The role of surface glycoconjugates in *Leishmania* midgut attachment examined by competitive binding assays and experimental development in sand flies

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SUMMARY

Binding of promastigotes to the sand fly midgut epithelium is regarded as an essential part of the *Leishmania* life cycle in the vector. Among *Leishmania* surface molecules putatively involved in attachment to the sand fly midgut, two GPI-anchored molecules are the most prominent: lipophosphoglycan (LPG) and promastigote surface protease gp63. In this work, we examined midgut attachment of *Leishmania* lines mutated in GPI-anchored molecules and compared results from 2 different techniques: *in vivo* development in sand flies and *in vitro* competitive binding assays using fluorescently labelled parasites. In combination with previous studies, our data provide additional support for (1) an LPG-independent parasite-binding mechanism of *Leishmania major* within the midgut of the permissive vector *Phlebotomus perniciosus*, and provide strong support for (2) the crucial role of *L. major* LPG in specific vector *Phlebotomus papatasi*, and (3) a role for *Leishmania amazonensis* gp63 in *Lutzomyia longipalpis* midgut binding. Moreover, our results suggest a critical role for GPI-anchored proteins and gp63 in *Leishmania mexicana* attachment to *L. longipalpis* midguts, as the wild type (WT) line accounted for over 99% of bound parasites.

Key words: phlebotomine sand flies, *Leishmania*, lipophosphoglycan, zinc protease gp63.

INTRODUCTION

Leishmaniasis is a vector-borne disease transmitted by phlebotomine sand flies. Development of *Leishmania* in female sand flies starts upon the release of amastigotes from infected macrophages ingested by the fly during the blood meal, which then transform into motile procyclic promastigotes with short flagella (reviewed by Bates and Rogers, 2004; Kamhawi, 2006). These forms grow within the blood meal surrounded by a semi-permeable peritrophic matrix. After the peritrophic matrix begins to become degraded (Sadlova and Volf, 2009) procyclic promastigotes transform into highly motile elongated nectomonad forms, whose function is to escape from the endoperitrophic space and anchor themselves to the midgut epithelium. Such binding of promastigotes to the midgut epithelium is regarded as an essential part of the life cycle in the sand fly vector, enabling the parasites to persist beyond the initial blood-meal phase. Laboratory studies examining the development of different *Leishmania* in a range of sand fly species have revealed that so-called specific (restricted or non-permissive) vectors, for example, *Phlebotomus papatasi* and *Phlebotomus sergenti*, support the growth of only the parasites they normally transmit in nature, while other sand fly species, for example, *Lutzomyia longipalpis*, support the growth of various *Leishmania* spp. and are called permissive vectors. These two groups of sand flies differ in their mechanisms of parasite attachment to the midgut epithelium (Volf and Myskova, 2007).

Among *Leishmania* surface molecules, putatively involved in attachment to sand fly midgut, two GPI-anchored molecules are prominent: promastigote surface protease gp63 and lipophosphoglycan (LPG). Gp63 (also known as leishmanolysin) is a zinc proteinase of molecular mass 58–65 kDa with a wide substrate specificity and pH optimum (Yao *et al*. 2003). LPG is a major surface glycoconjugate which has been implicated in a wide range of functions both in the mammalian host and vector, including adhesion to sand fly midgut epithelium lectins (Kamhawi *et al*. 2004). Species-specific differences in the structure of LPG are thought to be at least partially responsible for the variation

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in sand fly vectorial competence for different *Leishmania* species (Sacks and Kamhawi, 2001).

Two major techniques for studying *Leishmania* attachment in the sand fly midgut have been used to date: *in vivo* development in sand flies and *in vitro* binding assays. Recently we developed an improved *in vitro* method, the competitive binding assay, where 2 promastigote populations are labelled with different fluorescent dyes and compete for binding to dissected sand fly midguts (Wilson *et al*. 2010). The advantage of this assay is a direct microscopic examination of a mixture of 2 differentially stained parasite populations bound to individual sand fly midguts dissected from flies at an appropriate time post-blood feeding. Assays are performed in Grace's Insect Medium in which guts do not change their structural integrity and remain amenable to manipulation for several hours. Parasites are counted from one microscope field of view per gut instead of homogenizing the whole gut and counting the released parasites on a haemocytometer, which, due to differences in sizes of the guts and quality of dissections, introduces a significant source of variation. In this study, we have used parasites mutated in LPG and gp63 and examined their role in parasite attachment to midguts of various sand fly species using both competitive *in vitro* binding assays and *in vivo* development in the sand fly midgut.

