

A Role for Insect Galectins in Parasite Survival

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Summary

Insect galectins are associated with embryonic development or immunity against pathogens. Here, we show that they can be exploited by parasites for survival in their insect hosts. PpGalec, a tandem repeat galectin expressed in the midgut of the sand fly *Phlebotomus papatasi*, is used by *Leishmania major* as a receptor for mediating specific binding to the insect midgut, an event crucial for parasite survival, and accounts for species-specific vector competence. PpGalec is thus identified as a key molecule controlling vector competence for the most widely distributed form of cutaneous leishmaniasis in the Old World. In addition, these studies demonstrate the feasibility of using midgut receptors for parasite ligands as target antigens for transmission-blocking vaccines.

Introduction

Leishmania produce a spectrum of diseases in humans, including cutaneous, mucocutaneous, and visceral forms determined in large part by the *Leishmania* species transmitted. Cutaneous leishmaniasis due to *L. major* is the most common leishmanial disease in the Old World, widely distributed from West Africa to South Asia and particularly in the Middle East. The distribution of *L. major* and more than 20 other species and subspecies of *Leishmania* and the diseases they produce are determined by the availability of competent vectors. Phlebotomine vectors of leishmaniasis are also diverse and, in the case of some sand fly species, will only permit the complete development of the species of *Leishmania* they transmit in nature. Thus, *P. papatasi*, the natural vector of *L. major*, is refractory to the development of other *Leishmania* species. *Leishmania* undergo a digenetic life cycle as intracellular amastigotes in macro-

phages of mammalian hosts and as extracellular promastigotes within the digestive tract of competent insect vectors (Figure 1A). Amastigotes ingested by the sand fly during blood feeding transform to dividing promastigotes, which attach to the insect midgut epithelium (Figure 1A). The bound promastigotes undergo numerous divisions before differentiating into free-swimming infective metacyclics (Kamhawi, 2002; Sacks, 2001). Attachment of promastigotes to the midgut lining of the sand fly is crucial to the successful completion of their life cycle in the fly, serving to prevent excretion of developing parasites along with the bloodmeal remnants.

Lipophosphoglycan (LPG), the major glycoconjugate on the surface of *Leishmania* promastigotes, has been implicated as the ligand-mediating midgut attachment (Sacks and Kamhawi, 2001). LPG is a tripartite molecule, consisting of a phosphoglycan domain linked via a hexasaccharide glycan core to a 1-O-alkyl-2-lyso-phosphatidylinositol lipid anchor (Figure 1B). The phosphoglycan moieties of all LPG studied to date share a common backbone consisting of repeating disaccharide units of PO₄-6Gal(β1-4)Manα1, where the C-3 position of the Gal residue can either be unsubstituted (*L. donovani*, Sudan), partially substituted with glucose side chains (Indian *L. donovani*, *L. mexicana*, and *L. chagasi*), or completely substituted with side chain sugars that terminate primarily in galactose (*L. major*) or glucose and arabinose (*L. tropica*) (McConville et al., 1995; Thomas et al., 1992; Turco and Descoteaux, 1992). Figure 1B shows the polymorphic nature of LPG in representative parasite species. It is postulated that the composition, length, and extent of sugar residues branching off the disaccharide backbone repeats have developed in response to a selection pressure applied by the nature and diversity of sand fly midgut receptors. The involvement of LPG side chain sugars in the species-specific attachment of *Leishmania* parasites to their vectors has been most clearly demonstrated for *L. major* and *P. papatasi* (Pimenta et al., 1994). Oligosaccharide fragments corresponding to the terminal side chain Gal(β1-4) linked to the galactosyl residue of the PO₄-6Gal(β1-4)Man backbone unit, and the tetrasaccharide formed by side chain substitution of the backbone sequence with Gal(β1-3)Gal(β1-3), inhibited binding of procyclic *L. major* promastigotes to *P. papatasi* midguts in vitro (Pimenta et al., 1992). In addition, *L. major* mutants deficient in β1-3 galactosyltransferase activity and whose LPG lack galactose side chains lost their ability to attach to the midgut of *P. papatasi* (Butcher et al., 1996).

The nature of the LPG receptor in sand flies has been of obvious interest but remains largely undefined. Several publications have reported the presence of lectin-like molecules within crude midgut lysates that are able to agglutinate *Leishmania* parasites (Svobodova et al., 1996; Volf et al., 1998, 2002; Wallbanks et al., 1986). Dillon and Lane (1999) identified a microvillar protein from the midgut of *P. papatasi* that binds the LPG of *L. major*, though its final identity was not confirmed.

Here, we report the finding of a tandem repeat galectin in the midgut of the sand fly *P. papatasi* and demonstrate

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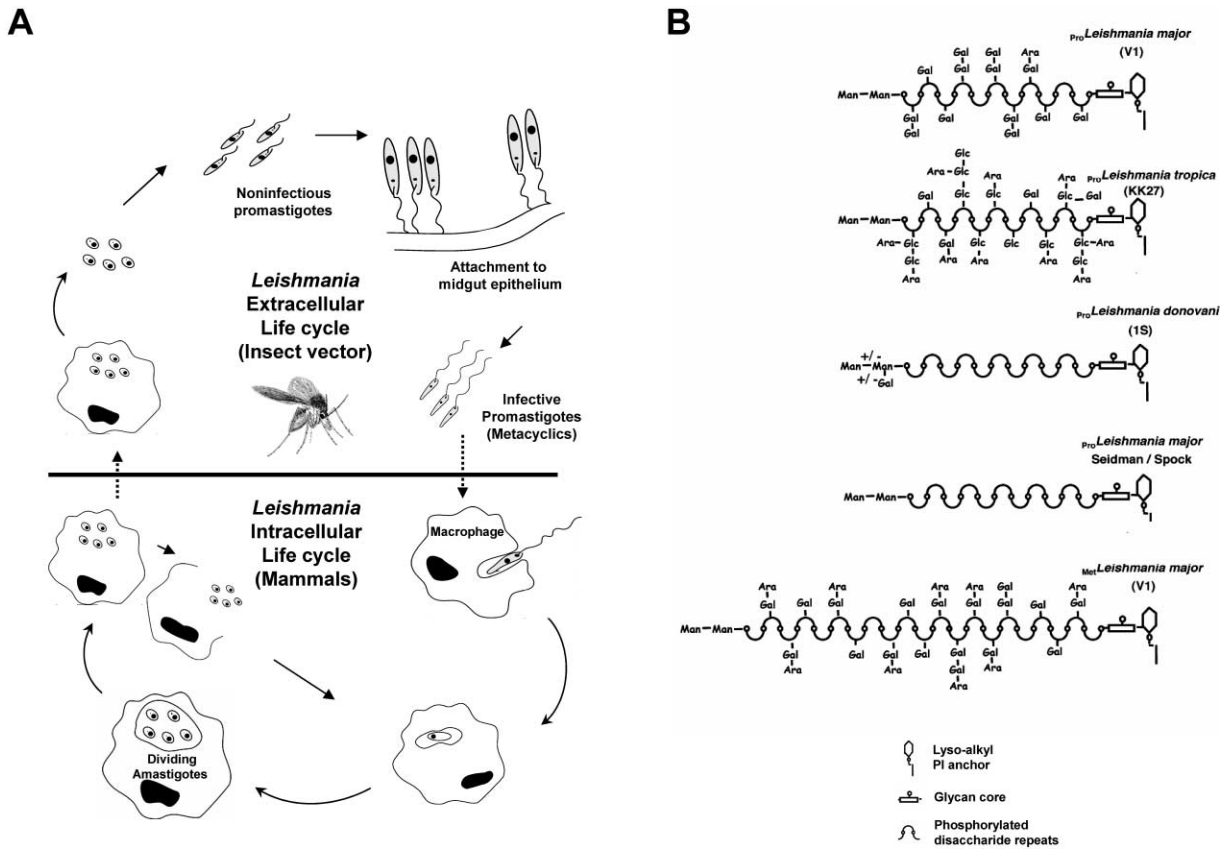


Figure 1. *Leishmania* Life Cycle and Representative LPG Structures of Several Parasite Species

(A) Macrophages containing amastigotes are ingested by sand flies during a bloodmeal. Amastigotes transform to promastigotes, which attach to the insect midgut epithelium. Bound promastigotes multiply and differentiate into free-swimming infective metacyclics available for transmission.

