Detection of *Leishmania* lipophosphoglycan binding proteins in the gut of the sandfly vector

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SUMMARY

Binding to the midgut microvillar surface in the sandfly *Phlebotomus papatasi* is a prerequisite for successful development of *Leishmania major* within the gut of the vector. This paper describes a method for detecting microvillar-associated proteins which act as ligands for the parasite surface glycoconjugate lipophosphoglycan (LPG). Adhesion of LPG to midgut proteins was visualized by probing midgut extracts with LPG using a Western ligand blotting technique. Procyclic *L. major* LPG bound to a microvillar polypeptide band of 65 kDa (estimated in the non-reduced state) and bound variably to several lower molecular weight bands, probably degradation products or subunits of the primary binding polypeptides. Specificity of binding was confirmed by co-incubating biotinylated LPG with an LPG-specific mAb which resulted in a great reduction in binding.

Key words: *Leishmania major*, lipophosphoglycan, *Phlebotomus papatasi*, microvilli, midgut.

INTRODUCTION

Lipophosphoglycan (LPG) is the major surface molecule found on *Leishmania* promastigotes during their development in the gut of the sandfly vector. The LPG consists of a series of phosphorylated saccharide repeats linked to a lipid anchor via a carbohydrate core (Turco & Descoteaux, 1992). The molecule undergoes structural changes in the saccharide repeats associated with development from the log phase (procyclic) to the metacyclic, mammalian infective form, of the promastigote (McConville *et al*. 1992). Both forms of LPG are expressed during development of the *Leishmania major* promastigote in the natural vector, *Phlebotomus papatasi* (Davies et al. 1990; Lang *et al*. 1991).

LPG is critical to the parasite's survival; strains of parasite lacking the intact surface coat fail to develop beyond the stage of bloodmeal digestion at which meal remnants are excreted (Pimenta *et al*. 1994). Two potential roles have been described for the LPG coat; protection against the hydrolytic proteases associated with bloodmeal digestion (Borovsky & Schlein, 1987) and binding of the parasite to the midgut wall thus preventing ejection from the gut with the digested bloodmeal (Sacks *et al*. 1994).

L. major infections do modulate sandfly protease activities (Borovsky & Schlein, 1987; Dillon & Lane, 1993*a*) but there is no evidence of direct inhibition of protease by LPG and an LPG-defective strain remained viable in the presence of purified sandfly protease (Dillon & Lane, unpublished observations). This does not, however, discount the possibility that LPG is indirectly inhibiting proteases or other hydrolases.

In vitro, attachment of the logarithmic, procyclic *L. major* promastigote to the midgut of *P. papatasi* is mediated via the terminally exposed galactose residues of the LPG (Pimenta *et al*. 1992). Thus parasites escaping from the digested bloodmeal, as the peritrophic matrix breaks down, attach to the microvillar surface of the midgut. Nothing is known of these binding sites. Subsequently the terminal saccharide residues are modified during metacyclogenesis so that the metacyclic form of the LPG binds less causing the parasite to be released from the surface and enabling its migration to the anterior part of the gut prior to transmission to the mammalian host. Interspecific differences in the terminal saccharides of the LPG could account, at least in part, for the specificity *Leishmania* species show to different sandfly species (Pimenta *et al*. 1994; Sacks *et al*. 1995; Butcher *et al*. 1996).

The purpose of this study was to detect adhesion molecules in the sandfly midgut to which parasites attach via LPG. Previous electron microscope studies revealed that the promastigotes bind to the microvillar membrane via the flagella-associated LPG (Lang *et al*. 1991; Saraiva *et al*. 1995). Therefore, instead of using crude midgut extracts, midgut microvillar (MV) proteins were selectively enriched (compared to other membrane and cyto-

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plasmic proteins) for use in Western ligand blots using calcium ion precipitation and differential centrifugation (Ferreira & Terra, 1980). Binding was visualized by probing the blots with LPG.

MATERIALS AND METHODS

Enrichment of microvillar proteins

Initial extracts were prepared (Ferreira & Terra, 1980; Houk, Arcus & Hardy, 1986) using 50 midguts from 3 to 4-day-old female *P. papatasi* (colony originated from the Jordan Valley, Israel and reared according to the method described by Dillon & Lane, 1993*a*). The midguts were ground in an ice-cold 200 μl vol. glass homogenizer with isolation buffer (300 mM mannitol, 100 mM Tris–HCl, pH 7·2, 1 mM phenylmethanesulfonyl fluoride (PMSF)) and made up to a final volume of 500 μl of isolation buffer with 10 mM $CaCl_2$. After incubation on ice for 15 min the suspension was centrifuged at 6000 ***g*** for 15 min at 4 °C. The pellet was resuspended in fresh isolation buffer with $CaCl_2$ and treated as above. The 2 supernatants were combined and centrifuged for 30 min at 19000 ***g***, the resulting pellet was used as the MV extract.

