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A novel fibronectin type III module binding motif identified on C-terminus of *Leptospira* immunoglobulin-like protein, LigB

Yi-Pin Lin^a, Alex Greenwood^c, WeiWei Yan^a, Linda K. Nicholson^c, Yogendra Sharma^d, Sean P. McDonough^b, and Yung-Fu Chang^{a,*}

^a Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA

^b Department of Biomedical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA

^c Department of Molecular Biology and Genetics, College of Agriculture and Life Science, Cornell University, Ithaca, NY 14853, USA

^d Center for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India

Abstract

Infection by pathogenic strains of *Leptospira* hinges on the pathogen's ability to adhere to host cells via extracellular matrix such as fibronectin (Fn). Previously, the immunoglobulin-like domains of *Leptospira* Lig proteins were recognized as adhesins binding to N-terminal domain (NTD) and gelatin binding domain (GBD) of Fn. In this study, we identified another Fn-binding motif on the C-terminus of the *Leptospira* adhesin LigB (LigBCtv), residues 1708–1712 containing sequence LIPAD with a β -strand and nascent helical structure. This motif binds to 15th type III modules (15F₃) ($K_D = 10.70 \mu\text{M}$), and association ($k_{on} = 600 \text{ M}^{-1} \text{ s}^{-1}$) and dissociation ($k_{off} = 0.0129 \text{ s}^{-1}$) rate constants represents a slow binding kinetics in this interaction. Moreover, pretreatment of MDCK cells with LigB_{1706–1716} blocked the binding of *Leptospira* by 39%, demonstrating a significant role of LigB_{1706–1716} in cellular adhesion. These data indicate that the LIPAD residues (LigB_{1708–1712}) of the *Leptospira interrogans* LigB protein bind 15F₃ of Fn at a novel binding site, and this interaction contributes to adhesion to host cells.

Keywords

Leptospira interrogans; Fibronectin; Type III modules; LigB

Introduction

Leptospirosis, caused by infection with pathogenic spirochete *Leptospira* spp., is a serious zoonosis with world-wide distribution [1]. These spirochetes usually penetrate through mucosal surfaces and/or skin wounds and disseminate to several organs, especially the kidney, liver, and lungs. Infection may lead to Weil's disease, which results in liver failure (jaundice), renal failure (nephritis), pulmonary hemorrhage and/or meningitis [1]. Human leptospirosis is often waterborne, and most commonly occurs in tropical areas of the world [1] and reemerging in the United States [2].

*Corresponding author. Fax: +1 607 253 3943. yc42@cornell.edu (Y.-F. Chang).

Many microbial pathogens produce Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM) to facilitate their colonization of host tissues during initial infection [3]. Pathogenic leptospires bind to mammalian cells, such as MDCK cells often via the extracellular matrix (ECM) and several adhesion molecules have been identified from *Leptospira* spp. [4–6].

Leptospiral immunoglobulin-like proteins, LigA and LigB, possess 12 and 13 immunoglobulin-like (Ig-like) domains are recognized as MSCRAMMs [7,8]. The binding of LigB to Fn is also modulated by calcium [9]. These studies indicate that Lig proteins are pivotal virulence factors of pathogenic *Leptospira* spp.

In previous studies, strong and mild affinity Fn-binding sites were located on LigB within Ig-like domains of LigB (LigBCen) and the C-terminal variable (LigBCtv) regions, respectively (Fig. 1A) [4,5]. Here, we have further localized the Fn-binding motif of LigBCtv to amino acids (AA) 1708–1712, this sequence binds to the 15F₃. This significant inhibition of cell binding by this short peptide demonstrates the importance of this sequence in adhesion of the pathogen to target tissues.

Materials and methods

Bacterial strains and cell culture

Leptospira interrogans serovar Pomona (NVSL1427-35-093002) was used as previously described [10]. Leptospires were grown in EMJH medium at 30 °C for less than five passages; growth was monitored by dark-field microscopy [10]. Madin–Darby canine kidney (MDCK) cells (ATCC CCL34™) were cultured in Dulbecco's minimum essential medium (DMEM) containing 10% fetal bovine serum (GIBCO Laboratories, Grand Island, NY). Cells were grown at 37 °C in a humidified atmosphere with 5% CO₂.

