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Lepstospira interrogans shotgun phage display identified LigB as a heparin-binding protein

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ABSTRACT

LigB is an adhesin from pathogenic *Leptospira* that is able to bind to extracellular matrix and is considered a virulence factor. A shotgun phage display genomic library was constructed and used for panning against Heparan Sulfate Proteoglycan (HSPG). A phage clone encoding part of LigB protein was selected in panning experiments and showed specific binding to heparin. To validate the selected clone, fragments of LigB were produced as recombinant proteins and showed affinity to heparin and to mammalian cells. Heparin was also able to reduce the binding of rLB-Ct to mammalian cells. Our data suggests that the glycosaminoglycan moiety of the HSPG is responsible for its binding and could mediate the attachment of the recombinant protein rLB-Ct. Thus, heparin may act as a receptor for *Leptospira* to colonize and to invade the host tissue.

1. Introduction

Leptospirosis is an important zoonotic disease affecting people and animals caused by pathogenic spirochetes of the genus *Leptospira*. The transmission of leptospirosis involves the maintenance of pathogenic *Leptospira* spp. in their hosts, where the spirochetes colonize target organs after penetrating them, usually through directly or indirectly contact with urine of infected animals [1]. Pathogenic *Leptospira* species invade host tissues through dermal abrasions or mucous membranes. *Leptospira*'s ability of invasion is due to its capacity of multiplying in blood, adhere to endothelial and epithelial cells, and penetrate into tissues, such as the kidney, liver and lung [2].

The first step in the establishment of a bacterial infection is the interaction between pathogenic bacteria and host cells. In this context, bacterial adhesins contribute to the extracellular binding onto host cells, thus initiating the infection. These proteins located on the microbe surface are generally known as Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) [2]. Several extracellular matrix (ECM) molecules that bind *Leptospira* spp. proteins have been identified; these include Lig proteins [3,4], lipoproteins [5–7], endostatin-like proteins [8], surface adhesins [9–14], transmembrane outer membrane protein [15] and TlyC [16].

Leptospiral immunoglobulin (Ig-like) protein B (LigB) is a member of the family of bacterial proteins containing immunoglobulin-like (Big) repeats that have been characterized as relevant adhesins to microbial pathogenesis [3]. LigB presents 12 Ig-like domains followed by a variable C-terminal domain and it is expressed on the surface of pathogenic *Leptospira* species [17]. It expression is induced by environmental signals such as osmolarity or temperature changes [18,19]. LigB is also considered to be a *Leptospira* MSCRAMMs involved in interaction with a host [3], contributing to the binding of *Leptospira* to extracellular matrix (ECM) proteins such as fibronectin, fibrinogen, laminin, collagen and elastin [3,20–23]. In this work, a shotgun phage display library constructed using genomic DNA from *Leptospira interrogans* serovar Copenhageni was screened against Heparan Sulfate Proteoglycan (HSPG), resulting in the identification of LigB protein. The recombinant LigB protein fragments were produced and assayed to bind to ECM molecules and mammalian cells.

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2. Material and methods

2.1. Materials and library construction

Escherichia coli strain TG1 (SupE thiΔ(lac-proAB) F́[traD36 proAB+ LacIq lacZΔM15]) were grown in liquid or on solid Luria–Bertani (LB)-medium, supplied with 50 μg/ml ampicillin. Helper phage M13K07 were from New England Biolabs. MaxiSorp microwell plates (Nunc) were used for immobilization of Heparan Sulfate Proteoglycan (HSPG) (Sigma–Aldrich) for panning. Libraries were constructed by ligation of *L. interrogans* serovar Copenhageni strain Fiocruz L1–130 DNA fragments in the range of 0.12–2.5 kbp into a phagemid vector PG3DSS [24] as described [25]. Two libraries were constructed using different sizes of genomic DNA fragments, with 1.5 and 0.3 kbp average sizes respectively.

