leishmania parasite glycocalyx is rich in galactose-containing glycoconjugates that are synthesized by specific glycosyltransferases which use UDP-galactose as a glycosyl donor. UDP-galactose biosynthesis is thought to be predominantly a *de novo* process involving epimerization of the abundant nucleotide sugar UDP-glucose by the UDP-glucose 4-epimerase, although galactose salvage from the environment has been demonstrated for *L. major*. Here we present the characterization of a *L. major* UDP-sugar pyrophosphorylase able to reversibly activate galactose-1-phosphate into UDP-galactose thus proving the existence of the Isselbacher salvage pathway in this parasite. The ordered bisubstrate mechanism and high affinity of the enzyme for UTP seems to favor the synthesis of nucleotide sugar rather than their pyrophospholysis. Although *L. major* UDP-sugar pyrophosphorylase preferentially activates galactose-1-phosphate and glucose-1-phosphate, the enzyme is able to act on a variety of hexose-1-phosphates as well as pentose-1-phosphates but not hexosamine-1-phosphates and hence presents a broad *in vitro* specificity. The newly identified enzyme exhibits a low but significant homology with UDP-glucose pyrophosphorylases and conserved in particular is the pyrophosphorylase consensus sequence and residues involved in nucleotide and phosphate binding. Saturation Transfer Difference (STD) Nuclear Magnetic Resonance (NMR) spectroscopy experiments confirm the importance of these moieties for substrate binding. The described leishmanial enzyme is closely related to plant UDP-sugar pyrophosphorylases and presents a similar substrate specificity suggesting their common origin.

INTRODUCTION

Trypanosomatid parasites of the genus *Leishmania*, the causal agent of the human disease leishmaniasis, are characterized by a digenetic life cycle with a promastigote stage in the sand fly vector and an amastigote stage in mammalian macrophages. According to World Health Organization reports more than 20 million people are infected worldwide (http://www.who.int/leishmaniasis/en/) and present manifestations ranging from self-healing cutaneous lesions to fatal visceral forms.

Leishmania parasites are coated by a dense glycocalyx composed of GPI-like structures which is essential for parasite survival in the sandfly vector and, at least for some species, for promastigote infectivity in the mammalian host (1). This glycocalyx is particularly rich in galactose occurring either in the pyranosic form (Gal) or the more unusual furanosic form (Gal*f*). Its biosynthesis depends thus on the availability of the nucleotide activated sugar UDP-galactopyranose (UDP-Gal) which can be interconverted into UDP-galactofuranose (UDP-Gal*f*) by the specific enzyme UDP-galactopyranose mutase (2;3). Consequently, mutants deficient in the formation of UDP-Gal*f* or in the transport of UDP-Gal into the secretory pathway organelles present an altered glycocalyx associated with parasite attenuation (4-7). A route to UDP-Gal formation is via epimerization of the abundant nucleotide sugar UDP-glucose (UDP-Glc) by the UDP-Glc 4-epimerase (8). The biosynthesis of UDP-Gal is thus intimately linked to glucose metabolism (Fig. 1). Since the trypanosomatid parasites *Trypanosoma brucei* and *Trypanosoma cruzi* are unable to take up galactose from the environment (9;10), the UDP-Glc 4-epimerase is indispensable for biosynthesis of UDP-Gal and derived glycoconjugates in these organisms and is essential for their survival (11-14). In contrast, a salvage pathway for UDP-Gal synthesis is known to occur in *Leishmania* since radiolabeled Gal is taken up by promastigotes and incorporated into surface molecules (15). Gal most likely enters cells by a family of hexose transporters (16) before being converted into galactose-1-phosphate (Gal-1-P) by the putative galactokinase present in the genome (*LmjF*35.2740). Deletion of three of these hexose transporters in *Leishmania mexicana* revealed their importance in the growth, infectivity and survival of the parasite underlining the importance of monosaccharide salvage in both promastigotes and amastigotes (16;17).

Gal-1-P is usually activated into UDP-Gal by a UDP-glucose:α-D-galactose-1-phosphate uridylyltransferase enzyme (EC 2.7.7.12 encoded by the gene GALT) via the Leloir pathway. A clear homologue of this activating enzyme is however not found in the *Leishmania major* genome. Alternatively, incorporation of Gal-1-P into uridine nucleotide by a pyrophosphorolytic reaction has been reported in mammals and constitutes the Isselbacher pathway (18) (Fig. 1) although a UTP:α-D-galactose-1-phosphate uridylyltransferase (EC 2.7.7.10) has never been

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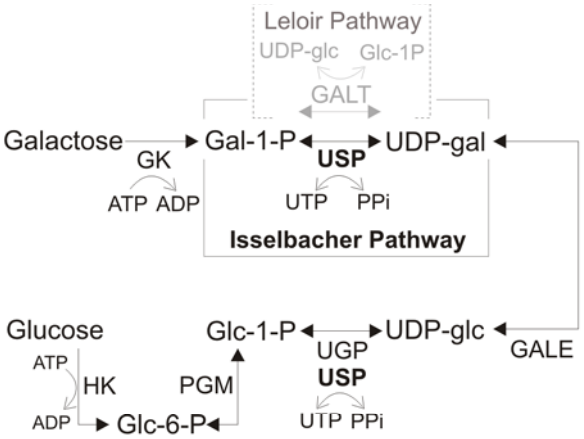

FIGURE 1. **UDP-galactose synthesis in *Leishmania major*.** GK: Galactokinase (EC 2.7.1.6), USP: UDP-sugar pyrophosphorylase (EC 2.7.7.64), GALT: UDP-glucose:α-D-galactose-1-phosphate uridylyltransferase (EC 2.7.7.12), GALE: UDP-galactose 4-epimerase (EC 5.1.3.2), UGP: UDP-glucose pyrophosphorylase (EC 2.7.7.9), PGM: Phosphoglucomutase (EC 5.4.2.2), HK: Hexokinase (EC 2.7.1.1). The *GALT* gene is absent from *Leishmania major* genome suggesting the absence of the Leloir pathway.

identified. In stark contrast, plants exhibit an enzyme with broad specificity called UDP-sugar pyrophosphorylase (USP; EC 2.7.7.64) that has been recently involved in this alternative pathway for Gal activation (19-21).

Analysis of a *L. major* UDP-glucose pyrophosphorylase (UGP) deletion mutant (Lamerz et al., unpublished work) revealed the presence of Gal containing molecules underlining the existence of a UDP-Gal biosynthetic pathway independent of UDP-Glc biosynthesis. Herein, we report the identification, cloning, and characterization of a *Leishmania major* UDP-sugar pyrophosphorylase (USP) (EC 2.7.7.64) with broad substrate specificity including Gal-1-P and glucose-1-phosphate (Glc-1-P). The enzyme identified by homology with its plant orthologues (19;21) suggests the presence of the Isselbacher pathway in *Leishmania*.

EXPERIMENTAL PROCEDURES

Cloning, Expression and Purification of His_6-tagged L.major *USP* – The entire open reading frame of *L. major* UDP-sugar pyrophosphorylase (*LmjF*17.1160) was amplified with the primer set ACL115 (CTG ACT CCA TAT GAC GAA CCC GTC CAA CTC C) and ACL116 (CTT AGC GGC CGC ATC AAC TTT GCC GGG TCA GCC G), containing integrated restriction sites for NdeI and NotI, respectively and inserted into a pET22b expression vector (Novagen), containing a C-terminal His_6-tag. For recombinant expression the vector was transformed into Ca^{2+}-competent *E.coli* BL21(DE3) via heat shock. Cells were grown in Power Broth (AthenaES) at 37°C to an OD of 1.0, transferred to 15°C and the expression induced at 1.2 OD by addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside. After 20 h the cells were harvested by centrifugation (6000 x g, 15 min, 4 °C) and washed with phosphate-buffered saline.

A bacterial pellet obtained from 500 mL Power Broth solution was resuspended in 15 mL Ni^{2+}-chelating buffer A_{Ni} (50 mM Tris/HCl pH 7.8, 300 mM NaCl) including protease inhibitors (40 µg/mL bestatin (Sigma), 4 µg/mL pepstatin (Sigma), 0.5 µg/mL leupeptin (Serva) and 1 mM phenylmethylsulfonyl fluoride (Roche Applied Science). Cells were lysed by sonication with a microtip (Branson Sonifier, 50% duty cycle, output control 5, eight 30 s pulses for 8 min) and cell debris were removed by centrifugation (20.000 x g, 15 min, 4 °C). The soluble fraction was loaded onto a 1 mL HisTrap HP Ni^{2+}-chelating column (GE Healthcare). After a 20 mL wash with buffer A_{Ni} (50 mM Tris/HCl pH 8, 300 mM NaCl), the column was eluted with 20 mL buffer A_{Ni} containing 40 mM imidazole followed by a final elution step of 5mL buffer A_{Ni} containing 300 mM imidazole. The fractions containing *L.major* USP were pooled and passed over a HiPrep 26/10 desalting column (GE Healthcare) to exchange buffer A_{Ni} to buffer A_Q (50 mM Tris/HCl pH 8.0). The sample was then loaded on a 1 mL Q-Sepharose FF anion exchange column (GE Healthcare) that was successively washed and eluted with 20 mL buffer A_Q, 20 mL buffer A_Q containing 100mM NaCl and a final final volume of 5 mL buffer A_Q containing 300 mM NaCl. Again, the fractions containing the recombinant *L. major* USP were pooled and exchanged to standard buffer (Tris/HCl pH 7.8, 10 mM $MgCl_2$) via HiPrep 26/10 column. Purified samples were snap-frozen in liquid nitrogen and stored in standard buffer at -80 °C.

Complementation of E.coli DEV6 galU Mutant – Complementation of the *E.coli* DEV6 galU mutant strain was performed as previously described by Lamerz et al (22).

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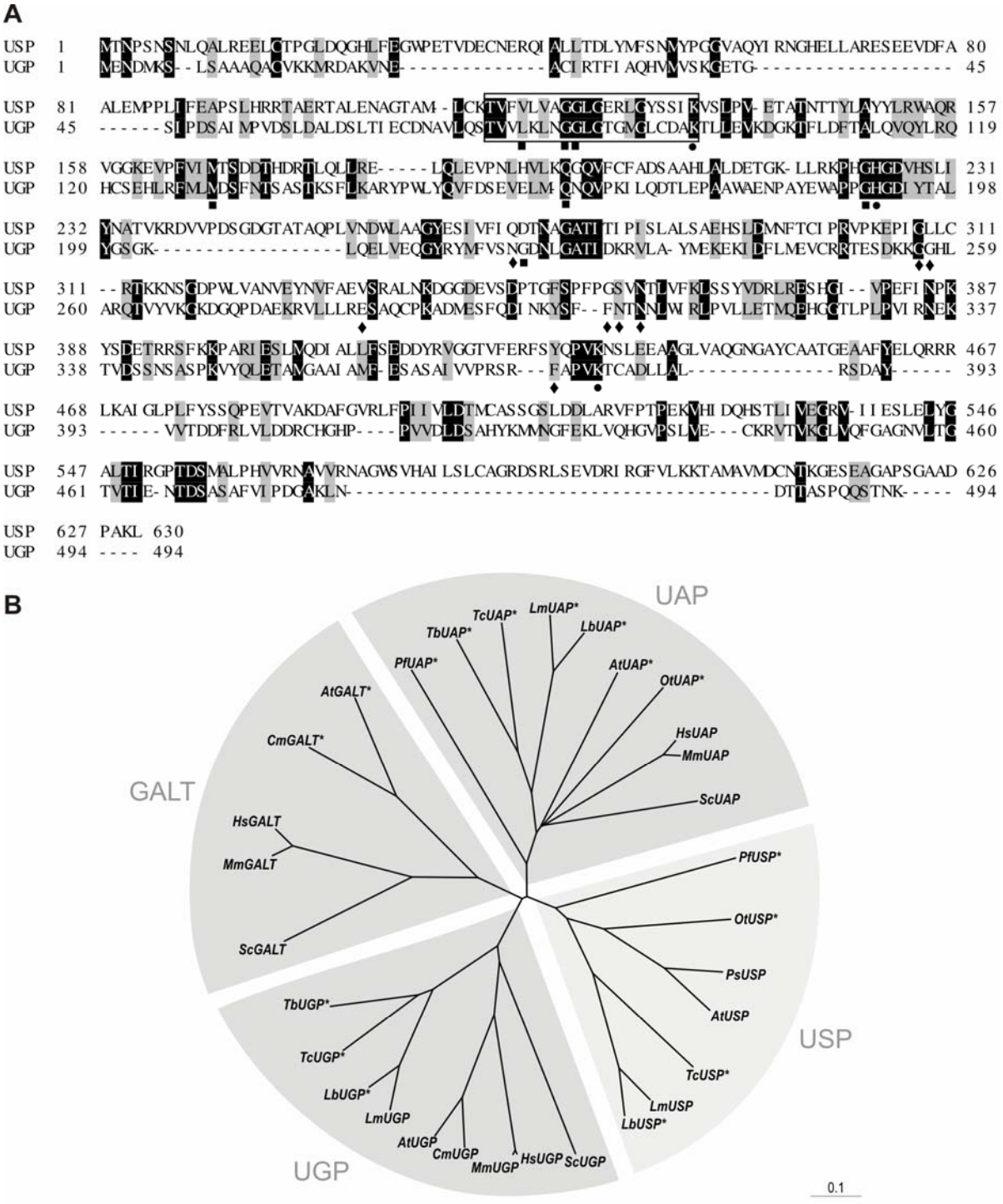

FIGURE 2. **Relation of *L. major* UDP-sugar pyrophosphorylase with UDP-glucose pyrophosphorylases, UDP-GalNAc pyrophosphorylases and UDP-glucose:α-D-galactose-1-phosphate uridylyltransferases.** *A*. Amino acid sequence alignment of *L. major* USP and UGP. Residues involved in UDP-glucose binding of UGP are marked below for contact with the nucleotide (square), glucose (diamond), or phosphate (circle); the pyrophosphorylase consensus sequence is boxed. *B*. Unrooted phylogenetic tree of selected sequences (EBI-ClustalW2 multiple alignment). USP: UDP-sugar pyrophosphorylase (EC 2.7.7.64), UGP: UDP-glucose pyrophosphorylase (EC 2.7.7.9), GALT: UDP-glucose:α-D-galactose-1-phosphate uridylyltransferase (EC 2.7.7.12), UAP: UDP-GalNAc pyrophosphorylase (EC 2.7.7.23). At: *Arabidopsis thaliana*, Cm: *Cucumis melo*, Hs: *Homo sapiens*, Lb: *Leishmania braziliensis*, Lm: *Leishmania major*, Mm: *Mus musculus*, Ot: *Ostreococcus tauri*, Pf: *Plasmodium falciparum*, Sc: *Saccharomyces cerevisae*, Tb: *Trypanosoma brucei*, Tc: *Trypanosoma cruzi*. Non characterized putative proteins are marked with an asterisk.

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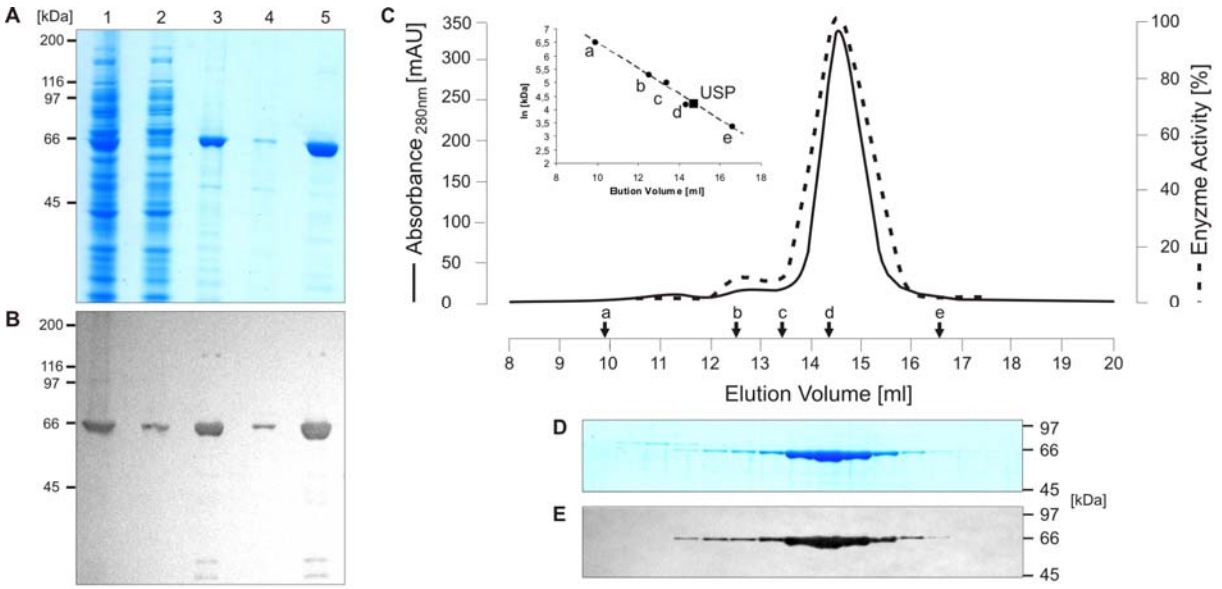

FIGURE 3. **Purification and oligomerization status of recombinant *L.major* UDP-sugar pyrophosphorylase.** Purification of *E. coli* expressed *L. major* USP-His_6 was followed by SDS-PAGE with Coomassie staining (A) and anti-His_6 Western-blotting (B), 1: bacterial lysate, 2: flow through of Ni-column, 3: desalted eluate of Ni-column, 4: flow through of anion-exchange column, 5: desalted eluate of anion-exchange column. Determination of quaternary organization was performed by size-exclusion chromatography (C). Protein standards are indicated by arrows (a-e: 669, 200, 150, 66, 29 kDa) and the apparent molecular mass of *Lmj*USP-His_6 was determined by standard curve (inset). *Lmj*USP-His_6 elutes at a retention volume of 14.65 mL (solid line), corresponding to a protein of 69.7 kDa. The activity pattern (dotted line) confirms that the monomer is the active form. Elution was traced by SDS-PAGE Coomassie staining (D) and anti-His_6 Western-blotting (E) of fractions.

Size Exclusion Chromatography – Size exclusion chromatography on a Superdex 200 10/300 GL column (10 × 300 mm) (GE Healthcare) was used to determine the quaternary organization of the recombinant *L. major* USP. The column was equilibrated with 50 mL of standard buffer (50 mM Tris/HCl, pH 7.8, 10 mM $MgCl_2$, loaded with 100 µL of one of the following standard proteins, bovine carbonic anhydrase (3 mg/mL), bovine serum albumin (10 mg/mL), yeast alcohol dehydrogenase (5 mg/mL), potato β-amylase (4 mg/mL), and thyroglobulin (3 mg/mL) (protein standard kit; Sigma) or with purified recombinant His_6-tagged *L. major* USP (4 mg/mL) and eluted at a flow rate of 1mL/min. The apparent molecular weight was determined by standard curve.

In vitro Enzyme Assays – The formation of pyrophosphate in the forward reaction was detected with the EnzChek® Pyrophosphate Assay Kit (Molecular Probes). The assay medium contained 50 mM Tris/HCl pH 7.8, 10 mM $MgCl_2$, 1 mM DTT, 0.2 mM 2-amino-6-mercapto-7-methylpurine ribo-nucleoside (MESG), 0.03 units APP, 2.0 units PNP and varying amounts of sugar-1-phosphate and UTP ranging from 0.5 to 3 mM. Enzyme reactions were performed at 25°C in a total volume of 100 µL and started by the addition of USP. A control without USP was used for normalization.

