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The Flagellar Protein FLAG1/SMP1 is a Candidate for *Leishmania*–Sand Fly Interaction

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Abstract

Leishmaniasis is a serious problem that affects mostly poor countries. Various species of *Leishmania* are the agents of the disease, which take different clinical manifestations. The parasite is transmitted by sandflies, predominantly from the *Phlebotomus* genus in the Old World and *Lutzomyia* in the New World. During development in the gut, *Leishmania* must survive various challenges, which include avoiding being expelled with blood remnants after digestion. It is believed that attachment to the gut epithelium is a necessary step for vector infection, and molecules from parasites and sand flies have been implicated in this attachment. In previous work, monoclonal antibodies were produced against *Leishmania*. Among these an antibody was obtained against *Leishmania braziliensis* flagella, which blocked the attachment of *Leishmania panamensis* flagella to *Phlebotomus papatasi* guts. The protein recognized by this antibody was identified and named FLAG1, and the complete FLAG1 gene sequence was obtained. This protein was later independently identified as a small, myristoylated protein and called SMP1, so from now on it will be denominated FLAG1/SMP1. The *FLAG1/SMP1* gene is expressed in all developmental stages of the parasite, but has higher expression in promastigotes. The anti-FLAG1/SMP1 antibody recognized the flagellum of all *Leishmania* species tested and generated the expected band by western blots. This antibody was used in attachment and infection blocking experiments. Using the New World vector *Lutzomyia longipalpis* and *Leishmania infantum chagasi*, no inhibition of attachment *ex vivo* or infection *in vivo* was seen. On the other hand, when the Old World vectors *P. papatasi* and *Leishmania major* were used, a significant decrease of both attachment and infection were seen in the presence of the antibody. We propose that FLAG1/SMP1 is involved in the attachment/infection of *Leishmania* in the strict vector *P. papatasi* and not the permissive vector *L. longipalpis*.

Key Words: *Leishmania*—*Lutzomyia longipalpis*—*Phlebotomus papatasi*—Flagellum—FLAG1/SMP1—Leishmaniasis—Sand fly—Vector–parasite interaction.

Introduction

Phlebotomine (Diptera: Psychodidae) sand flies are the main vectors of leishmaniasis, a disease ranging from self-healing skin lesions to fatal, if left untreated, visceral disease. Different *Leishmania* species are associated with distinct disease outcome, and from the hundreds of sand flies identified to date only a limited number have been proven to be *bona fide* vectors of *Leishmania* (Killick-Kendrick 1990). Some sand fly species are considered permissive (*e.g.*, *Lutzomyia longipalpis*) because they can be infected by several *Leishmania*, whereas other vectors can be infected only with the *Leishmania* species they carry in nature and are thus considered restrictive (*e.g.*, *Phlebotomus papatasi*) (Sacks and Kamhawi 2001, Volf and Myskova 2007). Several parasite and vector molecules allow the parasite's infection, survival, and multiplication within the midgut of the sand fly and eventual transmission to a vertebrate host during a blood meal. *Leishmania* can manipulate the sand fly potentially threatening digestive proteases activity (Borovsky and Schlein 1983, Schlein et al. 1983, Dillon and Lane 1993, Schlein and Jacobson 1998, Telleria et al. 2010) and also can cause damage

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to the stomodeal valve of the fly (Schlein et al. 1992, Rogers et al. 2008), potentiating transmission. On the other hand, sand flies can mount an immune response to *Leishmania* infection (Boulanger et al. 2004, Ramalho-Ortigão et al. 2007, 2010, Jochim et al. 2008, Pitaluga et al. 2009, Diaz-Albiter et al. 2012, Telleria et al. 2012).

Although *Leishmania* do not invade the midgut cells, adhesion to epithelial cells is well documented (Killick-Kendrick and Rioux, 1991). In the case of the *P. papatasi* attachment of *Leishmania major* can be promoted by the lipophosphoglycan (LPG) that covers the parasite (Sacks and Kamhawi 2001), for which the midgut galactose-binding protein PpGalec was shown to be a receptor (Kamhawi et al. 2004). The function of LPG in attachment was confirmed in strict vector–parasite pairs by the use of LPG-deficient *Leishmania* that failed to adhere to midguts *ex vivo* and *in vivo* after blood digestion (Sacks et al. 1995, Sacks et al. 2000). However, in permissive vectors, LPG-deficient *Leishmania* infected the insects normally, indicating an alternative attachment mechanism (Svárovská et al. 2010, Jecna et al. 2013). LPG-independent midgut binding has been suggested in association with the degree of glycosylation of proteins expressed by midgut epithelial cells (Myskova et al. 2007).