(B) Polymorphic structures of LPGs from Old World *Leishmania* species. Oligosaccharide core and lipid anchor domains are conserved between species. Developmental modification of LPG expressed by *L. major* metacyclic promastigotes is also shown.

its function as the receptor of *L. major* parasites. Galectins, a β -galactoside binding family of lectins, have gained wide recognition as multifunctional molecules important in development, homeostasis, and immune regulation (Hughes, 2001; Pace and Baum, 2004; Rabinovich et al., 2002; Yang and Liu, 2003; Young and Meeusen, 2004). Recently, a role for galectins in the establishment of parasitic diseases in mammalian hosts was demonstrated (Young and Meeusen, 2004). *L. major* LPG was shown to bind to galectin-3 and to galectin-9, promoting parasite interaction with its macrophage host cell (Pelletier et al., 2003; Pelletier and Sato, 2002). *Trypanosoma cruzi* was also shown to bind galectin-3, facilitating its mobility through the extracellular matrix (Moody et al., 2000). In insects, the number, nature, and function of galectins are less known (Pace and Baum, 2004). To our knowledge, only one tandem repeat galectin, involved in embryogenesis and host defense, has been fully characterized from *Drosophila melanogaster* (Pace et al., 2002). Other reports describe the presence of galactose-specific lectins that function in host defense (Dimopoulos et al., 1998; Mello et al., 1999), with one study suggesting a role for galactose-specific lectins in the attachment of *T. rangeli* to the salivary glands of its vector, *Rhodnius prolixus* (Basseri et al., 2002). The present study, on the interaction of the protozoan

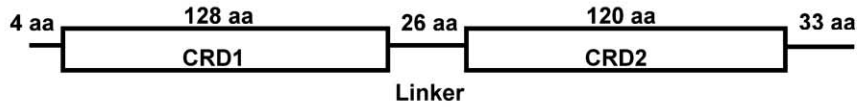
parasite *L. major* with its sand fly vector *P. papatasi*, provides evidence for the role of insect galectins in the establishment of parasites within disease vectors.

Results

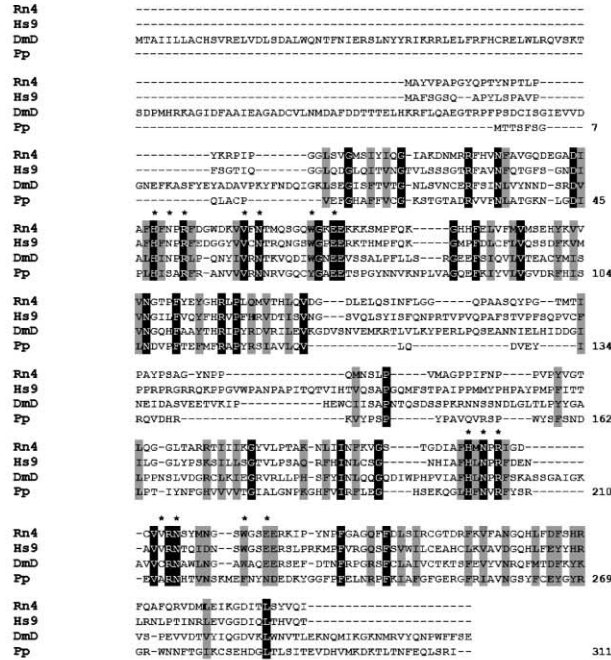
Identification of PpGalec as a Tandem Repeat Galectin

A nonamplified cDNA library from the midgut of female *P. papatasi* sand flies was plated, and 700 plaques were randomly picked and sequenced. The resulting sequences were clustered using BlastN with a cutoff of $10E^{-60}$ obtaining 380 unique clusters of related sequences. All sequences within each cluster were compared with the nonredundant (NR) protein database using the BlastX program (Altschul et al., 1997). Sequences were analyzed for signal secretory peptides and for transmembrane domains. A cDNA with high similarities to a galactose binding protein was identified and named PpGalec (GenBank accession number AY538600). This cDNA was relatively abundant in the midgut library of *P. papatasi*, representing cluster 19 (with four identical cDNA) of 380 clusters (total of 672 cDNA); only the first 24 clusters had more than three cDNA per cluster. PpGalec cDNA encodes a 311 amino acid protein with a predicted molecular weight of 35.4 kDa. This protein lacks a signal

A



B



C

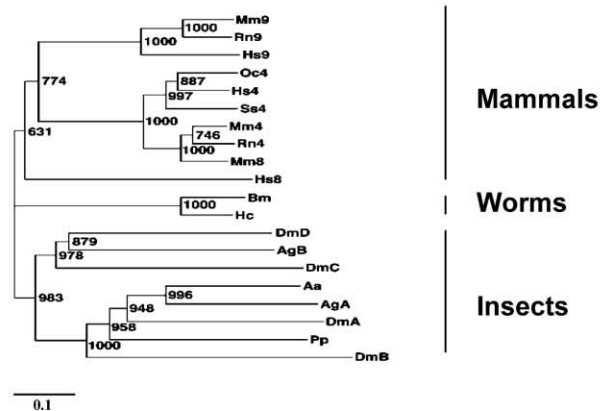


Figure 2. The Identification of PpGalec as a Tandem Repeat Galectin

(A) Schematic representation of the protein, showing the position of the two carbohydrate recognition domains (CRD) and the linker region. (B) ClustalW alignment of tandem repeat galectins of *Phlebotomus papatasi* (Pp) (AY538600, present study), *Rattus norvegicus* galectin-4 (Rn4) (NP037107), *Homo sapiens* galectin-9 (Hs9) (NP033665), and *Drosophila melanogaster* (DmD) (AF338142). Identical and similar amino acids are shaded in black and gray, respectively. Residues involved in β -galactoside binding are marked with an asterisk. (C) A phylogenetic tree comparing full-length PpGalec (AY538600) from *P. papatasi* (Pp) to selected tandem repeat galectins of different vertebrate and invertebrate species. Hs4, *H. sapiens* galectin-4 (AAH03661); Hs8, *H. sapiens* galectin-8 (NP_006490.1); Hs9, *H. sapiens* galectin-9 (NP_033665.1); Ss4, *Sus scrofa* galectin-4 (Q29058); Oc4, *Oryctolagus cuniculus* galectin-4 (AF091738); Rn4, *R. norvegicus* galectin-4 (NP_037107); Rn9, *R. norvegicus* galectin-9 (NP_037109.1); Mm4, *Mus musculus* galectin-4 (AAH21632); Mm8, *M. musculus* galectin-8 (AAH40243); Mm9, *M. musculus* galectin-9 (NP_034838.1); Hc, *Haemonchus contortus* (AAD11972); Bm, *Brugia malayi* (AF237486); DmA, *D. melanogaster* (AAL28440); DmB, *D. melanogaster* (NP_608553); DmC, *D. melanogaster* (CG11374); DmD, *D. melanogaster* (AF338142); AgA, *Anopheles gambiae* (XP_310776); AgB, *A. gambiae* (XP_319586); Aa, *Aedes aegypti* (EST-7403, TIGR database). Scale represents 0.1 nucleotide substitutions per site.

peptide and a transmembrane domain. A BLAST analysis of PpGalec amino acid sequence defined it as a tandem repeat galectin with two carbohydrate recognition domains (CRD) with affinity for β -galactose-bearing glycoconjugates, in which the CRDs occur within one molecule, separated by a linker region (Figure 2A). Both CRDs of PpGalec share consensus sequences with tandem repeat galectins from *D. melanogaster*, rat galectin-4, and human galectin-9 (Figure 2B). Most amino acids considered to be involved in galactose binding (Sujatha and Balaji, 2004) are highly conserved in PpGalec, including H₄₈, R₅₂, V₆₀, N₆₂, and E₇₃ for CRD1; and H₂₀₂, N₂₀₄, R₂₀₆, and N₂₁₅ for CDR2 (Figure 2B). There were, however, amino acid substitutions in galactose binding sites on the PpGalec protein. These divergences occurred on amino acids S₅₀ (from N₅₀) and Y₆₈ (from W₆₈) on the CRD1 domain and A₂₁₃ (from V₂₁₃), F₂₂₄ (from W₂₂₄), and N₂₂₇ (from E₂₂₇) on the CRD2 domain (Figure 2B). Analysis of the phylogenetic relationship between representative tandem repeat galectins shows that PpGalec separates into a distinct cluster, supported by a high bootstrap value, together with four other putative nonannotated insect tandem repeat galectins (Figure 2C).