UTP produced in the reverse reaction, was converted into one equivalent of inorganic phosphate by *E.coli* Cytidine Triphosphate (CTP)-synthase in presence of ATP, L-Gln and the cofactor GTP. Inorganic phosphate was then quantified using the EnzChek® Pyrophosphate Assay Kit (Molecular Probes) but omitting the first coupling enzyme. For these experiments, the CTP-synthase gene was recombinantly cloned from *E. coli* XL1-blue in a pET22b expression vector with a primer set including Nde I and Not I restriction sites (SD13: CTT ACA TAT GCA TCA TCA TCA TCA TCA CGC TAG CGG ATC CAT GAC AAC GAA CTA TAT TTT TGT GAC C, SD14: CTT AGC GGC CGC TTA CTT CGC CTG ACG TTT CTG G). The N-terminal His-tagged CTP-synthase was expressed and purified as described above for the USP, but without anion exchange chromatography. The assay mixture for the reverse reaction contained 50 mM Tris/HCl pH 7.8, 10 mM $MgCl_2$, 1 mM DTT, 0.2 mM MESG, 1 mM ATP, 1 mM L-Gln, 0.25 mM GTP, 3 µg CTP-synthase, 2.0

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A forward reaction (+ UTP)

Sugar-1-phosphate	*relative activity [%]*	
Gal-1-P	100	± 2.6
Glc-1-P	88	± 6.8
Xyl-1-P	12	± 1.0
GalA-1-P	1.6	± 0.1
GlcNAc-1-P	0.6	± 0.03
Man-1-P	0.007	± 0.004

B reverse reaction (+ PPi)

UDP-sugar	*relative activity [%]*	
UDP-Gal	83	± 1.2
UDP-Glc	100	± 4.1
UDP-L-Ara(p)	33	± 0.3
UDP-GalA	7.1	± 0.1
UDP-GalNAc	0.013	± 0.0015

C forward reaction (+ galactose-1-phosphate)

NTP	*relative activity [%]*	
UTP	100	± 13
CTP	0.2	± 0.2
ATP	0.7	± 0.2
GTP	0.3	± 0.6

TABLE 1. ***L. major* UDP-sugar pyrophosphorylase substrate specificity.** Relative enzymatic activities of purified *Lmj*USP were determined in the presence of different acceptor substrates. All acceptor substrates were used in rate non-limiting concentrations (3 mM sugar-1-phosphates, 0.8 mM UTP, 2 mM UDP-sugars and pyrophosphate). The highest activity obtained was set to 100%. Errors are given as standard deviations from triplicate measurements.

units PNP and 2 mM of UDP-sugar and pyrophosphate in a final volume of 100 µl. The reaction was initiated by addition of USP and normalized to buffer control.

Measurements were performed in 96-well half-area flat-bottom microplates (Greiner Bio-One) with the Power-WaveTM340 KC4 System (Bio-Tek). To exclude cross reactions all substrates and cofactors of coupling enzymes were tested against USP inhibition or competition and vice versa (data not shown). The determinations of K_M and V_{max} values were performed using varying substrate concentrations up to twelve triplicates, whereas the second substrate was set to a constant saturating concentration. The initial linear rates (y) were plotted against the substrate concentrations (x) and the Michaelis-Menten-kinetic was analysed in PRISM using nonlinear-regression ($y = V_{max} \cdot x / (K_M + x)$).

SDS-PAGE Analysis and Immunoblotting – SDS-PAGE was performed according to Laemmli. Protein samples were separated on SDS-polyacrylamide gels composed of a 5% stacking gel and a 10% separating gel. Protein bands were visualized by Coomassie brilliant blue staining. For Western blot analysis, proteins were transferred to nitrocellulose membranes (Schleicher & Schüll GmbH). His_6-tagged proteins were detected using the penta-His antibody (Qiagen) at a concentration of 1 µg/mL and a goat anti-mouse Ig alkaline phosphatase-conjugate (Jackson ImmunoResearch).

STD NMR – All STD NMR experiments were performed on a Bruker Avance DRX 600 MHz spectrometer equipped with a triple axis cryoprobe at 298 K in 50 mM deuterated TRIS buffer, pH 7.8 and 10 mM $MgCl_2$. The protein was saturated with a cascade of 40 selective Gaussian-shaped pulses of 50 ms duration with a 100 µs delay between each pulse resulting in a total saturation time of ~2 s. The on- and off-resonance frequency was set to 0.7 ppm and 40 ppm, respectively. In a typical STD NMR experiment, 0.5 µM recombinant USP was used and all investigated ligands were added at a molecular ratio (protein/ligand) of 1:100. A total of 1024 scans per STD NMR experiment were acquired, and a WATERGATE sequence was used to suppress the residual HDO signal. A spin lock filter with strength of 5 kHz and duration of 10 ms was applied to suppress protein background. Relative STD effects were calculated according to the equation $A_{STD} = (I_0 - I_{sat}) / I_0 = I_{STD} / I_0$ by comparing the intensity of the signals in the STD-NMR spectrum (I_{STD}) with signal intensities of a reference spectrum (I_0). The STD signal with the highest intensity was set to 100%, and other STD signals were calculated accordingly (23).

RESULTS

Identification of a putative UDP-sugar pyrophosphorylase in Leishmania genome – Although enzymatic epimerization of UDP-glucose is clearly not the sole source of UDP-galactose in *Leishmania major* (15) (Lamerz et al., unpublished work), the genome of this parasite lacks an obvious UDP-glucose:α-D-galactose-1-phosphate uridylyltransferase

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A forward reaction

Substrate	K_M [µM]	v_{max} [µmol/min/mg]	k_{cat} [s^{-1}]	Efficiency k_{cat}/K_M [$M^{-1}s^{-1}$]	Efficiency [%]
Gal-1-P	860 ± 34	190 ± 3	219 ± 3	2.59×10^5 ± 1.42×10^4	12 ± 0.7
Glc-1-P	1706 ± 144	166 ± 6	191 ± 6	1.14×10^5 ± 1.34×10^4	5 ± 0.6
UTP (Gal-1-P)	98 ± 10	175 ± 7	201 ± 8	2.09×10^6 ± 2.80×10^5	100 ± 13.4
UTP (Glc-1-P)	116 ± 18	181 ± 11	209 ± 13	1.83×10^6 ± 3.90×10^5	88 ± 18.7

B reverse reaction

Substrate	K_M [µM]	v_{max} [µmol/min/mg]	k_{cat} [s^{-1}]	Efficiency k_{cat}/K_M [$M^{-1}s^{-1}$]	Efficiency [%]
UDP-Gal	148 ± 8	134 ± 2	154 ± 2	1.07×10^6 ± 7.18×10^4	51 ± 3.4
UDP-Glc	174 ± 9	157 ± 3	180 ± 3	1.06×10^6 ± 7.18×10^4	51 ± 3.4
UDP-L-Ara_p	373 ± 48	63 ± 3	72 ± 4	1.98×10^5 ± 3.60×10^4	9 ± 1.7
UDP-GalA	790 ± 193	11 ± 1	13 ± 1	1.67×10^4 ± 5.61×10^3	1 ± 0.3
PPi (UDP-Gal)	307 ± 18	152 ± 3	174 ± 4	5.80×10^5 ± 4.51×10^4	28 ± 2.2
PPi (UDP-Glc)	383 ± 11	161 ± 2	185 ± 2	4.92×10^5 ± 1.91×10^4	24 ± 0.9
PPi (UDP-L-Ara_p)	1018 ± 112	70 ± 5	81 ± 5	8.12×10^4 ± 1.40×10^4	4 ± 0.7
PPi (UDP-GalA)	728 ± 113	13 ± 1	15 ± 1	2.08×10^4 ± 4.88×10^3	1 ± 0.2

TABLE 2. ***L. major* UDP-sugar pyrophosphorylase kinetic parameters.** Indicated substrate parameters wer determined in presence of rate non-limiting and constant co-substrate concentrations (0.8 mM UTP, 2 mM Gal-1-I 3 mM Glc-1-P, 2 mM pyrophosphate and 2 mM UDP-sugars), in order to generate a pseudo first-order reaction typ The highest efficiency obtained was UTP set to 100% for both directions (*A*+*B*). Errors are given as standa deviations from triplicate measurements.

enzyme (EC2.7.7.12). BLAST searches of the *L. major* genome revealed however the existence of a gene (*LmjF*17.1160) displaying approximately 32 % identity with pea sprout USP (19) and 15 % identity with *L. major* UDP-glucose pyrophosphorylase (UGP) (22;24). This gene is extremely conserved amongst *Leishmania* species and is also found in *Trypanosoma cruzi* but not in *Trypanosoma brucei*. In *L. major*, the encoded protein referred to here as *L. major* USP or *Lmj*USP, contains 630 amino acids and has a theoretical molecular weight of 69 kDa. An alignment of *Leishmania major* USP and UGP is presented in figure 2A and highlights the conservation of residues essential for catalytic activity of pyrophosphorylase and for nucleotide sugar binding (25;26). The *Lmj*USP basic residues K134, H224 and K434 (corresponding to *Lmj*UGP K95, H191 and K380) are strictly conserved and predicted to be involved in phosphate binding (26). Similarly, with the exception of V120 which is a lysine residue in UGPs, the amino acids involved in uridine binding are preserved. In contrast, only two of the residues of UGPs interacting with the glucose moiety (G256 and N308) are conserved in USPs (Fig. 2A, Fig. S1).

The phylogenetic tree presented in figure 2B demonstrates that *Lmj*USP is clearly distinct from the UDP-Glc synthesizing enzyme UGP as well as the UDP-*N*-acetylglucosamine pyrophosphorylase (UAPs) and UDP-glucose: α-D-galactose-1-phosphate uridylyltransferase (GALT). Trypanosomatid USPs clusters with the plant and algal USPs but constitute a separate branch.

Leishmania major USP is involved in biosynthesis of UDP-glucose and/or UDP-galactose. To demonstrate the role of *L. major* putative USP in the metabolism of galactose, we first investigated its ability to complement the growth defect of *E. coli* galU mutant strain DEV6. This specific bacterial strain is unable to grow on agar containing galactose as the only carbohydrate source due to a mutation in UGP which prevents synthesis of UDP-Glc and subsequent depletion of the cytotoxic Gal-1-P by the UDP-glucose:α-D-galactose-1-phosphate uridylyltransferase enzyme (EC2.7.7.12). Upon transformation with *Lmj*USP cDNA, the ability of these bacteria to grow on galactose containing media was restored (Fig. S2) indicating the involvement of *L. major* USP in the activation of Gal-1-P either dependently (via the Leloir pathway) or/and independently of UDP-Glc biosynthesis (via the Isselbacher pathway) (Fig. 1).

Leishmania major USP is a monomer exhibiting broad substrate specificity – Oligomerization has been shown to regulate the activity of barley UGP (27) but not that of *L. major* UGP (22). We have thus investigated the oligomerization state of *L. major* C-

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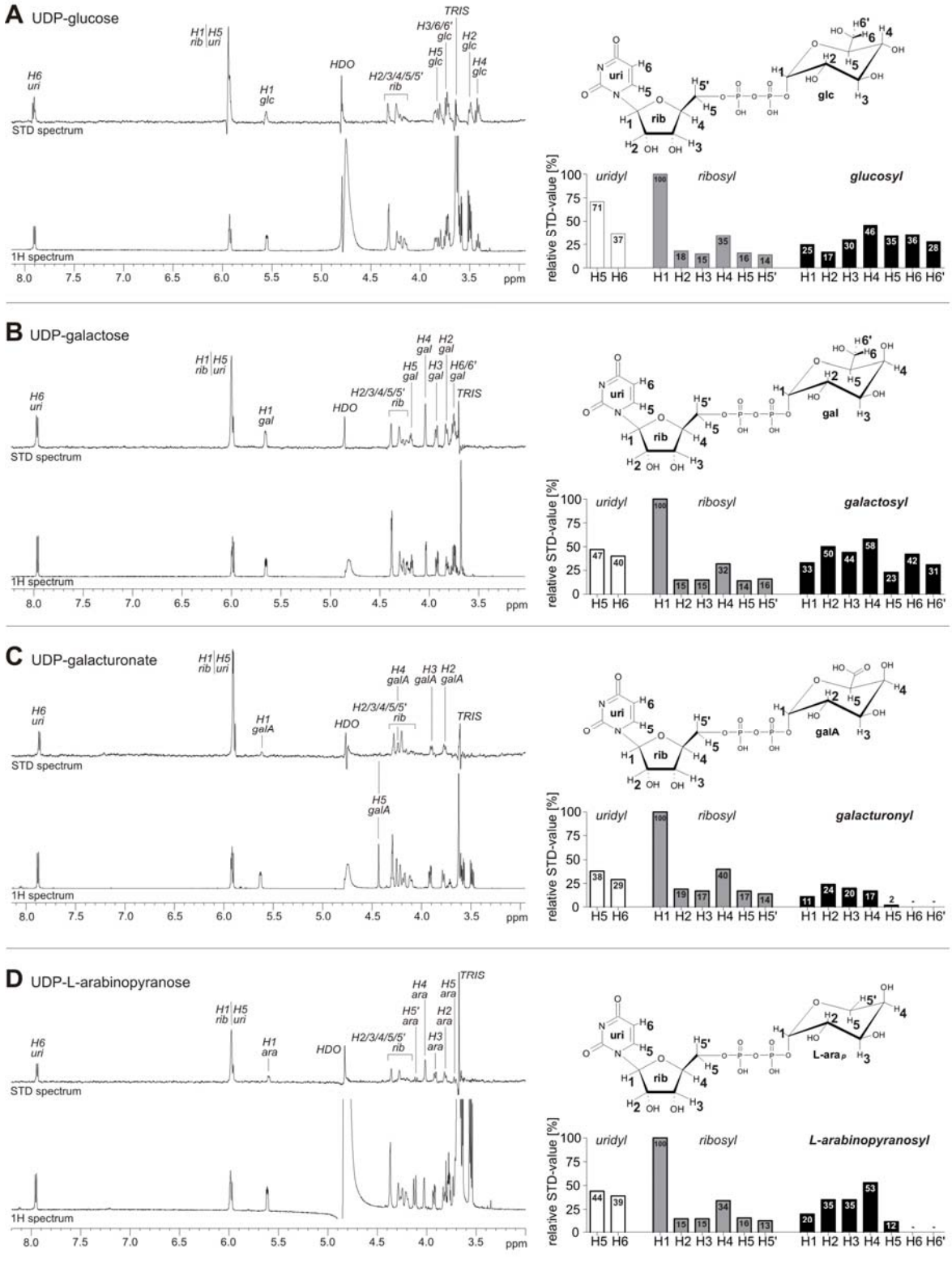

FIGURE 4. **STD NMR spectra of UDP-sugars complexed with *L. major* UDP-sugar pyrophosphorylase.** STD NMR spectra are shown for UDP-glucose (*A*), UDP-galactose (*B*), UDP-galacturonate (*C*), and UDP-L-arabinopyranose (*D*). All spectra were recorded at 298 K, 600 MHz using deuterated TRIS buffer (50 mM, pH 7.8) and $MgCl_2$ (10 mM) in D_2O. The on-resonance frequency was set to 0.7 ppm and the off-resonance to 40 ppm. The residual water signal was removed by applying a WATERGATE sequence. Epitope maps were constructed by calculating relative STD NMR effects according to the formula $A_{STD} = (I_0 \times I_{sat})/I_0 = I_{STD}/I_0$ (see supporting information) using the $H1_{rib}$ proton to 100% (bar chart). Each of the analysed UDP-sugars shows strong STD NMR signals.

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terminally His-tagged USP (*Lmj*USP-His_6) expressed in *E. coli* BL21 (DE3) cells and purified to homogeneity via nickel affinity, anion-exchange and by size exclusion chromatographies (Fig. 3). The recombinant *L. major* USP eluted as a single peak with a retention time of 14.65 mL corresponding to an apparent molecular mass of 69.7 kDa calculated from the Log molecular weight versus retention volume plot (Fig. 3C, inset). The theoretical mass (70.4 kDa) to apparent molecular mass ratio of 1.01 clearly indicates that the recombinant His_6-tagged *Lmj*USP is a monomer. This result was confirmed using untagged *Lmj*USP expressed in *E. coli* and partially purified by anion exchange- and size exclusion-chromatography (data not shown). Moreover, we demonstrated that the monomeric form (identified by PAGE and Western blotting in figure 3D and E, respectively) is the active form of the enzyme by assaying each fraction for their ability to synthesize UDP-glucose with the assay described below (Fig. 3C).

Because of its homology with plant USPs, *Lmj*USP was anticipated to have broad substrate specificity; versatile enzymatic assays that allow testing of the forward or reverse reaction with various substrates were thus established. The synthesis of UDP-Glc, UDP-Gal or other UDP-sugar from their respective sugar-1-phosphate and UTP (forward reaction) generates pyrophosphate as by-product which can be monitored using the Invitrogen Enz-Chek Pyrophosphate Kit. This system is based on hydrolysis of pyrophosphate and subsequent enzymatic reaction of inorganic phosphate with 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) to produce ribose-1-phosphate and 2-amino-6-mercapto-7-methylpurine absorbing at 360 nm. In pilot experiments, the possibility of ribose-1-phosphate cross-reactions with *Lmj*USP was, therefore, excluded by competitive testing with the substrate Glc-1-P in a different NADH based assay system (28) (data not shown). Alternatively, the formation of UTP was followed to analyze the synthesis of sugar-1-phosphate from nucleotide sugar and pyrophosphate (reverse reaction). In this assay, UTP was utilized by *E. coli* CTP-synthase (29) generating free inorganic phosphate which could be detected using the same principle. The enzyme's substrate specificity was determined by one of the employed *in vitro* assays (Table 1). Gal-1-P and Glc-1-P appeared to be the main substrates of *Lmj*USP, in agreement with the ability of the enzyme to functionally complement *E. coli* DEV6 galU mutants.

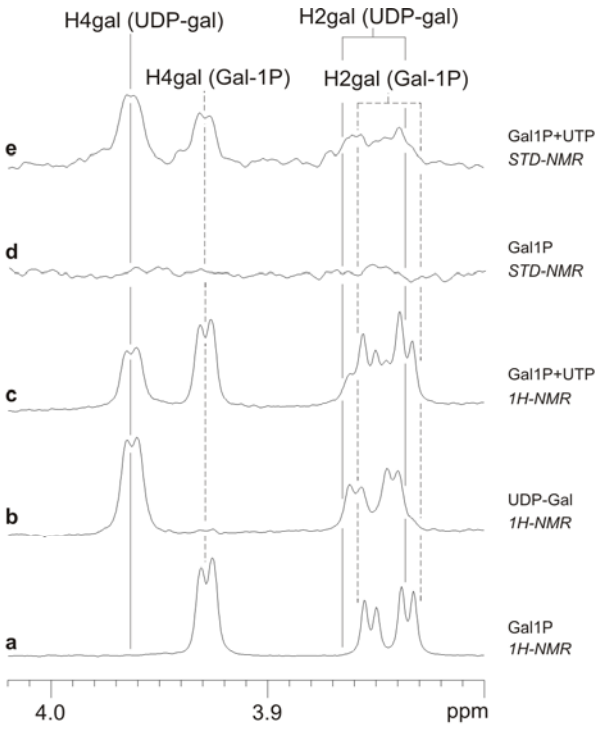

FIGURE 5. **Ordered bi-substrate mechanism of *L. major* UDP-sugar pyrophosphorylase.** STD NMR spectrum for Gal-1-P does not present any signal demonstrating absence of binding to *L. major* USP (panel d). In presence of UTP, Gal-1-P binds to the enzyme and UDP-Gal is formed as shown by relative STD NMR signals for these compounds (panel e). ^{1}H-NMR spectra of Gal-1-P (panel a); UDP-Gal (panel b) and a mixture of Gal-1-P and UTP showing conversion into UDP-Gal (panel c) were acquired for reference.

Like UGP, *Lmj*USP acts reversibly *in vitro* and could efficiently use the substrates UDP-Gal and UDP-Glc to produce Gal-1-P and Glc-1-P, respectively. Importantly, significant activity, albeit at lower level, was also detected when D-xylopyranose-1-phosphate (Xyl-1-P), UDP-β-L-arabinopyranose (UDP-L-Ara) or UDP-α-D-galacturonate (UDP-GalA) were used as substrates while GlcNAc-1-phosphate and UDP-GalNAc were not converted. Similarly, α-D-mannose-1-phosphate (Man-1-P) and α-L-fucose-1-phosphate (Fuc-1-P) were not activated by *Lmj*USP in the presence of GTP. Our results clearly establish that the leishmanial enzyme exclusively utilizes UTP for activation of Gal-1-P in accordance with expectations. Together, these data establish the pyrophosphorylase activity of the candidate protein and demonstrate its broad substrate specificity that includes Gal-1-P and Glc-1-P.