It is possible that other unknown molecules also have a role in midgut attachment. We have previously shown that a monoclonal antibody developed against *Leishmania braziliensis* flagella (Ismach et al. 1989) was capable of inhibiting attachment of *Leishmania panamensis* or L. major to dissected guts of *P. papatasi* (Warburg et al. 1989). We identified the 15-kD flagellar protein FLAG1/SMP1 recognized by this antibody (Córdova-Rojas 1998). Later, this protein was identified as a small myristoylated protein (SMP1) by another group (Tull et al. 2004). Here we show that the *Leishmania* flagellar protein FLAG1/SMP1 has a role in parasite interaction with the vector, in the case of the strict vector *P. papatasi*.

Materials and Methods

Leishmania

Leishmania infantum chagasi (MHOM/BR/1974/PP75), *L. infantum* (MHOM/ES/00/UCM-1), *L. pifanoi* (MHOM/VE/1975/LL1), *L. amazonensis* (MHOM/BR/1967/PH8), *L. major* (MHOM/SU/1973-ASKH), *L. donovani* (MHOM/ET/1967/HU3), and *L. mexicana* (MHOM/BZ/1982/BEL21) were obtained from the Instituto Oswaldo Cruz *Leishmania* Culture Collection. Promastigote-stage parasites were maintained by weekly transfers using M199 medium (pH 7.0) supplemented with 10% fetal bovine serum (FBS). *L. pifanoi* amastigotes were maintained at 30°C in F29 medium supplemented with FBS (Pan 1984). To obtain a metacyclic enriched population of parasites, a Ficoll (PM400, Sigma) gradient was used as described (Späth and Beverley 2001, Yao et al. 2008).

Sand flies and infection with Leishmania

L. longipalpis originating from Lapinha Cave, Minas Gerais, Brazil, F_1 adults or insects from a laboratory colony maintained at the Department of Entomology, Kansas State University for several years (LLLP strain), were used in the experiments. Insects were sugar fed on 30% sucrose solution *ad libitum* and blood fed directly on an anesthetized male hamster when needed. *P. papatasi* from colonies originating from Israel were maintained in a similar way. Three- to five-day-old sand flies were fed on mouse blood alone of blood containing 5×10^6 *L. major* promastigotes/mL or 1×10^7 *L. i. chagasi* promastigotes/mL using a feeding apparatus. All procedures involving live animals were approved by the FIOCRUZ bioethics committee (CEUA, protocol number P0-116-02) and by the Committee on Institutional Animal Care and Use of the Kansas State University (KSU-IACUC) (protocol numbers 3080 and 3081). All sand fly feedings on animals and all bleeds were performed on animals under anesthesia, and all efforts were made to minimize suffering.

Isolation and characterization of FLAG1

The FLAG1 protein was purified from *Leishmania* flagella preparations using previously described methods (Pereira et al. 1977, Ismach et al. 1989). The flagellar-enriched fraction was extracted using Brij-97, and nonsoluble material was eliminated by centrifugation at $39{,}000 \times g$ for 1 h at 4°C. The supernatant was then diluted in cold 20 mM sodium phosphate buffer (pH 7.0) containing protease inhibitors (1 mM of iodoacetamide, 1 μg/mL of 1,10-phenanthroline, and 5 μg/mL phenylmethylsulfonyl fluoride) purified on a F-2 monoclonal antibody immunoaffinity column. The FLAG-1 protein was eluted with 0.1 M glycine-HCl (pH 2.7), which was immediately neutralized with 1 M Tris-HCl (pH 7.2). Purity was assessed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The sample was subjected to isoelectric focusing (first dimension) followed by SDS-PAGE, as described by O'Farrell (1975).

The two-dimensional SDS-PAGE fractionated protein was electrotransferred onto Immobilon-P membranes (Millipore, Bedford, MA) as described (Towbin et al. 1979) and sent for amino acid composition and peptide sequencing at the Yale University School of Medicine Protein and Nucleic Acid Chemistry Facility. Peptides were generated by chemical cleavage with cyanogen bromide (CNBr) (Jirikowski, 1985) or digested enzymatically with trypsin (Lee and Forstner 1985), and isolated by high-performance liquid chromatography (HPLC) using a reverse-phase Vydac column C18 (Nest Group Inc., Southborough, MA). Selected isolated peptide peaks were sequenced using gas-phase Applied Biosystems models 470 and 477, as described (Matsudaira 1987).

