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Leishmania chagasi heparin-binding protein: Cell localization and participation in *L. chagasi* infection

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ABSTRACT

Visceral leishmaniasis is a fatal human disease caused by the intracellular protozoan parasite *Leishmania chagasi* that is captured by host cells in a process involving classics receptors mediated phagocytosis. The search for molecules involved in this process is important to design strategies to disease control. In this work, we verified the presence of heparin-binding protein (HBP) in *L. chagasi* promastigotes forms. HBP is a lectin of the group of ubiquitous proteins, whose main characteristic is to bind to carbohydrates present in glycoproteins or glycolipids, which is poorly studied in *Leishmania* species. *L. chagasi* HBP (HBP*Lc*) was purified by affinity chromatography using heparin–agarose column in FPLC automated system. Its localization in the parasite was assessed by immunolabeling and electronic transmission microscopy tests using anti-HBP*Lc* polyclonal antibodies, which showed HBP spread over the parasite outer surface and internally next to the kynetoplast. In addition, we verified that HBP*Lc* participates in the process of parasite infection, since its blocking with heparin generated a partial reduction in the internalization of *Leishmania* by RAW macrophages "in vitro". According to these results, it is believed that, in further "in vivo" studies, interference on this parasitic protein may provide us prophylactic and therapeutic alternatives against visceral leishmaniasis.

1. Introduction

Neglected tropical diseases refer to infectious diseases that occur in tropical regions of the world, affecting the world's poorest people [1]. Among these diseases is leishmaniasis, an infectious disease caused by the protozoa *Leishmania* sp., which is an obligate intracellular parasite that infects humans and other mammalian species. This disease can cause a wide spectrum of clinical presentations [2] and is responsible worldwide for the second highest number of deaths from parasitic infections [3]. Visceral leishmaniasis (VL) or kala-azar is the most severe clinical form of leishmaniasis, and is responsible for the higher incidence of deaths associated with the disease, hence representing a serious public health problem. It is clinically characterized by chronic low-grade fever, weight loss, hepatosplenomegaly, and laboratory findings which included pancytopenia, low albumin levels, and hypergammaglobulinemia; these symptoms render it fatal if not properly treated [4,5]. VL ranks second in mortality and fourth in morbidity among tropical diseases, with a mortality rate of approx. 20,000–40,000 deaths per year—reviewed in [6]. The treatment of VL is generally limited to the use of pentavalent antimonials as first-line drugs and pentamidine or amphotericin B as second-line drugs. However, an increased resistance to the first-line drugs as well as several side effects and high toxicity of the second-line drugs have been detected [7,8]. Thus, there is a need for the development of new drugs and alternative strategies to block, control, and prevent the disease [9]. Therapeutics that can disrupt certain mechanisms important for the virulence of the microorganism also becomes a potential target, aiming to control the established infection. In leishmaniasis, the characterization of parasite-specific molecules that contribute to their virulence mechanisms (called virulence factors), such as antigenic potential, is the subject of several research groups. These molecules include the *Leishmania* homolog of receptors for activated C-kinase (LACK) [10,11], the lipophosphoglycan (LPG), the

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gp63 glycoprotein [12–15], the cistein-peptidases [16], enzymes that participate in the catabolism of extracellular ATP [17], and proteinases, enzymes that hydrolyze peptide bonds and thus have the potential to degrade proteins and peptides that participate in a broad range of biological functions, including the infection process [18].

During the blood meal, the insect vector deposits metacyclic promastigotes in the skin of the mammalian host. These promastigotes are infective forms of *Leishmania* that interact with macrophages and dendritic cells, in a process mediated by classic receptors that initiate phagocytosis [18]. The intervention in the adhesion and penetration processes is important to prevent the infection. Some surface molecules of both the parasites and macrophages are responsible for this interaction: complement receptors (CR) such as CR 1 and CR3 (Mac-1); the fibronectin receptors in promastigotes; and the fucose–mannose receptors (FMRs) on the surface of macrophages play important roles in the binding of promastigotes to the host cells; binding to multiple receptors is required for parasite uptake or to trigger a protective immune response [19,20]. Other parasitic molecules potentially involved in infectious diseases are the lectins, a group of ubiquitous proteins whose main feature is to bind to soluble carbohydrates present in glycolipids or glycoproteins. This feature has aroused interest on the possible role of lectins in the access of pathogenic microorganisms to mammalian cells. Previous several studies demonstrate the involvement of host cell lectins in the recognition of carbohydrates present on the surface of different pathogenic species [21,22], such as parasites of the genus *Leishmania* [23,24]. The results of these studies prove that the modulation of the host immune response is due to the activation of signaling pathways which is triggered by the stimulation of surface lectins of the immune system cells by the carbohydrates present in pathogens. Interestingly, in some cases, lectins are involved in therapeutic purposes, including inhibition of HIV infection [25]. Parasitic lectins are also involved in infectious diseases caused by protozoan parasites that cause important diseases in humans: heparin-binding proteins (HBP) present on the surface of infectious forms of *Trypanosoma cruzi* are involved in the adhesion of amastigotes and epimastigotes forms to the host cells and intestinal epithelium of triatomines, respectively [26–28]; galactose and *N*-acetyl-galactosamines-binding lectins present in the surface of *Entamoeba histolytica* are important virulence factors for this protozoan parasite, which is responsible for the third highest number of deaths caused by parasitic diseases in the world, after malaria and schistosomiasis [29–31]. Several aforementioned studies on vertebrate hosts, plants or microorganisms have been conducted, which consider the biological effects of lectins on infectious agents [25,32,33]. However, the number of studies that involve profiling of the expression of surface lectins in *Leishmania* parasites which mainly consider the participation of these molecules in cellular adhesion or their influence over the mammalian host immunological response is less. Nevertheless, certain studies based on the investigation of the presence of HBP in *Leishmania braziliensis* promastigotes provide evidences of the expression of these molecules, thus, suggesting the participation of surface lectins in the interaction of the parasite with intestinal cells in *Lutzomyia* species as well as in the life cycle of the parasite [34–36].