Developmental Expression and Tissue

Specificity of PpGalec

PpGalec was expressed at low levels throughout the larval and pupal stages of development but was strongly upregulated in adult females (Figure 3A). Adult males showed a low level of gene expression. In adult females, the expression of PpGalec was shown to be constitutive, remaining constant up to 72 hr postbloodmeal (Figure 3B). Moreover, its expression appears to be specific to midgut tissues, as shown by its absence from the carcass (Figure 3B).

Restricted Distribution of PpGalec in Phlebotomine Sand Flies

A genomic DNA dot blot showed that PpGalec was present in *P. papatasi* and *P. duboscqi*, a sister species of *P. papatasi*, both belonging to the subgenus *Phlebotomus*. It was absent from two other species of the genus *Phlebotomus*: *P. sergenti* (subgenus *Paraphlebotomus*) and *P. argentipes* (subgenus *Euphlebotomus*). It was also absent from *Lu. longipalpis* and *Lu. verrucarum*, two species belonging to the New World genus *Lutzomyia* (Figure 3C). PpGalec was also present in PP9 (a *P. papatasi* cell line). It was absent from LL5 (a *Lu. longipalpis* cell line) (Figure 3C) and genomic DNA preparations of *D. melanogaster* and *A. gambiae* (data not shown). As a control, the same dot blot was stripped and rehybridized with a *Lu. longipalpis* S6 ribosomal protein cDNA probe (LLS6), which recognized the genomic DNA of all species tested (Figure 3D).

A mouse antiserum raised against purified recombinant PpGalec was found to specifically recognize a native protein of approximately 35 kDa in *P. papatasi* and *P. duboscqi* midguts but not in *P. sergenti*, *P. argentipes*, *P. perniciosus*, or *Lu. longipalpis* midguts (Figure 3E).

In Vitro Binding of Leishmania Species to Recombinant PpGalec

When viable log phase (procyclic) *L. major* "V1" promastigotes were incubated with recombinant PpGalec

(rPpGalec), strong agglutination was observed above 300 ng/ μ l of protein (data not shown). For flow cytometric analyses of binding, the subagglutination concentration of 300 ng/ μ l of rPpGalec was used. *L. major* V1 procyclics whose LPG bears typical side chains containing one or two β 1-3 linked galactose residues homogeneously bound His-tagged rPpGalec (Figure 4A) with a significant ($p < 0.05$) 4-fold increased binding over control (anti-His stained parasites not incubated with rPpGalec) (Figure 4B). As an additional negative control, *L. major* V1 did not bind a His-tagged recombinant 35 kDa salivary protein of *Lu. longipalpis* (data not shown). Promastigotes of two other *Leishmania* species, *L. donovani* "1S", whose LPG is unsubstituted, and *L. tropica* "KK27", which has an extensively branched LPG with side chains terminating mainly with glucose and arabinose, showed no significant binding of rPpGalec (Figure 4B). Moreover, *L. major* NIH/SD, a West African geographic isolate that naturally lacks galactose-containing side chains (Joshi et al., 1998), as well as "Spock," a mutant derived from *L. major* V1 and selected for absence of side chains (Butcher et al., 1996), both failed to bind rPpGalec (Figure 4B). *L. major* "LV39," which bears LPG side chains consisting of elongated galactose polymers, some of which are capped with arabinose (Dobson et al., 2003), also did not bind PpGalec above background. Importantly, *L. major* V1 metacyclics, which express twice the number of repeat units as procyclic LPG and which replace many of their side chain terminal sugars with arabinose (McConville et al., 1992; Sacks et al., 1990), lost their capacity to bind rPpGalec (Figure 4B). When parasites were stained with the monoclonal anti-LPG WIC79.3, which specifically recognizes the galactose-containing side chains of *L. major* LPG (Kelleher et al., 1994), we observed a correlation between anti-LPG staining of parasites and binding of rPpGalec (Figure 4C).

Distribution and Surface Expression of Native PpGalec in the Midgut of P. papatasi

Unfed *P. papatasi* midguts were opened and incubated either with preimmune serum or anti-PpGalec monospecific antibody. Compared to controls, anti-PpGalec brightly stained the entire insect midgut (Figure 5A). Anti-PpGalec did not stain *Lu. longipalpis* midguts (Figure 5B). Examination of luminal surface sections by confocal microscopy revealed that the expression of PpGalec varies among midgut cells (Figure 5C). To investigate the distribution of PpGalec across the midgut epithelium, multiple confocal sections were obtained and merged into four groups representing the luminal, middle, basal, and muscle planes (Figure 5C). *P. papatasi* midgut cells expressed PpGalec on their luminal surface (Figure 5C, lumen). These cells have regular geometrical shapes (usually pentagons or hexagonal), and their nuclei are centered and roughly equidistant from each other (Figure 5C, middle). A fine actin network can be observed in the basal plane (Figure 5C, basal) immediately above the muscle layer. In a lateral view, it is clear that PpGalec is expressed in the cytoplasm but accumulates along the luminal surface of the cell (Figure 5D).

