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Quantitation of *Leishmania* lipophosphoglycan repeat units by capillary electrophoresis

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Abstract

The glycosylphosphatidylinositol (GPI)-anchored lipophosphoglycan (LPG) of *Leishmania* is the dominant cell surface glycoconjugate of these pathogenic parasites. LPG is structurally characterized by a series of phosphoglycan repeat units. Determining the number of repeat units per LPG molecule has proven difficult using current technologies, such as mass spectrometry. As an alternative method to quantitate the number of repeat units in LPG, a procedure based on capillary electrophoretic analysis of the proportion of mannose to 2,5-anhydromannose (derived from the nonacetylated glucosamine of the GPI anchor of LPG) was developed. The CE-based technique is sensitive and relatively rapid compared to GC-MS-based protocols. Its application was demonstrated in quantitating the number of LPG repeat units from several species of *Leishmania* as well as from two life-cycle stages of these organisms.

Keywords: *Leishmania*; Lipophosphoglycan; Capillary electrophoresis

1. Introduction

Protozoan parasites of the genus *Leishmania* are the causative agents of leishmaniasis, a disease that afflicts millions of people world-wide. The life-cycle of the pathogens alternates between an aflagellated amastigote form in the mammalian macrophage and a flagellated promastigote form in the midgut of its insect vector, the sand fly [1]. Within parasite development in the latter stage, there exists two entities of promastigotes. Procyclic promastigotes originate when a sand fly takes up an infected bloodmeal and attach to the midgut epithelial cells as the bloodmeal is degraded by fly's digestive enzymes. After digestion of the bloodmeal and excretion of the bloodmeal waste by defecation, the procyclic promastigotes avoid the elimination process by attaching to the epithelial cells that line the midgut. As the sand fly prepares to feed again, parasites differentiate into the infectious metacyclic stage and detach from the midgut epithelium to allow transmission with the next bite. Coordination of the transition between adhesive and free parasite forms is thus critical for parasite transmission from the fly to the mammalian host.

Stage-specific adhesion of *Leishmania* promastigotes in the sand fly midgut is mediated by structural variation involving the abundant cell surface glycoconjugate lipophosphoglycan (LPG). This glycoconjugate, along with other surface molecules, contributes to parasite survival in the hydrolytic midgut environment [1]. The basic LPG structure in all *Leishmania* species consists of a 1-*O*-alkyl-2-*lyso*-phosphatidyl(*myo*)-inositol lipid anchor, a heptasaccharide glycan core, a long phosphoglycan (PG) polymer composed of (6Galβ1,4Manα1-PO_4) repeat units ($n \sim 10-40$), and a small oligosaccharide cap (Fig. 1). This basic structure of LPG with unsubstituted repeat units is abundantly expressed in *L. donovani* [2]. In other species the PG repeat units contain additional substitutions that mediate key roles in stage-specific adhesion. For example, in the *L. major* strain Friedlin V1, the LPG phosphoglycan $[Gal\text{-}Man\text{-}P]_n$ backbone repeat units bear (β1,3)galactosyl side chain modifications [3], which form the ligand for the sand fly midgut galectin PpGalec [4]. In *L. chagasi*, the $[Gal\text{-}Man\text{-}P]_n$ repeat units contain 1 or 2 (β1,3)glucosyl residues that branch off the backbone units [5]. As procyclic parasites differentiate into the infectious metacyclic stage, LPG increases in size due

Abbreviations: LPG, lipophosphoglycan; GPI, glycosylphosphatidylinositol; CE, capillary electrophoresis; APTS, 8-aminopyrene-1, 3,6-trisulfonic acid

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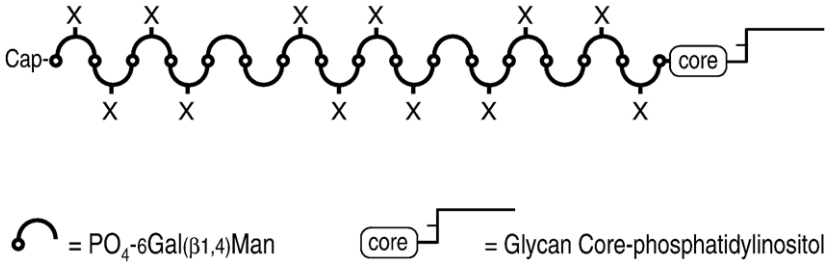

Fig. 1. Schematic diagram of a generalized LPG structure of *Leishmania*. The structure of the glycan core is Gal(α1,3)Gal(α1,3)Gal$_f$(β1,3)[Glc(α1-PO$_4$)-6]-Man(α1,3)Man(α1,4)GlcN(α1,4) linked to 1-*O*-alkyl-2-*lyso*-phosphatidylinositol anchor. The repeat units contain -PO$_4$-6Gal(β1,4)Man(α1) as a backbone structure. The "X" in the repeat units represents additional sugars that can branch off the repeat units of LPG from some species of *Leishmania*. In *L. donovani*, there are no additional branching sugars. The precise location of the various side chains in the repeat units is not known.

to an approximate doubling in the number of repeat units (Fig. 1) [6]. Along with structural changes of LPG side chains [7], the overall modifications that occur in LPG profoundly affect the ability of a given sand fly species to transmit *Leishmania* [7].