2.2. ECM components

All macromolecules, including the control protein bovine serum albumin (BSA) were purchased from Sigma–Aldrich. Laminin-1 and HSPG were derived from the basement membrane of Engelbreth-Holm-Swarm mouse sarcoma, plasma fibronectin was isolated from human plasma, and matrigel was from BD™ which is composed of a mixture of extracellular matrix proteins (laminin, collagen IV, Heparan Sulfate Proteoglycan, entactin/nidogen). Heparin was purchased from Roche and Sigma–Aldrich.

2.3. Panning procedures

Panning against 100 μg/mL of HSPG (Sigma–Aldrich) was carried covering a Nunc MaxiSorp microtiter plate and coated at 4 °C for 16 h. Then, the plate was blocked with 2.0% BSA for 1 h at room temperature. The panning procedures were performed essentially as described [25] with exception on the first cycle of selection in which the library was pre-incubated twice with 10 μg/mL BSA at 37 °C for 1 h each time and then incubated with HSPG for selection. The library used for panning procedures presented 1×10^9 CFU (colony forming units) per mL.

2.4. Analysis of selected phage (clones)

Selected clones of each round were submitted to DNA extraction of phagemid and then were sequenced on an ABI 3100 sequencer using BigDye2 kit (Applied Biosystems, Foster City, CA). Similarity searches were performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and confirmations of the insert ligands using bioinformatic tools (Vector NTI, Invitrogen).

2.5. Phage clone amplification and binding assays

In order to determine the specificity of the selected clones, each selected clone was amplified as described in [26] and stocked until use.

2.6. Cloning, expression, purification of recombinant proteins and antisera production

LigB (LIC10464) gene was separated in two regions: rLB-Nt, corresponding to the N-terminal 630 amino acids without the complete signal sequence resulting in an expected 64.2 kDa polypeptide and rLB-Ct, corresponding to the C-terminal amino acid residues from position 631–1156, resulting in an expected 55.6 kDa polypeptide. The gene fragments were amplified by PCR from genomic DNA of *L. interrogans* serovar Copenhageni strain Fiocruz L1–130 using the primer pairs listed in Supplementary Table 1. PCR fragments were cloned into the pGEM-T Easy vector (Promega Corp., Madison, WI) and transformed into *E. coli* DH5α. Following digestion with appropriate restriction enzymes, fragments were subcloned into the pAE vector [27] for the expression of recombinant proteins with an N-terminal 6×His tag. All constructs were verified by DNA sequencing with appropriate vector-specific primers. The expression and purification of the recombinant proteins were performed as described [28]. Antisera against rLB-Nt and rLB-Ct were produced as described [28].

2.7. ECM-binding assays

The binding of the recombinant proteins to ECM components was performed as described [29]. All experiments were performed in triplicate and repeated three times.

2.8. Adhesion of recombinant proteins to mammalian cell culture

The cell lines A31 (fibroblast cells derived from mouse - *Mus musculus*), LLC-PK1 (pig kidney epithelial cells – *Sus scrofa*) and VERO (monkey kidney epithelial cells – *Cercopithecus aethiops*) were obtained from the ATCC and were grown at 37 °C under 5% CO_2 in Dulbecco's modified Eaglés medium (Gibco®) supplemented with 10% heat-inactivated fetal bovine serum, 5mM L-glutamine (Gibco®) with 100 μg/mL ampicillin and streptomycin. The confluent cells were washed three times with 5.0 mL of PBS (phosphate-buffered saline), followed by trypsinization with a solution of 3.0 mL of PBS containing 300 μL of Gibco® Trypsin (0.25%) for 10 min at 37 °C. The cells were removed from culture flasks, centrifuged twice at 1500*g* and resuspended in PBS to a density of approximately 10^6 cells/mL. Then, 200 μL of each cell suspension were gently fixed for 10 min at 37 °C on 96-well tissue culture plates (Nunc) with a 0.1% solution of formaldehyde prepared in PBS. After washing with PBS, cells were blocked for 1 h with 2% BSA, washed and incubated for 2 h with 10 μg/mL of each recombinant protein. For competition experiments, proteins were incubated with 10 μg/mL of each ECM (heparin, fibronectin or laminin) for 1 h before the incubation with the cells. These binding assays were measured by ELISA using the parameters described [29]. Dilutions of mouse antisera against each recombinant protein were 1:5000 and 1:8000 was the dilution used for horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G.