Reagents and antibodies

Horseradish peroxidase (HRP)-conjugated streptavidin or goat anti-hamster antibody was purchased from Zymed (San Diego, CA) or Biosource (Camarillo, CA), respectively. FITC-conjugated streptavidin was purchased from Molecular Probes (Eugene, OR). Cell binding domain (CBD, 120 kDa) or 40 kDa domain (40 kDa) of Fn were purchased from Chemicon International (Temecula, CA). Anti-*L. interrogans* antibodies were previously prepared in hamsters from the challenge controls [10]. Full length human plasma Fn, N-terminal domain (NTD) or gelatin binding domain (GBD) of Fn were purchased from Sigma (St. Louis, MO), and Biotin labeling kit were purchased from Pierce (Rockford, IL).

Plasmid construction and protein purification

Rat 12th–13th, 14th, or 15th type III modules were purified as maltose binding protein (MBP) fusion proteins (Fig. 3A) [11]. The DNA fragments of LigBCon and BBK32 (AA 56–205) were inserted into pGEX-4T-2 (GE, Piscataway, NJ) [10]. LigBCtv was analyzed with Jpred to predict the secondary structure for truncation construction. Constructs for the expression of GST fused with LigBCtv1 (AA 1418–1632), LigBCtv2 (AA 1633–1889), LigBCtv3 (AA 1633–1723) and LigBCtv4 (AA 1724–1889) were generated using the vector pGEX-4T-2 (Fig. 1A). Relevant fragments of DNA were amplified by PCR using primers (Supplemental Table 1) based on the *ligB* sequence [7]. Primers were engineered to introduce a BamHI or SalI site at the 5' end of each fragment and a stop codon followed by a SalI or NotI site at the 3' end of each fragment. PCR products were digested sequentially with Bam-HI/SalI or SalI/NotI for the indicated fragment and then ligated into pGEX-4T-2. In this study, we purified the soluble form of the four GST fusion peptides from *Escherichia coli* as previously described [7].

Binding assays by ELISA

To determine the binding of truncated LigB (LigBCtv1, LigBCtv2, LigBCtv3, or LigBCtv4) or LigB peptides to Fn, 1 μ g of Fn or BSA (negative control) was coated onto microtiter plate wells described previously [4,5]. Then, various concentrations (as indicated in Figs. 1 and 3) of biotinylated LigB truncated protein, LigB peptide (Figs. 1B, C, and E and 3), scrambled peptide (negative control, Supplemental Table 2), BBK32 (56–205) (positive control, [12] or LigBCon (negative control, [5]) was added to each microtiter plate wells for 1 h at 37 °C. To measure the binding of biotinylated LigB protein, HRP-conjugated streptavidin was added into microtiter plate wells (1:1000).

To identify the binding site of LigB_{1706–1716} on Fn, 1 μ g of each full length Fn, NTD, GBD, CBD, 40 kDa domain of Fn, or MBP fused with 12th–13th type III modules (MBP-12–13F₃), 14th type III modules (MBP-14F₃), or 15th type III modules (MBP-15F₃) of Fn and MBP (negative control) were coated onto microtiter plate wells. Then, various concentrations (as indicated in Fig. 3B and C) of biotin-conjugated LigB_{1706–1716} (biotin-LigB_{1706–1716}) or biotin (negative control, data not shown) were added to microtiter plate wells. To measure the binding of LigB_{1706–1716} to MDCK cells, MDCK cells (10⁵) were incubated with various concentrations (as indicated in Fig. 4A) of biotin-LigB_{1706–1716} or biotin (negative control) in 100 μ l PBS for 1 h at 37 °C. To measure the binding inhibition of *Leptospira* to MDCK cells treated with LigB_{1706–1716}, MDCK cells (10⁵) were pretreated with various concentrations (as indicated in Fig. 4B) of biotin-LigB_{1706–1716} or biotin (negative control) in 100 μ l PBS for 1 h at 37 °C. Then, the following steps such as the addition of *Leptospira*, plate washing, and the incubation and usage of antibodies were described previously [5]. To detect the binding of biotin-LigB_{1706–1716} or biotin to Fn or MDCK cells, HRP-conjugated streptavidin (1:1000) was added and incubated for 30 min at 25 °C. The measurement of the binding by ELISA was described previously [5].