FLAG1/SMP1 sequencing

L. i. chagasi FLAG1/SMP1 was amplified from parasites DNA using primers Flag F (5′-GGA TCC GGC TGC GGT GCT TCT TCT-3′) and Flag R (5′-AAG CTT CTT TTC CTT CTC CGC CTG-3′) and sequenced in the Instituto Oswaldo Cruz (FIOCRUZ) PDTIS Facility.

Bioinformatics analyses

The FLAG1/SMP1 sequences aligned were collected at the National Center for Biotechnology Information (NCBI) genes database with the exception of *L. i.* chagasi, which was obtained from Sanger sequencing in our laboratory. The protein sequences were predicted with the ExPASy translation tool. For the alignments, we used the program Muscle WS with the score matrix Blossum62 through the platform Jalview. Phylogenetic analysis was performed using the

neighbor-joining method, with a bootstrap of 1000 and the model p-distance, and using the MEGA 5.1 program.

Real-time PCR

Total RNA from *L. i. chagasi* and *L. major* (promastigotes) and *L. pifanoi* (amastigotes and promastigotes) was extracted using TRIzol reagent (Invitrogen), according to manufacturer's instructions, followed by DNase I (Promega) treatment.

First-strand cDNA was synthesized from 5 μg of total RNA using SuperScript III First-Strand Synthesis (Invitrogen). Real-time polymerase chain reaction (qPCR) was performed using SYBR Green PCR Master Mix (Applied Biosystems) and the following primers FLAG_Rtime_Fwd (5′-AGT GGG TAG CCT CCG TGG TGG TGT A-3′), FLAG_Rtime_Rev (5′-CTC CGA CAG CGG CAA GGC GTC CAT C-3′), LeishActin_Fwd (5′-GTG GTC GAT AAA GCC GAA GGT GGT T-3′), LeishActin_Rev (5′-TTG GGC CAG ACT CGT CGT ACT CGC T-3′). Expression levels of FLAG1/SMP1 were determined through $\Delta\Delta$Ct, normalized using actin gene expression, yielding the relative expression value (Pfaffl 2001).

Immunofluorescence

Leishmania were concentrated to 10^8 parasites in 20 μL of phosphate-buffered saline (PBS), applied to poly-L-lysine *Poly-Prep*™ slides from Sigma Diagnostics, fixed in methanol at −20°C, and blocked in PBS + 3% bovine serum albumin (BSA) for 45–60 min. The slides were incubated with the anti-FLAG1/SMP1 antibody (Ismach et al. 1989) in a 1:100 dilution and then with a 1:1000 dilution of an anti-mouse rhodamine-coupled antibody (Jackson ImmunoResearch) in blocking solution for 45–60 min. Images were obtained in the confocal microscope LSM 510 META from the FIOCRUZ, PDTIS Facility.

Western blot

Samples corresponding to 25 μg of *Leishmania* extracts were separated on 15% SDS-PAGE gels at 120V for 2 h. Proteins were transferred to nitrocellulose membranes (BioRad) at 100V for 1.5 h at 4°C. The membranes were blocked with 5% nonfat milk Tris-buffered saline (TBS) + 0.1% Tween 20 (TBST) for at least 1 h. The membranes were then incubated with anti-FLAG1/SMP1 monoclonal antibody (Ismach et al. 1989) (1:3000) for 2 h in the same solution. After three washes of 10 min in TBST, the membranes were incubated with anti-mouse secondary antibody (Jackson ImmunoResearch) at a 1:10,000 dilution for 1 h. Three more washes were performed before the incubation of the membrane with the detection system Pierce SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific).

Ex vivo *inhibition of parasite binding by anti-FLAG1/SMP1 antibody*

Leishmania culture promastigotes (5×10^6) were incubated at room temperature for 1 h with anti-FLAG1/SMP1 antibody (1:500) or PBS. Midguts from 3- to 5-day-old non–blood-fed sand flies were dissected and washed in ice-cold PBS. Each midgut was opened longitudinally to expose the midgut epithelium and then incubated for 1 h at room temperature with the FLAG1/SMP1-treated parasites. Individual midguts were then washed three times with cold PBS, and homogenized in 30 μL of PBS followed by parasite counting using a hemocytometer.

Inhibition of infection by anti-FLAG1/SMP1 antibody

L. longipalpis and *P. papatasi* females were artificially fed with blood containing 10^7 *L. i. chagasi* or 5×10^6 *L. major*. The same volumes of anti-FLAG1/SMP1 antibody or PBS were added to the blood-containing parasites. After 72 h, groups of approximately seven females were examined and dissected in cold PBS. Individual midguts were homogenized in 30 μL of cold PBS, and parasites were counted using a hemocytometer. Total number of insects used in each experiment is shown in the figures (see below), where each dot represents one insect.

