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The C-terminal variable domain of LigB from *Leptospira* mediates binding to fibronectin

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Adhesion through microbial surface components that recognize adhesive matrix molecules is an essential step in infection for most pathogenic bacteria. In this study, we report that LigB interacts with fibronectin (Fn) through its variable region. A possible role for LigB in bacterial attachment to host cells during the course of infection is supported by the following observations: (i) binding of the variable region of LigB to Madin-Darby canine kidney (MDCK) cells in a dose-dependent manner reduces the adhesion of *Leptospira*, (ii) inhibition of leptospiral attachment to Fn by the variable region of LigB, and (iii) decrease in binding of the variable region of LigB to the MDCK cells in the presence of Fn. Furthermore, we found a significant reduction in binding of the variable region of LigB to Fn using small interfering RNA (siRNA). Finally, the isothermal titration calorimetric results confirmed the interaction between the variable region of LigB and Fn. This is the first report to demonstrate that LigB binds to MDCK cells. In addition, the reduction of Fn expression in the MDCK cells, by siRNA, reduced the binding of LigB. Taken together, the data from the present study showed that LigB is a Fn-binding protein of pathogenic *Leptospira* spp. and may play a pivotal role in *Leptospira*-host interaction during the initial stage of infection.

Keywords: adhesion, Fn, *Leptospira*, LigB, MDCK cell, siRNA

Introduction

Leptospirosis is a zoonotic disease caused by pathogenic spirochetes in the genus *Leptospira* [22]. The disease occurs widely in developing countries and is reemerging in the United States [29]. The clinical features are variable and include subclinical infection, a self-limited anicteric febrile illness and severe, potentially fatal disease [22]. In the severe form of leptospirosis (Weil's syndrome), the symptoms include an acute febrile illness associated with multi-organ damage with liver failure (jaundice), renal failure (nephritis), pulmonary hemorrhage, and meningitis [10]. If not treated, the mortality rate may exceed 15% [49]. Furthermore, *Leptospira* infection can trigger autoimmune diseases in horses as well as humans [36,41]. Several factors associated with virulence have been proposed for *Leptospira* spp., including the sphingomyelinases, serine proteases, zinc-dependent proteases, collagenase [3], LipL32 [59], the novel factor H-binding protein LfhA [54], and lipopolysaccarides [56].

Pathogenic spirochetes have evolved a variety of strategies to infect host cells such as evasion of the innate as well as adaptive immunity [54]. Attachment to host cells is an essential step for colonization by bacterial pathogens. *Leptospira* has been shown to bind to mammalian cells, such as Madin-Darby canine kidney (MDCK) cells [2] via the extracellular matrix (ECM) [15]. Several adhesion molecules in the pathogenic spirochetes have been identified including a Fn binding protein (36 kDa protein) [30], a laminin binding protein (Lsa24) [1], and Lig proteins [25,33,34] from *Leptospira spp.,* decorin-binding proteins (Dbp A and B) [37] and Fn-binding proteins (BBK 32 and 47 kDa) [21,38] from *Borrelia spp.* and MSP, Tp0155, Tp0483, Tp0751 from *Treponema* spp. [4,5,9]. Lig proteins (Lig A, B and C) possess immunoglobulin-like domains with 90 amino acid repeats that have been identified in other adhesion molecules, such as the intimin of *Escherichia coli* and the invasin of *Yersinia pseudotuberculosis* [14,17]. Interestingly, the N-terminal 630 amino acid sequences of LigA and B are identical, but the C-terminal amino acid sequences are variable with only 34% identitify [33]. *ligB* also encodes a C-terminal, non-repeat domain of 771 amino acid residues [33]. On the other hand, the *ligA-ligB* intergenic regions from *L. kirschneri* and *L. interrogans* are 943 bp and 1347 bp in length respectively, and *ligC* is not linked to the *ligA-ligB* locus [25]. The expression of LigA and LigB is controlled by a

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key environmental signal, osmolarity, to enhance the binding of *Leptospira* to host cells [26,27].

It has been shown that the *lig* genes are present exclusively in pathogenic *Leptospira* spp [25,33]. LigA and LigB are weakly expressed in low passage, but not in high passage cultures of this organism [25,33]. Importantly, we have shown that LigA and LigB expression is upregulated *in vivo* in the kidneys of *Leptospira*-infected hamsters [34]. Recently, LigA and LigB have been reported to bind to extracellular matrix proteins including collagens type I and IV, laminin, fibronectin, and fibrinogen [6,24]. These data indicate that Lig proteins may play an important role in attachment of pathogenic leptospires to host cells.

Although there are three copies of *lig* genes (*ligA, B* and *C*) in *L. interrogans* serovar Pomona and *L. interrogans* serovar Copenhageni [31,33,34], only *ligB* is present in most pathogenic *Leptospira* spp. *ligA* is absent in *L. interrogans* serovar Lai [42], *ligC* is truncated (a pseudogene) in *L. kirschneri* serovar Grippotyphosa [25] and both *ligA* and *ligC* are absent in *L. borgpetersenii* serovar Harjo [3]. Therefore, we focused on LigB in this study and report that the variable region of LigB binds with high affinity to Fn, suggesting that this fragment is crucial for bacterial adhesion to host cells.

Materials and Methods

Bacterial strains and cell culture

L. interrogans serovar Pomona (NVSL1427-35-093002) was used in this study [35]. All experiments were performed with virulent, low-passage strains obtained by infecting golden syrian hamsters as previously described [35]. *Leptospires* were grown in EMJH medium at 30°C for less than 5 passages and growth was monitored by dark-field microscopy. The MDCK cells (ATCC CCL34) were cultured in Dulbecco minimum essential medium containing 10% fetal bovine serum (GIBCO, USA) and were grown at 37°C in a humidified atmosphere with 5% CO_2.

Reagents and antibodies

Horseradish peroxidase (HRP)-conjugated goat anti-hamster antibody, HRP-conjugated goat anti-mouse antibody and HRP-conjugated goat anti-rabbit antibody were purchased from Zymed (USA). Rabbit anti-glutathione S-transferase (GST) antibody, Alexa 594-conjugated goat anti-hamster antibody, Alexa 488-conjugated goat anti-hamster antibody, and FITC-conjugated goat anti-mouse antibody were purchased from Molecular Probe (USA). Anti-Fn (MAB1932) and anti-actin mouse antibodies (MAB1501) were purchased from Chemicon International (USA). Human plasma Fn was purchased from GIBCO (USA). Anti-*L. interrogans* antibodies were prepared in hamsters as previously described [35].