NMR sample preparation and experiments

2.51 mM of cysteine conjugated LigB_{1703–1717} in PBS (pH 6.0) in either H₂O or D₂O was applied to NMR. NMR spectra were recorded at 25 °C on a Varian Inova 600 MHz spectrometer using the States-Haberkorn hyper-complex method of frequency discrimination [12]. Two-dimensional homonuclear total correlation spectroscopy (TOCSY) [13] and nuclear Overhauser enhancement spectroscopy (NOESY) [14] spectra were recorded with spectral widths of 6 kHz in *t*₁ (1024 real + imaginary data points in the TOCSY and H₂O NOESY experiments, 800 real + imaginary in the D₂O NOESY experiment) and 8 kHz in *t*₂ (2048 real + imaginary data points). Two-dimensional TOCSY spectra were acquired with a DIPSI-2 [15] isotropic mixing period (*t*_{mix}) of 55 ms, and NOESY spectra were acquired with a *t*_{mix} of 200 ms. For both experiments, the spectra were processed to a final data size of 1024 by 2048 real points.

NMR data were processed and analyzed using the software tools nmrPipe and nmrDraw [16]. Solvent subtraction was obtained by subtraction of a fourth-order polynomial fit in the time domain. The free induction decay (FID) was generally multiplied by a 72° shifted squared sine-bell function before zero-fitting and Fourier transformation. Linear baseline correction was applied in the acquisition dimension of all spectra. Analysis of the resulting TOCSY and NOESY spectra yielded proton resonance assignments for all 16 LigB-derived residues (Supplemental Table 3). Cys1 and Thr2 lack δ_{HN} assignments because of fast proton exchange of the N-terminal amino group.

Fluorescence spectrometry

Intrinsic fluorescence emission spectra were measured on a Hitachi F4500 spectrofluorometer (Hitachi, San Jose, CA). All conditions of recorded spectra were described previously [6]. For

LigB_{1706–1716} titration, various concentrations of LigB_{1706–1716} (as indicated in Fig. 3D) was mixed with 1 μ M of 15F₃ of Fn and spectra were recorded after 5 min. To determine the dissociation constant (K_D), the fluorescence intensities at 350 nm were recorded and fitted by using KaleidaGraph software (Version 2.1.3 Abelbeck software). All of the measurements were corrected for dilution and for inner filter effect.

Stopped-flow measurements

Stopped-flow measurements were performed using a KinTek stopped flow system (Austin, TX). Trp fluorescence emission above 320 nm was monitored using an excitation wavelength of 295 nm and a 320 nm cut-off filter at 25 °C. The stopped-flow experiments were performed by mixing Fn 15F₃ in PBS after mixing with increased concentrations of LigB_{1706–1716}; the time course of fluorescence intensity change was recorded by 1000 pairs of data in each experiment, and sets of data from three experiments were averaged. The apparent observed rate (k_{obs}) was obtained by fitting the stopped-flow trace (average of five repeated runs) with a single exponential equation as shown follows.

$$F_t = A \exp(-k_{obs}t) + F_{\infty} \quad (1)$$

where F_t is the fluorescence observed at any time, t , and A is the signal amplitude. F_{∞} is the final value of fluorescence, and k_{obs} is the observed first order rate constant. The residuals were measured by the differences between the calculated fit and the exponential data. To calculate k_{on} and k_{off} of the binding reaction, the concentrations of LigB_{1706–1716} were at least 25-fold higher than that of 15F₃ to fulfill the requirement of pseudo-first order so k_{obs} obtained from different concentrations of LigB_{1706–1716} could fit the following equation to determine the value of k_{on} and k_{off} .

$$k_{obs} = k_{on}[\text{LigB}_{1706-1716}] + k_{off} \quad (2)$$

Binding assays by confocal laser-scanning microscopy (CLSM)

MDCK cells (10^6) were preincubated with 10 μ M of biotin-LigB_{1706–1716} or biotin (negative control) as previously described [5]. Then, 150 μ l of FITC-conjugated streptavidin was added (1:250). Fixation and immunofluorescence staining were followed as previously described [5]. Three fields were selected to count the number of binding organisms counted by an investigator blinded to the treatment group. To measure the reduced attachment of *Leptospira*. All studies were repeated three times.