Statistical analysis

The Wilcoxon signed-rank test was used to analyze *in vitro* binding and *in vivo* infection. Values were considered significant at the 95% confidence interval.

Results

Sequencing of FLAG1/SMP1

FLAG1/SMP1 protein was isolated and sequence determined (Córdova-Rojas 1998). Briefly, the FLAG1/SMP1 protein was isolated from detergent-solubilized flagellar preparations from *L. amazonensis* promastigotes using immunoaffinity chromatography and the F-2 monoclonal antibody (Ismach et al. 1989). Peptides from CNBr-trypsin–digested FLAG1/SMP1 protein were isolated by HPLC and sequenced. Peptide sequences are shown in Table 1, and their location on the FLAG1/SMP1 complete sequence is shown in Figure 1.

Sequencing of the L. i. chagasi FLAG1/SMP1 *gene*

Degenerate PCR primers were designed and synthesized based on these internal peptide sequences (Table 1), and partial nucleotide sequences were obtained (Córdoba-Rojas 1998). A complete sequence was obtained as described above and this was aligned with other *Leishmania* and one *Trypanosoma cruzi* sequence (Fig. 1). *FLAG1/SMP1* is highly conserved among all investigated trypanosomatids, with a higher degree of conservation among the *Leishmania*, as expected.

Expression of FLAG1/SMP1

Expression of the *FLAG1/SMP1* gene was assessed by qPCR in different developmental stages of *L. major*, *L. i. chagasi*, and *L. pifanoi*, the only *Leishmania* species for which axenic amastigotes were available. The axenic amastigote forms of

Table 1. Peptide Sequence Data from Cyanogen Bromide-Trypsin-Digested FLAG1/SMP1

Elution peak number	*Peptide sequence*
33	FIEGR
52	a/s/gGxMDALPLSEEYxQ
55	(d)KMDALPLSEEYR
57	DALPLSEEY
70	xFEKDNGLLF
71	GFEKD