Plasmid construction and protein purification

Constructs for the expression of GST, GST fused with the conserved region of LigB (LigBCon; amino acids 1-630) and GST fused with the central variable region of LigB (LigBCen; amino acids 631-1417) were previously generated using the vector pGEX-4T-2 (Amersham Pharmacia Biotech, USA) [33]. GST fused with the C-terminal variable region of LigB (LigBCtv; amino acids 1418-1889) was generated using the vector pET41A (Novogen, USA). Relevant fragments of DNA were amplified by PCR using primers based on the *ligB* sequence [33]. Primers were designed to introduce a *Sal*I site at the 5' end of each fragment and a stop codon followed by a *Not*I site at the 3' end of each fragment. The PCR products were digested sequentially with *Sal*I and *Not*I and then ligated into pGEX-4T-2 or pET41A cut with *Sal*I and *Not*I. We purified the soluble form of GST-LigBCon, GST-LigBCen and GST-LigBCtv from *E. coli* as previously described [34,35].

Binding assays by ELISA

To measure the binding of *Leptospira* to the ECM components, 1 mg of each ECM component (as indicated in Fig. 1A) in 100 µl PBS (pH 7.2) was coated onto microtiter plate wells. For the dose-dependent binding experiments, different concentrations of Fn (as indicated in Fig. 1B) were coated onto the microtiter plate wells. The plates were incubated at 4°C for 16 h and subsequently blocked with blocking buffer (50 µl/well) containing 3.5% BSA in 50 mM Tris (pH 7.5)-100 mM NaCl-1 mM $MgCl_2$, $MnCl_2$, and $CaCl_2$ at room temperature (RT) for 2 h. Then, the *Leptospira* (10^7) were added to each well and further incubated at 37°C for 6 h. To determine the inhibition of *Leptospira* binding to the MDCK cells by Fn, the *Leptospira* (10^7) were pre-incubated at 37°C for 1 h with various concentrations of Fn (as indicated in Fig. 1C) prior to the addition of the MDCK cells (10^5) and finally incubated for 6 h at 37°C. The percentage of adhesions was determined relative to the attachment of the untreated *Leptospira* binding to the MDCK cells. For all experiments, the same concentration of BSA was used as a negative control. To determine the binding of LigBCen or LigBCtv to Fn, 10 nM of GST-LigBCen, GST-LigBCtv or GST (negative control) was added to 96 well microtiter plates coated with various concentrations of Fn (as indicated in Fig. 3A) or BSA (negative control and data not shown) in 100 µl PBS for 1 h at 37°C.

To measure the binding inhibition of *Leptospira* to Fn, various concentrations of GST-LigBCen, GST-LigBCtv (as indicated in Fig. 3B) or GST (negative control) in 100 µl PBS was added to Fn or BSA (negative control and data not shown) (1 mg in 100 µl PBS) coated wells at 37°C for 1 h, then the *Leptospira* (10^7) were added to each well and incubated at 37°C for 6 h. To measure the binding of LigBCen or LigBCtv to the MDCK cells, the MDCK cells