Aminopeptidase activities and protein determinations were performed as previously described (Dillon & Lane, 1993*a*).

Preparation of LPG

Leishmania major LV39 (MRHO/SU/59/P-strain, originally from *Rhombomys opimus*, Uzbekistan; Scott & Sher, 1986) was cultured from stocks of amastigotes stored in liquid nitrogen. Log phase promastigote cultures (4×10^6/ml) were grown at 26 °C using α-MEM medium supplemented with foetal calf serum (Kar *et al*. 1990). The LPG was purified from log phase promastigotes using octyl-Sepharose (Pharmacia Biotech, Herts, UK) (McConville *et al*. 1987). Fractions containing LPG were determined by probing dot blots using the mAb CA7AE (CedarLane Labs, Ontario, Canada) which recognizes *L. major* LPG (Tolson *et al*. 1989). The quantity of LPG (stored at −70 °C) is given as the dry weight of material obtained after freeze drying the appropriate fractions.

LPG was prepared for biotinylation by incubation in ice with 10 mM sodium periodate in acetate buffer, pH 5·5. The solution was ultrafiltered at 4 °C using a 10 kDa cut-off filter (Centricon 10, Amicon, UK), retentate was resuspended in buffer and refiltered 3 times. Biotin hydrazide (Pierce, UK) was mixed with the retentate to a final dilution of 4 mM and incubated for 1 h at 20 °C. The suspension was ultrafiltered 3 times as described above with 10 mM phosphate buffer (pH 7·2) as the washing buffer. The efficacy of LPG biotinylation was checked by Western blot of LPG and biotinylated LPG.

Western ligand blot with LPG

MV extracts were prepared for gel electrophoresis by suspension in sample buffer, containing 1 mM PMSF without reducing agent, and heating for 20 sec at 95 °C. Unless stated otherwise, 5 μl samples (0·2 μg protein, equivalent to MV extract from 5 midguts) were loaded per lane and separated on 4–20% SDS–polyacrylamide mini gels. The separated samples were transferred onto nitrocellulose membrane (0·45 μm) using a semi-dry blotter (Multiphor Novablot, Pharmacia Biotech) with a constant current of 42 mA for 1–1·5 h. The membrane was blocked overnight at 4 °C with 3% crystalline bovine serum albumin (BSA), 0·01% Tween 20 and 150 mM NaCl in 100 mM Tris–HCl, pH 7·5. Lanes selected for total polypeptide detection were blocked overnight in 0·1% Tween 20 and 150 mM NaCl in 100 mM Tris–HCl, pH 7·5 (TTBS) and stained using Protogold with silver enhancement (British Biocell, Cardiff, UK).

To detect binding the nitrocellulose strips were incubated for 3 h with a solution of LPG extract (6 μg/ml) in 3% crystalline BSA, and 150 mM NaCl in 50 mM Tris–HCl, pH 7·5 and washed for 30 min with 4 changes of TTBS. Blots were probed with anti-LPG mAb CA7AE or WIC79.3. Probing with CA7AE was for 1 h at a dilution of 1:2000 in TTBS, the blot was washed with TTBS for 30 min (4 changes) and incubated with 1:1000 alkaline phosphatase-conjugated secondary antibody goat anti-mouse IgM (Calbiochem-Novabiochem, San Diego) with 3% normal goat serum in Tris-buffered saline (TBS). Other blots were incubated with WIC79.3 (De Ibarra, Howard & Snary, 1982) using 1:20 diluted hybridoma culture supernatant, washed and incubated with 1:4000 alkaline phosphatase-conjugated secondary antibody anti-mouse IgG (Southern Biotechnology, Alabama). After a final 30 min wash in TTBS (4 changes), blots were developed with nitroblue tetrazolium/BCIP (5-bromo-4-chloro-3-indolyl phosphate) substrate (Vector Labs, UK).