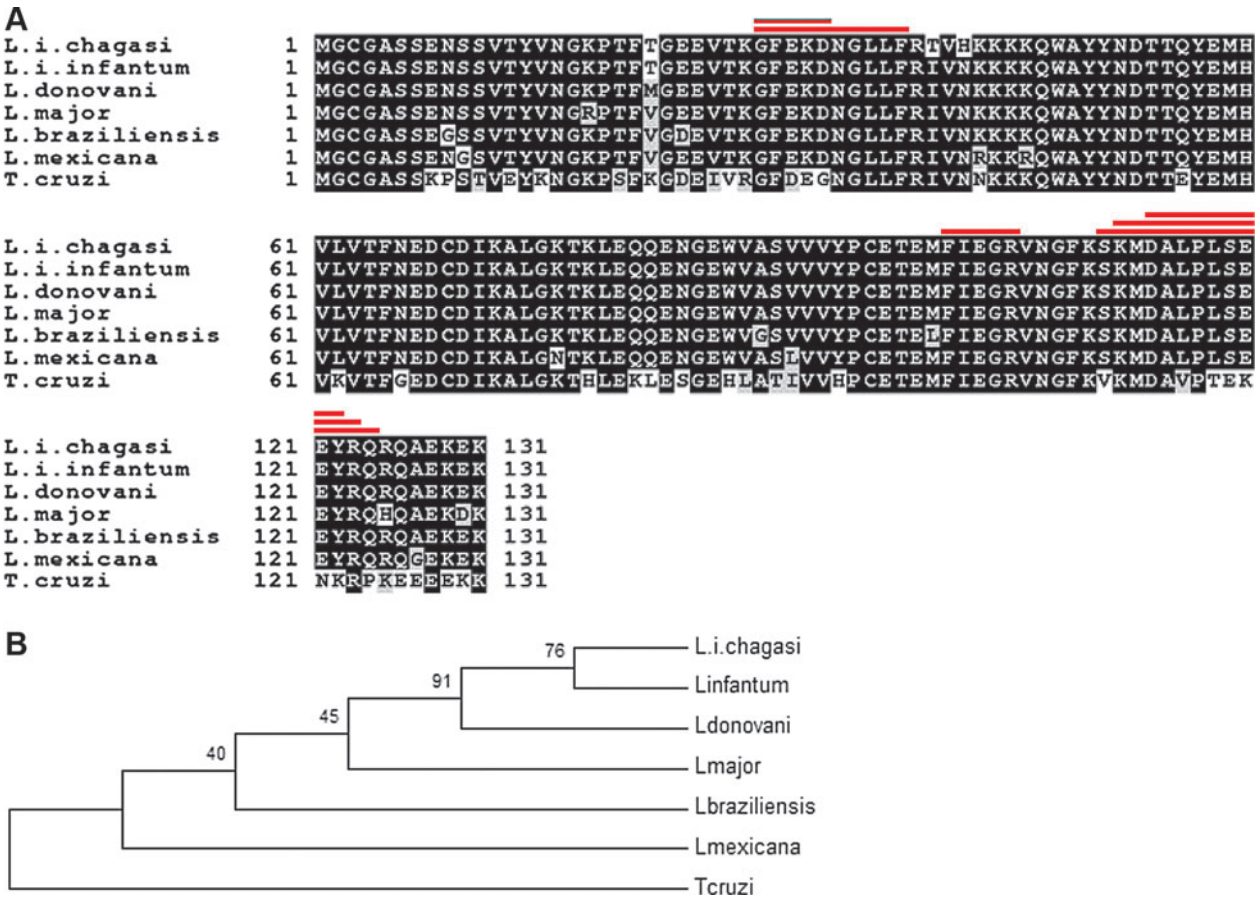

FIG. 1. Alignment of various *Leishmania* species and *T. cruzi* FLAG1/SMP1 sequences. (**A**) Alignment of *L. i. chagasi*, *L. infantum* (XP_001465265.1), *L. donovani* (XP_003860476.1), *L. major* (AAV59017.1), *L. braziliensis* (XP_003723102.1), *L. mexicana* (CBZ26701.1), and *T. cruzi* (XP_806365.1) FLAG1/SMP1-deduced protein sequences. The red lines above the aligned sequences show the position of peptides from Table 1. (**B**) Phylogenetic tree of the different trypanosomatid FLAG1/SMP1 proteins.

L. pifanoi presented a significantly lower expression when compared to promastigote forms (Fig. 2A).

There was no significant difference in *FLAG1/SMP1* mRNA expression between promastigote and metacyclic forms of the parasites, although *L. i. chagasi* and *L. major* metacyclics presented a tendency toward lower expression (Fig. 2B, C).

Detection of FLAG1/SMP1 in different Leishmania *species by western blot and immunofluorescence*

The presence and localization of FLAG1/SMP1 was investigated by western blot and indirect immunofluorescence assays, using the anti-FLAG1/SMP1 monoclonal antibody. FLAG1/SMP1 is expressed in *L. i. chagasi*, *L.pifanoi*, *L. amazonensis, L. major*, *L. mexicana*, and *L. donovani* (Fig. 3A) and is localized along the entire length of the flagellum in all species investigated, namely *L. amazonensis*, *L. i. chagasi*, and *L. pifanoi* (Fig. 3B).

Attachment/infection inhibition experiments

In both *ex vivo* and *in vivo* attachment/infection inhibition assays using the pair *L. longipalpis* and *L. i. chagasi,* no effect of the antibody was seen on the adhesion/infection process (Fig. 4C, D). Similar results were obtained using F_1 *L. longipalpis* (from the Lapinha Cave) as well as its counterpart maintained in laboratory colonies for many generations (LLLP).

However, *ex vivo* and *in vivo* attachment/infection inhibition experiments performed with *P. papatasi* and *L. major* showed a different outline. There was a significant decrease in the number of parasites attached or infected insects when they were pretreated with anti-FLAG1/SMP1 monoclonal antibody (Fig. 4A, B).

Discussion

It is widely accepted that a close interaction of the parasite *Leishmania* to the gut of the sand fly vector, as first seen by Killick-Kendrick et al. (1974a, b), is fundamental for a successful infection. It is also believed that the flagellum is involved in such interactions with a role in the adhesion, both through hemidesmosomes formation with the ectodermic cuticle of the gut (Walters et al. 1989) and through the flagellum insertion between microvilli of the midgut (Molyneux 1977, Walters et al. 1989). Interestingly, promastigotes move anteriorly using the flagellum; thus, as parasites migrate from the midgut toward the anterior gut parts of the sand fly during metacyclogenesis, the salient hypothesis that the