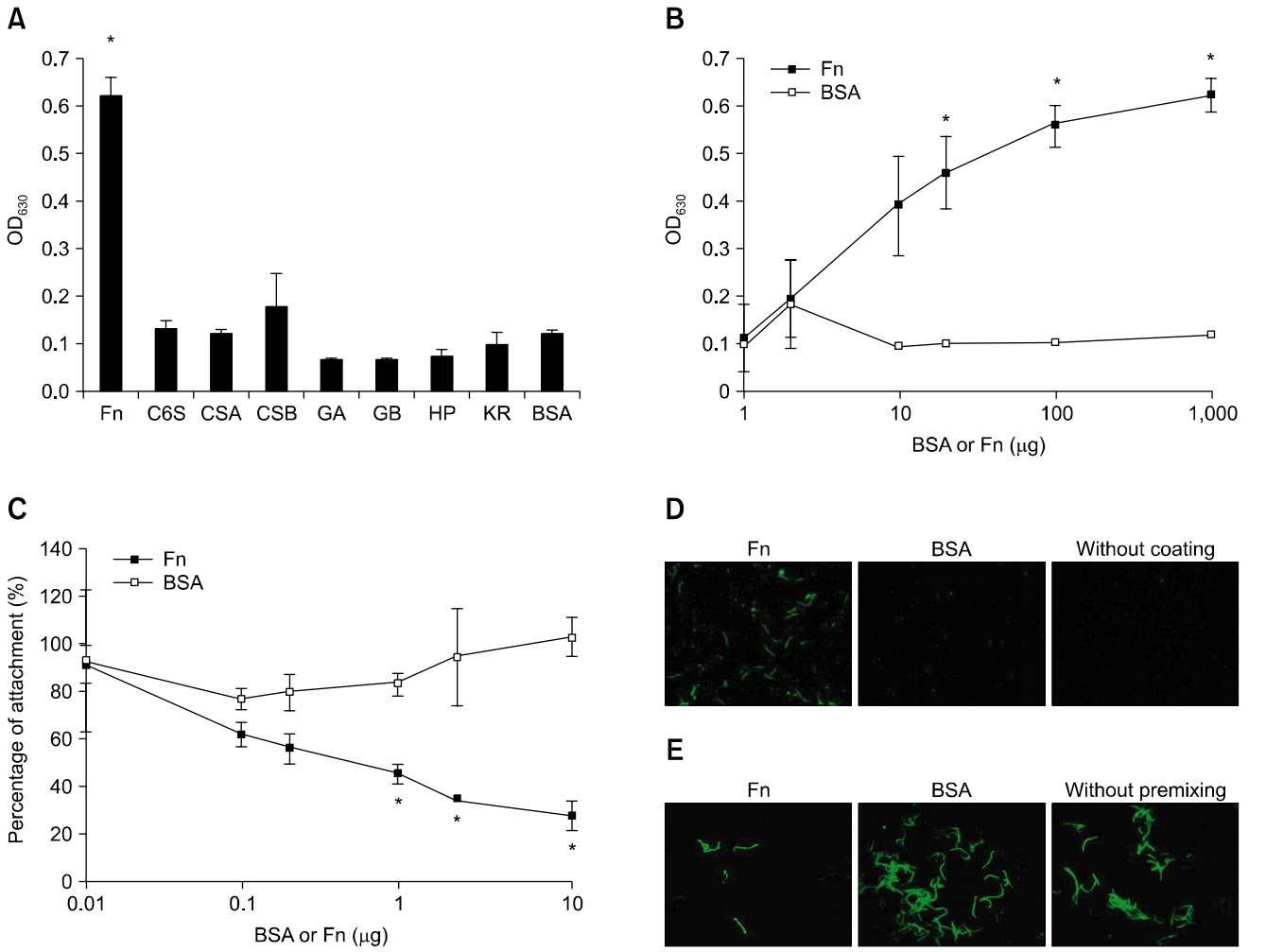

Fig. 1. The binding of *L. interrogans* serovar Pomona (NVSL 1427-35-093002) to Fn (A). Binding of *Leptopsira* to various immobilized ECM components. *Leptospira* (10^7) were added to wells coated with each ECM (1 mg in 100 μl PBS) including Fn, chondroitin-6-sulfate (C6S), chondroitin sulfate A (CSA), chondroitin sulfate B (CSB), gelatin A (GA), gelatin B (GB), heparin (HP), keratin (KR), or BSA (negative control). (B). Binding of *Leptospira* (10^7) to various concentrations of Fn (0, 10, 20, 100 or 1,000 μg in 100 μl PBS). BSA served as a negative control. (C). Fn inhibits the binding of *Leptospira* to the MDCK cells. *Leptospira* (10^7) were treated with various concentrations of Fn (0, 0.01, 0.1, 0.2, 1, 2, or 10 μg) or BSA (negative control) prior to addition to the MDCK cells (10^5). The percentage adhesion was determined relative to the attachment of untreated *Leptospira* onto the MDCK cells. (D). Binding of *Leptospira* to immobilize Fn. *Leptospira* (10^8) were cultured in Fn or BSA (negative control) coated (1 mg in 100 μl PBS) or un-coated wells (negative control). (E). Fn inhibited the binding of *Leptospira* to the MDCK cells. *Leptospira* (10^8) were pre-treated with 10 μg of Fn or BSA (negative control) prior to addition to the MDCK cells (10^6). Un-treated *Leptospira* was used as a negative control. The binding of *Leptospira* to ECMs or Fn or the adhesion of *Leptospira* to the MDCK cells was measured by ELISA (A, B, and C) or EPM (D and E). For all experiments, each value represents the mean ± SE of three trials performed in triplicate samples. Statistically significant ($p < 0.05$) differences are indicted by an asterisk. The EPM settings were identical for all captured images (D and E).

(10^5) were incubated with various concentrations (as indicated in Fig. 4A) of GST-LigBCen, GST-LigBCtv or GST (negative control) in 100 μl PBS for 1 h at 37°C. To measure the binding inhibition of *Leptospira* to the MDCK cells treated with LigBCen or LigBCtv, the MDCK cells (10^5) were pretreated with various concentrations (as indicated in Fig. 4B) of GST-LigBCen, GST-LigBCtv or GST (negative control) in 100 μl PBS for 1 h at 37°C. Then, the *Leptospira* (10^7) were added to each well and incubated for 6 h at 37°C. Following the incubation, the plates were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST). To measure the binding of the *Leptospira*, hamster anti-*Leptospira* (1 : 200) and HRP-conjugated goat anti-hamster IgG (1 : 1,000) were used as primary and secondary antibodies, respectively. To detect the binding of GST-

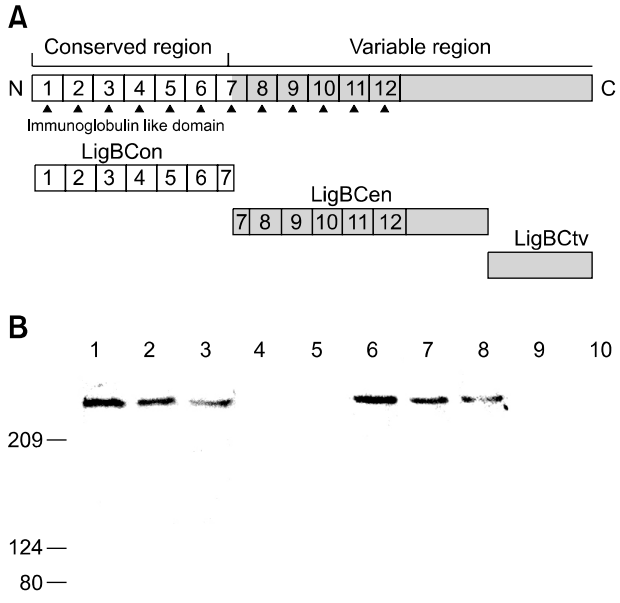

Fig. 2. The interaction between LigB and Fn by the GST-pull down assay (A) A schematic diagram showing the structure of LigB and the truncated LigB protein used in this study. (B). Human plasma Fn (lane 2 to lane 5) or cell lysates of the MDCK cells (lane 7 to lane 10) was applied to the GST beads pre-immobilized by GST, GST-LigBCon, GST-LigBCen, or GST-LigBCtv at 4°C for 3 h. The pull down complex was analyzed by immunoblot analysis using Fn antibodies. Lane 1 and lane 6 contain 1 μg of human plasma Fn and the cell lysate from 1×10^6 MDCK cells, respectively, to serve as a positive reference. Lane 2 and lane 7 are GST-LigBCen, lane 3 and lane 8 are GST-LigBCtv, lane 4 and lane 9 are GST-LigBCon, and lane 5 and lane 10 are GST. The molecular mass of the human Fn and canine Fn (MDCK cells) was 261 kDa and 271 kDa, respectively, and the relative positions of the standards are given in kDa on the left.

LigBCen, GST-LigBCtv, or GST to Fn or the MDCK cells, rabbit anti-GST (1 : 200) and HRP-conjugated goat anti-rabbit IgG (1 : 1,000) were used as primary and secondary antibodies, respectively. After washing the plates three times with PBST, 100 μl of TMB (KPL, USA) was added to each well and incubated for 5 min. The reaction was stopped by adding 100 μl of 0.5% hydrofluoric acid in each well. Each plate was read at 630 nm by an ELISA plate reader (Bioteck EL-312; BioTeck, USA). Each value represents the mean ± standard error of the mean (SEM) of three trials performed in triplicate samples. Statistically significant ($p < 0.05$) differences are indicated by asterisks.