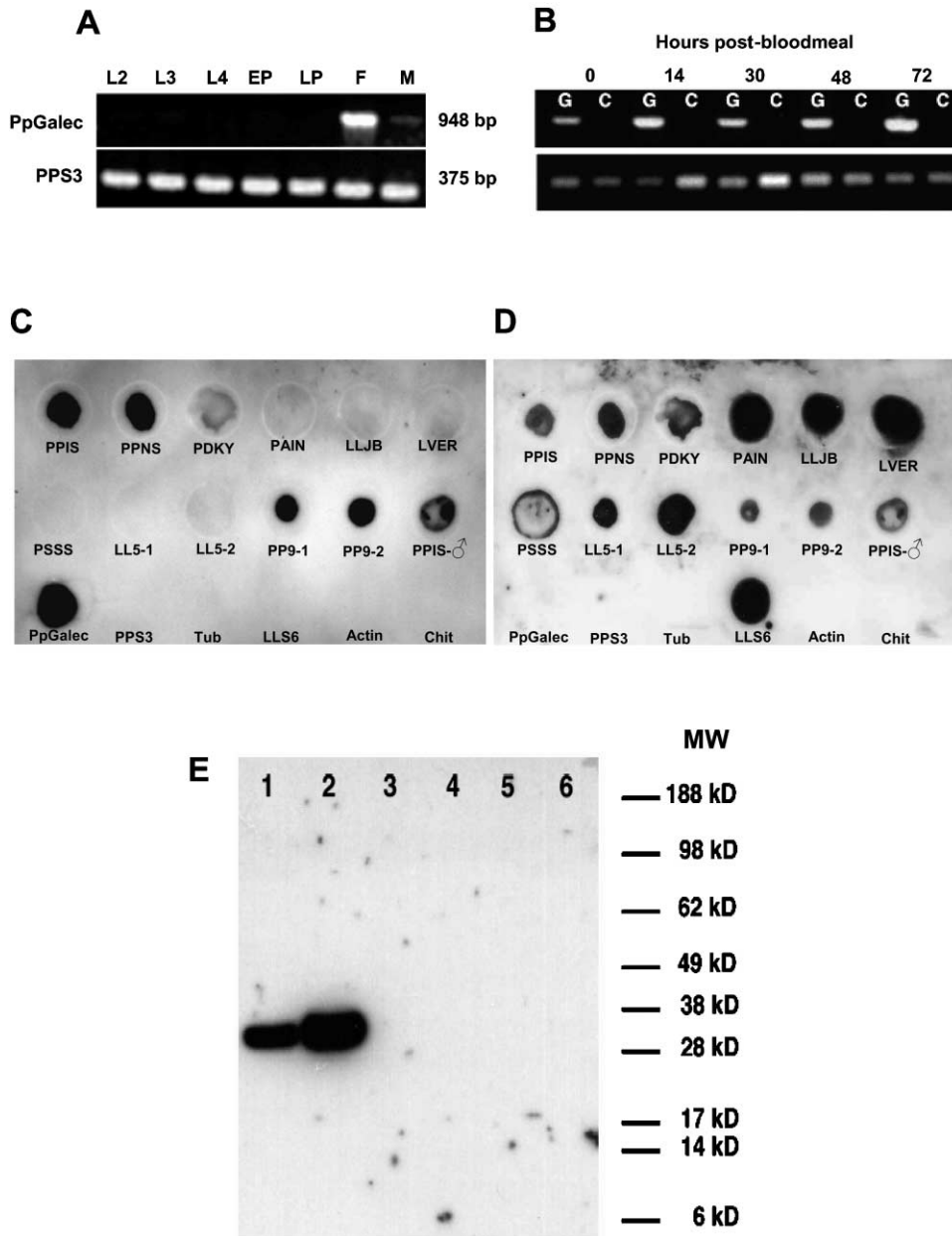


Figure 3. Developmental Expression and Tissue Specificity of *PpGalec* in *Phlebotomus papatasi*

(A) RT-PCR of larval stages (L2–L4), early pupae (EP), late pupae (LP), adult females (F), and adult males (M). RNA was isolated from 20 specimens of each stage. One of two representative experiments.

(B) Midguts of 5–6 females were dissected at 0, 14, 30, 48, and 72 hr post-blood feeding. RNA was isolated from both the midgut and carcasses for RT-PCR. G, gut; C, carcass. One of two representative experiments.

(C) Dot blot of genomic DNA isolated from various sand fly species and probed with DIG-labeled *PpGalec* cDNA: *P. papatasi* Israeli strain (PPIS), *P. papatasi* North Sinai strain (PPNS), *P. duboscqi* (PDKY), *P. sergenti* (PSSS), *P. argentipes* (PAIN), *Lutzomyia longipalpis* (LLJB), *Lu. Verrucarum* (LVER). DNA of sand fly cell lines LL5 and PP9 were also tested (1, 1 µg; 2, 2 µg DNA). As controls, ribosomal protein S3 (PPS3), tubulin (Tub), actin and midgut chitinase (Chit) from *P. papatasi*, and *Lu. longipalpis* ribosomal protein S6 (LLS6) were included. One of three representative experiments.

(D) Dot blot of genomic DNA in (C) stripped and reprobed with DIG-labeled control DNA (LLS6).

(E) Expression of *PpGalec* in various sand fly species: *P. papatasi* (1), *P. duboscqi* (2), *P. sergenti* (3), *P. argentipes* (4), *P. perniciosus* (5), and *Lu. longipalpis* (6). Equivalent of one midgut was loaded per well. One of three representative experiments.

Ex Vivo and In Vivo Inhibition of *L. major* Parasite Binding to *P. papatasi* Midgut by Anti-*PpGalec* Antibody

Anti-*PpGalec* antibodies were used to test the role of *PpGalec* in LPG and parasite binding to the midgut

ex vivo. Midguts that were preincubated with anti-*PpGalec* followed by incubation with procyclic *L. major* V1 PG (delipidated LPG) and detection with monoclonal anti-LPG showed an almost complete inhibition of PG binding (Figure 6A, panel 1). In contrast, midguts incu-

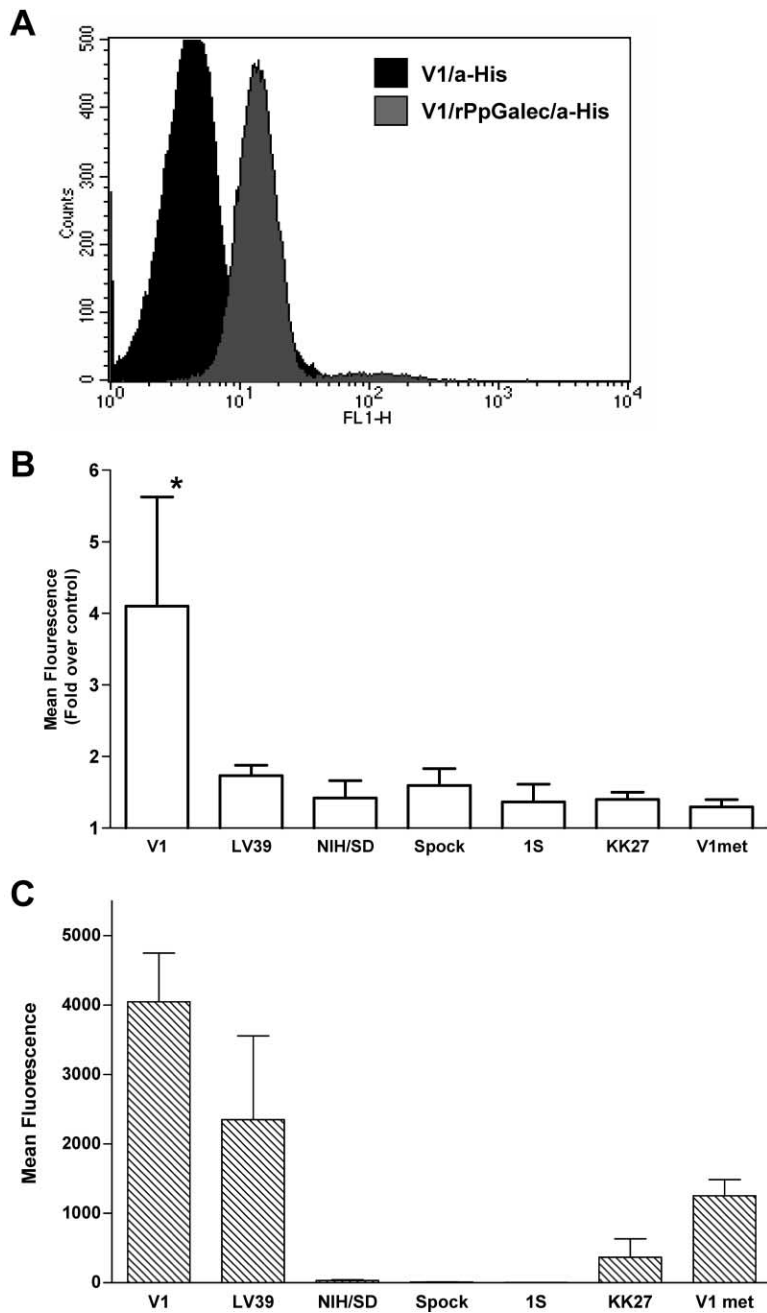


Figure 4. In Vitro Binding of *Leishmania* Species to His-Tagged Recombinant PpGalec

(A) Mean fluorescence of *L. major* (V1) incubated with FITC anti-His alone (black) or with recombinant PpGalec (rPpGalec) followed by FITC anti-His (gray).

(B) Mean fluorescence of various *Leishmania* strains bound to His-tagged rPpGalec shown as fold-over controls incubated with FITC anti-His alone. Strains used were the following: V1, *L. major* V1; LV39, *L. major* "LV39"; NIH/SD, *L. major* "Seidman"; 1S, *L. donovani* "1S"; KK27, *L. tropica* "KK27"; Spock, *L. major* "Spock"; V1 met, *L. major* metacyclics. On average, 50,000 cells were acquired for each sample. Mean of four experiments \pm SEM.

(C) Mean fluorescence of the parasites noted above stained with monoclonal anti-LPG WIC79.3, which specifically recognizes the galactose-containing side chains of *L. major* LPG.

bated with preimmune serum stained brightly with anti-LPG (Figure 6A, panel 2). Recognition of native PpGalec in the midgut of *P. papatasi* by anti-PpGalec is shown in panel 3 of Figure 6A. These results indicate that native PpGalec is available to LPG ligands in the lumen and suggest that it is an essential receptor for LPG-mediated binding of *L. major* promastigotes. This was confirmed by ex vivo binding studies using the parasites themselves, in which midguts preincubated with anti-PpGalec showed a 72% inhibition in the number of bound parasites ($p = 0.0006$) compared with midguts preincubated with pre-immune serum (Figure 6B).