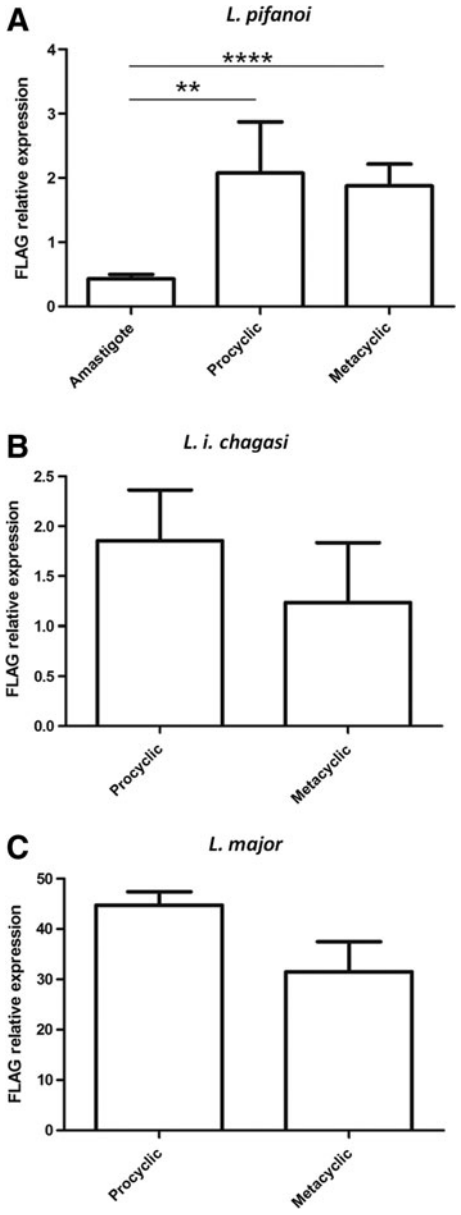

FIG. 2. FLAG1/SMP1 relative expression in developmental forms of *Leishmania*. The expression of FLAG1/SMP1 in procyclic, metacyclic and amastigotes forms of *L. pifanoi* (**A**), *L. i. chagasi* (**B**), and *L. major* (**C**) was determined by qPCR. (****) p less than sign 0.0001; (**) $p < 0.001$.

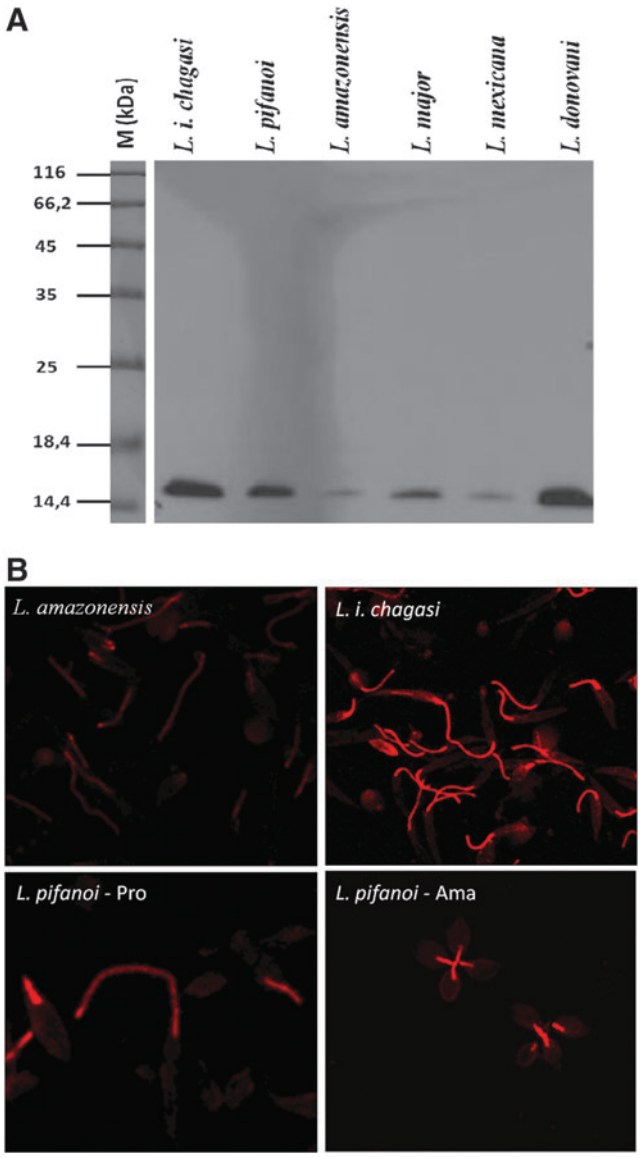

FIG. 3. Presence of FLAG1/SMP1 in different *Leishmania* species. (**A**) Western blot of parasite total protein extracts. (**B**) Indirect immunofluorescence using anti-FLAG1/SMP1 antibody.

flagellum touches, senses, and transduces signals is very appealing. These assumptions were reinforced when Cuvillier et al. (2003) demonstrated that *L. amazonensis* overexpressing the ADP-ribosylation factor-like protein 3A (LdARL-3A) lacked a flagellum and was incapable of developing in *L. longipalpis*. In contrast, more recent work indicated that a flagellum-less *L. braziliensis* isolated from a patient was capable of infecting this same vector (Zauli et al. 2012). These apparently contradicting results suggest a possibly complex interplay of different components during the *Leishmania*–sand fly interaction and need to be investigated further.