Leishmania major USP follows simple Michaelis-Menten kinetics – The kinetic parameters of the

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purified enzyme were determined for the main substrates of the forward (Gal-1-P, Glc-1-P, and UTP) and reverse (UDP-Gal, UDP-Glc, and pyrophosphate) reaction. Additionally, UDP-L-Ara and UDP-GalA which represent analogues of UDP-Gal in which the C6-group is either absent or substituted were analyzed. *L. major* USP followed Michaelis-Menten kinetics with all substrates tested (Fig. S3). The calculated kinetic parameters V_{max}, K_M, k_{cat} and derived catalytic efficiency (k_{cat}/K_M) are summarized in table 2. Interestingly, Gal-1-P seems to be preferred over Glc-1-P (K_M = 860 µM and 1706 µM, respectively) suggesting a slight influence of the C4 hydroxyl group stereochemistry in substrate binding. Nevertheless, the affinity of *L. major* USP for these two hexose-1-phosphates is rather low and remarkably lower than the affinity of the enzyme for UTP (K_M = 860 and 1706 µM versus 98 or 116 µM), the latter being comparable to the affinity of various UGPs for UTP (22;30;31). This striking difference is reflected by the low efficiency of the enzyme with hexose-1-phosphates when compared to the co-substrate UTP. In contrast, the affinity and maximum velocity of the enzyme with UDP-Gal and UDP-Glc are comparable (K_M = 148 or 174 µM and V_{max} = 134 or 157 µmol/min/mg respectively) resulting in an identical efficiency of the enzyme with these two substrates of 51% when compared to the efficiency obtained with UTP. Assuming that the sugar moiety of hexose-1-phosphate and UDP-hexose binds to the same site, the higher affinity of *Lmj*USP for the UDP-sugar suggests that the nucleotide moiety plays a major role in substrate binding. This hypothesis is consistent with the high K_M for UTP. As expected, the affinity and turnover for UDP-L-Ara and UDP-GalA are low in agreement with the observation that these nucleotide sugars are poorer substrates (Tables 1 and 2) (19;20;32). Thus the absence of the C6 hydroxyl group and particularly its substitution leads to a drastic reduction of the enzyme efficiency.

Because of its high affinity for *Lmj*USP, UTP might bind first to the enzyme followed by Gal-1-P or Glc-1-P thus favoring the forward reaction despite the low affinity of the enzyme for Gal-1-P and Glc-1-P. In line with this assumption, the turnover rates measured for the hexose-1-phosphates were similar to the turnover rate measured for UTP (~200 s^{-1}) and higher than the one of UDP-Gal, UDP-Glc or pyrophosphate (~170 s^{-1}). Altogether the kinetic data and their comparison with data obtained with *L. major* UGP (24) suggest an ordered bi-bi substrate mechanism.

Leishmania major USP follows an ordered bi-bi mechanism – To gain insight into substrate binding to *L. major* USP and substantiate a sequential binding mode of substrates to the enzyme, protein-ligand interactions were investigated by Saturation Transfer Difference Nuclear Magnetic Resonance spectroscopy (STD NMR). This powerful method (33;34) is capable of identifying ligand binding epitopes of a ligand when bound to a target protein. Ligand protons that are in close contact with the enzyme receive a higher degree of saturation via the protein resulting in the observation of stronger STD NMR signals compared to ligand protons that do not interact with the protein surface and are solvent exposed. ^{1}H NMR spectra were first recorded to assign signals and ensure that the enzyme was active in the forward and reverse reactions under the applied assay conditions. STD NMR experiments revealed strong STD NMR signals for UTP whereas the related nucleotides ATP, GTP and CTP result in low, if any, STD NMR signal intensity (Fig. S4). This result strongly suggests that ATP, GTP and CTP have low affinity for USP. The clear signals arising from the ribosyl proton $H4_{rib}$, uridyl protons $H5_{uri}$ and $H6_{uri}$ and in particular from the ribosyl proton $H1_{rib}$ of UDP-Gal indicate their close vicinity to the protein surface. Interestingly, UDP and UMP show little affinity for *Lmj*USP emphasizing the importance of the UTP γ-phosphate in binding (Fig. S5). In contrast, the STD NMR spectra of UDP-Gal, UDP-Glc, UDP-GalA and UDP-L-Ara display strong STD NMR effects, indicating high affinity (Fig. 4) for *Lmj*USP. The STD NMR spectrum of UTP revealed close proximity of the $H1_{rib}$, $H4_{rib}$, $H5_{uri}$ and $H6_{uri}$ protons when bound to *Lmj*USP while the $H2_{rib}$, $H3_{rib}$ and $H5/H5'_{rib}$ protons seem less important in the binding event. This interaction is strengthened by the sugar moiety notably in the case of UDP-Gal and UDP-Glc. Remarkably most of the Gal protons are in close vicinity of the protein surface with the $H4_{gal}$, $H2_{gal}$, $H3_{gal}$ and $H6_{gal}$ protons making the most significant contributions to the binding event. In comparison, the glucose moiety of UDP-Glc receives less saturation suggesting a lesser involvement in the binding event to the enzyme. The relative STD NMR effects of the $H1_{glc}$ (25%) $H2_{glc}$ (17%) and $H3_{glc}$ (30%) protons clearly demonstrate a less intimate contact with the protein and therefore a likely poorer affinity of UDP-Glc for USP. Despite their different stereochemistry, the $H4_{gal}$ and $H4_{glc}$ protons show no drastic difference in relative STD NMR effects, in good agreement with

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the specificity of the enzyme. Finally, the STD NMR spectra of UDP-Ara and UDP-GalA emphasize the importance of the $H6_{gal/glc}$ and $H6'_{gal/glc}$ proton contacts with the protein surface. UDP-Ara seems to bind to the enzyme in a similar manner as UDP-Gal but loss of H6 and H6′ results in a reduced affinity. In contrast, the steric hindrance and/or negative charge of the carboxylic group of GalA appears to strongly influence binding of the sugar as only weak STD NMR effects in complex with the enzyme are observed.

Interestingly, no binding of Gal-1-P, Glc-1-P or Xyl-1-P to L*mj*USP was observed by STD NMR spectroscopy. However, addition of UTP to sugar-1-phosphate:*Lmj*USP mixture resulted in strong and specific STD NMR signals for H4 and H2 protons of Gal-1-P (Fig. 5). This phenomenon, suggestive of an ordered bi-substrate mechanism with UTP binding preceding the hexose-1-phosphate entry, was previously observed with the L*mj*UGP and a conformational change was proposed (22). The subsequently determined x-ray crystal structure clearly revealed a conformational change upon complexion with UTP (26). It is therefore not unreasonable to assume that USP binds UTP in a similar mode. Interestingly, STD NMR signals could be clearly observed for various UDP-sugars even in the absence of pyrophosphate. In the reverse reaction, the data suggests a sequential binding mode with the nucleotide sugar binding prior to pyrophosphate supported by the observation that the affinity of the enzyme for pyrophosphate seems to be influenced by the nucleotide sugar (Table 2).

DISCUSSION

Although *Leishmania* promastigotes are known to incorporate Gal taken from the environment into surface molecules (15), enzymes involved in the salvage pathway for UDP-Gal synthesis had not yet been reported. The activation of this monosaccharide was lately shown to be independent from UDP-Glc synthesis since a *L. major* UGP deletion mutant still expresses Gal containing molecules (Lamerz et al., unpublished work). Herein we report the identification and characterization of a *L. major* UDP-sugar pyrophosphorylase able to reversibly activate Gal-1-P into UDP-Gal constituting the Isselbacher pathway for UDP-Gal synthesis.

Leishmania major USP presents a clear homology with plant USPs and a modest but significant homology with UGPs and UAPs over the entire sequence. In particular, the pyrophosphorylase glycine rich consensus motif (25;26) essential for catalysis is highly conserved and additional residues involved in uridine and phosphate binding. As highlighted by STD NMR spectroscopic studies, interactions of the uridine moiety of nucleotide sugars or UTP with *Lmj*USP are similar to those observed with *Lmj*UGP and play a significant role in substrate binding. This leading role of the nucleotide moiety is observed in many enzymes involved in glycosylation for example UDP-galactopyranose mutase (35), sialyltransferases (36), and pyrophosphorylases from *E. coli* (37) and might even hold true for nucleotide sugar transporters (38).

Intriguingly, residues interacting with the glucose moiety in UGPs are not conserved in USPs which probably accounts for the broader specificity toward monosaccharide-1-phosphates and UDP-sugars of the latter. Like plant USPs, *Lmj*USP is able to convert reversibly and efficiently both Glc-1-P and Gal-1-P with a slight preference for Gal-1-P. Pentose-1-phosphates such as Xyl-1-P and Ara-1-P can also be activated *in vitro* by *Leishmania* or plant USPs albeit with a reduced efficiency reflecting their lower affinity for the enzyme, and underlining the contribution of the hexoses H6 and H6′ protons to binding. In contrast, GalA-1-P is a poor substrate of *Lmj*USP. It is reasonable to assume that the carboxylic acid group of GalA creates either steric hindrance or more likely an unfavoured electrostatic potential leading to weak interactions of the uronic acid with the leishmanial enzyme. Different to the plant enzymes, all USPs identified in *Leishmania* species present an 18 amino-acid insertion near the uridine binding site that contains the conserved residues G223H224. Although not yet proven, this insertion might be responsible for the substrate differences observed for plant enzymes. The role of these additional amino acids awaits a crystal structure of *Lmj*USP.

Despite its lower affinity for Ara-1-P, *Arabidopsis thaliana* USP seems to play a central role in the salvage of this pentose *in vivo* (21). In *Leishmania*, however, where D-Ara is present, the monosaccharide is exclusively activated by GDP and a putative GDP-Ara pyrophosphorylase has been identified in the genome (8). In addition to GDP-α-D-Ara, *L. major* promastigote synthesizes UDP-Glc, UDP-Gal (in the pyranosic and furanosic form), UDP-GlcNAc, GDP-Man and low amounts of GDP-Fuc but neither UDP-Xyl nor its precursor UDP-GlcA are produced (8). Considering the specificity of *Lmj*USP for UDP-

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activated sugars, its inability to act on hexosamine-1-phosphate and the characterization or presence in the genome of specific pyrophosphorylases for the activation of GDP-activated sugars and UDP-GlcNAc (8;39), *Leishmania* USPs most likely play a role in the salvage of galactose and glucose exclusively. Moreover and despite the fact that USP is able to act reversibly, the ordered bi-bi mechanism of the enzyme and its high affinity for UTP, a naturally abundant metabolite, presumably ensures the synthesis of nucleotide sugar rather than their pyrophospholysis. Remarkably, *Lmj*USP seems to have evolved a slight preference for Gal-1-P over Glc-1-P in good agreement with the presence of galactose (Gal) in many of their surface glycoconjugates.

In contrast to *Leishmania* parasites, the trypanosomatids *Trypanosoma brucei* and *Trypanosoma cruzi* are thought to rely exclusively upon epimerization of UDP-Glc for synthesis of UDP-Gal since the hexose transporters of these parasites are unable to transport Gal (9;10). Nevertheless the genome of *T. cruzi* contains homologues of galactokinase and USP which might be involved in recycling galactose originating from degradation of glycoconjugates in the endo-lysosomal compartment or plays a role in salvage pathways of other sugars. *T. cruzi* is the only one of the three trypanosomatids that synthesizes UDP-Rha, UDP-Xyl and its precursor UDP-GlcA (8). Like the plant enzyme, *T. cruzi* USP might be involved in the synthesis of these nucleotide sugars. In *Arabidopsis*, USP is particularly important in pollination and possibly converts Gal-1-P, Ara-1-P and Rha-1-P secreted by the pistil (21). In *T. cruzi* however the precise function of the Xyl and Rha metabolism is still unclear.

The trypanosomatid USPs are closely related to plant USPs and hypothetical proteins of the diatoms *Phaedactylum tricornutum* and *Thalassiosira pseudonana* and green algae *Micromonas pusilla*, *Ostreococcus tauri*, *Ostreococcus lucimarinus* and *Chlamydomonas reinhardtii* which suggest the common origin of these genes. Moreover, USP homologues are found in ciliate protozoa (*Paramecium tetraurelia*, *Tetrahymena thermophila*) and Apicomplexa (*Toxoplasma gondi*, *Cryptosporidium sp*. and *Plasmodium sp*.) but seems absent from Percolozoa, Loukozoa and Metamonada. The discovery of a plant-like enzyme common to several pathogens opens new perspectives for the development of a pesticide like drug as already proposed for the apicomplexan parasites (40). Like in mammals (41) and yeast (42), accumulation of Gal-1-P might reveal toxic for the parasite.

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The abbreviations used – Ara, arabinose; CTP-synthase, cytidine triphosphate synthase; Fuc, fucose; Gal, galactopyranose; Gal*f*, galactofuranose; GalA, galacturonuic acid; GALT, UDP-glucose:α-D-galactose-1-phosphate uridylyltransferase; GalNAc, *N*-actetylgalactosamine; Glc, glucose; GPI, glycosyl-phosphatitylinositol; LPG, lipophosphoglycan; Man, mannose; MESG, 2-amino-6-mercapto-7-methylpurine ribonucleoside; STD, Saturation Transfer Difference; UAP, UDP-*N*-acetylglucosamine pyrophosphorylase; UGP, UDP-glucose pyrophosphorylase; USP, UDP-sugar pyrophosphorylase; Xyl, Xylose.

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Supplementary Material

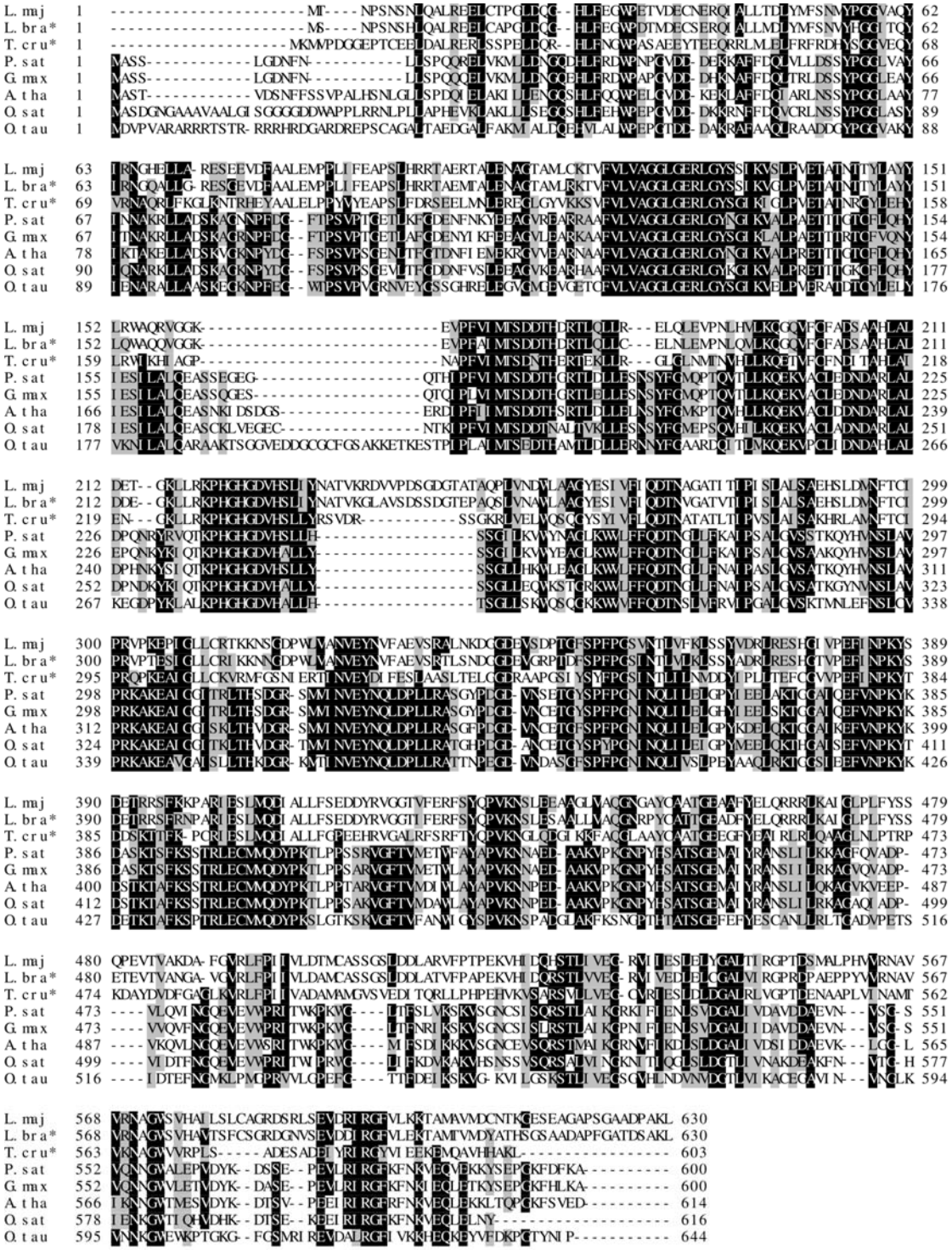

FIGURE S1. **Sequence alignment of UDP-sugar pyrophosphorylases.** The alignment was performed using ClustalW algorithm. Abbreviations: L.maj: *Leishmania major*, L.bra: *Leishmania braziliensis*, T.cru: *Trypanosoma cruzi*, P.sat: *Pisum sativum*, G.max: *Glycine max*, A.tha: *Arabidopsis thaliana*, O.sat: *Oryza sativa*, O.tau: *Ostreococcus tauri*. These organisms belong to the family of Trypanosomatidae and the kingdom of plants. Hypothetical proteins are indicated by an asterisk (*). Identical amino acids are shaded black, whereas similar amino

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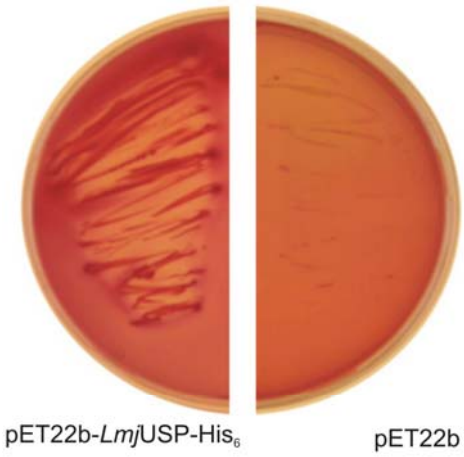

FIGURE S2. **USP complements the growth defect of *E. coli* galU mutant strain DEV6.** Lacking a functional UDP-glucose pyrophosphorylase gene *E. coli* DEV6 *gal*U mutants are unable to grow on galactose-containing MacConkey agar, due to accumulation of galactose-1-phosphate. Mutants transformed with the pET22b-*Lmj*USP-His$_6$ plasmid recover the ability to grow on galactose containing media. Mock control cells were unable to circumvent the cytotoxic effects of high galactose-1-phosphate concentrations.