MATERIALS AND METHODS

Parasites and sand flies

The following parasite isolates were used in this study: *Leishmania major* LV39 clone 5 (MRHO/SU/1959/Neal P) wild type (WT) and an isogenic LPG-deficient mutant ($lpg1^{-/-}$), which specifically lacks LPG through deletion of the *LPG1* galactofuranosyltransferase gene required for synthesis of the LPG core (Späth *et al*. 2000); 2 similar lines of *Leishmania infantum chagasi* (MHOM/BR/76/M4192): WT and $lpg1^{-/-}$, generated for the purposes of this study (see below); 2 lines of *Leishmania amazonensis* LV78 (MPRO/BR/72/M1845): transfectants P6·5 (control) and P6·5/1·9R down-regulated for expression of gp63 (Chen *et al*. 2000); and 3 lines of *Leishmania mexicana* (MNYC/BZ/62/M379): WT, LPG (Ilg *et al*. 2001) and $gpi8^{-/-}$ lacking expression of all cell surface GPI-anchored proteins (Hilley *et al*. 2000). *Leishmania infantum* $lpg1^{-/-}$ was generated essentially as described in Späth *et al*. (2000) by sequential removal of both alleles and replacement with blasticidin and hygromycin resistance genes (Brooks *et al*. 2000).

All parasites were maintained as promastigote forms at 26 °C on Medium 199 supplemented with 20% fetal calf serum (Gibco) and gentamicin (25 μg mL^{-1}). For the mutated lines, selection antibiotics were added to the culture medium as follows: hygromycin (15 μg mL^{-1}) and puromycin (11 μg mL^{-1}) for the *L. major* $lpg1^{-/-}$ mutant; blasticidin (50 μg mL^{-1}) and hygromycin (50 μg mL^{-1}) for the *L. infantum chagasi* $lpg1^{-/-}$ mutant; tunicamycin (10 μg mL^{-1}) for *L. amazonensis* transfectants; and nourseothricin (25 μg mL^{-1}) and puromycin (10 μg mL^{-1}) for the *L. mexicana* $gpi8^{-/-}$ mutant.

Laboratory colonies of 4 sand fly species were used: *Phlebotomus papatasi* (origin from Turkey), *P. perniciosus* (Spain), *P. sergenti* (Israel) and *Lutzomyia longipalpis* (Jacobina, Brazil). Sand flies were maintained at 26 °C on 50% sucrose under conditions described previously (Volf and Volfova, 2011).

Leishmania *development in sand flies*

Sand fly females were fed through a chick-skin membrane on heat-inactivated rabbit blood containing 10^6 promastigotes per mL of blood. Prior to sand fly experimental feeding parasites were washed by centrifugation and re-suspended in saline solution. Engorged sand flies were separated and maintained at 26 °C and dissected 2, 4 and 8 days after the bloodmeal. Parasite loads were graded as described previously by Cihakova and Volf (1997) as light (< 100 parasites per gut), moderate (100–1000 parasites per gut) and heavy (> 1000 parasites per gut). Data from 2 experiments were pooled and evaluated statistically by means of χ^2 test using the S-PLUS 2000 program.

Competitive in vitro *binding assay*

Lutzomyia longipalpis, P. perniciosus, P. papatasi and *P. sergenti* midguts were dissected in Grace's Insect Medium (GIM, Sigma) 4–6 days after bloodfeeding. By this time all species had defecated blood meal remnants. Midguts were carefully opened longitudinally using a fine glass needle and the assays performed as described previously (Wilson *et al*. 2010). Briefly, 4-day-old cultures of promastigotes parasites, in which the nectomonads and leptomonads were the predominant forms, were labelled for 1 h at 26 °C with 0·04% (v/v) Syto21 Green or 0·2% Syto40 Blue fluorescent dyes (Invitrogen), washed 3 times in M199 medium (Sigma) and twice in GIM, then re-suspended in GIM at a cell density of 10^8 cells mL^{-1}. Each midgut was placed in the well of a microscope chamber slide and incubated at RT for 45 min in a humid chamber with a 10 μL mixture of green- and blue-fluorescent parasites. The guts were then individually washed 5 times by placing them in successive drops of GIM and placed in 2 μL of cooled CyGel (Biostatus Limited) on a microscope slide, carefully flattened and mounted in Prolong Gold anti-fade reagent (Invitrogen). Bound parasites