A series of studies were conducted on *P. papatasi* fed on bloodmeals containing *L. major* amastigotes and

reconstituted with serum from PpGalec-immunized mice. At day 3 postinfection, prior to excretion of the digested bloodmeal, there was no significant difference in the number of parasites in sand flies fed on anti-PpGalec or preimmune serum (Figures 6C and 6D). At day 6 postinfection, however, when the bloodmeal was lost, there was an 86% decrease ($p = 0.001$) in the number of parasites retained in the midgut of sand flies fed on anti-PpGalec compared with controls, and infections appeared to be completely lost in half of the anti-PpGalec fed flies (Figure 6C). The inhibitory effect of anti-PpGalec on parasite survival was clearly timed with bloodmeal excretion, as indicated by the comparison of parasite loads in day 5-infected flies that had passed

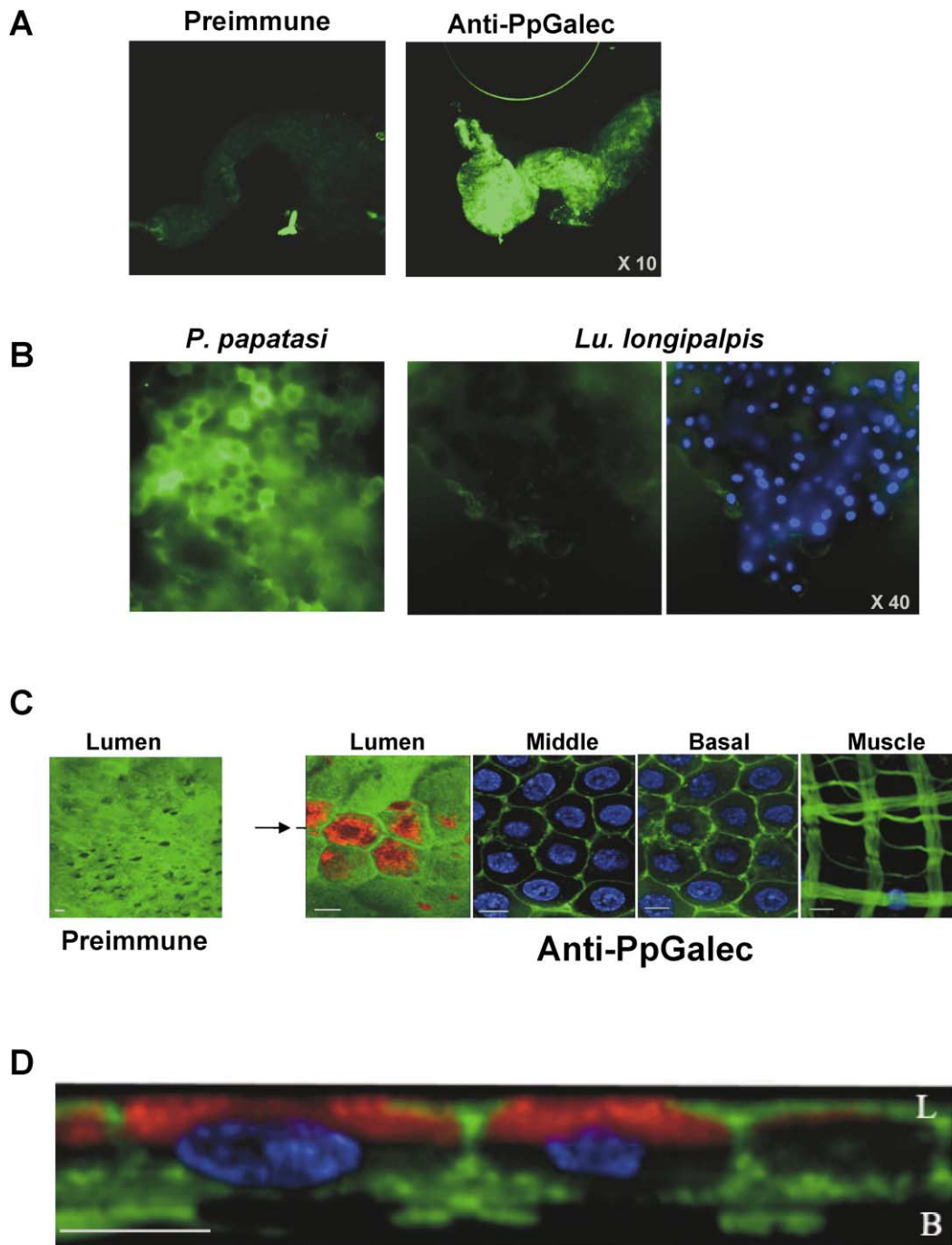


Figure 5. Distribution and Surface Expression of PpGalec on *Phlebotomus papatasi* Midgut

(A) Midguts incubated with preimmune serum or with anti-PpGalec. One of three representative experiments.

(B) Midguts of *P. papatasi* and *Lu. longipalpis* incubated with anti-PpGalec. A Dapi staining of *Lu. longipalpis* midgut is shown. One of three representative experiments.

(C) Midguts were incubated with either preimmune or anti-PpGalec sera and detected with Cy-3-conjugated anti-mouse (red). Alexa 488-conjugated phalloidin (green) and Dapi were used to stain actin and nuclei, respectively. Multiple confocal sections were obtained and merged into four groups, representing the lumen, middle, basal, and muscle planes of the midgut epithelium. Scale bars, 5 μ m. Arrow indicates the position of the horizontal section shown as a side view in the bottom panel.

(D) Side view of merged sections from the luminal surface (L) to the basal layer (B) of the midgut epithelium. Scale bar, 5 μ m.

or not yet passed their bloodmeal remnants (Figure 6D). The massive loss of infection at this early stage prevented the development of mature infections in the ante-

rior midgut, such that only three of 27 of the flies (11%) examined at day 14 harbored metacyclic promastigotes capable of initiating infection in the vertebrate host. By