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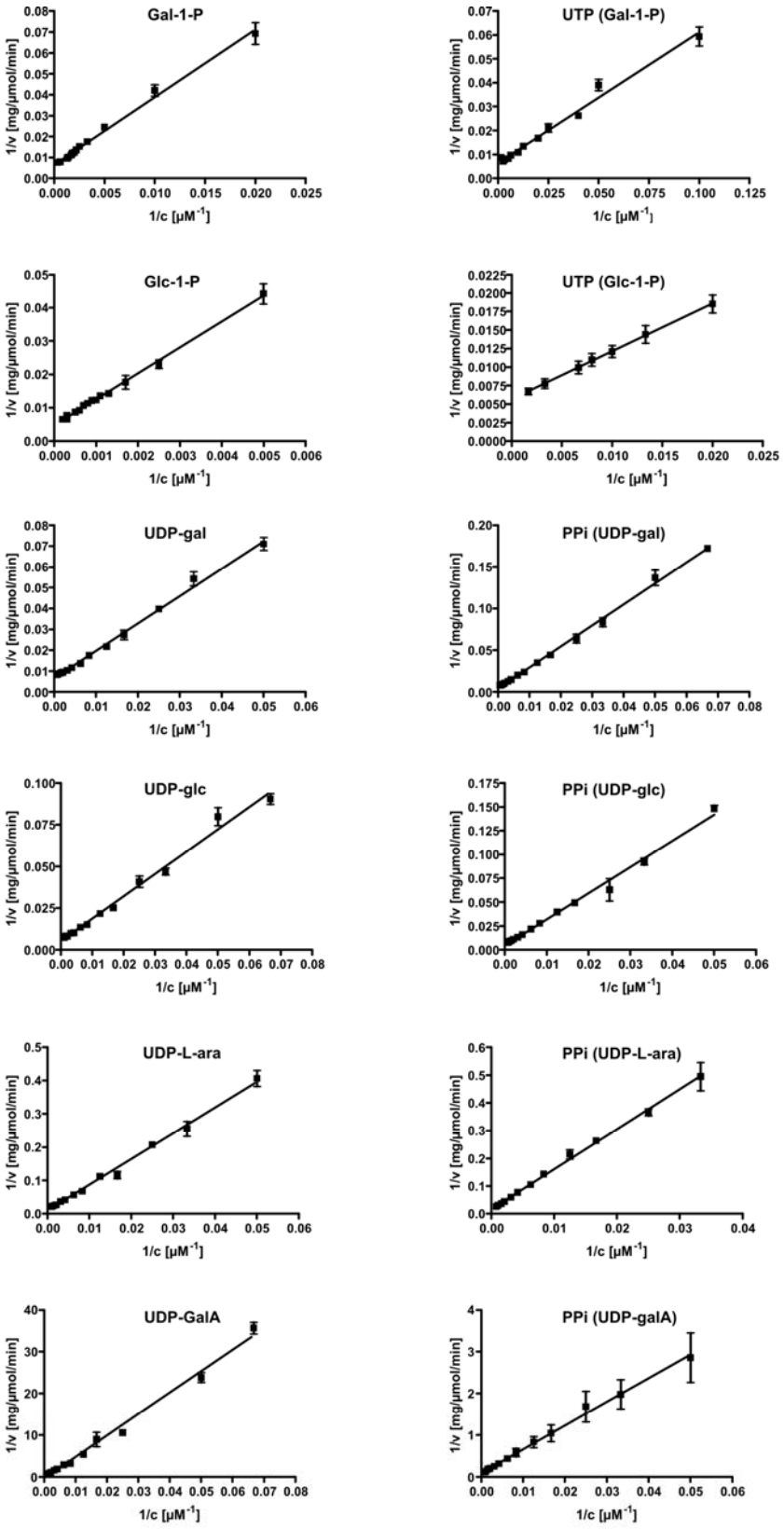

FIGURE S3. **Lineweaver-Burk plots indicating Michaelis-Menten kinetics.** Substrate parameters were determined in presence of rate non-limiting and constant co-substrate concentrations (0.8 mM UTP, 2 mM Gal-1-P, 3 mM Glc-1-P, 2 mM pyrophosphate and 2 mM UDP-sugars), in order to generate a pseudo first-order reaction type. The highest efficiency obtained was UTP set to 100% for both directions. Errors are given as standard deviations from triplicate measurements.

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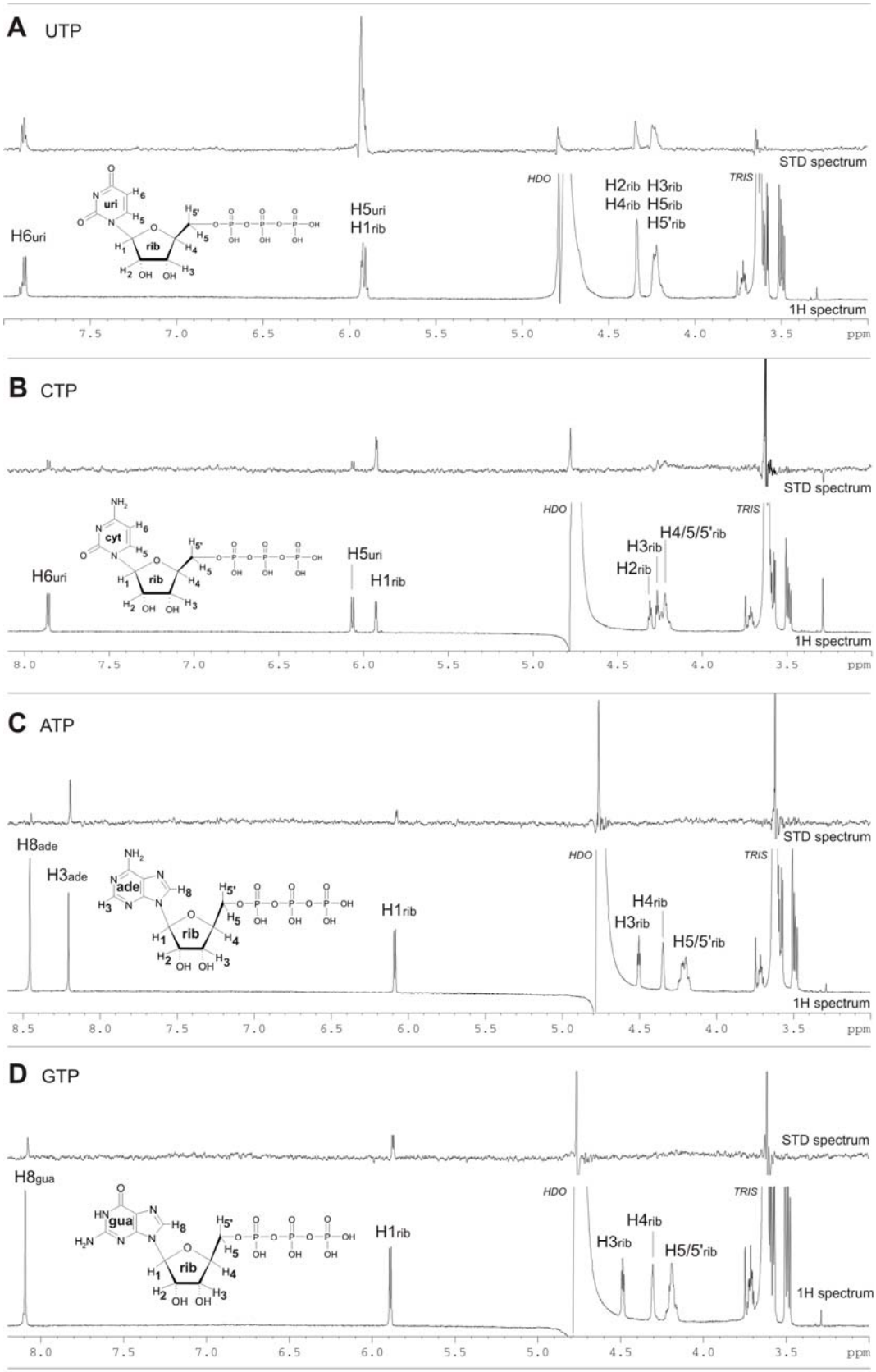

FIGURE S4. **UTP but not CTP, ATP and GTP binds to *L. major* UDP-sugar pyrophosphorylase.** STD NMR spectra for UTP (*A*), CTP (*B*), ATP (*C*), and GTP (*D*) displaying strong STD NMR signals for UTP and weak binding of other nucleotides. ^{1}H-NMR reference spectra, used to correctly assign STD signals, are given at the bottom of each panel. Spectra are truncated, showing the significant peaks of the nucleotide.

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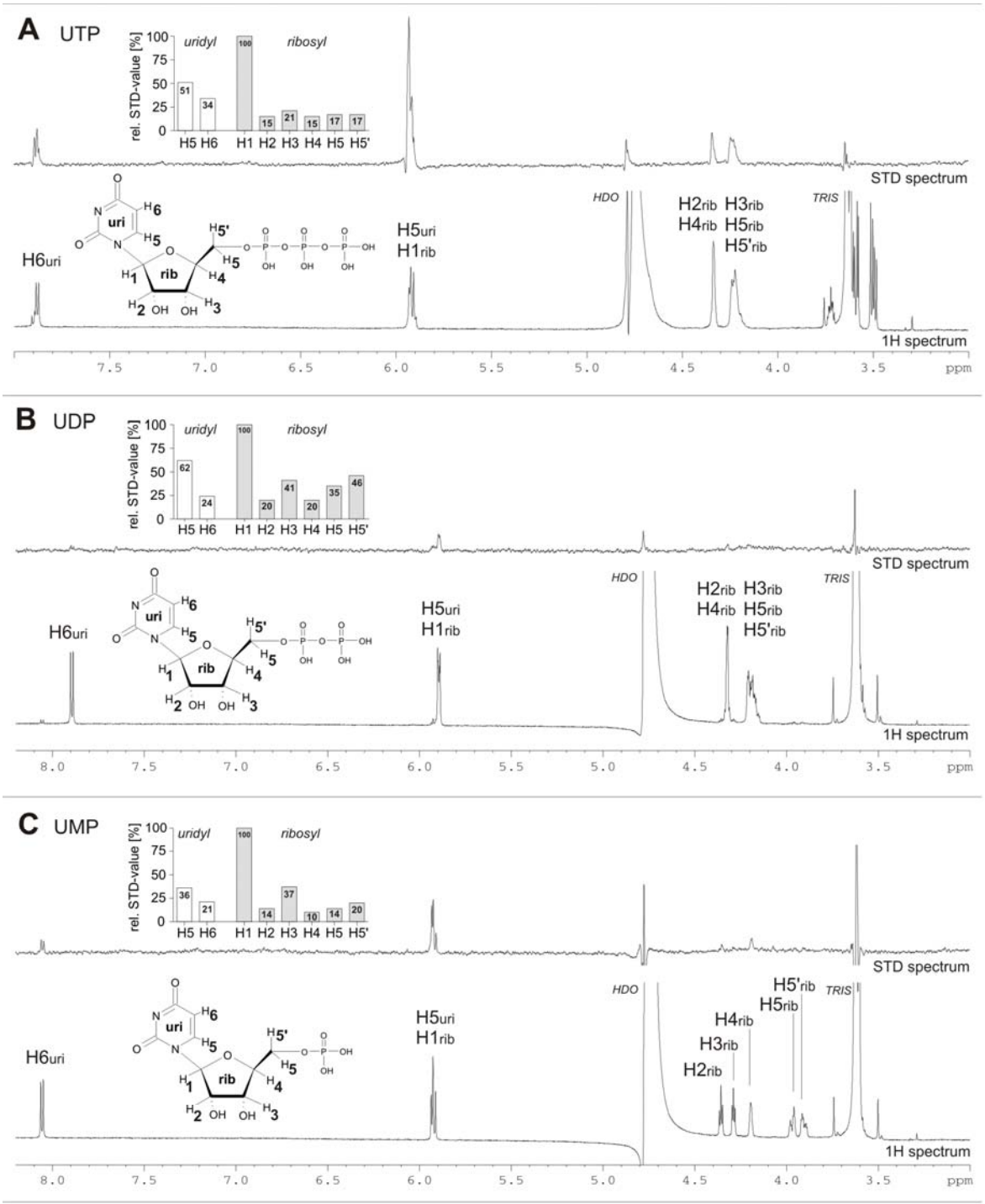

FIGURE S5. **Importance of γ-phosphate of UTP for binding to *L. major* UDP-sugar pyrophosphorylase.** STD NMR spectra for UTP (*A*), UDP (*B*) and UMP (*C*) demonstrating strong binding of UTP to *L. major* USP and weak binding of UDP and UMP. ^{1}H-NMR reference spectra, used to correctly assign STD signals, are given at the bottom of each panel. Relative STD NMR signals were calculated for each proton and depicted in a bar chart (inset). Spectra are truncated, showing the significant peaks of the nucleotide.

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Chapter 4 – Evaluating the Role of UDP-sugar Pyrophosphorylase in the Protozoan Parasite *Leishmania major*

Manuscript

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Preface – About this manuscript

This chapter of my work is dedicated to the evaluation of the role of UDP-sugar pyrophosphorylase (USP) within *L. major*. First, an *L. major usp* deletion mutant (Δ*usp*) was generated to demonstrate the role of USP in galactose salvage, as postulated from the previous studies, and its importance for the parasite evaluated. Moreover, in order to measure the contribution of this enzyme to residual UDP-sugar production in UDP-glucose pyrophosphorylase null mutants (Δ*ugp*), the generation of a mutant deficient in USP and UGP was attempted. Finally, the subcellular localization of USP, UGP and its metabolic neighbors was analyzed, since trypanosomes seem to situate their UDP-Gal metabolism in specialized compartments.

We succeeded in generating two *L. major* mutant strains: the Δ*usp* strain, in which both alleles of *L. major usp* were replaced by two resistance marker genes using homologous recombination and the Δ*ugp*/*usp*$^{+/-}$ strain, in which one allele of *usp* is replaced by an adequate resistance marker within the Δ*ugp* mutant. An episomal expression vector ('add-back') was introduced into the Δ*usp* mutant (Δ*usp*/+*USP*), in order to reconstitute the wild type situation. The strains were analyzed for the presence of the characteristic surface determinant lipophosphoglycan by a combination of electrophoretic, immunochemical and enzymatic techniques. Viability parameters were recorded concerning cell morphology and growth behavior. In addition, several techniques to determine subcellular localization were employed, such as immunofluorescence, permeablization experiments and isopycnic ultracentrifugation.

This chapter summarizes the unpublished data that I obtained in the last months and highlights my ongoing work.

***Leishmania major* UDP-sugar pyrophosphorylase (USP) catalyzes the reaction of a sugar-1-phosphate and its stringent nucleotide donor, UTP, forming UDP-sugar and pyrophosphate. The broad substrate diversity of the leishmanial enzyme is a characteristic of this class of enzyme (EC 2.7.7.64) that utilizes similar sugar-1-phosphates such as galactose-, glucose-, xylose-, arabinose-, galacturonic acid- or glucuronic acid-1-phosphate. The *in vitro* characteristics of USP are in perfect agreement with a postulated function of this enzyme in galactose salvage. In agreement with this role first analyses of an *L. major* USP deficient mutant suggest a reduction of side chain galactosylation of the abundant cell surface polysaccharide LPG. Furthermore, a heterozygous deletion of *USP* in recently characterized UDP-glucose pyrophosphorylase (UGP) knockout mutants, revealed high impact on residual LPG expression, strengthening the role of USP in the UDP-galactose pathway. Interestingly, USP as well as several other enzymes involved in nucleotide sugar metabolism seem to be situated in the cytoplasm despite the presence of peroximal targeting sequences, highlighting a major difference from the closely related trypanosomes.**

INTRODUCTION

Recently, we biochemically characterized a recombinantly expressed UDP-sugar pyrophosphorylase (*Lmj*USP) from *L. major*, which was until then only precisely characterized in plants. Genomic database screening revealed that *Lm*USP has no homologue in the animal kingdom and groups with putative USPs from plants and algae. In particular *Lmj*USP displays an enzymatic homology to characterized plant USPs from *Pisum sativum* and *Arabidopsis thaliana*, since a similar range of substrates with equal activities are utilized (1).

In contrast to the broad substrate spectrum of *Lm*USP, which activates predominantly D-galactose-1-phosphate, D-glucose-1-phosphate, but also xylose-, L-arabinose- and galacturonic acid-1-phosphate with UTP to form its respective UDP-sugar, the recently characterized *L. major* UDP-glucose pyrophosphorylase (*Lmj*UGP) is very specific in uridinylating glucose-1-phosphate (2). *Lmj*UGP is thought to play a major role in UDP-galactose synthesis, via the interplay with a genetically assigned UDP-galactose 4-epimerase (GALE) in *L. major*. Interestingly, the expression of the characteristic *L. major* surface and secreted phosphoglycan structures, which are heavily loaded with galactose, was however only modestly affected in *Lm*UGP gene deletion (Δ*ugp*) mutants and residual activity for UDP-Glc production was detected. The galactose salvage pathway known to take place in *Leishmania* is thus independent from UDP-Glc biosynthesis in agreement with the lack of UDP-glucose:α-D-galactose-1-phosphate uridylyltransferase (GALT) enzyme mediating the classical Leloir pathway. The *in vitro* characteristics of USP allow postulating a role of this enzyme in salvage pathway.

The UDP-Gal pathway from the related trypanosomatids *Trypanosoma brucei* and *Trypanosoma cruzi* differ from the one of *Leishmania.* Indeed the hexose transporters of these trypanosomes are unable to transport galactose and thus the only route for UDP-Gal formation is via epimerization of UDP-Glc (3; 4). Deletion of *GALE* is lethal in *T. brucei* and *T. cruzi (5-7).* In contrast, *L. major GALE* deficiency could theoretically be compensated by direct activation of imported galactose via galactokinase and USP.

Trypanosomatid parasites (including *Trypanosoma* and *Leishmania* species) possess specialized peroxisomes termed glycosomes. This unique organelle compartmentalizes various metabolic pathways, including glycolysis and gluconeogenesis. In trypanosomatid parasites unlike in most eukaryotes, the activities of hexokinases are not regulated by feedback inhibition (8; 9). Several studies have suggested that, due to low ATP concentrations inside the glycosome, kinase activities are kept in check, which in turn protects these cells from uncontrolled glycolytic flux (10-12). Moreover accumulation of glucose-6-phosphate has been shown to be toxic in *Leishmania* like in *Trypanosoma* (12; 13). Enzymes involved in nucleotide sugar metabolism were also localized in the glycosomes in *T. cruzi* or *T. brucei (14; 15).*

In this study we address the role of USP within *L. major* parasites by targeted gene deletion. Preliminary results are consistent with a role of USP in galactose salvage. In addition, localization of several enzymes involved in UDP-Glc and UDP-Gal metabolism suggests that *Leishmania* and *Trypanosoma* differ in their regulation of the carbohydrate metabolism.

EXPERIMENTAL PROCEDURES

Parasite culture, transfection and growth curves – Promastigote cultures of *L. major* MHOM/ SU/73/5ASKH and respective mutant cell lines were grown at 27°C in M199 medium (Invitrogen) supplemented with 10% heat inactivated fetal calf serum, 40 mM Hepes pH 7.5, 0.1 mM adenine, 0.0005 % hemin, 0.0002 % biotin and 50 U/mL penicillin/streptomycin. Cultures were diluted 10-fold every 3-4 days. Transfection of parasites was performed by electroporation (16) and allowed to grow in M199 medium for 24 hours before transfer to semi solid media containing 1% Noble agar (Becton Dickinson), M199 medium and the doubled concentrations of appropriate antibiotics. Individual colonies were picked and grown in selective M199 liquid media. The antibiotics phleomycin, hygromycin B, puromycin and nourseothricin were obtained from InvivoGen, G418 (neomycin) from Sigma. Leishmania growth was recorded by measuring the OD at 615 in 1 ml-cuvettes, while promastigote cultures were provided with sugar deficient RPMI 1640 medium (Gibco, R1383) plus 10% dialyzed FBS (Gibco), which was either supplemented with 50 mM glucose, galactose, glucose/galactose mixture, or xylose. No selective antibiotics were added.

Antibody Preparation – Three New Zealand rabbits (#222, #223, #244) were immunized with *L. major* USP antiserum by subcutaneous injection with 500 µg of recombinantly purified protein. For the first injection, protein was mixed with complete Freund's adjuvant (Difco), followed by six injections at 6-week intervals using the incomplete Freund's adjuvant (Difco). Blood was collected 10 days after last injection. The prepared serum can be diluted up to 1:20 000, specifically recognizing both recombinant and native forms of *Lmj*USP. No cross-reactivity to other His_6-tagged proteins could be recorded.

Immunofluorescence microscopy – For fluorescence microscopy the cells were washed in PBS and immobilized on poly(L)-lysine coated cover slides using 4% paraformaldehyde. To visualize endogenous *Lmj*USP, wild type parasites were incubated with 50 mM NH4Cl for 15 min and permeabilized with 0.1% Saponin. The samples were sequentially incubated with polyclonal rabbit anti-serum USP#223 (see above) at a dilution of 1:500 and 1:1000 and the secondary antibody Alexa 488 goat anti rabbit mAb (Molecular Probes) at dilutions of 1:500 and 1:100, respectively. Nucleus and kinetoplast DNA was stained with 8µg/ml 4,6-Diamidino-2-phenylindole (DAPI, Sigma) in parallel to secondary antibody incubation. The same protocol was performed for detection of UGP using similar dilutions of a recently prepared antiserum (2).