Table 1. *Competitive binding assays of various* Leishmania *species and mutants defective in expression of surface molecules to the midguts from permissive and specific sand fly species*

(The numbers are mean ± standard deviation (S.D.) bound/midgut field of view from all examined midguts for a given vector species, where *n* is the number of midguts analysed and the figure in parentheses is the total number of parasites counted. The result from each set of comparisons is displayed as a% of the mutant parasites in a 1 : 1 mixture of mutant and wild type (WT). Equal binding of the mutant with WT will produce a% value close to 50%; values above this would indicate preferential binding of the mutant, values below indicate preferential binding of the WT.)

	Number of bound parasites			
Sand fly	*L. major* WT	*L. major* LPG	% LPG	Fold change
Phlebotomus perniciosus (*n* = 24)	466 ± 365 (15851)	409 ± 377 (13913)	47 ± 9	1·1 (*P* = 0·53)
Phlebotomus papatasi (*n* = 23)	285 ± 138 (6561)	18 ± 13 (418)	6 ± 4	8·3 (*P* < 0·001)
	L. infantum WT	*L. infantum* LPG	% LPG	Fold change
P. perniciosus (*n* = 14)	49 ± 19 (681)	8 ± 6 (110)	14 ± 9	3·6 (*P* < 0·01)
P. sergenti (*n* = 30)	3 ± 6 (86)	1 ± 2 (20)	Not specified	Not specified
	L. mexicana WT	*L. mexicana* LPG	% LPG	Fold change
Lutzomyia longipalpis (*n* = 11)	38 ± 20 (414)	12 ± 4 (137)	25 ± 6	2 (*P* < 0·001)
	L. mexicana WT	*L. mexicana* GPI8	% GPI8	Fold change
L. longipalpis (*n* = 23)	203 ± 92 (4673)	2 ± 2 (38)	1 ± 1	50 (*P* < 0·001)
	L. amazonensis P6·5	*L. amazonensis* P6·5/1·9R	% P6·5/1·9R	Fold change
L. longipalpis (*n* = 28)	197 ± 90 (5509)	86 ± 53 (2404)	30 ± 9	1·7 (*P* < 0·01)

of both populations were visualized using a fluorescence microscope at 400× magnification with a CFP/YFP-A filter set (Olympus). The numbers of bound parasites from each population were counted in 1 field of view per midgut. Each experiment was repeated with the colour labelling of the parasites switched and each pair of comparisons was performed at least twice. The difference between bound parasites of 2 lines was evaluated statistically by means of the ANOVA test.

RESULTS

Binding of L. major *LPG mutant to* P. perniciosus *and* P. papatasi *midguts*

The role of LPG in the binding of *L. major* to a permissive vector was studied by incubation of the *L. major* LPG-mutant and WT control with midguts of *P. perniciosus*; a similar comparison was made using the natural and specific vector of *L. major*, *P. papatasi* (Table 1). As expected, the LPG-mutant was not able to bind to midguts of *P. papatasi* and was strongly out-competed by WT parasites, the mutant accounted for only 6% of the total bound parasites in competition with the WT (*P* < 0·001). On the other hand, using midguts of the permissive vector

P. perniciosus the LPG-mutant bound equally well as *L. major* WT (47% of bound parasites were LPG-negative, showing no significant difference from that of the WT, *P* = 0·53).