Binding assays by epifluorescence microscopy (EPM) and confocal laser-scanning microscopy (CLSM)

To measure the binding of *Leptospira* to Fn by EPM, *Leptospira* (10^8) were added to each well (eight well culture slides) coated with 1 mg Fn or BSA (negative control) in 100 μl of PBS and incubated at 37°C for 6 h (Fig. 1D). To measure the binding inhibition of *Leptospira* to the MDCK cells by Fn, 10^8 *Leptospira* were pre-incubated with 10 μg of Fn or BSA (negative control) in 100 μl of PBS for 1 h at 37°C prior to the addition of 10^6 MDCK cells and incubated 6 h at 37°C (Fig. 1E). To measure the binding inhibition *Leptospira* to Fn by LigBCen or LigBCtv by EPM, 50 nM of GST-LigBCen, GST-LigBCtv or GST (negative control) in 100 μl PBS was added to each of the Fn or BSA (negative control and data not shown) (1 mg per 100 μl) coated wells for 1 h at 37°C. Then, the *Leptospira* (10^8) were added to each well and incubated for 6 h at 37°C (Fig. 3C). To determine the binding inhibition of *Leptospira* to the MDCK cells by LigBCen or LigBCtv by CLSM, the MDCK cells (10^6) were preincubated with 50 nM of GST-LigBCen, GST-LigBCtv or GST (negative control) in 100 μl of PBS for 1 h at 37°C respectively. Then, the *Leptospira* (10^8) were added to each well and incubated for 6 h at 37°C (Fig. 4C). For the detection of *Leptospira* binding in Figs. 1D, E, and Fig. 3C, hamster anti-*Leptospira* antibodies (1 : 100) and Alexa 488-conjugated goat anti-hamster IgG (1 : 250) were used as primary and secondary antibodies, respectively. To determine the attachment of *Leptospira* and the binding of GST-LigBCen, GST-LigBCtv or GST, Fig. 4C, rabbit anti-GST (1 : 250) and hamster anti-*Leptospira* antibodies (1 : 100) served as primary antibodies, and FITC conjugated goat anti-rabbit IgG (1 : 250) and Alexa 594-conjugated goat anti-hamster IgG (1 : 250) were used as secondary antibodies. Fixation and immunofluorescence staining were performed as previously described [44] with slight modifications. Briefly, *Leptopsira* and the MDCK cells were fixed in 2% paraformaldehyde for 60 min at RT. For the antibody labeling, fixed bacteria were incubated in PBS containing 0.3% BSA for 10 min at RT. The primary and secondary antibodies, in the PBS containing 0.3% BSA, were incubated sequentially for 60 min at RT. After incubation with the primary and secondary antibodies, the glass slides were mounted with coverslips using Prolong Antifade (Molecular Probe, USA) and viewed with a 60 × objective by EPM (Nikon, Japan) or CLSM (Olympus, Japan). An Olympus Fluoview 500 confocal laser-scanning imaging system, equipped with krypton, argon and He-Ne lasers on an Olympus IX70 inverted microscope with a PLAPO 60 × objective, was used. The settings were identical for all captured images. Images were processed using Adobe Photoshop CS2. For counting the attachment of *Leptospira* to the MDCK cells or Fn, three fields were selected to count the number of binding organisms. All studies were repeated three times and the number of *Leptospira* attached to the MDCK cells were counted by an investigator blinded to the treatment group.

GST pulldown assay

The GST pull-down assay was performed as previously described [57]. Purified proteins or GST (negative control) were loaded onto 0.5 ml glutathione-Sepharose beads