Subcellular fractionation – The method was based on a procedure described before (17; 18), but briefly performed as followed. All steps are accomplished at 4°C. Approximately $8x10^8$ cells of *L. major* promastigotes were harvested at late Log-phase by centrifugation (1000xg, 10 min), washed twice in 5 ml ice cold PBS and once in 2 ml ice cold hypotonic Buffer (2 mM EGTA 2 mM DTT, 1 mM PMSF, 1 µM Leupeptin, 1 µM Pepstatin, 1 µM Bestatin). After centrifugation cells were adjusted to $2x10^8$ cells/ ml with hypotonic buffer and incubated for 5 min on ice. Cell lysis was achieved by expulsion (10-times) of the suspension through a 27 gauge needle and status checked by light microscopy. Accordingly the lysate was made isotonic by addition of 4x stabilization buffer (100 mM HEPES-NaOH pH 7.4, 7.5 $\%_{(w/v)}$ sucrose, 1 mM ATP, 1 mM EGTA, 2 mM DTT, 1 mM PMSF, 1 µM Leupeptin, 1 µM Pepstatin, 1 µM Bestatin) and centrifuged 10 min at 500xg to remove cell debris and nuclear fraction. The postnuclear supernatant (approx. 5 ml) was layered on top of a linear sucrose gradient (12.5 ml), which was prepared using FPLC system (Amersham Pharmacia Biotech ÄKTA FPLC) setting up a gradient ranging from 15-80 $\%_{(w/v)}$ sucrose in an Ultraclear Centrifuge tube (Beckman). The organelles were fractionated by centrifugation at 170.000xg over night at 4 °C in a Beckman L-80 Ultracentrifuge using a SW41 rotor. Fractions (0.7 ml) were collected from the bottom of the tube by punctuation and densities calculated by measuring the refractive index. For each fraction protein concentrations were measured by using the BCA assay (Pierce). Samples were snap frozen in liquid nitrogen and subjected to -80 °C awaiting further analysis using coupled in vitro enzyme assays.

Selective permeabilization – Intact cells were titrated with growing concentrations of digitonin, and fractions analyzed enzymatically. Hence, depending on cell membrane destabilizing digitonin concentrations a characteristic set of enzymes is released. Therefore stationary phase *L. major* promastigote cultures ($5x10^7$ cells/ ml) were washed

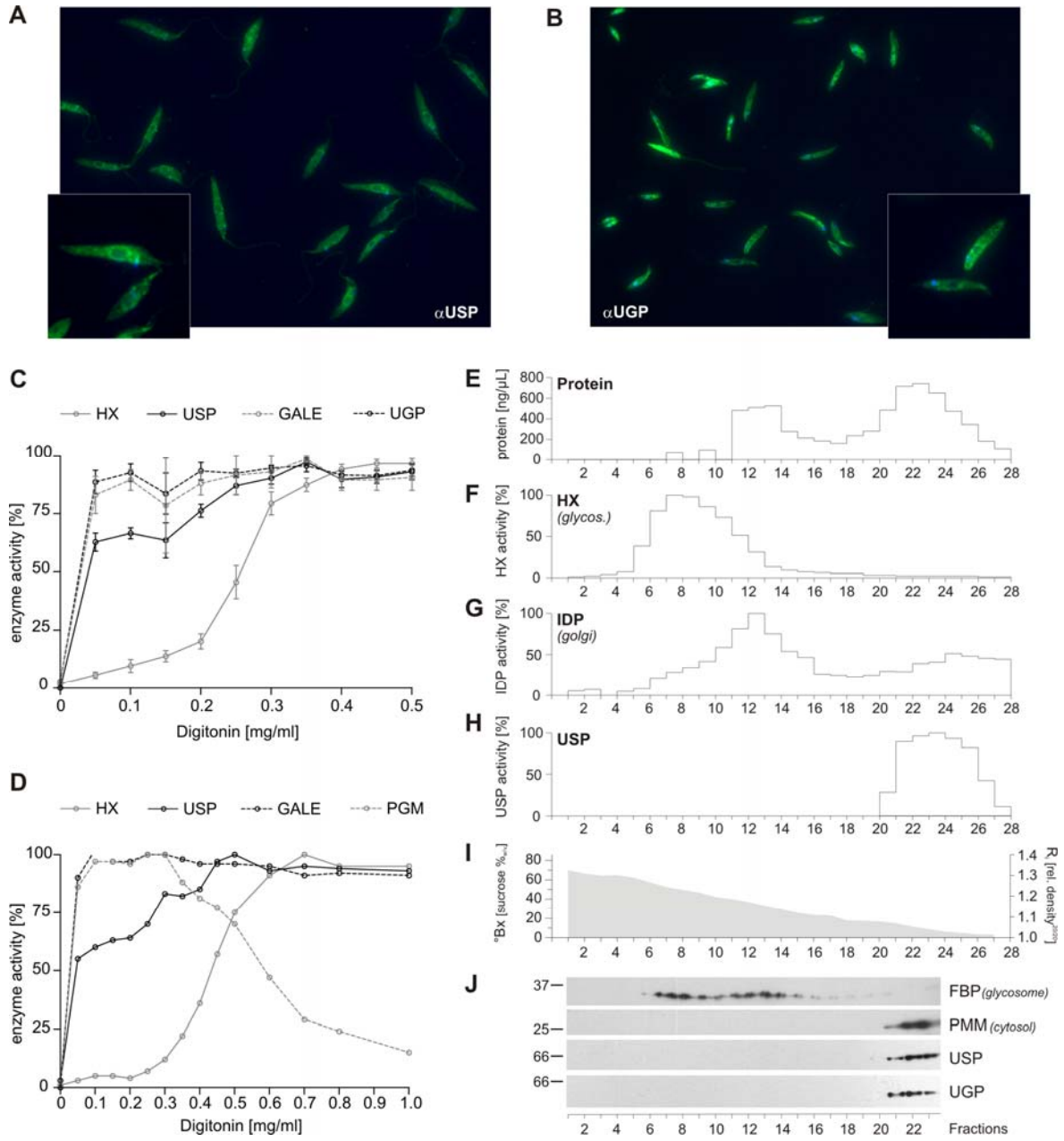

Figure 1. ***L. major*** **UDP-galactose and UDP-glucose metabolism is situated in the cytosol.** Immunofluorescence staining (**A+B**), successive permeabilization by digitonin titration (**C+D**) and cell fractionation by isopycnic ultracentrifugation (**E-J**) are described in detail in the experimental procedures section. Immunofluorescence: saponin permeabilized, stationary phase *L. major* promastigotes were detected either with rabbit αUSP (**A**) or αUGP (**B**) serum and visualized via mouse α-rabbit Alexa488 monoclonal antibody. Successive permeabilization: using promastigote cultures, increasing digitonin concentrations successively released enzyme markers representative of different subcellular compartments; **C**, $6x10^7$ cells/ml; **D**, with $1.2x10^8$ cells/ml, marker activities were detected enzymatically by the respective *in vitro* assay, HX: hexokinase, USP: UDP-sugar pyrophosphorylase, GALE: UDP-galactose 4-epimerase, UGP: UDP-glucose pyrophosphorylase, PGM: phosphoglucomutase. Cell fractionation: *L. major* promastigotes were lysed and the post-nuclear supernatant was subjected to isopycnic ultracentrifugation on a sucrose gradient; **E**, proteinconcentrations were determined with BioRad Bradford assay; **F**, the glycosomal marker hexokinase (HX), **G**, the golgi marker inositol diphosphatase (IDP), and (**H**) the UDP-sugar pyrophosphorylase (USP) were detected with a coupled enzyme assay for each fraction, and highest activity was set to 100%, respectively; **I**, degree Brix (°B) of the sucrose gradient was checked refractometrically and relative density R_i calculated; **J**, western blot analysis was performed with either α-fructose 1,6-bisphosphatase (αFBP) or α-phosphomannomutase (αPMM) serum used as subcellular markers for glycosomes or cytosol, similarly αUSP and αUGP serum was probed and all detected with an ECL Femto-Kit (Pierce).

twice with STE buffer (250 mM sucrose, 25 mM Tris-HCl pH 7.4, 1 mM EDTA, 150 mM NaCl) and adjusted to $2x10^8$ cells/ ml with STE buffer. A series of digitonin concentrations (0-1000 µg/ml) was prepared in a volume of 250 µl STE buffer, and each sample supplemented with 100 µl cell suspension. Probes were gently mixed by inversion, incubated for 5 minutes at room temperature and rapidly cooled down on ice. Accordingly, the samples were centrifuged at 13000xg for 2 min at 4 °C, the supernatant was recovered carefully, snap frozen in liquid nitrogen and stored at -80 °C for further analysis.

Enzyme Assays – Enzymatic activity was tested either in full cell extracts, or in samples derived from cellular fractionation by velocity centrifugation or digitonin titration. The assay buffer used was 50 mM Tris/HCl pH 7.8 and 10 mM $MgCl_2$, and according to the detected enzyme activity, supplemented with the respective substrates and coupled enzymes for NADH detection at 340 nm. Hexokinase (HX) activity was tested in assay buffer supplemented with 10 mM glucose, 1.5 mM NAD+, 1 mM ATP and 1 unit glucose-6-Phosphat dehydrogenease (G6P-DH) (*Leuconostoc mesenteriodes*, SIGMA). UDP-galactose 4-epimerase (GALE) activity was measured in additional 2 mM NAD+, 2 mM UDP-Gal and 0.08 U/ml UDP-glucose dehydrogenase (UG-DH) (bovine liver, Calbiochem). Phosphoglucomutase (PGM) reaction took place in the presence of 2 mM NAD+, 3 mM glucose-1-phosphate and 1 unit G6P-DH. *Lmj*USP activity was tested in a preparation supplemented with 2 mM NAD+, 2 mM galactose-1-phosphate, 1 mM UTP, 0.12 units/ml UDP-galactose 4-epimerase (*Streptococcus thermophilus*, Calbiochem) and 0.08 U/ml UG-DH (Calbiochem). Similarly the *Lmj*UGP activity was measured, but for testing in wild type, contaminating USP activity had to be taken into account, therefore pure UGP activity was additionally probed in Δ*usp* mutants, using the assay buffer supplemented with 2 mM NAD+, 3 mM glucose-1-phosphate, 1 mM UTP and 0.08 U/ml UG-DH (Calbiochem). A different detection procedure and assay buffer was used for the inosine 5'-diphosphatase (IDP, Golgi). At first each sample was incubated for 1 hour at 30 °C in 50 mM Tris/HCl pH 7.5, 1 mM $MgCl_2$, 3 mM Inosine 5'diphosphate and $0.3\%_{(w/v)}$ digitonin. Then the amount of phosphate produced by degradation of Inosine 5'diphosphate to Inosine 5'monophosphate could be measured using malachite-green detection mix (0,034% Malachit Green, e10 mM $(NH_4)_6Mo$, 1 M HCl, 3.4% EtOH, 0,1% Tween 20) plus a diluted sample of the first reaction. The mixture was incubated for 15 min and the end-point absorption recorded at 620nm. Each sample was tested as blank for background phosphate contamination, as well. All measurements were performed in 96-well half-area flat-bottom microplates (Greiner Bio-One) with the Power-WaveTM340 KC4 System (Bio-Tek).

Generation of L. major USP gene deletion mutants and USP add back strains – Gene replacement cassettes were constructed by double-joint PCR as described elsewhere (19), with adaptations performed as followed. Briefly, three PCR products were generated: 5'UTR, Resistance Marker (RM) and 3'UTR. The RM amplikon was additionally equipped with 50 bp overlapping regions homologous to either 5'UTR or 3'UTR amplikon. Accordingly, a fourth fusion PCR using the 5'UTR, RM and 3'UTR yielded the desired gene replacement cassette. This construct was amplified with nested flanking primers at the very ends. The 5' and 3' regions flanking the USP gene were amplified using genomic *L. major* DNA and the primers 5UTR_1_NotI(fw) (ctg act gaG CGG CCG CTT GCT GAT GAG GGA AGG ATC TGC) and 5UTR_1(rev) (AAG GCC GCG TGA CGA CAG AAA AGG) for the 5'UTR spanning 2.3 kb, while 3UTR_1(fw) (TTG TTG TTG AGA GGG CCC TTG C) and 3UTR_1_NotI(rev) (ctg act gaG CGG CCG CAC AGG AGC GAC CTG CGA CGA CG) produced a 3'UTR PCR product of 1.3 kb length. The RMs were amplified by overlapping primers used as followed: HYG_overlap(fw) (TTC CCC CCG CCG AGC CCC TCT GCT CTC TCC TTT TCT GTC GTC ACG CGG CCT TAT GAA AAA GCC TGA ACT CAC CGC), HYG_overlap(rev) (CAT TCA ACT ACA CTG GAA CAC CCA CAC TAG CAA GGG CCC TCT CAA CAA CAA CTA TTC CTT TGC CCT CGG ACG), PHLEO_overlap(fw) (TTC CCC CCG CCG AGC CCC TCT GCT CTC TCC TTT TCT GTC GTC ACG CGG CCT TAT GGC CAA GTT GAC CAG TGC), PHLEO_overlap(rev) (ATT CAA CTA CAC TGG AAC ACC CAC ACT AGC AAG GGC CCT CTC AAC AAC AAT CAG TCC TGC TCC TCG GCC ACG), PAC_overlap(fw) (TTC CCC CCG CCG AGC CCC TCT GCT CTC TCC TTT TCT GTC GTC ACG CGG CCT TAT GAC CGA GTA CAA GCC CAC GG), PAC_overlap(rev) (CAT TCA ACT ACA

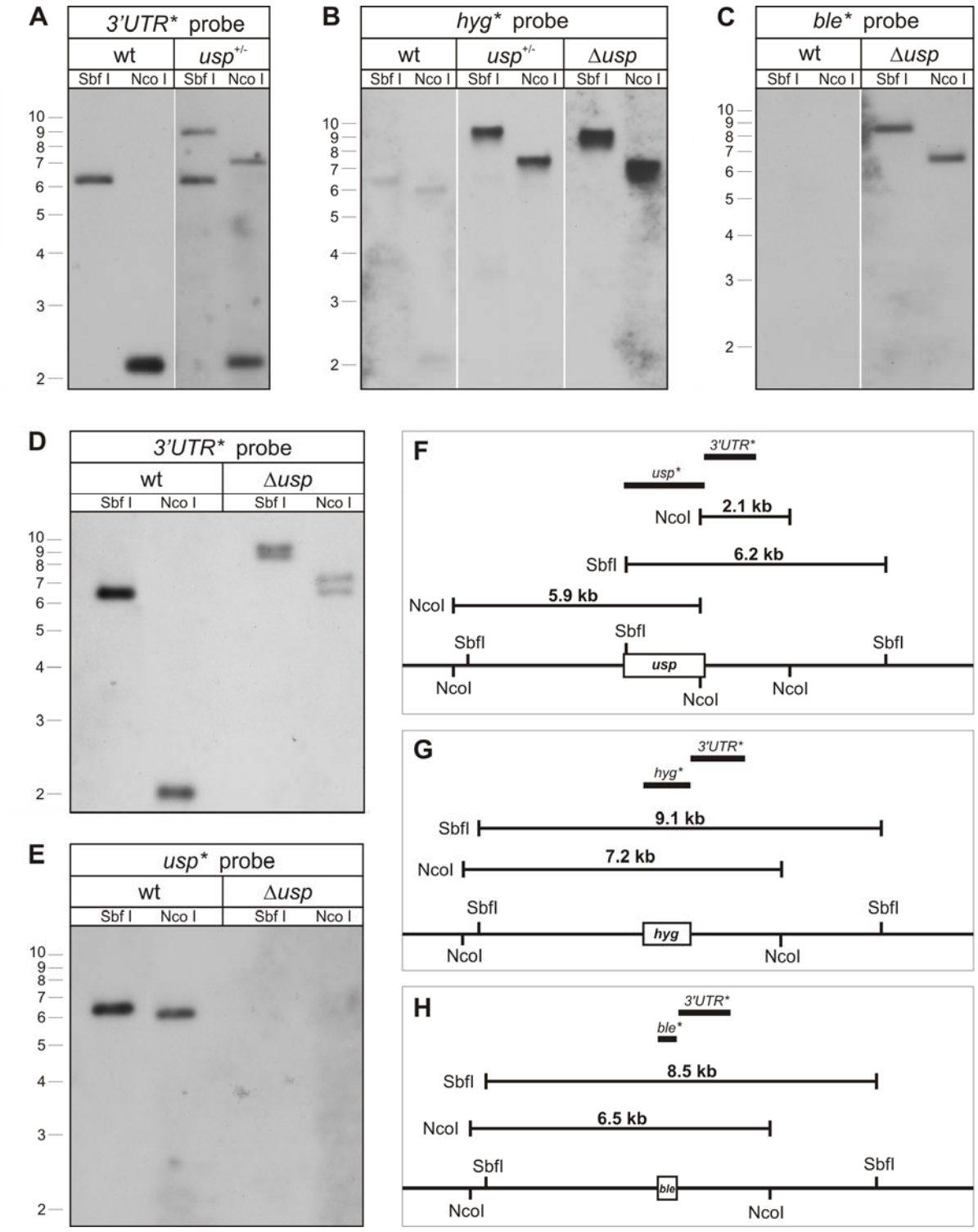

Figure 2. **Targeted gene replacement analysis of *usp* alleles in wild type cells by southern blot.** Southern blot of genomic DNA from wild type (wt), heterozygous mutant *usp*/Δ*usp*::*HYG* (*usp*$^{+/-}$), and homozygous mutant Δ*usp*::*HYG*/Δ*usp*::*BLE* (Δ*usp*) (**A-E**). DNA was digested with either Sbf I or Nco I, separated on agarose gel, blotted onto nylon membrane, and hybridized with digoxigenin-labeled probes *3'UTR**, *usp**, *hyg**, or *ble**. The differential probe labeled fragments can be assigned to the respective theoretical length in panel **F-H**, indicating correct gene replacement.

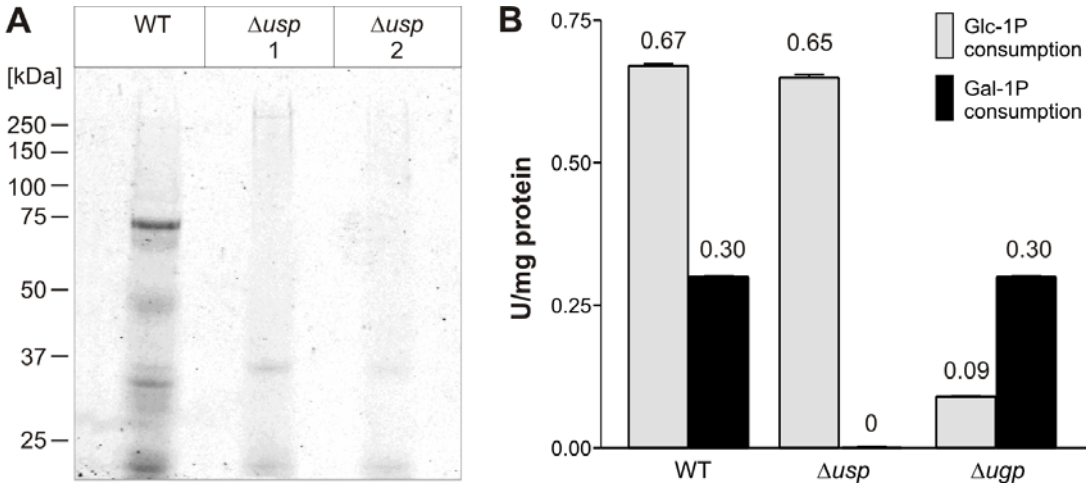

Figure 3. **Analysis of targeted gene replacement by Western blot and enzyme activity assay of Δ*usp* clones.** Southern blot validated Δ*usp* clones 1 and 2 were tested for absent USP protein expression, using ful cell lysates for SDS-PAGE and Western blotting together with primary αUSP serum and secondary α-rabbit-IR800 antibody for infrared detection on a LI-COR scanning facility (**A**). USP enzyme activity was tested in full cell extracts of wild type, Δ*usp* and Δ*ugp* mutants probing for glucose-1-phosphate (grey boxes) and galactose-1-phosphate (black boxes) uridinylating activity via the coupled enzymes UDP-galactose 4-epimerase (not for glc-1-P) and UDP-glucose dehydrogenase, producing an increase of NADH which can be followed at 340 nm (**B**).