Western ligand blot with biotin–LPG

Blots were prepared and blocked overnight as described above. Nitrocellulose strips were incubated for 3 h with 1:1000 dilution of biotin–LPG in a solution of 3% crystalline BSA, 0·01% Tween 20 and 150 mM NaCl in 100 mM Tris–HCl, pH 7·5. After washing in TTBS for 30 min the strips were incubated with 1:1000 streptavidin alkaline phosphatase (Vector Labs) in the 3% BSA-buffered blocking solution for 45 min, washed for 1·5 h in TTBS and developed with the substrate described above.

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RESULTS AND DISCUSSION

The total polypeptide profile for the blotted, non-reduced midgut microvillar extract from *P. papatasi* (Fig. 1, lane 6, Fig. 2, lane 1) revealed multiple bands including 2 major bands (121 and 85–87 kDa) and at least 9 other bands ranging from 19 to approximately 210 kDa. In comparison, reduced preparations of MV revealed major bands at 84–87 kDa (data not shown). Coomassie blue stained SDS–PAGE gels of female mosquito (*Culex tarsalis*) MV extracts revealed 2 major bands with approximately 100 and 70 kDa (Houk *et al*. 1986) under reducing conditions.

The method of calcium precipitation combined with differential centrifugation resulted in a 5·3-fold enrichment in MV proteins. Enrichment was estimated by comparing specific activities of the microvillar marker aminopeptidase (Terra & Ferreira, 1994) in the crude extracts ($3{\cdot}0 \pm 0{\cdot}2$ μM/μg protein) with activity in the microvillar extract ($16{\cdot}0 \pm 1{\cdot}5$ μM/μg protein). The method takes advantage of the highly charged nature of the MV membrane compared to other gut cell membranes (Ferreira & Terra, 1980). Although this process results in the loss of some MV protein, it greatly reduces contamination with irrelevant proteins associated, for example, with the basolateral membrane and thus enabled loading of a greater quantity of the proteins normally exposed to developing parasites.

Procyclic LPG from *L. major* consistently bound to 3–4 bands in Western ligand blots prepared from MV extracts of 6 different midgut preparations. Preliminary experiments established that increased LPG binding was obtained using non-reduced, in comparison to reduced, samples for SDS–PAGE probably because reduction of disulphide bridges generally results in the loss of ligand binding (Hossenlopp & Binoux, 1994). Overnight blocking was used to promote renaturation of the proteins.

The LPG binding was visualized initially with the anti-LPG IgM mAb CA7AE which recognizes the repeated phosphorylated galactose β-1,4-mannose disaccharide unit of *Leishmania* sp. LPG (Tolson *et al*. 1989). The LPG bound to proteins of 121, 86 and 65 kDa (estimated in the non-reduced state; Fig. 1, arrowed bands, lane 3). The amount of binding correlated with a reduction in the amount of microvillar extract loaded; 3 bands are clearly visible in lane 3 but are barely visible in lane 5 where the amount of protein loaded was one sixth of that loaded in lane 3. Control lanes consisted of membrane strips not incubated with mAb or LPG. There was a small amount of apparent cross-reactivity of the mAb towards the gut polypeptides with the 121 and 86 kDa bands (faint bands in lane 1).

Other blots were prepared and the LPG detected using WIC 79.3, an IgG mAb specific for the LPG of *L. major* (Handman, Greenblatt & Goding, 1984). Binding of the ligand was detected with 121, 86 and 65 kDa but also with 34 and 29 kDa (arrowed on Fig. 2, lane 5) and some faint bands of lower molecular weight. The amount of binding was also correlated with amount of protein loaded per lane as noted in the previous experiment. The absence of any visible bands in lane 3 shows that the amount of protein loaded was below the threshold of protein required to detect LPG binding.

Comparison of the main LPG blot (Fig. 2, lane 5) with those of the major polypeptide bands (lane 1) reveals a specific binding process which is not associated with the abundance of individual polypeptide bands; the 65 kDa LPG binding peptide gave the strongest signal but migrated just below a major 70 kDa band.