A potential alternative candidate for such interaction was identified several years ago by some of us following the development of an anti-flagellum monoclonal antibody (Ismach et al 1989) shown to block adhesion of *L. panamensis* flagella to *P. papatasi* midguts (Warburg et al. 1989). This antibody recognizes a protein called FLAG1 (Rojas 1997) which was independently characterized by another group and named SMP-1. Tull et al. (2004) demonstrated that SMP-1 is myristoylated and palmitoylated, and that these fatty acids are responsible for the flagellar location of the protein. SMP-1 was also shown to stabilize the flagellar membrane, and it is required for flagellar elongation and function. Deletion of the genes encoding SMP-1 led to the production of short flagella and defects in motility (Tull et al. 2009).

We were especially interested in the preliminary results indicating a function of this protein in attachment of parasites to the sand fly gut (Warburg et al. 1989). LPG has been clearly demonstrated to be critical in *Leishmania* binding to the so-

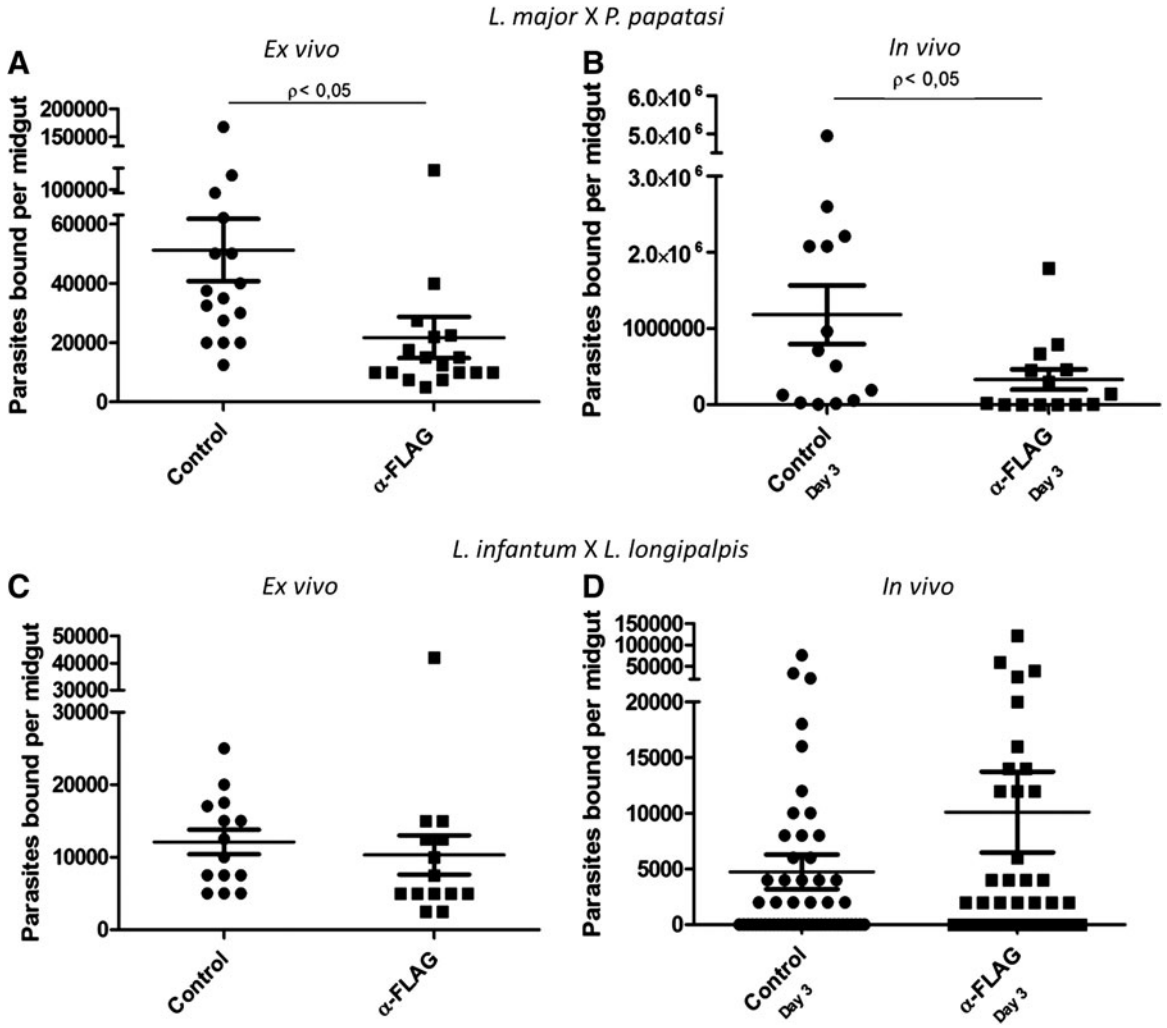

FIG. 4. Effect of anti-FLAG1/SMP1 antibody on parasite binding to the midgut and sand fly infection. Attachment of *L. major* to *P. papatasi* midgut epithelia *ex vivo* (**A**) and infection *in vivo* (**B**) in the presence or absence of anti-FLAG1/SMP1 antibody were investigated. Attachment of *L. infantum* to *L. longipalpis* midgut epithelia *ex vivo* (**C**) and sand fly *Leishmania* infection *in vivo* (**D**) in the presence or absence of anti-FLAG1/SMP1 antibody were investigated in the presence or absence of anti-FLAG1/SMP1 antibody. Each dot in the graphs represents one insect; the horizontal line indicates the mean, and the whisker plots represent the standard deviation.

called restrictive vectors (Sacks and Kamhawi 2001). However, in permissive vectors, molecules responsible for such binding remain elusive, in spite of the indication that the level of glycosylation of midgut molecules potentially is related to this binding (Myskova et al. 2007). Hence, we initially investigated the role of FLAG1/SMP1 in the attachment of *Leishmania* to *L. longipalpis*. Our results, however, did not support a role for this flagellar protein in attachment in this *Leishmania*–sand fly pair. On the other hand, our results suggest that FLAG1/SMP1 is involved in the binding of *L. major* to the restrictive vector *P. papatasi*. The fact that *L. major* LPG mutants failed to infect *P. papatasi* indicates that FLAG1/SMP1 *per se* is not sufficient to allow survival of the parasite in this vector. LPG covers the entire surface of the parasite whereas FLAG1/SMP1 is localized to the flagellum. The initial interaction of the parasite with the gut occurs by the flagellum, and classical depictions of this interaction show this organelle is deeply embedded into the gut microvilli (Killick-Kendrick et al. 1974a, b). We hypothesize that this initial interaction through the flagellum, via FLAG1/SMP1, may be essential for further and possibly stronger interaction through the LPG molecules.