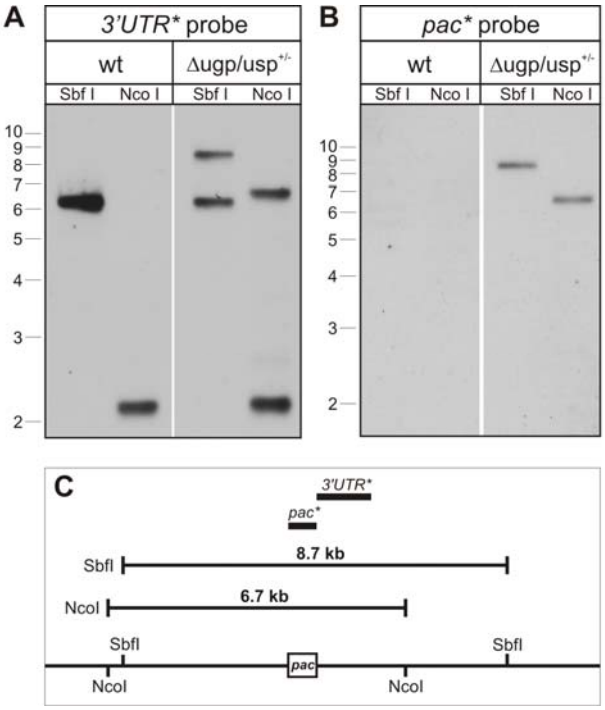

Figure 4. **Targeted gene replacement analysis of one *usp* allele in Δ*ugp* mutant cells by southern blot.** Southern blot of genomic DNA from wild type (wt) and heterozygous *usp*/Δ*usp*::*PAC* in Δ*ugp*::*HYG*/Δ*ugp*::*BLE* mutants (Δ*ugp*/*usp*$^{+/-}$) (**A**+**B**). DNA was digested with either Sbf I or Nco I, separated on agarose gel, blotted onto nylon membrane, and hybridized with digoxigenin-labeled probes *3'UTR** and *pac**. The differential probe labeled fragments can be assigned to the respective theoretical length in panel **C**, indicating correct gene replacement.

CTG GAA CAC CCA CAC TAG CAA GGG CCC TCT CAA CAA CAA TCA GGC ACC GGG CTT GCG GGT C); the underscore marks the part of the respective RM. After amplikon fusion using 5'UTR, 3'UTR and RM, the desired gene deletion cassette was amplified with the nested primer pair 5UTR_3_NotI(fw) (ctg act gaG CGG CCG CAC GGT GCT GAG GAC TGC G) and 3UTR_3_NotI(rev) (ctg act gaG CGG CCG CTG CTG CAG CTC TGG CGA GC). The Not I restriction sites allowed sub-cloning of the fragments into pYESNTA plasmid for accurate multiplication of the construct in *E.coli* XL1-blue cells. The *USP*::*HYG*, *USP*::*PHLEO* and *USP*::*PAC* targeting constructs were digested with Not I and the corresponding fragments purified by gel extraction and subsequent ethanol precipitation. The deletion mutant was generated by two consecutive rounds of homologous recombination using the *USP*::*HYG* fragment in the first and the *USP*::*PHLEO* in the second round, whereas *USP*::*PAC* was used in Δ*ugp* clones[1]. Southern blotting standard techniques, were used to confirm the precise gene replacement. The obtained homozygous mutant was named Δ*usp* and the heterozygous mutant produced in Δ*ugp* clones was termed Δ*ugp*/*usp*$^{+/-}$. For episomal expression of USP in the Δ*usp* background, the construct pXG-USP was transfected into several clones, referred to as

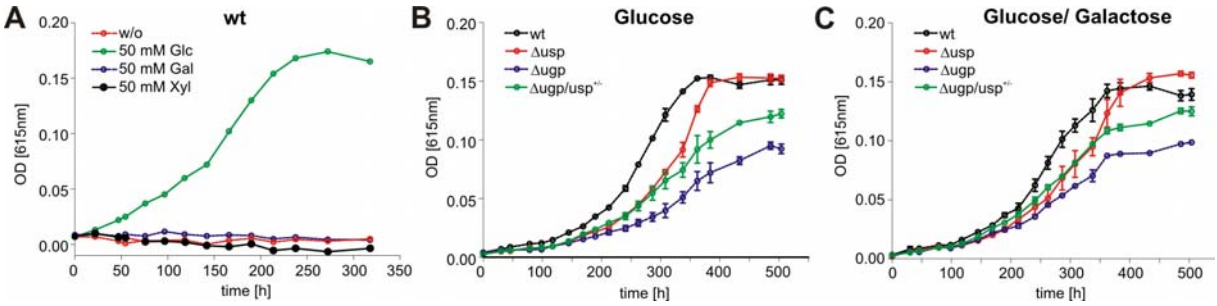

Figure 5. **Growth curves of different *L. major* cultures in varying conditions.** In a preliminary experiment wild type (wt) *L. major* promastigotes were cultured in RPMI 1640 and 10% dialyzed FBS without sugar in total, or supplemented with either 50 mM Glc, Gal, or Xyl, and OD was measured at 615 nm, showing that parasites do not grow on Gal or Xyl only (**A**). Another experimental setup displays growth of wt and Δ*usp*, Δ*ugp* or Δ*ugp*/*usp*$^{+/-}$ mutants, respecively. This setup is summarized, either corresponding to glucose or a glucose/galactose supplementation of the media (**B+C**). Deviations are derived from three separate cultures.

Δ*usp*/+USP. The plasmid was generated by sub-cloning the USP (equipped with a stop-codon) from a pET22b+ expression vector described previously (1), into an altered version of pXG-SAT (20), termed pXG-SAT/+His_6, via Nde I and Not I restriction sites (underlined, see as follows). pXG-SAT/+His_6 has an additional adapterprimer insertion (GGG CAT ATG CCT AGG CAA TTG TGG CCA GCG GCC GCg gca cca cca cca cca cca cTA GG) between the MCS restriction sites SmaI and BamHI of pXG-SAT, allowing the expression of a His_6-tagged protein, but without relevance for this study.

SDS-Page & Immuno Blotting – Whole cell lysates from early stationary phase *L. major* promastigote cultures were prepared in Lysis-Buffer (500µl 1M Tris HCl pH 7.8, 100µl 1M MgCl, 100µl 10% Triton X 100, 100µl 100mM PMSF, 20µl Leupeptin (2µg/ml), 50µl Pepstatin (5µg/ml), add 10 ml H2O) and after subsequent sonication (Branson Sonifier 450, output cycle 50, 4x 30sec) separated by SDS/PAGE and transferred onto nitrocellulose membranes (Whatmann Schleicher & Schüll). Equal protein loading and transfer efficiency was assessed by reversible staining in Ponceau S-solution (0.2 % Ponceau S, 3 %$_{(w/v)}$ trichloroacetic acid), or by running a separate SDS/PAGE, following Coomassie brilliant blue protein staining. Infrared detection on Li-Cor Odyssey Imager was performed after incubation with mAb WIC79.3 (ascites fluid) and goat anti-mouse IgG IR800Dye800CW (Li-Cor) at dilutions of 1:4000 and 1:20000 respectively. *Lmj*USP was detected using a 1:20000 dilution of polyclonal rabbit anti-serum USP#244 and either goat anti-rabbit IgG IR800Dye800CW (Li-Cor) or AP-conjugated goat-anti-rabbit antibody (1:2000, Dianova).

RESULTS and DISCUSSION

Localization studies – The *Leishmania* USP and UGP protein sequences described previously, display the putative C-terminal peroximal targeting signal (PTS-1) -AKL and -TNK respectively (21), which are assumed to mediate localization to the glycosomes. However, immunofluorescence studies using polyclonal rabbit αUSP- and αUGP-serum respectively, show that the enzymes are distributed over the whole cell, indicative of a cytoplasmic localization (**Fig. 1 A+B**). This cytoplasmic localization was confirmed by the selective permeabilization of membranes with digitonin. Low concentration of digitonin, leading to permeabilization of the plasma membrane, allowed detection of both USP and UGP whereas higher concentrations were required to detect the glycosomal enzyme hexokinase (HX) (**Fig. 1 C+D**). Interestingly, *Leishmania* phosphoglucomutase (PGM) and UDP-galactose 4-epimerase (GALE) seem also (at least partially) localized to the cytosol whereas they have been shown to be present in the glycosome in trypanosomes (14; 15).

Since many enzymes present a dual glycosomal and cytoplasmic localization (22-24), we have further investigated the possible presence of USP or UGP in the glycosome by isopycnic ultracentrifugation. Cell fractionation followed by enzymatic activity assays or staining of western blots with specific antibodies indicate again cytosolic localization of USP and UGP, while the marker HX and fructose-1,6-biphosphatase (FBP) were as expected present in the glycosome (**Fig. 1 E-J**) (8; 9; 17; 25). All together

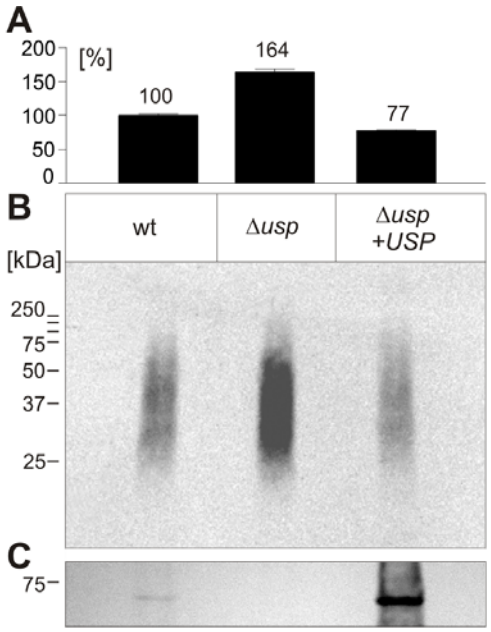

Figure 6 (above). **LPG phenotype characterization of Δ*usp* mutant and episomal reconstituted add-back.** Increased WIC79.3 phenotype of Δ*usp* mutants compared to wild type (wt) and signal reduction after USP add-back integration (Δ*usp*/+*USP*) (**A**+**B**); densitometrical calculated band intensities (**A**), the corresponding Western blot detected with WIC79.3 and infrared αmouse-IR800 antibody (**B**), Western blot using rabbit αUSP serum and monoclonal αrabbit-alkaline phosphatase antibody (**C**).

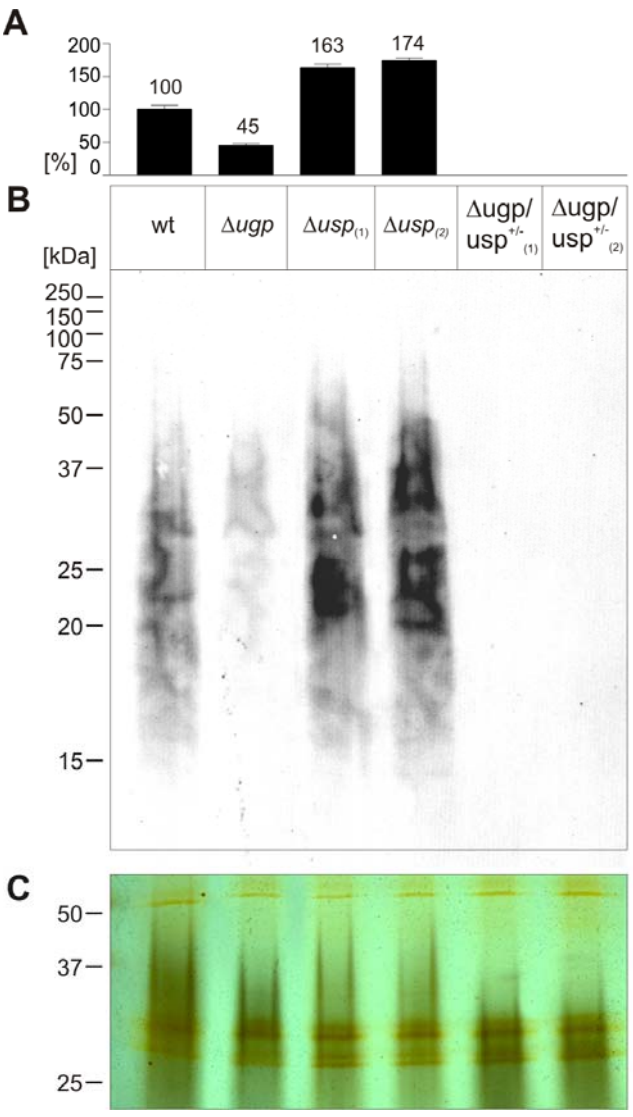

Figure 7 (right). **Δ*ugp*/*usp*$^{+/-}$ mutants are almost LPG negative in proteinase treated cell lysates for WIC79.3 blot and carbohydrate staining.** LPG phenotype comparison of proteinase digested cell lysates of wild type, Δ*usp*, Δ*ugp* and Δ*ugp*/*usp*$^{+/-}$ clones. Densitometrically calculated band intensities (**A**), the corresponding Western blot detected with WIC79.3 and infrared αmouse-IR800 antibody (**B**), and periodic acid silver stain (**C**).

these experiments argue against a glycosomal localization of *Lmj*USP and *Lmj*UGP, despite the presence of potential targeting sequences.

These findings challenge the assumption that *L. major* UDP-Glc and UDP-Gal metabolism take place within the glycosomes (21). In *Leishmania* like in *Trypanosoma*, accumulation of glucose-6-phosphate (Glc-6-P) in the cytoplasm is toxic (12; 13). The hexokinase and glucokinase are however not regulated by a feedback inhibition mechanism like in most eukaryotes (8; 9; 25-27) and are thus sequestrated in the glycosomes, where ATP level are balanced, with several other enzymes involved in glycolysis and gluconeogenesis. This compartmentalization avoids an uncontrolled glycolytic flux. Our experiments confirm the glycosomal localization of the hexokinase but strongly suggest that the phosphoglucomutase, USP and UGP are in the cytoplasm despite the presence of putative peroxisomal (glycosomal) targeting sequences (PTSs). Further studies, could address targeting to glycosome in *Leishmania* via PTSs using several PTS tagged GFP-fusion proteins.

In contrast to the glycolysis and gluconeogenesis and to the situation in trypanosomes, the nucleotide sugar metabolism of *Leishmania* appears thus to take place in the cytoplasm. The distribution of the enzymes involved in UDP-Glc synthesis, presented here, implies translocation of Glc-6-P from the glycosome into the cytoplasm which could be mediated by the glucose-6-phosphate transporter homolog *Lmj*F30.2680 (**Fig. 8**). Studies of the *in vitro* specificity of this transporter and its localization are currently under investigation.

It is noteworthy that PGM activity decreases from digitonin concentration of 0.3 mg/ml, where the glycosomal marker HX starts to increase (**Fig. 1 D**). Digitonin concentrations up to 1 mg/ml did not account for PGM inhibition, which was tested in a separate experiment (data not shown). Possibly PGM might be inhibited by the release of glycosomal substrates like fructose-1,6-bisphosphate, which is known to be a strong competitive inhibitor for PGM in other organisms like e.g. human (28), potato tuber (29) or *Bacillus subtilis* (30).

Generation of LmUSP deletion mutants – LmjUSP is a single copy gene (data not shown) located at chromosome 17. Using deletion constructs based on antibiotic resistance genes fused to *L.major usp* flanking regions of 1.7 kb and 1.2 kb for the 5'UTR and 3'UTR respectively, a *usp* null mutant (Δ*usp*::*HYG*/Δ*usp*::*BLE*) was obtained by two successive rounds of gene replacement. Southern blots of either Sbf I or Nco I digested genomic DNA from wild type, homozygote and heterozygote mutants were hybridized with different digoxigenin-labeled(*) probes binding to the *3'UTR*, *usp*, *hyg* or *ble* coding sequences and provided evidence of the correct integration of resistance marker genes and the absence of *usp* (**Fig. 2**). Successful mono-allelic *usp* deletion is visualized using *3'UTR** by the signals at 9.1 and 6.2 kb with Sbf I digestion and 7.2 and 2.1 kb with Nco I, corresponding to the integrated *hyg* resistance marker or the residual *usp* allele (**Fig. 2A**). This result was confirmed using a probe hybridizing to the integrated resistance marker gene *hyg** (**Fig. 2B**). Homozygous *usp* gene deletion was validated by the presence of bands at 9.1 (Sbf I) and 7.2 kb (Nco I) visualized with the *hyg* probe (**Fig. 2B**) or 8.5 (Sbf I) and 6.5 kb (Nco I) with the *ble* probe (**Fig. 2C**). Moreover, hybridization with a *3'UTR* probe showed no bands corresponding to *usp* but highlighted the presence of the respective resistance marker genes *hyg* and *ble* (**Fig. 2D**). Finally, the *usp** probe did not bind to digested genomic DNA after two rounds of targeted gene deletion, indicating the absence of *usp* in *L. major* parasites (Δ*usp* mutants) (**Fig. 2E**).

As expected, the distinct protein band at ~69 kDa detected in cell lysates from wild type parasites using polyclonal rabbit αUSP serum was absent from *L. major* Δ*usp* (**Fig. 3A**). Additionally, a coupled enzyme assay, based on UDP-galactose 4-epimerase and UDP-glucose dehydrogenase (producing NADH measured at 340 nm), confirmed the absence of activity catalyzing the conversion of galactose-1-phosphate and UTP to UDP-galactose and pyrophosphate in the Δ*usp* mutant (**Fig. 3B**).

Facing the next step in dissecting the UDP-galactose pathway, a single *usp* allele could be successfully replaced with the *PAC* gene conferring resistance against puromycin in the UDP-glucose pyrophosphorylase null mutant Δ*ugp* and was designated Δ*ugp*/*usp*$^{+/-}$ (Δ*ugp*::*HYG*/Δ*ugp*::*BLE; usp*/Δ*usp*::*PAC*). The southern blot analysis using a digoxigenin-labeled *3'UTR** and probe hybridizing to the *pac ORF* (*pac**) shows correct resistance marker insertion proving single allelic deletion of *usp* in Δ*ugp* mutants (**Fig. 4 A+B**).

Partial characterization of LmUSP deletion mutants – By light microscopy, all Δ*usp* clones appeared morphologically normal compared to the parental wild type strain. Moreover, no major effect on growth of Δ*usp* mutants was observed when the parasites were grown in 5% dialyzed FBS/RPMI media supplemented with glucose as single carbon source (**Fig. 5 B**) or a mixture of glucose and galactose (**Fig. 5 C**). Interestingly presence of galactose in the media did not result in poor *in vitro* growth arguing against a toxic accumulation of galactose-1-phosphate in the parasite in contrast to the situation in mammals or yeast (31; 32).

L. major promastigotes were moreover unable to grow in media with galactose as the sole carbon source strongly suggesting that this monosaccaride cannot enter glycolysis via USP, GALE, UGP and PGM (**Fig. 5A; Fig 8**) (33). The presence of an *L. major* inorganic pyrophosphatase in the cytosol (34), is in perfect agreement with this assumption, since pyrophosphate degradation is a driving force for pyrophosphorylase mediated reactions, like the one of UGP and USP, and thus preventing the reverse reactions of UDP-glucose to Glc-1-P.