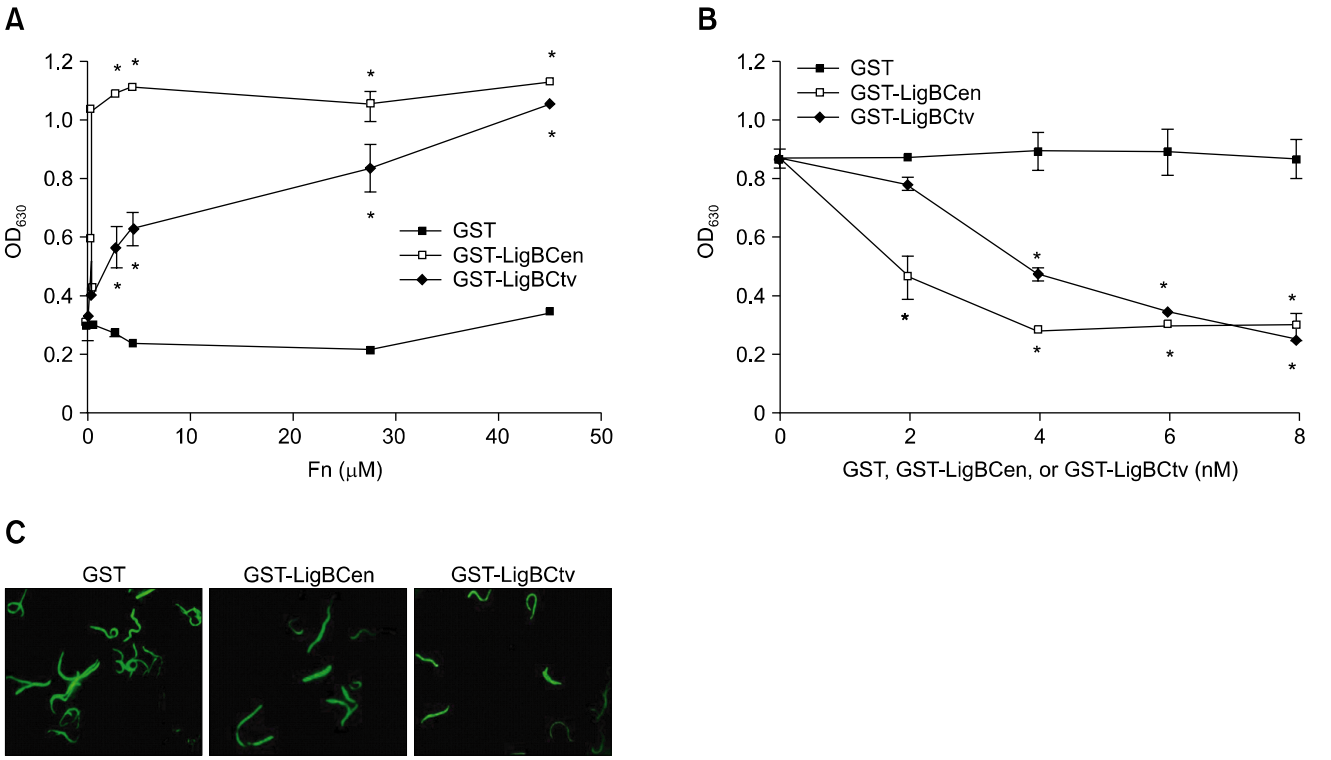

Fig. 3. LigBCen or LigBCtv binds to Fn and inhibits the binding of *Leptospira* to Fn (A). Binding of LigBCen or LigBCtv to various concentrations of immobilized Fn. Ten nM of GST-LigBCen, GST-LigBCtv or GST (negative control) was added to wells coated with various concentrations of Fn (0, 0.27 μM, 0.45 μM, 2.7 μM, 4.5 μM, 27 μM, or 45 μM) in 100 μl PBS. The binding of each of these proteins to Fn was measured by ELISA. (B) LigBCen or LigBCtv inhibited the binding of *Leptospira* to immobilized Fn. Various concentrations (0, 2, 4, 6, or 8 nM) of GST-LigBCen, GST-LigBCtv, or GST (negative control) were added to each well coated with Fn (1 mg in 100 μl PBS) prior to the addition of *Leptospira* (10^7). The attachment of *Leptopsira* to wells was measured by ELISA. The percentage of attachment was determined relative to the attachment of *Leptopsira* in the untreated Fn. (C) LigBCen or LigBCtv inhibited the binding of *Leptospira* to Fn. Fifty nM of GST-LigBCen, GST-LigBCtv or GST (negative control) was added to wells coated with Fn (1 mg in 100 μl PBS) prior to the addition of *Leptospira* (10^8). The binding of *Leptospira* to wells was detected by EPM. In (A) and (B), each value represents the mean ± SE of three trials performed in triplicate samples. Statistically significant differences ($p < 0.05$) are indicted by *. In (C), The EPM settings were identical for all captured images. Images were processed using Adobe Photoshop CS2.

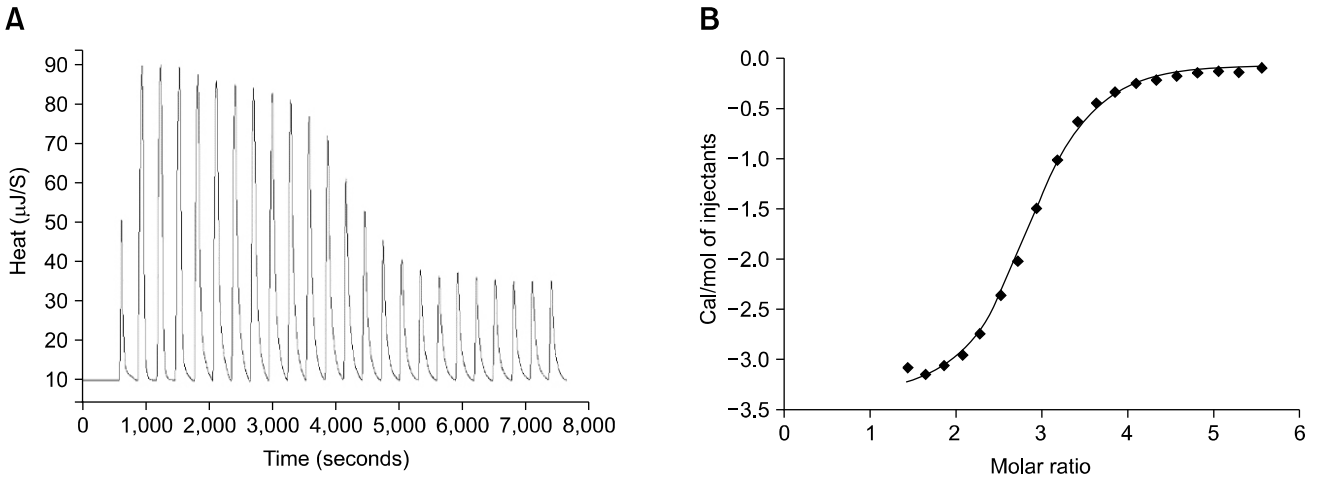

Fig. 4. Isothermal titration calorimetry (ITC) profile of LigBCtv with Fn as a typical ITC profile in this studyA: heat differences obtained from 25 injections. B: Integrated curve with experimental point (◆) and the best fit (—). The thermodynamic parameters are shown in Table 1.

(Amersham Biosciences Piscataway, USA) at 4°C overnight. The beads were then washed three times with the lysis buffer containing 30 mM Tris acetate, 10 mM sodium phosphate, pH 7.4, 0.1% Tween 20, 1 mM EDTA, 2 μg/ml leupeptin, 4 μg/ml aprotinin, 1 μg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The MDCK cells (10^6) were lysed in the lysis buffer and used immediately after lysis. A 500 μl aliquot of cell lysate or human plasma Fn (40 μg/ml) was incubated with purified proteins immobilized on glutathione-Sepharose beads at 4°C for 3 h. After incubation, the beads were separated by centrifugation, washed three times with the lysis buffer and boiled in Laemmli sample loading buffer consisting of 50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% sodium dodecyl sulfate, 0.25 mM PMSF, and 0.1% bromophenol blue in 20% glycerol. The eluted proteins were subjected to 6% SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes. The membranes were incubated in 5% skim milk in PBS/T overnight and then incubated with mouse anti-Fn antibody (1 : 1,000). The immunocomplexes were detected with an HRP-conjugated goat anti-mouse IgG antibody (1 : 5,000).

Small interfering RNA (siRNA) inhibition of LigB binding

The siRNA duplexes directed against the sequence 5'-gcagcacaacuuccaauua-3' of Fn and negative siRNA duplex, 5'-auucuaucacuagcgugac-3', were selected by the software, siDESIGN [43] and synthesized by Dharmacon (USA). The RNA duplexes were introduced into the MDCK cells by the method of lipofection [18], and 8×10^5 cells were transfected with 0.4 μg negative siRNA and Fn-siRNA. Adhesion assays were performed 72 h after lipofection [51]. The knockdown efficiency of endogeneous Fn expression was determined as previously described [57] with slight modification. The total protein contents of the MDCK cells (10^6) were analyzed using Western immunoblotting as described under 'GST pulldown assays'. The protein bands of actin derived from the MDCK cells were measured as a control using a mouse anti-actin antibody (1 : 5,000). The band intensity was measured by densitometry using the Image J software (National Institutes of Health, Bethesda, MD, USA) [53]. A LigB binding assay was performed 72 h after lipofection. To determine the binding of LigB fragments to Fn, each fragment (50 nM) was added to the MDCK cells (10^6) transfected with Fn or negative siRNA. To determine the binding of each fragment and the expression of Fn in the MDCK cells, rabbit anti-GST (1 : 250) and mouse anti-Fn (1 : 250) served as the primary antibodies, and FITC-conjugated goat anti-mouse IgG (1 : 250) and Texas Red-conjugated goat anti-rabbit IgG (1 : 250) were used as secondary antibodies. Fixation, immunofluorescence staining, image detection, and processing were carried out as described in previous sections. All experiments were performed in triplicate.