2.9. Heparin affinity chromatography

A total of 0.2 mg/mL of rLB-Ct protein was diluted in 20 mM Tris Buffer pH 6.8 containing 0.15 M NaCl. The sample was loaded onto a column with 1.0 cm diameter (Amersham Pharmacia Biosciences) containing 1.0 mL of Heparin–Sepharose Fast Flow (GE Healthcare) pre-equilibrated with 20 mM Tris Buffer pH 6.8 containing 0.15 M NaCl. Following the adsorption of rLB-Ct, the column was washed with 20 mM Tris Buffer pH 6.8 containing 0.15 M NaCl (20 column volumes). Elution of the adsorbed proteins on Heparin–Sepharose resin was carried out with 3 column volumes of each 20 mM Tris Buffer pH 6.8 containing 0.2, 0.3, 0.4, 0.5, 1.0 M NaCl, respectively. The elution fractions were subjected to 12% SDS–PAGE gel and stained with silver.

3. Results

3.1. LigB was selected by panning the library against Heparan Sulfate Proteoglycan (HSPG)

In order to characterize new adhesins from *L. interrogans*, we have constructed a phage display genomic library using DNA

fragments from *L. interrogans* serovar Copenhageni. The leptospiral DNA fragments were size selected and cloned in phagemids. A random sequencing survey of clones showed the presence of leptospiral DNA inserts within the range of the selected size. This procedure yielded 2.8×10^5 transformants, 95% of which carried an insert. The library containing inserts of average size 1.5 kbp was used for panning against HSPG. After four rounds of panning, one in frame clone (F11) was selected. The F11 clone corresponded to part of LigB gene. It displayed a polypeptide (amino acids 998–1230) (Supplementary Fig. 1) containing the partial 11th and the full 12th Ig-like domains and part of the C-terminal domain of LigB (Fig. 1), which distinguish LigB from other leptospiral Lig proteins [30].

3.2. F11 shows affinity to heparin

Since clone F11 was identified after panning against HSPG, it was not possible, at this stage, to define if the binding of the phage product was due to the polypeptide core or to the glycosaminoglycan (GAG) moiety of the glycoprotein. Therefore, clone F11 was amplified and used for binding assays against heparin, a heparan sulfate structural analog [31]. Indeed, clone F11 showed binding against heparin, indicating that the GAG moiety of the heparan sulfate was probably responsible for its binding to F11 clone (Fig. 2A). M13 phage used as control did not exhibit significant binding to heparin.

3.3. rLB-Ct binds heparin

To validate the phage selection results that showed LigB would be a heparin-binding protein, two fragments of LigB were expressed and purified as a his-tagged protein. One, rLB-Nt, comprises the N-terminal portion of LigB (amino acids 34–630) with the first 6th Big domains plus part of the 7th Big domain and the other, rLB-Ct, comprises part of the 7th Big domain and the 8th to 12th Big domains, as schematically indicated in Fig. 1. The rLB-Ct contains almost the entire sequence coded by phage F11 (Supplementary Fig. 1).

rLB-Nt and rLB-Ct were submitted to binding assays against heparin (Fig. 2B). rLB-Ct, but not rLB-Nt, was able to bind heparin. Even when higher amounts of heparin (up to 200 UI) were used in the binding assays, no significant binding to heparin was observed for rLB-Nt or to the control leptospiral proteins used (LIC10009 and TlyC). The first of these control proteins (LIC10009) was shown to not present adhesion properties and the other (TlyC) was described to bind laminin, collagen and fibronectin, respectively [16,29]. Binding assays against a panel of several ECM proteins as well as against HSPG and heparin confirmed the binding ability of rLB-Ct to matrigel, HSPG and also heparin. In our study we did not observe the binding of rLB-Nt to any ECM protein or to HSPG and heparin (Fig. 2C). Previous work with LigB amino-terminal fragments (Big 1–3 and Big 4–6) also showed that these regions do not bind to ECM [3].