Binding of L. infantum *LPG mutant parasites and their development in* P. perniciosus *and* P. sergenti

The existence of a non-LPG-mediated attachment mechanism that can support the development of *L. major* in a number of (unnatural) permissive vectors does not directly prove that such a mechanism is also used by parasites that are normally transmitted by such sand flies. To test this hypothesis directly, *L. infantum* LPG-mutants were generated and their abilities to develop in *P. perniciosus* and bind to midguts of this sand fly were investigated. Interestingly, the *L. infantum* LPG-mutant developed relatively poorly in *P. perniciosus* (Fig. 1). On days 4 and 8 post blood meal, LPG-mutants were present only in 50–60% of females and less than 15% of them had high parasite density in the midgut and colonized stomodeal valve. The differences in the positivity rate (% of *Leishmania*-positive females) and parasite loads of WT and LPG-negative parasites were statistically highly significant (*P* < 0·01). Competitive binding assays also revealed that *L. infantum* LPG-negative
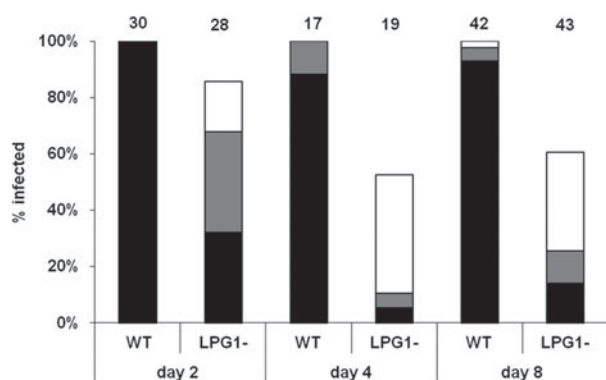
Fig. 1. Development of *Leishmania infantum* LPG-mutant in *Phlebotomus perniciosus*. *P. perniciosus* females were fed with *L. infantum* wild type (WT) or mutants lacking LPG ($lpg1^{-/-}$). Day 2 and Day 4 dissections were before defecation, Day 8 dissections were after defecation. Parasite loads were classified into three categories: heavy (more than 1000 parasites per gut) – black bars; moderate (100–1000 parasites per gut) – grey bars; light (1–100) – white bars. Numbers above the bars indicate the number of dissected females.

parasites competed poorly against the WT control for binding to *P. perniciosus* midguts, representing only 14% of the total population ($P < 0.01$; Table 1).

To explore the properties of the *L. infantum* LPG-mutant further, its ability to develop in and bind to midguts of the specific vector *P. sergenti* was investigated. On day 2 post blood feeding, both mutant and WT lines, initially produced moderate or heavy parasite loads in the abdominal midgut. However, after defecation, on days 5 and 8, no parasites were found in any dissected flies. Consistent with this finding, neither *L. infantum* WT nor LPG-negative parasites were found to bind to *P. sergenti* midguts in significant numbers (Table 1).

Binding of L. mexicana *LPG and GPI8 mutant parasites to* L. longipalpis

We examined the binding of *L. mexicana* LPG-negative parasites to *L. longipalpis* midguts. In this series of the experiment we showed that the mutants bound less well than WT controls, constituting only 25% of the total bound parasites (Table 1, $P < 0.001$).

To show the key role of the other *Leishmania* surface molecules in parasite attachment to the sand fly midgut, competitive binding assays experiments using *L. mexicana* GPI8- and WT lines in *L. longipalpis* were performed. The GPI8 null mutant was generated by targeted disruption of the *gpi8* gene that encodes the catalytic component of the GPI : protein transamidase complex, and does not express detectable levels of GPI-anchored proteins, including gp63, but have normal levels of other GPI-anchored molecules such as LPG and GIPLS (Hilley *et al*. 2000). Our results show that the WT line was found to account for over 99% of the parasites

bound ($P < 0.001$), indicating that WT parasites significantly out-competed GPI8 knockouts for binding sites (Table 1).

Binding of L. amazonensis *P6.5/1.9 R mutant*

Gp63 is the most highly expressed protein on the surface of *Leishmania* promastigotes. To investigate its potential role, binding of the *L. amazonensis* gp63 down-regulated transfectant P6.5/1.9R to *L. longipalpis* midguts was investigated. This cell line bound in significantly lower numbers ($30\% \pm 9$; $P < 0.01$) than the control line P6.5 (mutant with vector alone) (Table 1). This modest but statistically significant decrease suggests that the gp63 of *L. amazonensis* plays a role, albeit not critical, in the binding of its promastigotes to the *L. longipalpis* midgut.