One of the difficulties in LPG characterizations from various species and growth stages is accurately determining the average number of repeat units in this structurally heterogeneous glycoconjugate. In this manuscript, a capillary electrophoresis-based protocol was developed to quantitate the average repeat unit number. The method is sensitive, relatively rapid compared to current GC-MS-driven methods, and is likely applicable to analyses of other analogous glycoconjugates as well.

2. Materials and methods

2.1. Purification of LPG

Procyclic *L. donovani, L. major*, and *L. chagasi* were harvested at a concentration of 1×10^7 cell/ml promastigotes grown in M199 medium with Hanks salts. LPG was extracted and purified as described elsewhere [8]. In brief, the cells (1×10^{10}) were washed in phosphate-buffered saline, and pelleted by centrifugation. The cells were resuspended and partitioned twice in chloroform/methanol (3:2). The delipidated pellet was extracted twice with 4 mM magnesium chloride, three times with chloroform/methanol/water (10:10:3), and then the LPG was extracted four times with Solvent E (water/ethanol/diethylether/pyridine/NH_4OH, 15:15:5:1:0.017). The LPG was further purified by hydrophobic chromatography on phenyl-Sepharose.

2.2. Purification of metacyclic promastigotes

Metacyclic promastigotes were isolated from *L. donovani* and *L. chagasi* culture using negative selection with peanut agglutinin [9]. Briefly, promastigotes were grown to very late stationary phase ($3-5\times10^7$ cells/ml) in 500 ml of M199 medium. Cells were centrifuged and washed in phosphate-buffered saline. Cell pellets were resuspended in 50 ml of M199 medium containing 35 μg/ml of peanut agglutinin and incubated for 30 min at 25 °C with gentle shaking. The cell suspension was centrifuged at 1500×*g* for 5 min at 4 °C. Under these conditions, procyclic cells agglutinate with peanut agglutinin and are pelleted, while the metacyclic cells are not agglutinated and remain in the supernatant. The supernatant was further centrifuged at 2100×*g* for 10 min at 4 °C to pellet the metacyclic promastigotes.

Metacyclic promastigotes of *L. major* were purified using a 10–40% ficoll gradient protocol [10]. Parasites were harvested as mentioned above, and applied to a 15 ml plastic conical tube with 10% ficoll in water layered over 40% ficoll in M199 medium. The tubes were centrifuged at 1300×*g* for 10 min at 25 °C. The metacyclic promastigotes remain in 10% ficoll, which was removed and spun at 1900×*g* for 10 min at 25 °C to pellet these promastigote forms. The metacyclic promastigotes were resuspended in phosphate-buffered saline and LPG was extracted as above.

2.3. Preparation of monosaccharides for capillary electrophoresis

LPG samples were chemically delipidated by nitrous acid deamination [11,12]. Samples were incubated overnight with 0.25 M sodium acetate pH 4.0, and 0.5 M sodium nitrite in a 1:1 ratio at 37 °C. The lipid portion was removed from the samples by passages of the samples through C_{18}-Sepharose columns equilibrated in water. The aqueous-soluble material was dried by vacuum centrifugation and subjected to strong acid hydrolysis in 2 N trifluoroacetic acid for 3 h at 100 °C. The multiple PO_4–Gal linkages in LPG are refractory to hydrolysis under these strong acidic conditions. The substituents were dried using vacuum centrifugation and then labeled in a volume of 4 μl for 90 min at 55 °C with the fluorophore APTS (0.2 M) containing 1 M sodium cyanoborohydride, which labels the reducing termini of saccharides by reductive amination. The reaction was stopped by adding 46 μl of water, diluting the sample 10-fold. Prior to injection in CE, the samples were further diluted in H_2O at a ratio of 39:1.

2.4. Separation of monosaccharides by capillary electrophoresis

APTS-derivatized monosaccharides were separated using a P/ACE MDQ capillary electrophoresis system (Beckman-Coulter). An uncoated capillary measuring 52 ID, 361 OD and 40 cm long was conditioned using 5 min washes each of 0.1 N HNO_3, 0.1 N HCl, and 0.1 N NaOH. Each wash was followed by a 1-min H_2O wash. The washes were done at room temperature using 20 psi. Following the washes, the conditioning was completed with a methanol wash for 2.5 min and another H_2O wash for 1 min. Lastly, the capillary was primed with 30 mM sodium borate in 5% methanol, pH 9.4 at 20 psi for 5 min. A fluorescein standard (Beckman) was detected using a laser induced fluorescence (LIF) detector. Prior to application of samples, the capillary was rinsed for 1 min using H_2O at 20 psi, followed by a 2-min rinse with 0.1 N NaOH at 20 psi. Following another H_2O rinse for 2 min, the capillary was reprimed with the 30 mM sodium borate in 5% methanol, pH 9.4. The samples were injected for 4 s at 0.4 psi and separated for 20 min at 20 kV with the settings of 100 μA maximum current at 25 °C, with a threshold of 2 and peak width of 9. Normal filter settings of the laser used were 488 nm excitation and 520 nm emission.

2.5. Western blotting of L. donovani LPG

LPG purified as described above was subjected to electrophoresis on a SDS-PAGE gel using 5% and 12% polyacrylamide for the stacker and main gel, respectively. The transfer was done at 12 V for 1 h onto nitrocellulose. The membrane was blocked with 5% milk in phosphate-buffered saline. The primary mouse monoclonal antibody CA7AE, which recognizes the Gal-Man-P repeat units of LPG, was used at a 1:1000 dilution. A goat anti-mouse