Isothermal titration calorimetry

The experiments were carried out with CSC 5300 microcalorimeter (Calorimetry Science, USA) at 25°C as previously described [47]. In a typical experiment, the cell contained 1 ml of a solution of proteins, and the syringe contained 250 μl of a solution of Fn at a concentration that was 20 times higher than the protein concentration in the cell. Both solutions were in PBS pH 7.5. The titration was performed as follows: 15 to 25 injections of 10 μl (Table 1) with a stirring speed of 250 rpm, and the delay time between the injections was 5 min. Data were analyzed using Titration BindingWork 3.1 software (Calorimetry Science, USA) that was fit to an independent binding model. The concentration of Fn and LigB used in this study was based on our preliminary titration experiments (data not shown).

Statistical analysis

Statistically significant differences between samples were determined using the Student's t-test following logarithmic transformation of the data. Two-tailed *p*-values were determined for each sample, and a $p < 0.05$ was considered significant. Each data point represents the mean ± SE of a sample tested in triplicate. An asterisk indicates that the result was statistically significant.

Results

Attachment of *Leptospira* to the MDCK cells was mediated by fibronectin

The binding of leptospiral cells to various ECM compo-

Table 1. Thermodynamic parameters for the interaction of Fn and truncated LigB

Macromolecule	LigB Residues	[Macromolecule]	[Fn]	ΔH	ΔS	K_d
		μM	μM	kcal mol^{-1}	cal mol^{-1} K^{-1}	μM
LigBCon	1-630	1.25	25	n/f*	n/f*	n/f*
LigBCen	631-1,417	2	40	−2,002.67 ± 14	−6.68	0.011 ± 0.003
LigBCtv	1,418-1,889	2.82	56.4	−12,140 ± 557	−40.71	8.55 ± 0.75

*n/f: non-fittable.

nents was determined by ELISA. As shown in Fig. 1A, *Leptospira* were strongly bound to Fn, but not to other ECM molecules (Fig. 1A). Furthermore, the binding of *Leptospira* to Fn was dose dependent (Fig. 1B). When *Leptospira* were pretreated with Fn, binding to the MDCK cells was decreased (Fig. 1C). There was an approximately 3.5-fold increase in the immobilization of *Leptospira* in the Fn-coated wells compared to the controls (Fig. 1D). Moreover, Fn was observed to block the attachment of *Leptospira*, by approximately 47%, when the Fn treated *Leptospira* were added to the MDCK cells (Fig. 1E). Thus, Fn appears to mediate the attachment of *Leptospira* to the MDCK cells.

Interaction between LigB and Fn

To determine whether LigB interacts with Fn, we truncated the LigB protein into three parts, LigBCon, LigBCen and LigBCtv, (Fig. 2A) due to the difficulty of expressing and purifying the full length LigB [33]. First, we analyzed the interaction of each LigB fragment with Fn using a GST-pull down assay. Our results showed that both human plasma Fn and Fn derived from the MDCK cell lysates could bind both LigBCen and LigBCtv, but not LigBCon (Figs. 2B and C). Since LigBCen and LigBCtv showed a positive pull down result, the interaction between LigBCen and LigBCtv with Fn was further studied by ELISA. We found that both the binding of LigBCen and LigBCtv to Fn,