DISCUSSION

This study provides data on the role of *Leishmania* surface glycoconjugates comparing results of two techniques: *in vitro*-binding experiments and *in vivo* development of *Leishmania* in sand flies. Firstly, we tested the binding of the *L. major* LPG mutant to *P. papatasi* midguts by the *in vitro* binding assay. The observed poor binding of the mutated line in comparison with the WT is consistent with the established observation for the dependency of *L. major* binding on LPG, as shown previously using other assays (Sacks *et al*. 2000; Kamhawi *et al*. 2004; Myskova *et al*. 2007). On the other hand, the same *L. major* mutated line bound as efficiently as the WT when incubated with the midguts of a permissive vector *P. perniciosus*. *Phlebotomus perniciosus* is the proven vector of *L. infantum* in the western Mediterranean (Killick-Kendrick, 1999) and according to previous data belongs to the group of permissive vectors (Volf and Myskova, 2007), which is susceptible for development of a wide range of *Leishmania* species. Our observation is in line with the results of *in vivo* experiments reported in earlier studies. Svarovska *et al*. (2010) showed that *L. major* LPG-mutants are able to complete their development in *P. perniciosus* as well as the WT parasites. Similar results were observed from analysis of *L. major* in 2 other permissive species *Phlebotomus argentipes* (Svarovska *et al*. 2010) and *L. longipalpis* (Myskova *et al*. 2007; Secundino *et al*. 2010). Taken together, the use of the competitive binding assay in the current study confirmed the presence of an LPG-independent *L. major*-binding mechanism within the midgut of the permissive vector *P. perniciosus* and provides further strong support for the crucial role of LPG in the specific vector *P. papatasi*.

Interestingly, different results were observed in the case of *L. infantum*, the parasite species transmitted

by *P. perniciosus* in nature. The observed poor development of LPG-deficient *L. infantum* as well as their poor binding in comparison with the WT suggests an important role for LPG in the binding and development of this *Leishmania* species. Although the experimental approach was different, these data are consistent with a previously reported role of LPG for *Leishmania donovani* attachment in *P. argentipes* (Sacks *et al.* 2000). Unfortunately, as there are no add-back lines available for both of the mentioned mutants (Sacks *et al.* 2000; this work), the results gained by *P. perniciosus*–*L. infantum* and *P. argentipes*–*L. donovani* combinations should be treated with caution; at present we cannot exclude the possibility that mutagenesis and prolonged cultivation *in vitro* also introduced changes in some non-target molecules.

Previous work with *L. mexicana* LPG-mutants demonstrated that these parasites were able to complete their development in *L. longipalpis*, although not as efficiently as WT parasites (Rogers *et al.* 2004). The results of our *in vitro* binding study using this mutant line show that LPG of *L. mexicana* also plays some role in its binding to *L. longipalpis* midgut, although it appears to be less critical than for *L. infantum* in *P. perniciosus* or for *L. major* in *P. papatasi*.

In contrast to the intriguing data with *L. infantum* LPG mutant and *P. perniciosus*, it was not surprising to see that both the WT and the LPG-deficient *L. infantum* line failed to develop in the specific vector *P. sergenti*. The results obtained *in vivo* were clearly reflected in the poor *in vitro* binding of both *L. infantum* lines (WT line and LPG mutant) seen with *P. sergenti* midguts, which is in agreement with findings obtained by Killick-Kendrick *et al.* (1995) and Kamhawi *et al.* (2000) using other *Leishmania* species, confirming the specificity of *P. sergenti* for *Leishmania tropica* only.