To verify the results obtained with mAbs a biotin label was used as a more direct method of ligand binding detection. The biotin–streptavidin method not only removed any possibility of Ab cross-reactivity with midgut proteins but also gave a stronger signal. Biotin labelled and unlabelled LPG was detected using the CA7AE mAb to reveal a broad band characteristic of the glycoconjugate LPG (Fig. 3A). Incubation of microvillar blots with biotin–LPG (Fig. 3B) revealed binding to over 6 bands including the 64 kDa band previously detected with mAbs/LPG. Binding was strongest with lower molecular weight bands particularly a 19 kDa band. Some of the lower molecular weight LPG binding proteins might be degradation products, or subunits of the larger primary binding proteins. This might explain some of the variability in the number of bands detected with LPG binding activity. Another possibility is that a combination of specific and non-specific binding occurred, particularly with the bands which migrated at 121 and 86 kDa. However, substituting the wash post-LPG incubation with a high salt wash (650 mM NaCl in Tris–HCl pH 7·5 with Tween 20) to remove non-specific binding did not reduce LPG binding (data not shown).

The most conclusive evidence for specific LPG binding to midgut polypeptides is given (Fig. 3B, lane 3) where incubation of biotin–LPG with CA7AE (1:50) resulted in abolition of binding to both the 64 kDa band and the lower molecular weight bands. With the techniques available it was not possible to determine if the bands contained 1 or several proteins. Therefore we cannot conclude that there is only a single binding protein on the sandfly midgut.

However, there is no reason to expect that the parasite binds to only one or two specific receptors in the midgut. Indeed, it would be in the interests of the parasite to bind to a molecule that was both common in the gut as well as functionally important to the sandfly. Even the specific attachment to the macrophage by the *Leishmania* promastigote is

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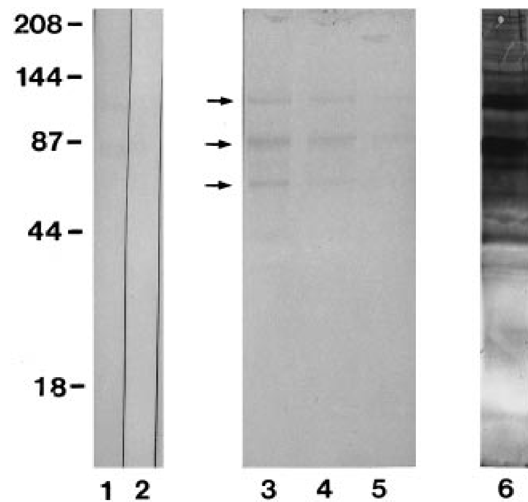

Fig. 1. Western ligand blot analysis of lipophosphoglycan-binding proteins in microvillar extracts from the midgut of *Phlebotomus papatasi*. Proteins separated under non-reducing SDS–PAGE were electroblotted and incubated with procyclic LPG from *Leishmania major*. LPG binding was detected using CA7AE anti-LPG specific mAb. SDS–PAGE 4–20% gradient gel. Molecular weight standards are given in kDa. Arrows denote the main LPG binding locations on the nitrocellulose membrane strips. Lane 1, 10 μl of extract/lane, control, no LPG. Lane 2, 10 μl of extract, control, no mAb. Lanes 3–5, LPG incubated with a strip containing decreasing quantities of microvillar extract per lane; 24 μl (lane 3), 12 μl (lane 4), 4 μl (lane 5). Lane 6, 5 μl of extract. Protein stained for microvillar extract.

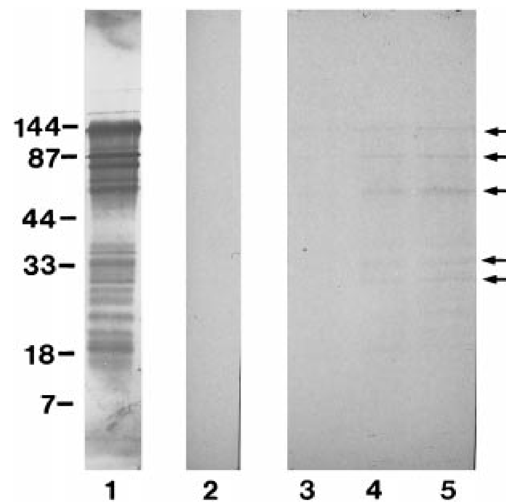

Fig. 2. Western ligand blot analysis of lipophosphoglycan-binding proteins in microvillar extracts from the midgut of *Phlebotomus papatasi*. LPG binding was detected using WIC79.3, anti-*Leishmania major* LPG specific mAb. SDS–PAGE 12% gel. Molecular weight standards are given in kDa. Arrows denote the main LPG binding locations on the nitrocellulose membrane. Lane 1, 5 μl of extract. Protein stained for microvillar extract. Lane 2, 12 μl of extract, control, no LPG. Lanes 3–5, LPG incubated with a strip containing increasing quantities of microvillar extract per lane; 4 μl (lane 3), 12 μl (lane 4), 24 μl (lane 5).