In view of the fact that Δ*usp* mutants are able to synthesize UDP-Gal via the *de novo* pathway, we anticipate a mild phenotype *in vitro*. The presence and possible modification of phosphoglycans was analyzed in different *L. major* stationary phase cultures, using the monoclonal antibody WIC79.3. This antibody recognizes galactose-substituted repeat units present in *L. major* phosphoglycans, like LPG and PPG. Western blotting of total *L. major* cell extracts labeled with WIC79.3 reveals a characteristic LPG smear of about 15-100 kDa, which can vary in intensity and range, indicating differential LPG

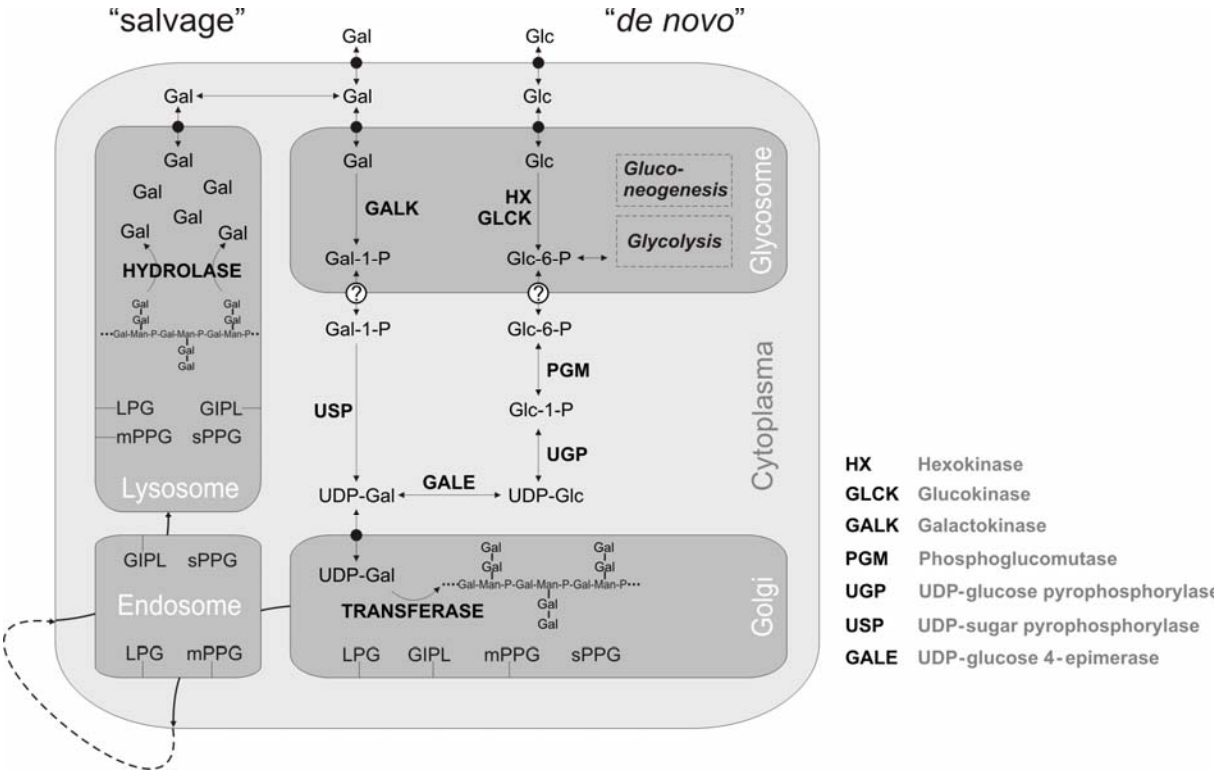

Figure 8. **UDP-galactose metabolism in *Leishmania major*.** Glucose (Glc) can either be incorporated or its activated form glucose-6-phosphate (Glc-6-P) is generated by gluconeogenesis (*de novo*). A putative Glc-6-P transporter homologue is a crucial player within this model, because PGM, GALE, UGP and USP are localized within the cytosol, challenging the model proposed by Opperdoes & Szikora 2006. Galactose can be incorporated from the extracellular lumen or could be salvaged by degraded glycanstructures from the lysosomes. Since galactose-1-phosphate is also assumed to be activated within the glycosomes its transport across the membrane could be anticipated.

expression or modification of side chains (**Fig. 6 A+B**). Interestingly, the USP deficient mutant displays an increased WIC79.3 reactivity when compared to wild type parasites (**Fig. 6 A+B**) and this effect could be reverted by episomal expression of the *Lmj*USP (Δ*usp*/+*USP)* (**Fig. 6 C**). Considering that the antibody has a strong preference for monogalactosylated repeat units (-6[Galβ1-3]Galβ1-4Manα1-P-), the elevated WIC79.3 signal in Δ*usp* mutants could arise from an increased level of monogalactosylated repeat units at the expense of polygalactosylated repeat units (-6[Galβ1-3Galβ1-3]Galβ1-4Manα1-P-) as previously observed in the *LPG5B*$^{-}$ mutant lacking a UDP-galactose transporter (35). This change could reflect a small decrease in the UDP-Gal pool in the golgi.

More impressively, the additional deletion of one *usp* allele in Δ*ugp* mutants (Δ*ugp*/*usp*$^{+/-}$) resulted in the quasi absence of LPG as observed by lack of carbohydrate staining in proteinase treated cell lysates and absence of labeling by the monoclonal antibody WIC79.3 (**Fig. 7**). This indicates that USP is responsible for the residual LPG expression observed in Δ*ugp* mutants[1] and support a potential involvement of USP in UDP-Gal production.

Like the Δ*ugp*, the Δ*ugp*/*usp*$^{+/-}$ mutants tended to form rosettes and displayed a similar delayed growth defect. Comparing the growth in single glucose or glucose/galactose supplemented media, Δ*ugp* and Δ*ugp*/*usp*$^{+/-}$ cultures expand significantly better in glucose/galactose supplemented media (**Supplemental Fig. 1C+D**). This effect could be allocated to an anabolic advantage given by USP activity.

More detailed analysis of LPG and other glycoconjugates are however necessary to confirm these modifications and thereby establish a role of USP in UDP-Gal production. The impact of USP and UGP absence on the UDP-Gal or UDP-Glc level will also be addressed by analysis of nucleotide-sugars extracted from *L. major* wild type and mutants by HPLC mass spectrometry as described by Turnock and Ferguson (36). Finally, a study of the hexose transporters in *Leishmania mexicana* demonstrates that these permeases are essential for the replication of

the parasites within macrophages and thus suggest an important role for monosaccharide salvage in the amastigote stage. The virulence of the Δ*usp* mutant will therefore be investigated in mice and macrophages.

Importantly, repeated attempts to create double null mutants remained unsuccessful, suggesting that either UDP-glucose and/or UDP-galactose production are essential in *L. major*. This observation strongly resembled the *T. brucei* and *T. cruzi* UDP-glucose 4-epimerase (GALE) deletion mutants, where inability of UDP-galactose formation turned out to be essential as well (5-7). Interestingly, trypanosomatid parasites DNA contains the hypermodified base J (β-D-glucosyl 5-hydroxymethyluracil) thought to be essential for parasite viability. This base would originate from glucosylation of the precursor hydroxyl-methyldeoxyuridine. It is thus reasonable to assume that UDP-Glc is the donor for base J biosynthesis and is thus essential for *Leishmania* survival (37).

[1]A.-C. Lamerz, S. Damerow, B. Kleczka, M. Wiese, G. van Zanbergen, A. Wenzel, J. Lamerz, F. F. Hsu, J. Turk, S. M. Beverley, and F. H. Routier, "*Deletion of UDP-glucose pyrophosphorylase reveals a UDP-glucose independent UDP-galactose salvage pathway in Leishmania major*", a revised and accepted version has been published in *Glycobiology,* vol. 20 no. 7 pp. 872–882, 2010.

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Supplementary Material

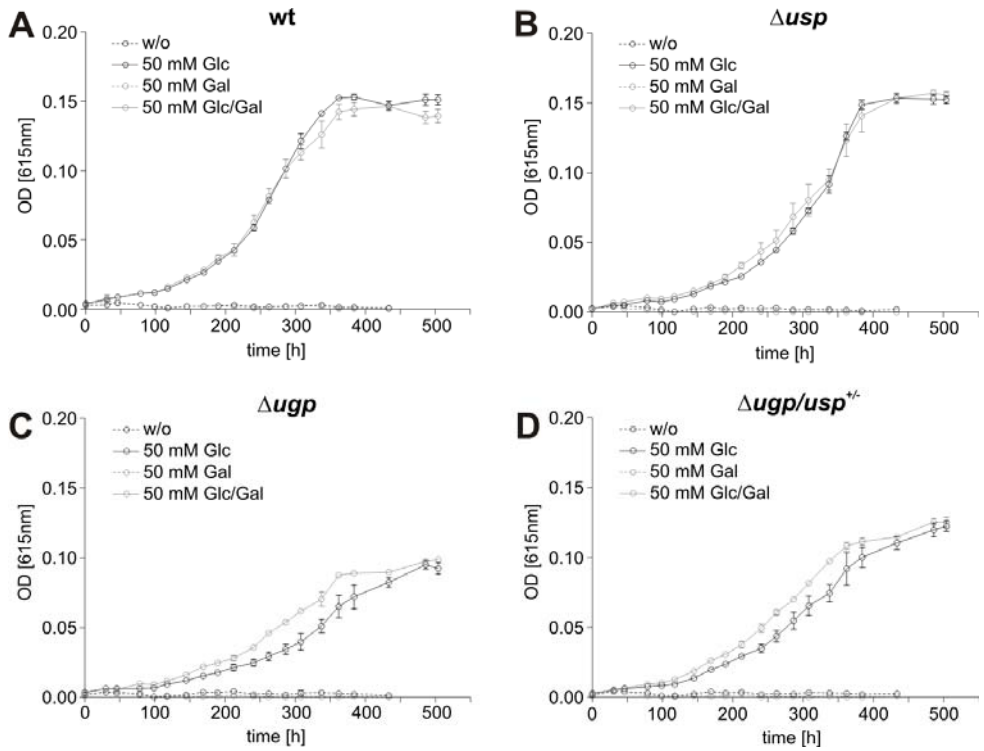

FIGURE S1 . **Growth curves of *L. major* cultures in varying conditions.** *L. major* promastigotes were cultured in RPMI 1640 and 10% dialyzed FBS without sugar in total, or supplemented with either 50 mM Glc, or Gal and OD was measured at 615 nm. **A**, wild type; **B**, USP gene deletion mutant Δ*usp*; **C**, UGP gene deletion mutant Δ*ugp*; **D**, UGP deletion mutant with single allelic deletion of USP Δ*ugp*/*usp*$^{+/-}$. Deviations are derived from three separate cultures. Δ*usp* shows no influence with Glc/Gal media, compared to Δ*ugp* and Δ*ugp*/*usp*$^{+/-}$.

CHAPTER 5 – General Discussion

Current therapy of leishmaniasis encompasses chemotherapeutic based treatment using the first-line drugs of pentavalent antimonal agents or the second-line drugs like the antibiotics pentamidine and paromomycin, or the fungicide amphotericin B in different combinations (*Ashutosh et al., 2007; Lee and Hasbun, 2003; Kshirsagar et al., 2005; Croft and Coombs, 2003; Kanyok et al., 1994; Maltezou, 2010*). The newly discovered miltefosine, originally developed as an anti-malignant, finds increasingly use as an antiprotozoal drug, having less severe side-effects compared to the other leishmanicidals (*Fischer et al., 2001*). Nevertheless, the development of drug resistant strains is fast. After approximately 50 years of pentavalent antimonal treatment, this drug has become almost useless in the majority of the affected countries (*Maltezou, 2010*). And resistance against miltefosine has also been reported, using cell culture experiments (*Perez-Victoria et al., 2006; Maltezou, 2010*). Moreover, prevalence of the disease is increasing with estimates of 12 million people currently infected worldwide, causing 80.000 deaths annually without mentioning the morbidity of the disease, which can cause severe disfigurement and lead to social isolation (*according to WHO statistics*). In answer to this worrying situation, predominantly found in third world countries, a wide field of *Leishmania* research is dedicated to the identification of new drug targets. Although several drug targets have been proposed during the last decades, only few of them seem to be *Leishmania*-specific and truly essential for virulence or survival. Nevertheless, *Leishmania* parasites present unique features, such as the rare sugar galactofuranose (*Bakker et al., 2005*), a special glycosylated desoxyribonucleotide called Base J (*van Leeuwen et al., 1998*), a subset of plant-like genes (*Hannaert et al., 2003*), peroxisome-like glycosomes (Opperdoes and Szikora, 2006) and a phosphoglycan rich glycocalyx (*Naderer et al., 2004*) that deserve close investigations.

The surface glycocalyx was one of the main subjects studied during the last three decades, since it represents the protecting interface between parasite and an always changing, hostile environment (*Naderer et al., 2004*). It was shown by several gene deletion experiments that *Leishmania* hampered in their glycocalyx biosynthesis are either avirulent or displayed an attenuated lesion phenotype, underlining the biological importance of this structure (*Sacks et al., 2000; Spath et al., 2000; Ilg, 2000b; Ilg, 2000a; Descoteaux et al., 1995; Kleczka et al., 2007; Zufferey et al., 2003; Garami and Ilg, 2001; Capul et al., 2007*). Since the phosphoglycans, that comprise the highly abundant lipophosphoglycans (LPG) and the secreted or membrane bound proteophosphoglycans (s/mPPG), are extremely rich in galactose, interfering with the production of UDP-Gal, the main building-block of phosphoglycan assembly, would profoundly affect the glycocalyx formation and the parasite virulence. However, the galactose metabolism of *Leishmania* still had a few surprises in store.

5.1 UDP-glucose pyrophosphorylase deletion revealed a UDP-Glc independent galactose salvage pathway in *L. major*

It is likely that the primary route of UDP-Gal formation in *L. major* is via epimerization of UDP-Glc by the UDP-galactose 4-epimerase (GALE) (*Turnock and Ferguson, 2007*). However, in contrast to *Trypanosoma cruzi* and *Trypanosoma brucei, Leishmania* species also possess a salvage pathway (*Turco et al., 1984*) that was assumed to occur by uridinylation of galactose-1-phosphate via UDP-glucose:α-D-galactose-1-phosphate uridylyltransferase (GALT), as described by Leloir (*Leloir, 1951*). The initial activation of UDP-glucose carried out by a UDP-glucose pyrophosphorylase (UGP) from glucose-1-phosphate and UTP (*Lamerz et al., 2006*) seemed thus essential for UDP-galactose biosynthesis. One goal was therefore to evaluate the importance of UDP-galactose biosynthesis in *L. major* by means of a UGP deficient mutant (*Lamerz, 2005*). Surprisingly, targeted gene replacement of UGP showed only modest effects on the synthesis of several key molecules of the glycocalyx, like LPG, protein linked phosphoglycans and glycoinositolphospholipids (GIPLs), and still displayed residual UDP-Glc formation by *in vitro* activity testing. However, the genome of *Leishmania major* does not contain any close UGP homologues. Conversely, a *leishmanial* homologue of the recently described plant UDP-sugar pyrophosphorylase (USP) was found. An enzyme that can utilize both glucose-1-phosphate or galactose-1-phosphate together with UTP to produce UDP-Glc or UDP-Gal and pyrophosphate (*Litterer et al., 2006b; Kotake et al., 2004; Kotake et al., 2007; Litterer et al., 2006a*). Such an enzyme would be able to fuel the UDP-Gal pool by direct activation of Gal-1-P and be responsible for the limited UDP-Glc production that takes place in the UGP deletion mutant. With the completion of the genome, it also became clear that no obvious GALT homologue exists in *trypanosomatids*, strengthening the potential involvement of USP in galactose salvage.

After completing this study, we have hence concentrated our effort on the biochemical characterization and evaluation of the metabolic importance of *Leishmania major* UDP-sugar pyrophosphorylase, an enzyme that was only described in the plant kingdom.

5.2 In vitro characteristics of a plant-like UDP-sugar pyrophosphorylase from *Leishmania major*

The distribution of the USP gene is quite limited and eventually depends on the ecological niche of its organism. Beside *Leishmania major*, the recently sequenced genomes of *L. brasiliensis* and *L. infantum* both contain a USP homologue. Interestingly, the slightly distant relative *T. cruzi* presents a putative USP gene as well, whereas *T. brucei* seems to be devoid of such enzyme. The *trypanosomatid* USPs are closely related to plant USPs and hypothetical proteins of the diatoms

Phaedactylum tricornutum and *Thalassiosira pseudonana* and green algae *Micromonas pusilla*, *Ostreococcus tauri*, *Ostreococcus lucimarinus* and *Chlamydomonas reinhardtii* which suggest the common origin of these genes. Moreover, USP homologues are found in ciliate protozoa (*Paramecium tetraurelia*, *Tetrahymena thermophila*) and apicomplexa (*Toxoplasma gondi*, *Cryptosporidium sp*. and *Plasmodium sp*.) but are absent from the fungal and animal kingdoms.
The strong homology between *trypanosomatid* and plant USPs might suggest that *Lmj* USP has been acquired by lateral gene transfer. It has indeed been suggested that *kinetoplastids* and *euglenoids,* which together form the phylum *Eugleonoza,* acquired plastids by endosymbiosis or phagocytosis of an alga before their divergence and that the former lineage subsequently lost the organelle and ability to photosynthesize but retained numerous plant-like genes (*Hannaert et al., 2003*). This hypothesis is however controversial (*Waller et al., 2004*). Although protist USPs show high homology to plant USPs, these constitute a separate phylogenic branch. USP gene might thus have been present in a common ancestor and lost during evolution. In this context, we should mention that USPs exhibit a modest but significant homology to UGPs and UDP-GlcNAc pyrophosphorylases (UAPs) over the entire sequence. In particular, the pyrophosphorylase glycine rich consensus motif (*Peneff et al., 2001; Steiner et al., 2007*) essential for catalysis is highly conserved, as well as residues involved in uridine and phosphate binding.

When compared to the plant enzymes, all USPs identified in *trypanosomatids* present a short amino-acid insertion near the uridine binding site. The primary sequence analysis tool ELM (http://elm.eu.org/) for prediction of functional sites and globular domains in proteins revealed glycosylation and phosphorylation motifs for both the 18aa insertion from *Leishmania spp.* and the 7aa insertion from *T. cruzi*. These short sequences are disordered and might be post-translationally modified. Determination of the crystal structure of USP might shed some light on the function of these additional amino acid residues.

STD NMR spectroscopic studies emphasized that interactions of *L. major* USP with UTP or the uridine moiety of nucleotide-sugars play a significant role in substrate binding as for *L. major* UGP (*Lamerz et al., 2006*). A finding bolstered by kinetic studies of USP and other pyrophosphorylases, showing that the affinity for UTP is the strongest, followed by the activated UDP-sugar (*Lamerz et al., 2006; Kotake et al., 2004; Weissborn et al., 1994; Turnquist et al., 1974*). This leading role of the nucleotide moiety is observed in many enzymes involved in glycosylation, for example UDP-galactopyranose mutase (*Gruber et al., 2009*), sialyltransferases (*Datta and Paulson, 1995*), as well as pyrophosphorylases from *E. coli* (*Thoden and Holden, 2007*) and might even hold true for nucleotide sugar transporters (*Maggioni et al., 2008*). Intriguingly, residues interacting with the glucose moiety in UGPs are not conserved in USPs which probably accounts for the broader specificity toward monosaccharide-1-phosphates and UDP-sugars of the latter. Like plant USPs, *L. major* USP is able to

convert reversibly and efficiently both Glc-1-P and Gal-1-P with a slight preference for Gal-1-P. Pentose-1-phosphates such as Xyl-1-P and Ara-1-P can also be activated *in vitro* by *Leishmania* or plant USPs, albeit with a reduced efficiency reflecting their lower affinity for the enzyme, and suggesting the contribution of the hexose H6 and H6′ protons to binding. In contrast, GalA-1-P is a poor substrate of *L. major* USP. It is reasonable to assume that the carboxylic acid group of GalA creates either steric hindrance or more likely an unfavoured electrostatic potential leading to weak interactions of the uronic acid with the *leishmanial* enzyme. Since *L. major* USP does not activate hexosamine- or N-acetylhexosamine-1-phosphates it clearly and metabolically differentiates itself from the enzymatic class of UAPs.