Statistical analysis

Significance between samples was 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 -value < 0.05 was considered significant. Each data point represents the mean $\pm$ standard error of the mean (SEM) of sample tested in triplicate. An (*) indicates the result was statistically significant.

Results

LigB residues 1708–1712 (LIPAD) possess Fn-binding activity

To identify the Fn-binding region, truncated LigBCtv including LigBCtv1, LigBCtv2, LigBCtv3, and LigBCtv4 (Fig. 1A) were assayed for Fn-binding capability using ELISA. Only

LigBCtv2 and LigBCtv3 bound Fn (Fig. 1B). The Fn-binding region was more finely mapped to LigB residues 1688–1723 using synthesized peptides corresponding to the LigBCtv sequence (Fig. 1C). Then, peptides corresponding to AA 1688–1723 were also synthesized with overlapping regions of five amino acids (Fig. 1D and Supplemental Table 2). Eventually, LigB_{1706–1716} displayed the highest Fn-binding activity, while both LigB_{1700–1710} and LigB_{1711–1720} had moderate Fn-binding affinities (Fig. 1D). Based on the alignment of these three peptides, the residues LIPAD (LigB_{1708–1712}) of LigBCtv were found to be essential for Fn binding (Fig. 1E).

β-Strand and nascent helical character are in the peptide containing the LIPAD motif

To reveal the structure of this Fn-binding motif, LigB_{1703–1717}, the peptide containing LIPAD residues, was investigated with NMR. As shown in Fig. 2A, nuclear Overhauser effect (NOE) cross-peaks between successive amide protons, or $d_{NN}(i, i + 1)$ NOEs, were observed for residues A₁₇₁₁D₁₇₁₂K₁₇₁₃A₁₇₁₄F₁₇₁₅ in the NOESY spectra of LigB_{1703–1717}. An additional cross-peak between the amide protons of D₁₇₁₂ and A₁₇₁₄, or $d_{NN}(i, i + 2)$ NOE, was also observed. This pattern of local NOEs is indicative of nascent helical structure, defined as an equilibrium between a series of turn-like structures and unfolded states [17]. Furthermore, the secondary structure of peptides can be elucidated by $\Delta\delta_{H\alpha}$ difference between an observed alpha protons chemical shift and the corresponding random coil value for a given amino acid residue [18]. As shown in Fig. 2B, $\Delta\delta_{H\alpha}$ values greater than +0.1 for sequential residues L₁₇₀₈ and I₁₇₀₉ (+0.19 and +0.54, respectively), indicating that this segment of the peptide may transiently adopt a β-strand conformation. This region is followed by a nascent helical region for residues A₁₇₁₁D₁₇₁₂K₁₇₁₃A₁₇₁₄ consistent with the nascent helical character deduced from the observed NOEs involving these residues as described above. The low value for cysteine, the first amino acid in the peptide (conjugated for mouse immunization) is most likely due to its position near the positive N terminus.

The LigB LIPAD motif binds to the 15th type III module of Fn with slow binding kinetics

ELISA was also performed with biotin-LigB_{1706–1716} and full length or truncated Fn including NTD, GBD, CBD, and 40 kDa to identify the LIPAD-binding site on Fn. The results show that LigB_{1706–1716} binds to Fn and 40 kDa (Fig. 3B). Then, the ELISA applied to biotin-LigB_{1706–1716} with 40 kDa and MBP-12–13F₃, MBP-14F₃, MBP-15F₃ further locate the LIPAD-binding site on 15F₃ (Fig. 3C) in agreement with the quenching of the intrinsic Trp fluorescence intensity of 15F₃ in the presence of LigB_{1706–1716} ($K_D = 10.70 \pm 2.23 \mu\text{M}$) (Fig. 3D). The kinetic rates of LigB_{1706–1716}-15F₃ determined by stop flow experiment ($k_{on} = 600 \pm 12 \text{ M}^{-1} \text{ s}^{-1}$ and $k_{off} = 0.0129 \pm 0.0003 \text{ s}^{-1}$) are comparatively slow (Fig. 3E). These results indicate that the LIPAD motif binds to the 15F₃ with a slow binding kinetics.