Leishmania possess a number of cell surface glycoconjugates in addition to LPG that could function as ligands/receptors in midgut binding. The majority of these molecules are GPI-anchored glycoproteins (McConville and Ferguson, 1993; Ferguson, 1994), including gp63/leishmanolysin, PSP2/gp42 and membrane proteophosphoglycan (mPPG). To investigate whether this class of molecules may be involved in LPG-independent midgut binding, assays were performed with a *L. mexicana* GPI8 null mutant (Hilley *et al.* 2000). The GPI8-mutant displayed normal growth in liquid culture, was capable of differentiating into replicating amastigotes within macrophages *in vitro* and was infective to mice (Hilley *et al.* 2000). These authors suggested that GPI-anchored proteins are not essential for parasite infectivity and survival in a mammalian host, but could play a role in a vector part of the life cycle. This suggestion is supported by the results of our study, in which we showed that GPI-anchored

proteins are essential for *in vitro* binding of *L. mexicana* promastigotes to *L. longipalpis* midguts.

The gp63 has been described as a virulence factor with several different roles in the vertebrate host (Chaudhuri *et al.* 1989; Brittingham *et al.* 1995; Kulkarni *et al.* 2006). On the other hand, its function in the vector is still unclear. Data reported by Joshi *et al.* suggest that gp63 is not essential for development and survival of *L. major* in *Phlebotomus duboscqi* and *P. argentipes* (Joshi *et al.* 1998, 2002). Hajmova *et al.* (2004) showed that the gp63 down-regulated transfectants *L. amazonensis* (P6·5/1·9R) developed at a lower rate and with lower parasite density during the early stage of development in *L. longipalpis*, suggesting that gp63 could protect promastigotes from degradation by the midgut digestive enzymes. Given the results described with the *L. mexicana* GPI mutant above, it is interesting to note that *L. amazonensis* is very closely related to *L. mexicana* (in the same species complex), and they are therefore likely to be using similar attachment mechanisms in *L. longipalpis*. In addition, the putative role of gp63/leishmanolysin in its interaction with its insect host is reinforced by the results of Sadlova *et al.* (2006) who observed that repeated passaging of *Leishmania* through sand flies leads to enhanced gp63 expression. To these observations we can now add a potential role of gp63 in *Leishmania* midgut attachment. *Leishmania amazonensis* gp63 down-regulated transfectants bound slightly less efficiently than the control line to the midguts of *L. longipalpis* in our *in vitro* binding assay.

A role for gp63 in parasite attachment has previously been proposed also in trypanosomatids other than *Leishmania*. Pereira *et al.* (2009, 2010) showed that monoxenic *Herpetomonas samuelpessoai* and *Leptomonas* species produce a 63-kDa metallopeptidase that has similar biochemical/immunological properties to *Leishmania* gp63 and contributes to the parasite adhesion to *Aedes aegypti* guts or *Aedes albopictus* cell line C6/36.

In conclusion, the results of this study have confirmed the important role of *Leishmania* LPG in the interaction of *L. major* with its specific vector *P. papatasi*. They also confirm the existence of an LPG-independent mechanism of attachment for *L. major* in *P. perniciosus*, and that midgut binding is a necessary, albeit insufficient, for the generation of transmissible infections. However, the molecules involved in the binding of promastigotes in permissive vectors appear to be multiple, including LPG itself, gp63 and, importantly, other GPI-anchored proteins.

These molecules may interact with one another, and are likely to differ in importance and composition among different vector-parasite combinations. Thus what emerges from these data is a more complex and nuanced definition for a *Leishmania*-permissive sand fly. Nevertheless, some important general

conclusions can be drawn. It appears that although an LPG-independent mechanism definitely exists, it is most clearly manifested when the midgut of a permissive sand fly is exposed to an unnatural parasite (*L. major* in *P. perniciosus*), whereas the natural parasite of the permissive sand fly may rely mainly, although not necessarily exclusively, on LPG for midgut binding (*L. infantum* in *P. perniciosus*). This has interesting evolutionary and epidemiological implications. It suggests that LPG-independent mechanism(s) could be advantageous for *Leishmania* in a chance-encounter with a new and permissive sand fly host by having multiple ligands alternative to LPG for evolutionary adaptation to survive in the midgut. The parasite may subsequently fine-tune its LPG for adaptation to evolve its utility as the main midgut binding ligand. This makes sense given that LPG is always the dominant cell surface glycoconjugate in *Leishmania* promastigotes. Thus, although multiplicity and complexity of the LPG-independent mechanisms render their analysis difficult they are important for further investigation as leishmaniasis continues to spread to new foci around the world.

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