In this study, we aimed to detect, purify, and determinate the localization of HBP in *Leishmania chagasi* promastigotes (HBP*Lc*), describing by the first time the molecular mass profile of this protein. Additionally, we investigated the participation of this lectin in the adhesion and internalization of *L. chagasi* promastigotes in mammalian cells. Once HBP*Lc* is identified and characterized, the next step may be directed to the study of the effects of blocking of protein on the development of infectious diseases, giving us perspectives to provide new strategies to control infection by *L. chagasi*.

2. Materials and methods

2.1. Ethics statement

C57BL/6 mice (4–8 weeks old) were obtained from the University's animal facility (Biotério Central—UFV, Viçosa, Brazil). Animals were given water and food "ad libidum". Animal experimentation was done respecting ethical principles of the Code of Professional Veterinarian, according to the opinion of the Ethics Committee for Animal Use (approved by CEUA/UFV – Research project – process number: 22/2011), based in the actual Brazilian Legislation (Law no. 11.794, October 08, 2008), in the Normative Resolutions edited by CONCEA/MCTI, as in the "Diretriz Brasileira de Prática para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos" (DBCA), following the orientations to the practices of euthanasia commended by CONCEA/MCTI.

2.2. Parasites

L. chagasi promastigotes, M2682 strain (MHOM/BR/75/M2682), were cultured in Grace's insect medium (GIBCO BRL, Grand Island, N.Y., USA) supplemented with 10% heat-inactivated fetal calf serum (FCS; LGC Biotecnologia, Cotia, SP, Brazil), 2 mM L-glutamine (GIBCO BRL) and 100 U/ml penicillin G potassium (USB Corporation, Cleveland, OH, USA), pH 6.5, at 26 °C. The cultures were started with cell density of 10^5 cells/ml. On the fifth day of culture, the material was centrifuged at 1540 × *g*/4 °C/10 min, the parasites were washed two times with phosphate buffered saline (PBS), pH7.2, and the pellet was stored frozen at −20 °C.

2.3. L. chagasi *promastigotes protein extract preparation*

Parasitic pellets were thawed and pooled. Amount corresponding to 1.5×10^{11} parasites were resuspended in 10 ml of 50 mM sodium phosphate/150 mM sodium chloride buffer, pH 7.0, and disrupted by sonication (ultrasonic Q) at a frequency of 6 KHz, performing six cycles of 15 s with 1 min intervals in ice cold. All resulting material was centrifuged at 7000 × *g*/4 °C/20 min and the supernatant of soluble protein was recovered, filtered through a 0.45 μM pore diameter membrane, and stored at 4 °C.

2.4. Purification of HBPLc

The soluble protein extract of *L. chagasi* was subjected to affinity chromatography on heparin column, using 50 mM sodium phosphate/150 mM sodium chloride buffer, pH 7.0 to equilibrate the column and to elute non-adsorbed fraction. The adsorbed fraction was eluted with the same buffer plus NaCl 2 M. Purification was performed on automated Fast Protein Liquid Chromatography (FPLC) system (Akta Purified, GE®) at a flow rate of 1 ml/min and monitored by absorbance reading at 280 nm. The fractions of interest were collected, pooled and subjected to size exclusion chromatography technique in a *D-salting* column (1 ml bed volume). The buffer used for elution was 50 mM sodium phosphate/150 mM sodium chloride, pH 7.0. The new samples were collected and submitted to protein dosage. Samples were aliquoted to be stored at −20 °C, subjected to freeze drying or submitted directly to the immunolocalization tests.

2.5. Electrophoretic analysis of HBPLc

Electrophoretic analysis was carried out on polyacrylamide gel under dissociating conditions (SDS-PAGE) performed in "Mini Ver-