LigB_{1708–1712} LIPAD residues mediate the attachment of *Leptospira* to MDCK cells

To investigate the role of the LIPAD residues in cell binding, various concentrations of biotin-conjugated LigB_{1706–1716} or biotin were added to MDCK cells. Our results show that biotin-LigB_{1706–1716} binds to MDCK cells in a dose-dependent manner (Fig. 4A and C). Pretreatment of MDCK cells with biotin-LigB_{1706–1716} reduced the attachment of *Leptospira* by approximately 39% compared to either untreated (data not shown) or biotin-treated MDCK cells in a dose-dependent manner (Fig. 4B).

Discussion

Recently, it was shown that the some bacterial Fn-binding proteins binds to NTD by β zipper, two antiparallel β-strands [19], but some binds to the cationic cradle of the 13F₃ [20]. Here, we show that the LIPAD motif of LigB binds to the 15F₃, and this motif can also found in other *Leptospira* serovars and zonadhesin, an eukaryotic ECM binding proteins [21]. Our study

describes for the first time a bacterial MSCRAMM binding to this module. These results suggest that LigB_{1706–1716} may exploit a novel Fn-binding mechanism.

The immunoglobulin-like repeat regions of LigA and LigB, including LigBCen2, strongly bind to the NTD and GBD [4,5]. This study identifies an Fn-binding LIPAD motif located in the C-terminal non-repeat region of LigB that slowly binds to the 15F₃ with moderate affinity ($K_D = 10.70 \mu\text{M}$, $k_{\text{on}} = 600 \text{ M}^{-1} \text{ s}^{-1}$, $k_{\text{off}} = 0.0129 \text{ s}^{-1}$) (Fig. 3). The kinetic rates of LigB_{1706–1716} binding to the 15F₃ are at least 100-fold slower than rates seen with other bacterial Fn-binding proteins [22]. The slow k_{off} may be an important factor in retention of the bound state in the biological system. In an *in vivo* system where the concentration of ligand is not constant, but rather influenced by factors such as absorption, distribution, metabolism, and excretion (ADME), the residence time of a receptor–ligand complex is not necessarily appropriately described by the equilibrium dissociation constant K_D [23]. Current experimental evidence has pointed to the dissociation rate constant k_{off} as a more relevant parameter in some cases [24]. This has led to arguments that the k_{off} may be an important parameter to consider in drug design and screening. In light of this, it is tempting to interpret the slow k_{off} of the LigB_{1706–1716}:Fn complex as an example of an evolutionarily optimized k_{off} for a bacterial adhesin/target complex, which is challenged by many of the same limitations to overall ligand concentration as drug/target complexes are.

It has been indicated a *ligB* mutant with an insertion containing a *Spc*^r cassette into the 3' end of the *ligB* gene is still virulent in a hamster model [25]. The reasons are unknown. However, there are several possibilities: (1) LigB is only needed for initial adhesion and invasion of *Leptospira* moving from the environment through mucous membranes and injured skin. (2) The presence of other putative adhesins with potentially redundant functions. To answer these questions, further studies are needed.

We have demonstrated that the sequence LIPAD located on the C-terminus of LigB is an Fn-binding motif that interacts with the 15th type III module of Fn with slow binding kinetics, mediates cell adhesion, and displays β -strand and nascent helix character when free in solution. Work to further elucidate this interaction is in progress in our lab.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:
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Fig. 1.