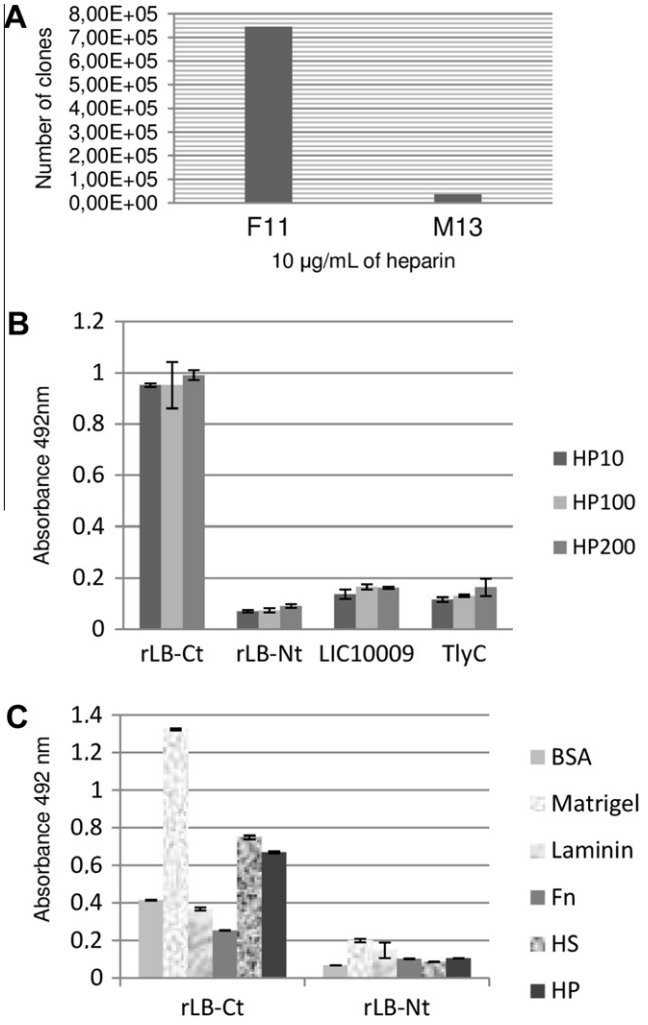

Fig. 2. The phage clone binding to heparin and the recombinant proteins binding to ECMs. (A) Number of recombinant phage F11 and wild type phage M13 (M13K07) eluted after panning against heparin. (B) Recombinant proteins and their binding to 10, 100 and 200 UI of heparin. (C) Binding of rLB-Ct and rLB-Nt to 1.0 µg of each different ECMs: BSA, Matrigel, Laminin, Fn (fibronectin), HS (HSPG), HP (heparin).

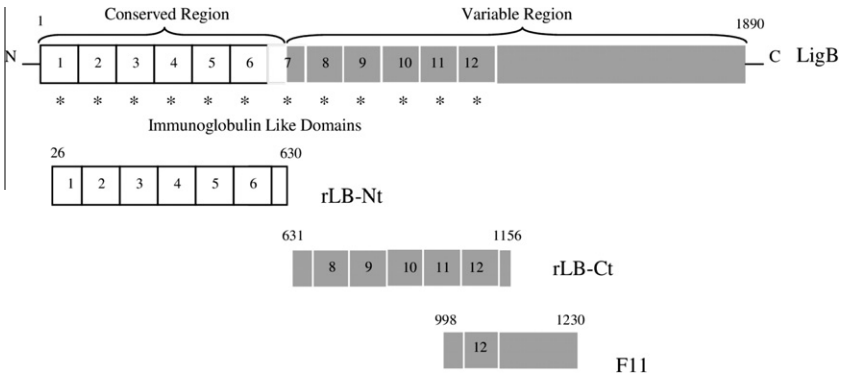

Fig. 1. Schematic view of Ig-like various domain, conserved and variable regions of LigB protein. rLB-Nt: recombinant protein with the amino-terminal of LigB, rLB-Ct: recombinant protein and their truncated carboxi-terminal LigB regions, F11: region expressed by the recombinant phage.