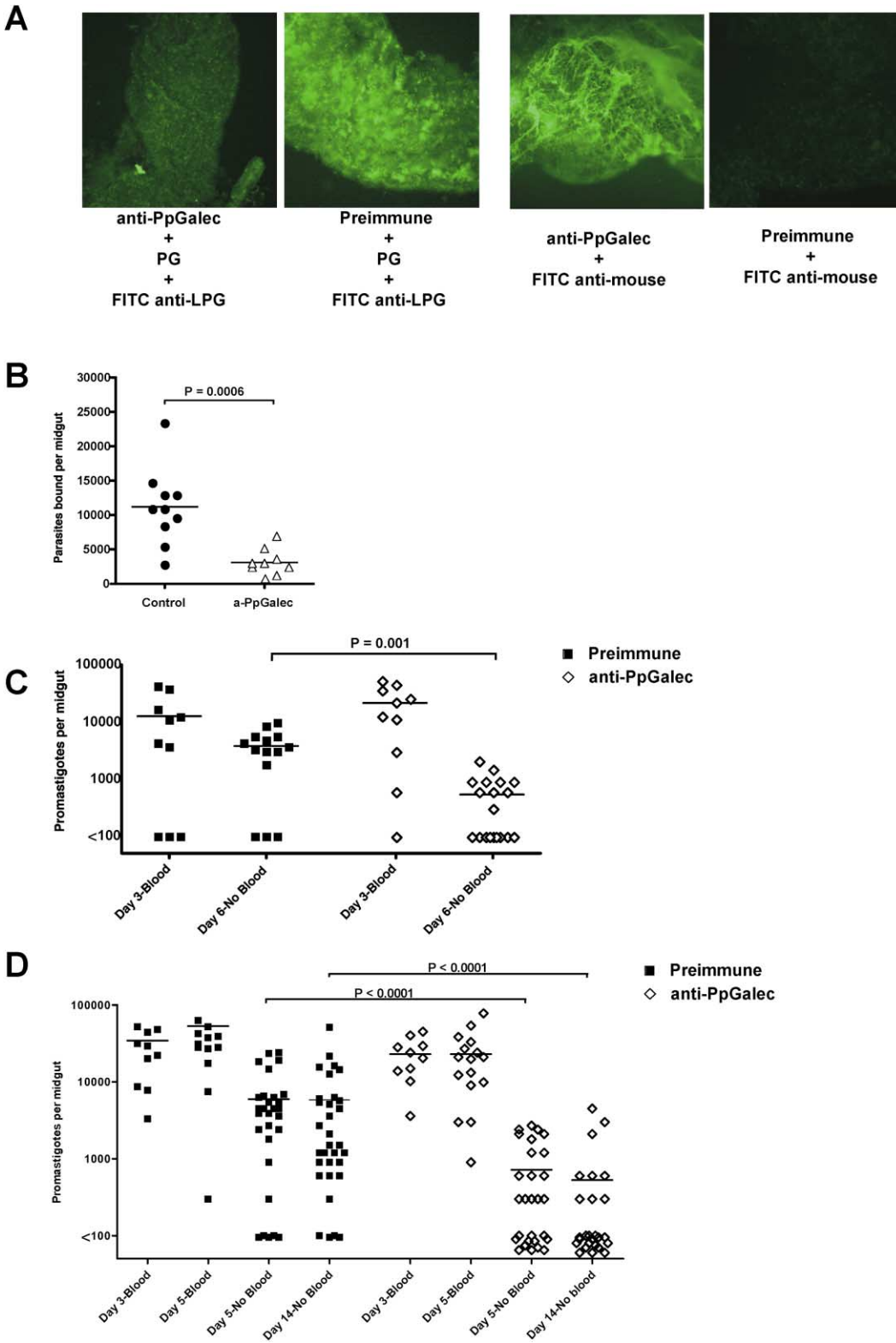


Figure 6. Effect of Blocking PpGalec on Parasite Binding and Survival

(A) Inhibition of *Leishmania major* procyclic PG binding by preincubation of midguts with anti-PpGalec. As controls, midguts were preincubated with either anti-PpGalec or preimmune serum and detected with FITC-anti-mouse antibody. One of two representative experiments.

(B) Ex vivo binding of *L. major* parasites to the midgut of *Phlebotomus papatasi* following preincubation with either preimmune (●) or anti-PpGalec (△) sera. One of three representative experiments.

(C) In vivo infection of *P. papatasi* with 3×10^6 *L. major* parasites/ml mixed with blood containing either preimmune (■) or anti-PpGalec (◇) sera. Parasite load was determined at day 3 and day 6 postinfection. One of two representative experiments.

(D) In vivo infection of *P. papatasi* with 3×10^6 *L. major* parasites/ml mixed with blood containing either preimmune (■) or anti-PpGalec (◇) sera. Parasite loads were determined at days 3, 5, and 14 postinfection. Data are pooled from two independent experiments.

contrast, 18 of 33 (55%) sand flies fed on preimmune serum developed metacyclics in numbers typical of mature, transmissible infections.

Discussion

Here, we report on the characterization of a galectin gene homolog from a cDNA library of the midgut of *P. papatasi* and demonstrate the function of the protein, named PpGalec, as a specific receptor for *L. major* procyclic LPG. The observed homology of PpGalec to galactose binding proteins, together with previous studies indicating that poly-Gal(β 1-3) side chains on the LPG of *L. major* are responsible for specific binding to *P. papatasi* midguts, suggested that PpGalec might be the midgut receptor for *L. major* in this sand fly species. This is supported by a number of lines of evidence presented in this report: (1) expression of PpGalec in *P. papatasi* is strongly upregulated in adult females (Figure 3A) and is restricted to midgut tissue (Figure 3B); (2) expression of PpGalec is restricted to *P. papatasi* and *P. duboscqi* (Figures 3C and 3E), sister species belonging to the subgenus *Phlebotomus*, each of which transmits *L. major* in nature; (3) the binding specificity of rPpGalec is restricted to *Leishmania* promastigotes bearing poly-Gal(β 1-3) side chains on their LPG (Figure 4B); (4) PpGalec is distributed throughout the abdominal and thoracic midgut and localized on the luminal surface of midgut cells (Figure 5); (5) antibodies against PpGalec inhibited *ex vivo* midgut binding of *L. major* PG and parasites (Figures 6A and 6B); and (6) PpGalec antibodies fed to *P. papatasi* severely impaired parasite development and survival in the insect midgut (Figures 6C and 6D).

PpGalec is a 35.4 kDa tandem repeat galectin with two nonidentical carbohydrate recognition domains (CRD) separated by a linker region (Figure 2A) (Bianchet et al., 2000; Cooper, 2002). Galectins, a widely distributed family of lectins reported from fungi to mammals, are implicated in cell-cell and cell-matrix interactions, growth and differentiation, migration, and apoptosis and immunity (Hughes, 2001; Pace and Baum, 2004; Rabinovich et al., 2002; Yang and Liu, 2003; Young and Meeusen, 2004). They share evolutionarily conserved sequences in CRD that have a cation-independent affinity to β -galactosides (Cooper, 2002; Hughes, 2001; Rabinovich et al., 2002; Yang and Liu, 2003) and typically bind to type I Gal β 1,3GlcNAc or type II Gal β 1,4GlcNAc units (Hughes, 2001; Rabinovich et al., 2002). Both CRDs of PpGalec are homologs to CRDs of other galectins; however, five amino acids involved in galactose binding (Sujatha and Balaji, 2004) exhibit some divergence (Figure 2B), possibly a reflection of the binding specificity of the molecule (Arata et al., 2001; Bianchet et al., 2000). Phylogenetic analysis (Figure 2C) suggests that PpGalec, together with putative nonannotated galectins from *D. melanogaster*, *Aedes aegypti*, and *An. gambiae*, may represent a novel family of galectins.

The abundant expression of PpGalec in adult female midgut tissue (Figures 3A and 3B) fulfills basic requirements of a receptor for *L. major*. Moreover, among the seven vector species examined, its expression is restricted to the known sand fly vectors for *L. major*, *P. papatasi*, and *P. duboscqi* (Figures 3C and 3E).

Binding of rPpGalec was restricted to *L. major* V1 bearing poly-Gal (β 1-3) side chains on its LPG (Figures 4A and 4B), emulating the species-restricted vector competence of *P. papatasi* to *L. major* infections. The absence of binding of rPpGalec to *L. major* strains NIH/SD and "Spock," lacking poly-Gal (β 1-3) side chains, reinforces the role of LPG-mediated binding to PpGalec in parasite survival, as these strains failed to maintain infection in *P. papatasi* (Joshi et al., 1998; Butcher et al., 1996).

Another strain of *L. major* "LV39" did not bind PpGalec above background despite its polygalactosylation. This may be explained by the fact that the side chains of LV39 LPG are not typical of *L. major* but contain β 1-3-linked galactose residues up to eight units in length, some of which are arabinose capped (Dobson et al., 2003). More critically, "LV39" promastigotes survive poorly in *P. papatasi* compared with V1 promastigotes, and a massive initial inoculum is required to establish post-bloodmeal infection in these flies (Sacks et al., 2000). It is possible that this structural variant arose spontaneously as a result of serial culture in the absence of sand fly selection, or else it reflects the structures of the natural isolates, transmitted by *P. papatasi* bearing a variant of PpGalec that can better accommodate their particular polygalactose epitopes, or expressing a coreceptor for parasite attachment.

It is pertinent to note that metacyclic promastigotes of *L. major* did not bind rPpGalec (Figure 4B). This is entirely consistent with the fact that the downregulation of polygalactose epitopes during metacyclogenesis results in the loss of LPG-mediated parasite binding to the midgut. This structural modification is thought to be crucial to the development of transmissible infections, since free-swimming infective metacyclics are necessary for successful transmission from the sand fly to the mammalian host (Pimenta et al., 1992; Sacks and Kamhawi, 2001).