Identification of Fn-binding residues on LigBctv. (A) A schematic diagram showing the truncated LigBctv used in this study. (B) The Fn-binding activity of LigBctv1, LigBctv2, LigBctv3, and LigBctv4 regions. (C) The Fn-binding activity of the 30mers peptides from LigBctv. (D) The synthetic peptide sequences (11mers) with overlapping regions used to identify the essential amino acids (indicated in bold) required for Fn binding. (E) The synthetic peptides indicated in (D) used in the Fn-binding assays. Various concentrations (40, 20, 10, 5, 2.5, 1.25, and 0.625 μ M) of each tested biotinylated-protein or -peptide, -BBK32₅₆₋₂₀₅ (positive control; B, C, and E), -LigBCon (negative control; B) or -scrambled peptide (negative control; C and E) were used in this study. Bound proteins or peptides were measured by ELISA.

Fig. 2.

(A) ^1H - ^1H region of the homonuclear NOESY spectrum ($t_{\text{mix}} = 200$ ms, pH 6.0) of LigB₁₇₀₃₋₁₇₁₇ in PBS in H_2O . The d_{NN} cross-peaks are labeled with residue numbers, and arrows indicate sequential connectivities. The NOEs between sequential residues in residues A₁₇₁₁ through F₁₇₁₅ indicate nascent helical character. (B) Plot of the difference in H^α chemical shifts, or $\Delta\delta_{\text{H}^\alpha}$, between observed and random coil values for LigB₁₇₀₃₋₁₇₁₇. (C) Indicated the first cysteine conjugated on LigB₁₇₀₃₋₁₇₁₇. A stretch of $\Delta\delta_{\text{H}^\alpha}$ values below -0.1 indicates nascent α -helical structure, and a stretch of values above 0.1 indicates preference for β -strand conformation.

Fig. 3.

Mapping and characterization of LigB₁₇₀₆₋₁₇₁₆ binding sites on Fn. (A) A chart presenting Fn and truncated Fn used in this study. (B,C) Various concentration (0.625, 1.25, 2.5, 5, 10, 20, 40 μM) of biotin-LigB₁₇₀₆₋₁₇₁₆ or biotin (negative control and data not shown) were added to 1 μg of (B) Fn, NTD, GBD, CBD, 40 kDa, or BSA (negative control) (C) 40 kDa, MBP-12-13F₃, MBP-14F₃, MBP-15F₃, MBP (negative control), or BSA (negative control) coated microtiter plate wells. Bound proteins were measured by ELISA. (D) 1 μM of 15F₃ in PBS in the presence of 0, 0.625, 1.25, 2.5, 5, 10, 20 and 40 μM of LigB₁₇₀₆₋₁₇₁₆ was excited at 295 nm to measure Trp fluorescence. Inner plot: K_D of 15F₃-LigB₁₇₀₆₋₁₇₁₆ determined by monitoring quenching fluorescence intensities at 350 nm. (E) Stop flow experiment of 15F₃

binding to LigB₁₇₀₆₋₁₇₁₆. The signal represents the total fluorescence emission above 320 nm by excited at 295 nm. Inner plot: The kinetic plot of k_{obs} versus concentration of 1 μM 15F₃ under different LigB₁₇₀₆₋₁₇₁₆ concentrations (25, 50, 100, 200, 400 μM).

Fig. 4.

The binding of LigB₁₇₀₆₋₁₇₁₆ to MDCK cells reduces leptospiral adhesion. (A) Various concentrations (0, 2, 4, 6, 8, or 10 μM) of biotin-LigB₁₇₀₆₋₁₇₁₆, or biotin (negative control) were added to MDCK cells (10⁵). (B,C) LigB₁₇₀₆₋₁₇₁₆ inhibits the binding of *Leptospira* to MDCK cells. (B) MDCK cells (10⁵) were incubated with various concentrations (0, 2, 4, 6, or 8, 10 μM) of biotin-LigB₁₇₀₆₋₁₇₁₆, or biotin (negative control) prior to the addition of *Leptospira* (10⁷). (A) The binding or (B) the reduced attachment was measured by ELISA. (C) The adhesion of *Leptospira* (10⁸) or the binding of 10 μM of biotin or biotin-LigB₁₇₀₆₋₁₇₁₆ to MDCK cells (10⁶) was detected by CLSM.