In *Arabidopsis*, USP is particularly important in pollination and possibly converts Gal-1-P, Ara-1-P and Rha-1-P secreted by the pistil. And despite its lower affinity for Ara-1-P, *Arabidopsis thaliana* USP seems to play a central role in the salvage of this pentose *in vivo* (*Kotake et al., 2007*). In *Leishmania*, however, where D-arabinose is present, the monosaccharide-1-phosphate is exclusively activated by GTP and a putative GDP-arabinose pyrophosphorylase has been identified in the genome (*Turnock and Ferguson, 2007*). In addition to GDP-α-D-Ara, *L. major* promastigotes synthesize UDP-Glc, UDP-Gal (in the pyranosic and furanosic form), UDP-GlcNAc, GDP-Man and low amounts of GDP-Fuc but neither UDP-Xyl nor its precursor UDP-GlcA are produced (*Turnock and Ferguson, 2007*). In addition, *L. major* parasites are neither able to catabolize D-xylose nor D-galactose, which excludes the role of *L. major* USP in energy extraction from rare sugars derived by plant nectars within the sand fly midgut. Considering the specificity of *L. major* USP for UDP-activated sugars, its inability to act on hexosamine-1-phosphate and the characterization or presence in the genome of specific pyrophosphorylases for the activation of GDP-activated sugars and UDP-GlcNAc (*Turnock and Ferguson, 2007; Garami and Ilg, 2001*), *Leishmania* USPs most likely play a role in the salvage of galactose and glucose exclusively. Remarkably, *L. major* USP seems to have evolved a slight preference for Gal-1-P over Glc-1-P in good agreement with the presence of galactose in many of their surface glycoconjugates. Moreover, although it is a bidirectional enzyme, the ordered bi-bi reaction mechanism of USP highlighted in this work, and its high affinity for UTP, which is known to be a naturally abundant metabolite, presumably ensure the synthesis of nucleotide sugars rather than their pyrophosphorolysis and support a putative role in galactose salvage. Galactose most likely enters cells by a family of hexose transporters before being converted into Gal-1-P by the putative galactokinase present in the genome (*LmjF*35.2740) and activated into UDP-galactose by USP.

In contrast to *Leishmania*, the *trypanosomatids Trypanosoma brucei* and *Trypanosoma cruzi* are thought to rely exclusively upon epimerization of UDP-Glc for synthesis of UDP-Gal since the hexose transporters of these parasites are unable to transport Gal (*Tetaud et al., 1997; Barrett et al., 1998*). While the genome of *T. brucei* is devoid of a USP and houses only a galactokinase pseudogene, *T. cruzi* contains four homologues of galactokinase and one USP. In the latter, USP might be involved in recycling galactose originating from degradation of glycoconjugates in the endolysosomal

compartment or plays a role in salvage of other sugars. For example, *T. cruzi* is the only one of the three *trypanosomatids* mentioned here that synthesizes UDP-Rha, UDP-Xyl and its precursor UDP-GlcA (*Turnock and Ferguson, 2007*). Like the plant enzyme, *T. cruzi* USP might be involved in the synthesis of these nucleotide sugars.

5.3 UDP-galactose metabolism in *L. major*

In eukaryotes, most of the nucleotide-sugars are synthesized in the cytosol. Remarkably, *trypanosomatids* seem to have emerged special features, since several enzymes of the carbohydrate metabolism were shown or predicted to reside within the glycosomes (*Opperdoes and Szikora, 2006*). This unique kinetoplastid organelle contains a large portion of plant-like genes and carries out pathways including glycolysis, purine salvage and pentose phosphate pathway, in addition to more typical peroxisome functions such as β-oxidation of fatty acids (*Hannaert et al., 2003; de Souza, 2002; Opperdoes and Szikora, 2006*).

In both *Leishmania* and *Trypanosoma*, cytosolic accumulation of glucose-6-phosphate is lethal (*Kumar et al., 2009; Haanstra et al., 2008*). The hexokinase and glucokinase are nevertheless not regulated by allosteric feedback inhibition like it is the case in most eukaryotes (*Nwagwu and Opperdoes, 1982*) but sequestrated in the glycosomes, where ATP level are balanced, with several other enzymes involved in glycolysis and gluconeogenesis. This compartmentalization avoids an uncontrolled glycolytic flux and moreover was proposed to serve as energy reservoir of organic-phosphates to circumvent short periods of starvation (Bakker *et al.*, 2000). Here, inability of allosteric regulation of kinases would contribute for an increased pool-size of organic-phosphates, since phosphorylation leads to intracellular fixation.

In *Trypanosoma,* several enzymes of the UDP-Gal and UDP-Glc biosynthetic pathways have also been located to the glycosomes (*Roper and Ferguson, 2003; Penha et al., 2009*). Likewise, *Leishmania* USP and UGP protein sequences display the putative C-terminal peroxisomal targeting signal (PTS1) -AKL and –TNK respectively, assumed to mediate localization to the glycosomes (*Opperdoes and Szikora, 2006*). However, challenging the assumption that *L. major* UDP-Glc and UDP-Gal metabolism takes place within these organelles, this study demonstrates the cytoplasmic localization of USP and UGP. Interestingly, *Leishmania* phosphoglucomutase (PGM) and UDP-galactose 4-epimerase (GALE) seem also localized to the cytosol, whereas they have been shown to be present in the glycosome of trypanosomes (*Roper et al., 2005; Penha et al., 2009*). These observations suggest a divergence in the nucleotide sugar metabolism between *Trypanosoma* and *Leishmania.* The sub-cellular distribution of the enzymes highlighted in this study would imply translocation of glucose-6-phosphate from the glycosome into the cytoplasm. This task could be mediated by the product of *Lmj*F30.2680 that shows strong homology to the plant glucose-6-phosphate transporter.

Studies of the *in vitro* specificity of this transporter and its localization are currently under investigation.

Similarly, *L. major* GDP-Man biosynthesis, which requires a phosphomannomutase and GDP-mannose pyrophosphorylase, takes place within the cytosol (*Penha et al., 2009; Opperdoes and Coombs, 2007*) and might require translocation of glycosomal activated mannose-6-phosphate (*Opperdoes and Coombs, 2007*). Finally, *Leishmania* putative galactokinase also displays a canonical peroxysomal targeting motif (PTS1). If this localization is confirmed, galactose-1-phosphate would also need to be translocated into the cytoplasm to be activated by USP. The data presented in this thesis therefore gave rise to a new model for the topology of the carbohydrate metabolism in *Leishmania* (page 70, compared to page 22). Further studies, such as the investigation of signals for targeting to *Leishmania* glycosomes, will challenge this model.

5.4 Importance of galactosylation for growth, virulence and viability in *L. major*

In *Leishmania* species the abundance of incorporated galactose is tightly connected to its surface glycoconjugate structures like LPG, PPG and type-2 GIPLs (*Ferguson et al., 1994; Mcconville et al., 1995; Ilg, 2000b*). Within the last years, a variety of approaches have been used to genetically disrupt *Leishmania* metabolism, in order to interfere with the glycocalyx biosynthesis and dissect the role of its components. Notably, disruption of the GDP-mannose pyrophosphorylase leading to disruption of the majority of the glycocalyx components resulted in viable but avirulent parasites demonstrating the importance of this cellular coat (*Stewart et al., 2005*). To date, the biological role of galactosylation has been mostly defined by three gene deletion mutants in *L. major*, these are a putative galactofuranosyltransferase (LPG1) (*Spath et al., 2000*), a UDP-galactopyranose mutase (GLF) (*Kleczka et al., 2007*), and a UDP-galactose transporter double knock-out ($LPG5_{A/B}$) (*Capul et al., 2007*) (see **table 1**, below). All of them are devoid of LPG, whereas phenotypical differences can be assigned to PPGs and GIPLs expression. The GIPLs stay generally unaffected except within the GLF mutant that lacks UDP-galactofuranose and consequently expresses truncated GIPLs, while PPGs are only affected within the $LPG5_{A/B}$ mutant. These three galactose based mutants all display an attenuated virulence in mice. In contrast, deletion of the *L. major* GDP-mannose transporter (LPG2) lacking both LPG and PPGs like $LPG5_{A/B}$, is totally avirulent within mice. To date this discrepancy remains unexplained. Moreover in the related *trypanosomatid* parasites *Trypanosoma cruzi* and *Trypanosoma brucei*, biosynthesis of UDP-Gal is essential for survival (*Roper et al., 2005; Macrae et al., 2006*). These observations encouraged us to refine our understanding of the galactose metabolism

in *Leishmania* and revisit the role of galactosylated molecules by targeting the UDP-galactose formation.

Code	Name	LPG	GIPLs	PPG	Virulence	Species
LPG1	Galactofuranosyl transferase	no	yes	yes	delayed	*L. major*
GLF	UDP-galactopyranose mutase	no	affected	yes	delayed	*L. major*
$LPG5_{A/B}$	UDP-galactose transporters	no	yes	no	delayed	*L. major*
LPG2	GDP-mannose transporter	no	yes	no	no	*L. major*
GDP-MP	GDP-mannose pyrophosphorylase	no	no	no	no	*L. mexicana*
UGP	UDP-glucose pyrophosphorylase	residual	yes	residual	affected	*L. major*
USP	UDP-sugar pyrophosphorylase	affected	?	?	?	*L. major*

Table 1. Gene deletion phenotypes assigned to nucleotide-sugar metabolism in *Leishmania*.

To demonstrate a role of USP in galactose metabolism and evaluate its importance in *L. major*, the *usp* single copy gene was targeted by homologous recombination and its two alleles replaced by selective resistance markers. The absence of activity (by USP or a similar enzyme) catalyzing the conversion of galactose-1-phosphate and UTP to UDP-galactose and pyrophosphate could be confirmed enzymatically in the Δ*usp* mutant. By light microscopy, all Δ*usp* clones appeared morphologically normal compared to the parental wild type strain; no body swelling was observed, which could have been indicative for osmotic stress by possible accumulation of Gal-1-P. There was no significant difference between the growth of wild type and mutant. In contrast to the situation in yeast or mammals (*Lai et al., 2003; de Jongh et al., 2008*), the suggested Gal-1-P accumulation in *L. major* seems not to be fatal.

Since the *L. major* USP mutant parasites are able to synthesize UDP-Gal via the *de novo* pathway, we anticipated a mild phenotype. Interestingly, while the amount of LPG exposed by carbohydrate staining seems unaffected by the lack of USP, staining by the monoclonal antibody WIC79.3 was noticeably increased when compared to wild type. This signal increase could thus be attributed to an increase of the monogalactosylated phosphoglycan repeat units (-6[Galβ1-3]Galβ1-4Manα1-P-) recognized by WIC79.3 at the expense of polygalactosylated repeat units (-6$[Gal\beta1\text{-}3]_{2\text{-}3}$Galβ1-4Manα1-P-) as previously observed in the *LPG5B*$^{-}$ mutant lacking a UDP-Gal transporter (*Capul et al., 2007*). Since *L. major* is known to express the most complex LPG with a highly galactosylated backbone of varying side chain lengths, high amounts of activated galactose are needed to fuel its biosynthesis (*Mcconville et al., 1990; Mcconville et al., 1995*). These preliminary results suggest thus an impact of USP on side chain galactosylation and ultimately on the Golgi UDP-Gal pool. More detailed analysis of LPG and other glycoconjugates are however necessary to confirm these observations and establish a role of USP in UDP-Gal production.

In comparison, a strong decrease in WIC79.3 reactivity was observed in the UGP deletion (Δ*ugp*) mutant and most likely reflects a strong decrease in the amount of LPG, as observed by carbohydrate

staining of total extracts, possibly combined with a decrease in side chain galactosylation. With respect to UDP-Glc, its abundance might be of importance concerning the substitution of the LPG core glycan by glucose-6-phosphate, which seems to be a prerequisite to further elongate this core by galactopyranose (*Ferguson MAJ., personal communication*). The reduction of LPG biosynthesis might in this case be a sign of a reduced UDP-glucose pool. Studies comparing the nucleotide sugar pools in both UGP and USP mutants would probably bring support to this hypothesis.

Leishmania major possibly maintained a salvage pathway in order to fuel its high demand on UDP-Gal needed for phosphoglycan side chain galactosylation among others. In this regard, the absence of USP could be most obstructive for the promastigote life style. Within the insect, *L. major* USP might be of importance to metabolize sugars released by hydrolysis of blood cell glycan structures or present in plant saps (*Pimenta et al., 1997*). In contrast, the phagolysosome surrounding the amastigote stage is thought to be poor in carbohydrates. Nevertheless, studies of hexose transporters in *Leishmania mexicana (Rodriguez-Contreras et al., 2007; Feng et al., 2009)*, demonstrated that these proteins are essential for the replication of the parasites into macrophages and suggest thus an important role for monosaccharide salvage in the amastigote stage. Investigation of the virulence of the Δ*usp* mutant in macrophages and mice will be undertaken and might bring an answer to some of these issues.

The contribution of USP to the UDP-Glc/UDP-Gal biosynthesis was further supported by the deletion of a USP allele in the Δ*ugp* mutant (Δ*ugp*/*usp*$^{+/-}$) and ensuing absence of LPG. Moreover, despite several attempts, deletion of the second USP allele remained unsuccessful suggesting a lethal phenotype. This observation strongly resembled the *T. brucei* and *T. cruzi* UDP-galactose 4-epimerase (GALE) deletion mutants, where inability of UDP-galactose formation turned out to be essential as well, whereas already single allele knockouts exhibited 30% reduction of their cell surface determinants in *T. brucei* and 60% reduction *in T. cruzi (Roper et al., 2005; Macrae et al., 2006)*. These examples of haploid insufficiency suggest that a similar regulation of biosynthetic enzyme level may also exist in *L. major* parasites, where the stepwise deletion of genes contributing to UDP-gal formation, accounts for a stepwise reduction of LPG expression and basal enzyme activity in cell lysates of *L. major* wild type, Δ*ugp* and Δ*ugp*/*usp*$^{+/-}$. This direct correlation between gene and enzymatic activity, implies that *Trypanosoma* GALE, or *Leishmania* UGP and USP are working at maximum activity, without any redundancy.
A recently published strategy that involves the use of a conditionally destabilized fusion domain to the protein of interest, allowing inducible degradation on protein level, might be used to obtain a Δ*ugp*/Δ*usp* deletion mutant. Indeed, no strategies for conditional gene knock out or use of RNAi are available for *Leishmania (Madeira et al., 2009)*.

If confirmed, the lethal phenotype of the Δ*ugp*/Δ*usp* deletion mutant could be attributed to either UDP-Gal and/or UDP-Glc. Besides its implication in UDP-Gal biosynthesis, UDP-Glc is the likely donor substrate for substitution of the LPG glycan core by a glucose-6-phosphate residue, as mentioned above. The importance of this modification found in approximately 30% of the molecules is currently unknown.

Furthermore, UDP-Glc is indispensable for glucosylation by the UDP-glucose:glycoprotein glucosyltransferase of the oligosaccharide $Man_{9-7}GlcNAc_2$ found on newly synthesized glycoproteins. The resulting monoglucosylated $Glc_1Man_{9-7}GlcNAc_2$ interacts with the chaperones calnexin and calreticulin that ensure proper folding and quality control in the endoplasmic reticulum. Although calreticulin-deficient mice die during embryonic development (*Mesaeli et al., 1999*), eukaryotic cells in culture can generally survive in the absence of calnexin or calreticulin. In the parasites *T. cruzi* and *T. brucei*, components of the ER glucosylation dependent quality control system can be deleted with only moderate effects on parasite growth, differentiation and infectivity (*Jones et al., 2005; Labriola et al., 1999; Conte et al., 2003*). In *Leishmania,* the importance of the calnexin calreticulin "quality control" cycle has not been addressed. However, the gene encoding the putative UDP-Glc transporter Hut1-like, required for this cycle, seems lethal in this organism (*Capul et al., 2007*).

Finally UDP-Glc is presumably involved in the biosynthesis of base J (β-D-glucosyl 5-hydroxymethyluracil), a hypermodified base found in the DNA of all kinetoplastid flagellates and some unicellular flagellates closely related to trypanosomatids (*van Leeuwen et al., 1998*). The biosynthesis of base J is thought to occur in two steps: first, a specific thymidine in DNA is converted into hydroxymethyldeoxyuridine, and then this compound is glucosylated. Strong indirect evidence suggests that the first step is catalyzed by two thymidine hydroxylases JBP1 and JBP2. JBP2 appears mainly responsible for *de novo* synthesis whereas JBP1 is a DNA binding protein mediating the maintenance of the J present. The glucosyltransferase catalyzing the subsequent transfer of the glucose residue remains elusive. The functions of base J are also still unknown. In all kinetoplastid flagellates, this base is predominantly found in the telomeres suggesting that it has a conserved telomeric function. In the case of *Leishmania*, more than 98% of base J is found in the telomeres and the function of base J is apparently essential since a knock out of JBP1 seems lethal. In consequence, the biosynthesis of UDP-glucose might be crucial for survival of *Leishmania*.

To dissect the role of UDP-Gal biosynthesis on one hand, and UDP-Glc biosynthesis on the other hand, the Δ*ugp*/Δ*usp* mutant lacking both nucleotide sugars might be compared to a mutant exclusively deficient in UDP-Gal obtained by deletion of USP and UDP-glucose 4-epimerase.

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Abbreviations

α	anti
Ara	arabinose
ADS	alkyldihydroxyacetonephosphate synthase
ble	phleomycin resistance gene
Da	dalton
e.g.	*exempli gratia*
et al.	*et alii*
ER	endoplasmatic reticulum
EtN	ethanolamine
FBP	fructose-1,6-biphosphatase
Fuc	fucose
Gal	galactose (galactopyranose)
GalA	galacturonic acid
Gal_F	galactofuranose
GALK	galactokinase
GalNAc	N-actetyl-galactosamine
GALE	UDP-galactose/glucose 4-epimerase
GALT	Galactose-1-phosphate:UDP-glucose
GDP-MP	GDP-mannose pyrophosphorylase
GFP	green fluorescent protein
GIPLs	glycosylinositolphospholipids
Glc	glucose
GlcA	glucoronic acid
GLCK	glucokinase
GlcN	glucosamine
GlcNAc	N-actetyl-glucosamine
GLF	gene coding for UDP-galactofuranose/pyranose
gp63	glycoprotein 63
GPI	glycosylphosphatidylinositol
hyg	hygromycin
HX	hexokinase
kb	kilobase
kDa	kilodalton
LPG	lipophosphoglycan
Lm/ Lmj	*Leishmania major*
mAb	monoclonal antibody
Man	mannose
MS	mass spectrometry

NAD^+	nictotinamide adenine dinucleotid (oxidized)
NADH	nictotinamide adenine dinucleotid (reduziert)
neo	neomycin
NMR	nuclear magnetic resonance
NS	nucleotide sugar
OD	optical density
pac	puromycin acetyltransferase
PFK	phosphofructokinase
PHLEO	phleomycin
PI	phosphatidylinositol
P_i	phosphate
PP_i	pyrophosphate/ diphosphate
PGM	phosphoglucomutase
PMM	phosphomannomutase
PPG	proteophosphoglycan
Rha	rhamnose
sAP	secreted acid phosphatase
sat	seothricin acetyl transferase
STD	saturated transfer difference
UDP	uridine-5'-diphosphate
UGM	UDP-galactofuranose/pyranose mutase
UGP	UDP-glucose pyrophosphorylase
USP	UDP-sugar (monosaccharide) pyrophosphorylase
UTP	uridine-5'-triphosphate
UTR	untranslated region
Xyl	xylose

Curriculum vitae

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Graduation	June 2005, Dipl.-Biochem.
Doctoral thesis	October 2005 – February 2010, under supervision or Prof. Françoise Routier, Dept. of Cellular Chemistry, Hannover Medical School.

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ICOP XIII - XIII International Congress of Protistology
Brazil, Rio de Janeiro, Armação dos Búzios, August 23-28, 2009
Title: “Identification and functional characterization of a multi-substrate specific UDP-sugar pyrophosphorylase from *Leishmania major.”*

Poster presentations

18th Joint Meeting Glykobiologie
France, Lille, November 4-6, 2007
Title: “Identification and Functional Characterization of a multi substrate specific UDP-Sugar Pyrophosphorylase from *Leishmania major*”

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