A prerequisite to the function of PpGalec as a midgut receptor of *L. major* is its abundance and surface expression on epithelial cells. Figure 5 shows the abundant distribution of PpGalec throughout the midgut of *P. papatasi*. In addition, analysis of midgut sections using confocal microscopy showed that PpGalec is present on the luminal surface of epithelial midgut cells (Figures 5C and 5D). This is consistent with the cell surface localization of other galectins, which, despite the absence of a signal peptide, are known to be secreted by nonclassical pathways to the cell surface, where they bind to appropriately glycosylated surface molecules (Cooper, 2002; Hughes, 2001; Rabinovich et al., 2002). Dillon and Lane (1999) reported the specific binding of *L. major* LPG to a microvillar peptide in the gut of *P. papatasi* and suggested aminopeptidase as a prime candidate for receptor. Danielsen and van Deurs (1997) demonstrated that galectin-4, a tandem repeat galectin lacking a signal peptide, is externalized via nonclassical pathways, where it directly associates and coimmunoprecipitates with aminopeptidase N, a transmembrane brush border enzyme in the small intestine of the pig. We propose that the digestive enzyme reported by Dillon and Lane (1999) may have been coprecipitated with PpGalec, and the latter was in fact responsible for the observed specific binding to *L. major* LPG.

Because anti-PpGalec recognized and could block the native protein on the luminal surface of midgut cells, the role of PpGalec in parasite attachment could be directly tested. Preincubation of *P. papatasi* midguts with anti-PpGalec resulted in a significant decrease in both *L. major* PG and promastigote binding (Figures 6A and 6B). This is strong evidence that PpGalec provides an essential recognition site for parasite attachment. Its potential as a transmission-blocking vaccine was confirmed in studies showing that flies fed on infective bloodmeals reconstituted with sera from PpGalec-immunized mice lost such a substantial number of parasites during bloodmeal excretion (Figure 6D) that they failed to recover mature, transmissible infections.

Identifying and targeting midgut molecules essential for parasite survival represents a novel strategy for development of transmission-blocking vaccines against vector-borne diseases.

Experimental Procedures

Phlebotomus papatasi cDNA Library Construction

A midgut library of *P. papatasi* was constructed from ten adult females using the Micro Fast Track mRNA isolation kit (Invitrogen) and the SMART cDNA library construction kit (BD Biosciences/Clontech) as described previously (Valenzuela et al., 2002a). Detailed description of the bioinformatic treatment of the data can be found elsewhere (Valenzuela et al., 2002b). Briefly, primer and vector sequences were removed from raw sequences, compared against the GenBank nonredundant (NR) protein database using the standalone BlastX program found in the executable package at (<ftp://ftp.ncbi.nlm.nih.gov/blast/executables/>) (Altschul et al., 1997), and searched against the Conserved Domains Database (CDD) (<ftp://ftp.ncbi.nlm.nih.gov/pub/mmdb/cdd/>), which includes all Pfam (Bateman et al., 2000) and SMART (Schultz et al., 1998, 2000) protein domains. Sequences were clustered using the BlastN program (Altschul et al., 1990) as detailed before (Valenzuela et al., 2002b). Cluster designations were assigned in order, cluster 1 having the largest number of representative sequences. Sequences were submitted to the SignalP server (<http://www.cbs.dtu.dk/services/SignalP-2.0/>) for verification of secretory signal peptide and to the TMHMM Server v. 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>) to search for transmembrane domains (Krogh et al. 2001).

Sequence Analysis of PpGalec

A BLAST search of PpGalec-predicted amino acid sequence was carried out against several databases including the National Center for Biotechnology Information (NCBI) databases, the Berkeley *Drosophila* Genome Project, and the Sanger Center *Anopheles* genome database. Sequence alignments and phylogenetic tree analysis used the ClustalW package (Thompson et al., 1997). Phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei, 1987). Bootstrapping of phylogenetic trees, corrected for multiple substitutions and excluding positions with gaps, was done with the Clustal package for 1000 trials. Phylogenetic trees were formatted with TreeView (Page, 1996) using the ClustalW output.

Expression of Recombinant PpGalec

PpGalec full-length cDNA was PCR amplified using the forward and reverse primers 5'-ATGACTACCTCATTCTCTGGTCAA-3' and 5'-CAGCGTGTGATATCAGATACGAGA-3', respectively. The PCR product was cloned into pCRT7NT TOPO expression vector (Invitrogen) according to manufacturer's instructions. rPpGalec was expressed as a His-tagged protein using the RTS proteomaster system following manufacturer's instructions (Roche Laboratories).

Purification of Recombinant PpGalec

Expression reaction (1 ml) was mixed with 1 ml of 50 mM Tris (pH 8.0), 20 mM EDTA, and Complete Protease Inhibitor Mix (Roche Laboratories) (TE 50/20/PI). The sample was centrifuged at 20,000 ×

g for 1 hr. The pellet was washed several times over 2 days in TE50/20/PI. The pellet was solubilized in 6 M guanidine-HCl, 100 mM Tris-HCl (pH 8.0), and 2 mM EDTA; incubated for 1 hr at RT; and centrifuged at 20,000 rpm for 1 hr at 4°C. DTT (10 mg/ml) was added to the supernatant and incubated at RT for 2 hr. The solution was added to a refolding buffer (0.1 M Tris-HCl [pH 9.0], 0.9 mM GSSG, 2 mM EDTA, 0.5 M NaCl, and 200 mM lactose, protease inhibitor mix) and incubated for 48–66 hr at 4°C. The recombinant protein was purified by reverse phase HPLC using a 40 min gradient of 10%–80% acetonitrile with 0.1% trifluoroacetic acid, at 2 ml/min using a 1 × 25 cm octadecyl silica column (Model 218TP510 from Vydac). Eluent was collected at 1 min intervals. Fractions were spotted on a PVDF membrane, and positive anti-His fractions were pooled. An aliquot of the positive anti-His fractions was dried and run in a SDS-PAGE 4%–20% to confirm the molecular weight and purity of recombinant protein. The rest of the sample was lyophilized in batches of 250 µg in the presence of 40 µg of bovine serum albumin.

Leishmania Parasites

Parasites were grown as previously described (Kamhawi et al., 2000). Procytic promastigotes were harvested from 1–2 day logarithmic phase cultures. The following strains of *Leishmania* were used in this study: the NIH Friedlin strain of *L. major*, clone V1 (NIH/V1) (MHOM/IL/80/FN); *L. donovani* (MHOM/SD/00/1S-2D); the West African NIH Seidman (NIH/SD) strain of *L. major* (MHOM/SN/74/Seidman); *L. tropica* (MHOM/AF/88/KK27); and *L. major* "Spock" mutant (Butcher et al., 1996). *L. major* (V1) metacyclics were purified by negative selection with 50 µg/ml peanut agglutinin from 5–6 day stationary cultures.

Insects

Sand fly colonies were reared at Walter Reed Army Institute of Medical Research and at The National Institutes of Health. The following sand flies species were used in this study: *Phlebotomus papatasi* from colonies originating from Israel (PPIS) and North Sinai, Egypt (PPNS); *P. duboscqi* from Kenya (PDKY); *P. sergenti* from South Sinai, Egypt (PSSS); *P. argentipes* from India (PAIN); *P. perniciosus* from Italy; *Lutzomyia longipalpis* from Brazil (LLJB); and *Lu. verrucarum* from Peru (LVER). *Anopheles gambiae* were reared in the Laboratory of Malaria and Vector Research at NIH. *Drosophila melanogaster* flies were kindly provided by Dr. Denise Montell, Johns Hopkins School of Medicine.

Sand Fly Cell Lines

Embryonic cell lines developed from eggs of *P. papatasi* (PP9) and *L. longipalpis* (LL5) (Tesh and Modi, 1983) were grown at 26°C in Mitsuhashi-Marmorosch medium (MM) supplemented with 20% heat-inactivated fetal bovine serum, 100 U/ml of penicillin, 100 µg/ml streptomycin, 200 mM L-glutamine, and 2 mM myo-inositol.

RT-PCR

RT-PCR was performed on total RNA extracted from pools of 20 L2, L3, and L4 larval stages, early pupae, late pupae, adult females and males (less than 5 hr old) using RNA STAT-60 solution (Tel-Test Inc.). For tissue specificity and response to blood feeding, total RNA was extracted from carcasses and midguts of six adult females at 0, 14, 30, 48, and 72 hr post-bloodmeal using the RNeasy Mini Kit (Qiagen). For RT-PCR, 0.5 µg of total RNA and 0.6 µM of PpGalec forward and reverse primers were added to a ready-to-use RT-PCR bead (Amersham Pharmacia Biotech). The reaction mixture was incubated at 42°C for 30 min and 95°C for 5 min. PCR conditions were 25 cycles of 94°C for 45 s, 50°C for 2 min, 72°C for 2 min, followed by 10 min extension at 72°C. PCR products were visualized in 1.2% ethidium bromide-stained agarose gels.