3.4. rLB-Ct binds to mammalian cells and is partially displaced by heparin

We have also evaluated the capacity of rLB-Nt and rLB-Ct to bind to mammalian cell cultures. As shown in Fig. 3A, only rLB-Ct was able to significantly bind to A31, LLC-PK1 and VERO cells. Neither rLB-Nt nor other control proteins with described adhesin activities such as TlyC and LipL32 [5,16] presented significant binding to these cells.

To evaluate the contribution of heparin in the binding of rLB-Ct to these mammalian cells, heparin was added to the incubation media. Fig. 3B shows that heparin was able to partially displace the binding of rLB-Ct to the A31, LLC-PK1 and VERO cells. This displacement was not observed when heparin is substituted by 1.0 μg of plasma fibronectin or laminin in the assay.

3.5. rLB-Ct is a heparin-binding protein

In order to explore the interaction between rLB-Ct and heparin, we performed a heparin affinity chromatography. rLB-Ct was loaded onto Heparin–Sepharose column. After the adsorption of rLB-Ct, the elution was proceeded by a NaCl gradient, showing that the rLB-Ct eluted from the column with 0.5 M of NaCl. This result indicates that rLB-Ct is a low affinity heparin-binding protein (Fig. 4).

4. Discussion

The adherence of the pathogen to their host surfaces is a key step in the host-pathogen interaction. Once adhered to host cell surface, the pathogen becomes able to initiate its specific processes that may result in the infection. Pathogenic species of *Leptospira* present several MSCRAMMs that had been described to bind ECM [1–15] and thus represent potential targets to vaccines, drugs and diagnostic assays. LigB is a leptospiral adhesin that binds to

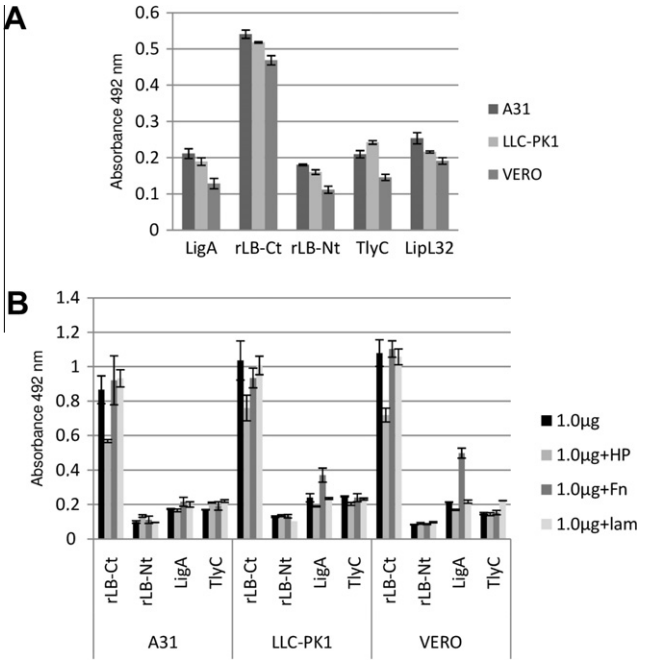

Fig. 3. The binding of recombinant proteins to different types of cell lines. (A) Binding of 1.0 μg of recombinant proteins to 10^6 cells/mL of each cell: A31, LLC-PK1, VERO. (B) Binding of 1.0 μg of recombinant proteins to 1.0 μg of ECMs prior the binding of different types of cell lines; ECMs: HP (heparin), Fn (fibronectin), lam (laminin).