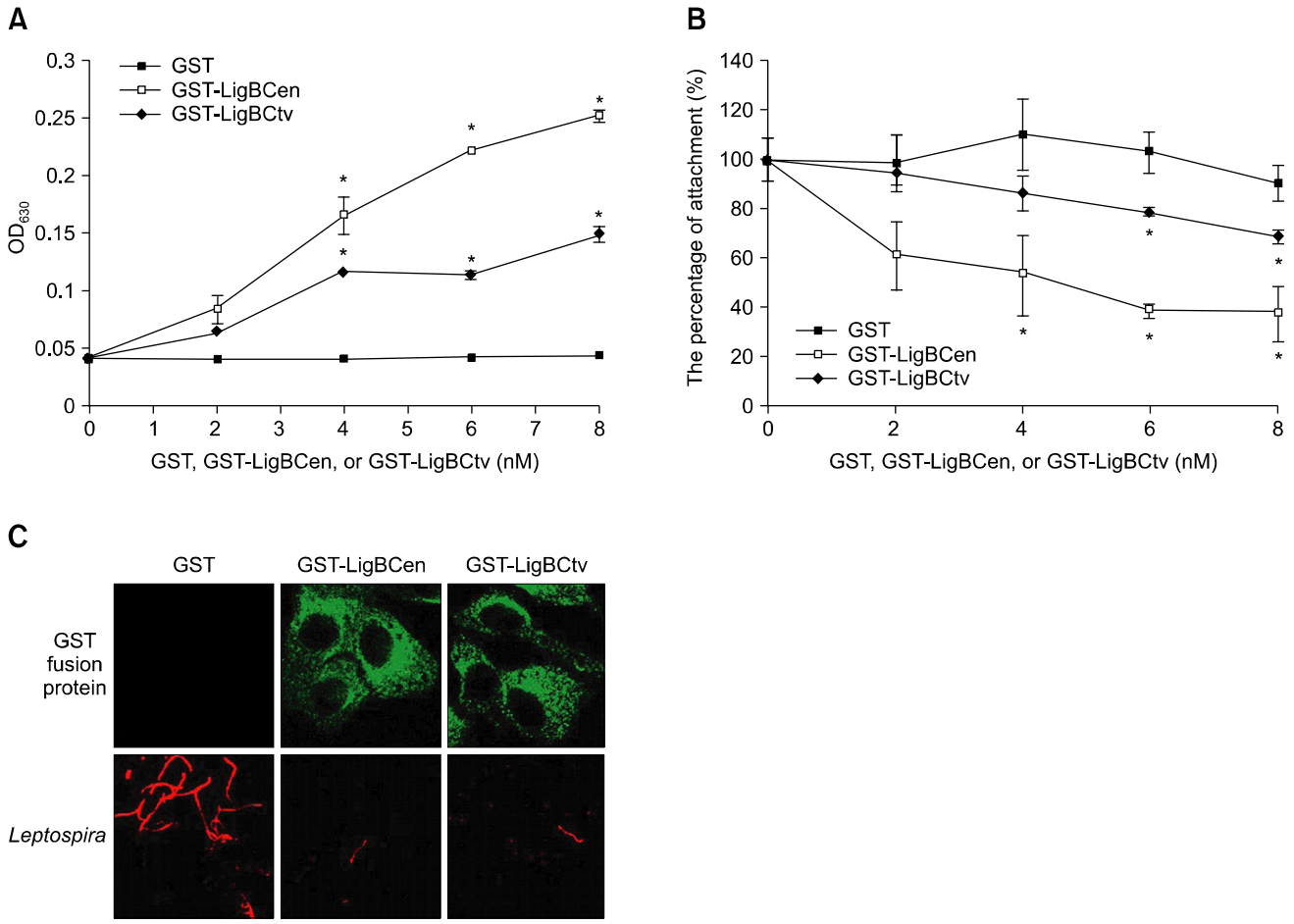

Fig. 5. The binding of LigBCen or LigBCtv to the MDCK cells reduced leptospiral adhesion (A) Binding of LigBCen or LigBCtv to the MDCK cells. Various concentrations (0, 2, 4, 6, or 8 nM) of GST-LigBCen, GST-LigBCtv or GST (negative control) was added to the MDCK cells (10^5). The binding of each of these proteins to the MDCK cells were measured by ELISA. (B) LigBCen or LigBCtv inhibits the binding of *Leptopsira* to MDCK cells. The MDCK cells were incubated with various concentrations (0, 2, 4, 6, or 8 nM) of GST-LigBCen, GST-LigBCtv or GST (negative control) prior to the addition of *Leptopsira* (10^7). The adhesion of *Leptospira* to the MDCK cells (105) was detected by ELISA. The reduced percentage of attachment was determined relative to the attachment of *Leptopsira* in the untreated MDCK cells. (C). LigBCen or LigBCtv inhibited the binding of *Leptopsira* to the MDCK cells. The MDCK cells (10^6) were pre-treated with 50nM of GST-LigBCen, GST-LigBCtv and GST (negative control) prior to the addition of the *Leptopsira* (10^8). The adhesion of *Leptospira* or the binding of these proteins to the MDCK cells were detected by CLSM. In (A) and (B), each value represents the mean± SEM of three trials in triplicate samples. Statistically significant values ($p < 0.05$) are indicted by *. In (C), the CLSM settings were identical for all the captured images. Images were processed using Adobe Photoshop CS2.

and the inhibition of the attachment of *Leptospira* to Fn by LigBCen and LigBCtv, were dose-dependent (Figs. 3A and B). Moreover, the EPM images revealed an up to 40% reduction in the attachment of *Leptospira* to Fn in the presence of LigBCen and LigBCtv (Fig. 3C). Finally, in order to quantitatively evaluate the binding affinity between Fn and LigB fragments, the dissociation constants (K_d) were measured by ITC (Table 1). Fig. 4 shows the data from a typical ITC experiment. The interaction appears to be exothermic with a favorable enthalpy and unfavorable entropy. The calculated K_d values for Fn binding to LigBCen and LigBCtv were 0.01 μM and 8.55 μM, respectively (Table 1). The binding of LigBCon could not be detected by ITC (data not shown). These findings are in agreement with our previous results. Altogether, these data indicate that Fn specifically interacts with LigBCen and LigBCtv fragments.

LigBCen and LigBCtv mediate the attachment of *Leptospira* to the MDCK cells

To determine if LigB is used by *Leptospira* to adhere to the MDCK cells, various concentrations of LigBCen or LigBCtv were added to the MDCK cells, and binding was detected by ELISA and immunofluorescence staining. Our results clearly showed that LigBCen and LigBCtv were bound to the MDCK cells in a dose dependent manner (Fig. 5A). Pretreatment of the MDCK cells with LigBCen or LigBCtv reduced the attachment of *Leptospira* by ~32%. The reduction of *Leptospira* attachment was also dose-dependent (Figs. 5B and C). We further elucidated the receptor role of Fn in the MDCK cells for its possible ligand, LigB on the surface of *Leptospira*, by RNA interference to decrease the endogeneous Fn expression in the MDCK cells. As shown in Fig. 6A, transfection of the cells with siRNA duplex specific for canine Fn resulted in a ~36% reduction of the Fn expression, relative to the control cells. The binding of LigBCen and LigBCtv to Fn siRNA-transfected MDCK cells was significantly reduced (Figs. 6B and C). These results suggest that Fn serves as a receptor for LigB that mediates *Leptospira* adhesion.

Discussion

Adhesion to host cells is pivotal for many pathogenic bacteria including *Leptospira* spp. Since pathogenic *Leptospira* spp. can infect a variety of tissues including liver, kidney and lung, study of the host-pathogen interaction is extremely important for improved understanding of leptospirosis. Recently, the leptospiral genome has been sequenced and a number of tentative virulence factors have been proposed [3,31,42]. However, their exact roles in leptospiral pathogenesis remain to be established. To date, several leptospiral adhesion molecules have been identified. These include a 36 kDa Fn-binding protein [30], a 24