Genomic DNA Dot Blot

Genomic DNA was isolated from PP9 and LL5 cell lines, from 2- to 5-day-old female sand flies of various species, and from *D. melanogaster* and *A. gambiae* using the Cells and Tissue DNA Isolation Kit (Amersham Pharmacia Biotech). DNA was resuspended into 10 µl hydration buffer and sonicated. The DNA concentration was determined spectrophotometrically, and 1–2 µg of DNA was blotted onto

a nylon membrane. A PCR product of PpGalec was used as positive control. Negative controls used were cDNA of ribosomal protein 3 (PPS3), tubulin, actin, and chitinase from *P. papatasi* as well as cDNA of ribosomal protein S6 from *Lu. longipalpis* (LLS6). DNA was denatured in 1.5 M NaCl/0.5N NaOH, neutralized in 0.5 M Tris/1.5 M NaCl, and crosslinked to the blots. Hybridization was performed overnight at 50°C with a digoxigenin-labeled PpGalec cDNA probe and detected by chemiluminescence according to manufacturer's protocols (Roche Molecular Biochemicals). For control reactions, the blot was stripped and hybridized to a digoxigenin-labeled LLS6 cDNA probe from *Lu. longipalpis*.

Mouse Anti-PpGalec Antibodies

Polyclonal anti-PpGalec antibodies were prepared by repeated immunization of five BALB/c mice with purified rPpGalec protein (Spring Valley Laboratories Inc.). Preimmune sera were used as controls.

Western Blots

The midguts of two 5-day-old female sand flies from each species were dissected and placed in 30 μ l PBS in groups of two. One gut equivalent of homogenates was separated on 4%–12% NuPAGE Bis-Tris gels using MES SDS running buffer (Invitrogen). Proteins were transferred onto a nitrocellulose membrane for 1 hr at 30V and blocked in 5% milk/0.1% TBS overnight at 4°C. Blots were incubated with polyclonal anti-PpGalec (1:200) for 2 hr at RT followed by incubation with anti-mouse IgG-HRP (1:5000) (Santa Cruz Biotechnology) for 40 min at RT. The blots were detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce) and exposed to BioMax ML film (Eastman-Kodak).

Immunostaining and Flow Cytometry

Lyophilized His-tagged rPpGalec was resuspended in PBS to a concentration of 300 μ g/ml. Round-bottom 96-well plates (Corning) were blocked with 1% BSA, and 500,000 *Leishmania* promastigotes of various species were added to each well in triplicate and centrifuged at 3500 rpm for 10 min. One set of wells was incubated with 50 μ l of PpGalec, the other two with PBS containing 0.1% bovine serum albumin (PBS/BSA), overnight at 4°C. The plates were maintained at 4°C during two washes with PBS/BSA and until they were fixed with 50 μ l of 4% paraformaldehyde for 15 min/RT. The parasite-PpGalec complexes and one of the two sets of wells incubated with PBS/BSA were stained with anti-His (C-term)-FITC antibody (Invitrogen) (1:500). The final set of wells was stained with Alexa Fluor 488 (BD Biosciences)-conjugated anti-LPG WIC79.3 (1:400). Both antibodies were incubated with parasites for one hr/RT, washed three times with PBS/BSA, and resuspended in 150 μ l PBS/BSA for FACS analysis. On average, 50,000 cells were acquired for each sample, and the data were collected and analyzed using CellQuest software and a FACSCalibur flow cytometer (Becton Dickinson).

Ex Vivo Staining of Sand Fly Midguts with Anti-PpGalec

Two- to five-day-old female sand flies, maintained on 50% sucrose, were dissected in PBS. For each fly, the crop, malpighian tubules, and hindgut were removed, leaving only the midgut. The midgut was opened along its dorsal surface to expose the inner side. The opened midguts were placed in the concave well of a microscope chamber slide in groups of eight to ten, fixed in 2% paraformaldehyde for 20 min at 4°C, washed twice in PBS, and blocked with 1% BSA for 1 hr at RT. The guts were incubated with anti-PpGalec or preimmune serum (1:20) for 1 hr at RT, washed four times in PBS, and incubated with FITC goat anti-mouse IgG (BD Pharmingen) (1:300) for 40 min at RT. After washing in PBS, the guts were mounted onto slides using VectaShield with Dapi (Vector Laboratories) and viewed under a fluorescent microscope.

Study of *P. papatasi* Midgut by Confocal Microscopy

P. papatasi midguts were dissected in PBS at 24–48 hr post-blood-meal, fixed in 4% paraformaldehyde for 1 min at RT, and placed on ice-cold PBS. Midguts were opened longitudinally to remove the bloodmeal, fixed for an additional 60 min, and blocked in PBT solution (1% BSA, 0.1% Triton X-100 in PBS [pH 7.2]) for 2 hr at RT.

Samples were incubated with either anti-PpGalec or preimmune serum (1:300) overnight at 4°C, followed by 4 hr at RT with Cy3-anti-mouse-conjugated antibody (1:500) (Amersham). Actin was stained by incubating the samples for 20 min at RT with 6.6 μ M Alexa488-conjugated phalloidin in methanol (Molecular Probes, Eugene, OR) diluted 1:40 in 1% BSA/PBS. Midguts were washed and mounted in Vectashield (Vector Laboratories, Inc.) containing Dapi. Final images were obtained using the confocal microscope DM IRE2 from Leica Microsystems, Inc. (Exton, PA).

Inhibition of *L. major* PG Binding by Anti-PpGalec

P. papatasi midguts were opened and fixed in 2% paraformaldehyde for 20 min at 4°C, washed twice in PBS, and incubated with anti-PpGalec or preimmune (1:20) for 1 hr at RT. After washing with PBS, the guts were incubated with 50 μ g/ml *L. major* phosphoglycan (PG) (Orlandi and Turco, 1987; Pimenta et al., 1992) for 1 hr at RT. After washing, the guts were stained with Alexa Fluor 488 (BD Biosciences)-conjugated anti-LPG WIC79.3 (1:100) for 40 min at RT. The guts were washed another five times in PBS and mounted on slides for fluorescence microscopy.

Inhibition of Parasite Binding Ex Vivo by Anti-PpGalec

P. papatasi midguts were opened and fixed as mentioned above and incubated with either anti-PpGalec or preimmune sera (1:10) for 1 hr at RT. The guts were washed three times with PBS and incubated with 2×10^6 parasites for 1 hr at RT. Individual midguts were washed four times in PBS and placed in 30 μ l of 1% BSA/PBS. Following homogenization, parasites were counted using a hemocytometer.

Inhibition of Parasite Binding In Vivo by Anti-PpGalec

P. papatasi females were membrane fed on a bloodmeal containing 3×10^6 amastigotes/ml. The blood consisted of red blood cells reconstituted with anti-PpGalec or preimmune sera. At each time point, ten to 15 specimens were examined for their bloodmeal status and dissected in PBS. Individual midguts were placed in 30 μ l of 1% BSA/PBS and homogenized. The number of promastigotes per midgut was counted using a hemocytometer.

Statistical Analysis

The Kruskal-Wallis test was used to analyze in vitro binding of *Leishmania* species to rPpGalec. Two-tailed Mann-Whitney test was used to compare binding and in vivo survival of parasites. Values were considered significant at the 95% confidence interval.

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Accession Numbers

The GenBank accession numbers for the PpGalec (Pp), *Brugia malayi* (Bm), *Drosophila melanogaster* (DmA), *Anopheles gambiae* (AgA), and *A. gambiae* (AgB) sequences reported in this paper are AY538600, AF237486, AAL28440, XP_310776, and XP_319586, respectively. The name given to the *Aedes aegypti* sequence reported in this paper according to the TIGR database is EST-7403.