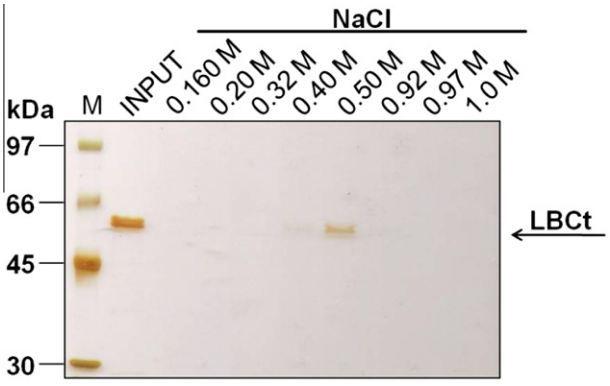

Fig. 4. Heparin–Sepharose chromatography carried by NaCl gradient elution: 12% SDS–PAGE gel stained with silver showed the rLB-Ct binding to heparin and its elution peak at 0.5 M NaCl. M: Molecular weight marker.

many ECM molecules [4,5,19–22] and this protein expression is controlled by environmental signals [18,19].

In this work, a shotgun phage display genomic library was constructed and used for panning against HSPG. Phage display libraries made from bacterial DNA are able, theoretically, to display all proteins encoded by the bacterial genome. Such libraries can be used to identify bacterial proteins, including adhesins, that bind to various targets [25,32–38]. F11, a phage clone encoding part of *Leptospira* LigB protein was selected by panning against HSPG and showed specific binding to heparin, suggesting that the GAG moiety of the heparan sulfate was responsible for its binding to F11 clone. The recombinant protein rLB-Ct, corresponding to the part of F11 that includes the 12th Big domain and the first 112 amino acids from the non repetitive C-terminal of LigB, binds heparin as well as it binds to HSPG. Recently, reports describing the binding of a LigB with ECMs showed the importance of the 12th immunoglobulin-like domain of LigB in the interaction with those molecules [20,39,40]. Moreover, they showed that the LigB protein mediate the attachment of leptospira to MDCK cells [20,21].

The pivotal feature of pathogenic *Leptospira* is its ability to invade the host and disseminate to every tissue especially the kidney, liver and lungs. Thus, the leptospires adhesion seems to involve the recognition of host surface-exposed molecules. Our results demonstrated that rLB-Ct is also able to bind to mammalian fibroblasts and kidney epithelial cells (A31, LLC-PK1 and VERO). Furthermore, when heparin was added to the rLB-Ct prior to the mammalian cells, it causes a decrease in the binding of the recombinant protein to the cells, reducing up to 73% the binding of rLB-Ct. These findings suggest that the GAG moiety can mediate the attachment of the recombinant protein rLB-Ct, acting as a receptor for the *Leptospira* to colonize and invade the host tissue.

Heparan sulfate (HS) is a major GAG component of proteoglycans (PGs). HSPGs are integral components of plasma membranes and are ubiquitously distributed among cell populations of animal tissues, where they are thought to be involved in cell–cell and cell–ECM interactions [41]. Several bacterial pathogens exploit proteoglycans as adhesion receptors, binding to the GAG moiety of proteoglycans and promoting their attachment and internalization [42,43]. Thus, the interaction of specific proteins (adhesins) on the surface of microorganisms with some receptors (carbohydrate chains on the glycoconjugate) allows the pathogens to trigger their first step towards establishing an infection, causing the disease [44].

Studies with *Borrelia burgdorferi*, another pathogenic spirochete, showed the binding of the bacteria to heparin, heparan sulfate, chondroitin sulfate B and mammalian endothelial and kidney cells [45]. Later works described *B. burgdorferi* interaction with

GAG-binding protein, including a heparin-binding protein [46,47]. Together with our results, these findings reinforce the suspect that GAGs participate, in some way, in the infection of the mammalian host, thus promoting the physical association of bacteria with the host tissues during the establishment of infection. Further studies related to the importance of GAGs in the adhesion of pathogenic *Leptospira* to the host may assist us in the understanding of the progression of leptospirosis. This is the first report about the interaction of heparin and LigB protein, suggesting that heparin mediates bacterial-host attachment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.137.

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