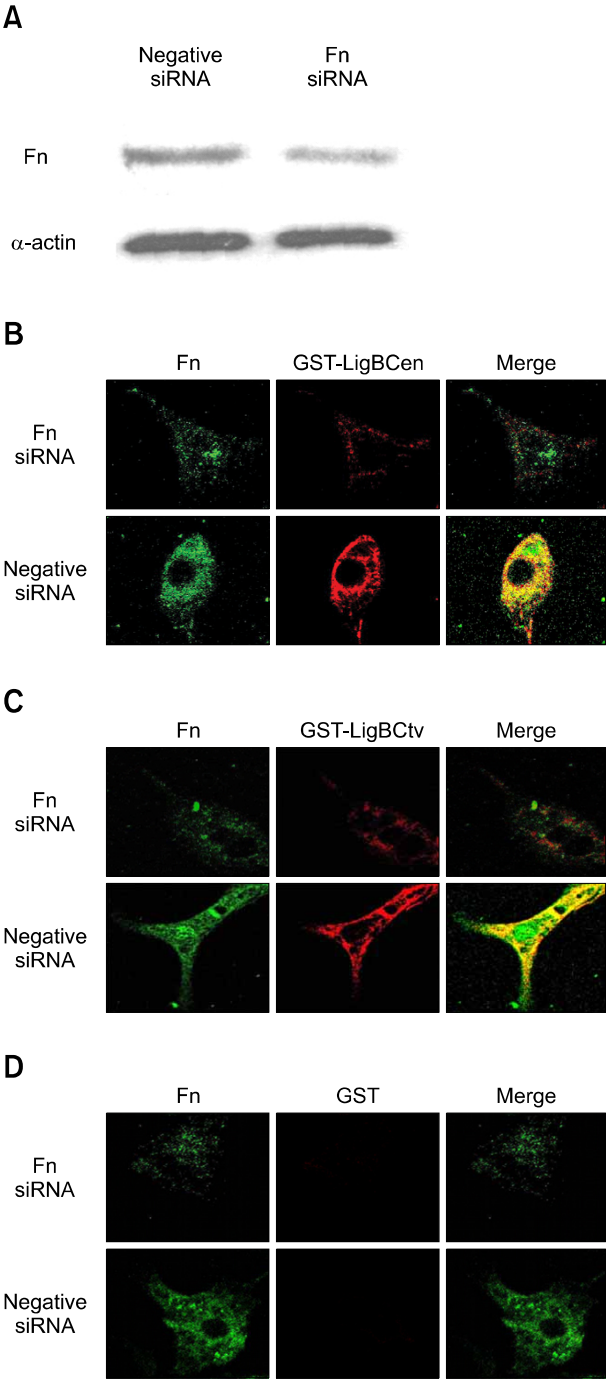

Fig. 6. The binding of LigBCen or LigBCtv to Fn siRNA transfected MDCK cells was reduced (A). Detection of the expression of Fn and actin in the MDCK cells 72 h after transfected by Fn or negative siRNA. Fn and α-actin were detected by immunoblotting probed by actin antibody or Fn antibody. (B) Binding of GST-LigBCen or (C) GST-LigBCtv was reduced by the siRNA transfected cells. (D) GST served as a negative control. Fifty nM of GST-LigBCen, GST-LigBCtv or GST was added to Fn or the negative siRNA transfected MDCK cells. Expression of Fn and the binding of these proteins to the MDCK cells were detected by CLSM. The CLSM settings were identical for all the captured images. Images were processed using Adobe Photoshop CS2.

kDa laminin-binding protein [1] and LigA, LigB and LigC proteins [25,33,34]. These molecules may play an important role in the pathogenesis of leptospiral infection since they are able to bind to ECMs such as collagens I and IV, laminin and fibronectin [6,24].

Pathogenic *Leptospira* spp. have been previously reported to adhere to extracellular matrices [15,16] including Fn. Fns are dimers of two similar peptides linked at their C-termini by two disulfide bonds [8] and serve as receptors for several bacteria, including spirochetes [7,11,12,19,20, 23,28,32,38,40,46,50,55]. Our results showed that Fn immobilized *Leptospira*. In addition, Fn was observed to block the attachment of *Leptospira* to MDCK cells if the *Leptospira* were pre-treated with Fn. These results support the recent report that Fn might be an important molecule involved in the pathogenic adherence of *Leptospira* spp. to host cells [6,24].

We demonstrated the interaction between LigB and Fn. It was shown that the LigBCen and LigBCtv fragments were bound to Fn, by GST-pulldown assays, ELISA and ITC measurements. The low K_d values for LigBCen indicated that the LigB-Fn interaction was specific. This evidence strongly suggests that LigB is a Fn-binding protein. A study reported by Choy *et al.* [6] showed that LigB U1 and LigB U2 (LigBCen equivalent) could strongly bind to Fn, while the LigB CTD (LigBCtv equivalent) binds weakly to Fn. However, the Kd values of LigBCen and LigBCtv to Fn that we obtained were slightly different than those reported by Choy *et al.* [6]. The differences in the obtained K_d values could be explained by (i) the protein fragments evaluated in this study (LigBCen and LigBCtv) were not exactly the same length fragments (LigBU1, LigBU2 and LigBCTD) and (ii) the method we used (ITC) to measure the K_d differed from that of Choy *et al.* [6].

Since pathogenic *Leptospira* spp. adheres to renal tubular epithelial cells and induces a severe tubulointerstitial nephritis leading to renal failure [58], it is possible that LigB is responsible for the binding of *Leptospira* to the renal tubular epithelium. Our results indicated that LigB binds to the MDCK cells via the LigBCen or the LigBCtv fragments. However, the LigBCen was observed to bind to both the MDCK cells and Fn with a greater affinity than the LigBCtv. The microscopic images also showed that not all of the Fn was co-localized with the LigB. This result suggests that LigB might bind to two or more receptors. Our results elucidate the process of *Leptospira* attachment to the MDCK cells, as noted in a previous study [52], and demonstrated how Fn can block leptospiral attachment to the MDCK cells.

Our results clearly confirm that LigB is one of the microbial surface components that recognize adhesive matrix molecules (MSCRAMM) members that bind to the ECM including Fn. The transmembrane domain of LigB is predicted to reside within the conserved region, with only the variable region exposed on the surface [33,34]. These results support our data that Fn-binding domains of LigB are localized in the variable regions. This is not surprising since similar findings have been reported for other MSCRAMMs [13,37,39]. In *Borrelia*, the binding motifs in the decorin-binding proteins, DbpA and B, are located in the central regions, which vary among the different *Borrelia* strains (*B. burgdorferi, B. garnii,* and *B. afzeli*) [37]. The Fn-binding domain of the Fn-binding protein, BBK32 is also variable among the different *Borrelia* strains [39]. The repetitive D1, D2 and D3 elements of *Staphylococcus aureus* Fn-binding protein, which bind the N-terminal 29 kDa of Fn, also vary [13].

Since both LigBCen and LigBCtv bind to Fn, but with different affinities, this suggests that there is more than one potential Fn-binding domain. In *Mycobacterium avium*, two Fn-binding domains are located on two non-contiguous segments of 24 amino acids in the Fn attachment protein-A [45]. The FnBPA of *Staphylococcus aureus* contains three repetitive elements, D1, D2 and D3 and each binds the N-terminal 29 kDa fragment of Fn [13]. Seven additional Fn-binding elements are located in the N-terminal of the D repeats [48]. In *Streptococcus dysgalactiae*, there are five Fn-binding segments within the C-terminus of the Fn binding protein F1/(FnBB) [47,48]. Therefore, it is likely that several binding sites might be present in the LigB variable region. However, we were unable to identify a similar Fn-binding motif in the other known Fn-binding proteins.

In conclusion, we have shown that LigBCen and LigBCtv bind to Fn and have confirmed that LigB is a member of the MSCRAMMs. Since pathogenic *Leptospira* spp. initially attaches to mucosal epithelial cells prior to entry into the bloodstream and subsequent dissemination to multiple organs such as the kidney, liver and lung, Lig proteins may play a pivotal role in the pathogenesis of leptospirosis. Fn is one of the most important ECMs on epithelial cells and serves as a receptor for leptospiral adherence [6,15,24]. Thus, further studies into the interaction of Lig proteins and ECMs are warranted.

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