FLAG1/SMP1 was found to be a well-conserved protein present in all *Leishmania* investigated so far. By immunofluorescence, the anti- FLAG1/SMP1 monoclonal antibody reacted with the flagellum of *L. braziliensis*, *L. i. chagasi*, *L. donovani*, *L. guyanensis*, *L. major*, *L. mexicana*, *L. panamensis*, and *L. pifanoi* (Córdova-Rojas 1998, Tull et al. 2010; our results). Western blots of *L. amazonensis*, *L. i. chagasi*, *L. donovani*, *L. major*, *L. mexicana*, and *L. pifanoi* using the anti-FLAG1/SMP1 antibody produced the expected band of approximately 15 kD. Differences in intensity observed both in the immunofluorescence and western blots may be reflective of the abundance of protein present in different parasites or due to differences in epitope recognition of the different FLAG1/SMP1 proteins by the antibody. Expression of the FLAG1/SMP1 gene was compared among different developmental forms of some parasite species. As expected, there was lower mRNA expression in axenic amastigotes of

L. pifanoi and a slight but nonsignificant decreased expression in metacyclic versus procyclic forms of *L. pifanoi*, *L. i. chagasi*, and *L. major*. When the FLAG1/SMP1 sequences available in the database were compared, a high degree of homology between the various sequences was evident, suggesting a strong functional conservation. It is interesting to speculate how such a conserved protein might have a strikingly different function in vector–parasite interactions. This might be due to the small sequence differences observed among *Leishmania* species, although these do not seem to cause big structural changes in the molecule, as can be seen in Figure 1.

Conclusions

We have shown that FLAG1/SMP1 is involved in the attachment of *Leishmania* to the strict vector *P. papatasi* and not to the permissive vector *L. longipalpis*. We are currently attempting to identify molecule(s) in the restrictive *P. papatasi* sand fly that are able to interact with FLAG1/SMP1. We predict that such a molecule will be present in significantly greater concentrations in this fly in comparison to *L. longipalpis*, or altogether absent in the latter.

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Author Disclosure Statement

No competing financial interests exist.

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