thought to be mediated by binding to a variety of receptors (see e.g. Green *et al*. 1994). Perhaps the microvillar surface glycoproteins exposed to the luminal environment have developed common structural configurations which enable them to resist the hydrolytic mileau and consequently LPG is binding to a site common to several different proteins. Biotinylated *T. brucei* also binds many proteins from midgut homogenates derived from the tsetse gut (van den Abbeele *et al*. 1996). Another possibility is that the LPG also binds to some cytoskeletal elements associated with the microvillal extract in addition to cell surface molecules. *Leishmania* promastigotes will attach to collagen for example (Lira, Rosales-Encina & Arguello, 1997). However, attempts to remove the cytoskeletal elements from the microvillar membranes using hyperosmotic Tris (Jordão, Terra & Ferreira, 1995) are impractical in the present context because it would require prodigious quantities of insects.

Several studies have sought to identify lectins in the sandfly midgut using a strategy of *in vitro* agglutination of promastigotes with whole midgut extracts and inhibition by selected sugars (Wallbanks, Ingram & Molyneux, 1986; Volf & Killick-Kendrick, 1996; Svobodova, Volf & Killick-Kendrick, 1996; Palanova & Volf, 1997). The studies

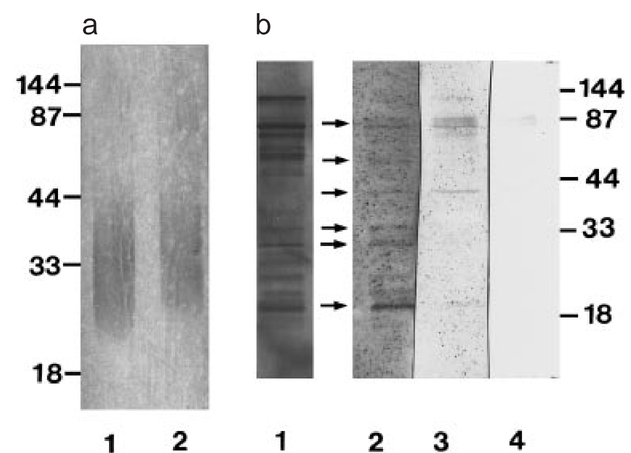

Fig. 3. Western ligand blot analysis of lipophosphoglycan-binding proteins in microvillar extracts from the midgut of *Phlebotomus papatasi*. SDS–PAGE 12% gel. Biotinylated LPG used with streptavidin. Molecular weight standards are given in kDa. Arrows denote the main LPG binding locations on the nitrocellulose membrane. (A) Blot of LPG (lane 1) and biotinylated LPG (lane 2) detected with anti-LPG mAb CA7AE. (B) Lane 1, 5 μl of extract. Protein stained for microvillar extract. Lane 2, blot incubated with biotin–LPG. Lane 3, blot incubated with biotin–LPG and CA7AE. Lane 4, no biotin–LPG control.

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suggest that haemagglutination activity, located throughout the body (Palanova & Volf, 1997), is greatest in the midgut lumen during bloodmeal digestion (Volf & Killick-Kendrick, 1996) and is almost entirely lost after defaecation. It is clear that these lectin-like compounds cannot be involved in the specific binding of the *Leishmania* parasite to the microvilli.

Information on the biochemical structure of insect microvilli and the associated carbohydrate-rich glycocalyx is limited (Lane, Dallai & Ashhurst, 1996). However, it is known that the dominant integral proteins on the MV membrane of Dipteran insects are hydrolases involved in the terminal processes of digestion (Jordão *et al*. 1995). In Dipteran sandflies there are highly active α-glucosidases (Dillon & El Kordy, 1997) and the MV preparations are enriched more than 8-fold for α-glucosidase activity (Dillon & El Kordy, unpublished observations). One possibility is that LPG sugars are binding to glycosidases. Other hydrolases, 100–120 kDa glycoprotein aminopeptidases, have been identified as the MV receptor for the endotoxins of the insect pathogen *Bacillus thuringiensis* in Lepidoptera (Knight, Crickmore & Ellar, 1994; Valaitis *et al*. 1997). Aminopeptidase is present on the MV membrane in sandflies before and after bloodfeeding (Dillon & Lane, 1993*b*) and is another potential binding protein.

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