Calcium Binds to LipL32, a Lipoprotein from Pathogenic *Leptospira*, and Modulates Fibronectin Binding*

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The abbreviations used are: OMP, outer membrane protein; ECM, extracellular matrix; PBS, phosphate-buffered saline; MAD, multiformanelogous dispersion; ITC, isothermal titration calorimetry; MOPS, 4-morpholinepropanesulfonic acid; ELISA, enzyme-linked immunosorbent assay; polyD, polyaspartate.

Tubulointerstitial nephritis is a cardinal renal manifestation of leptospirosis. LipL32, a major lipoprotein and a virulence factor, locates on the outer membrane of the pathogen *Leptospira*. It evades immune response by recognizing and adhering to extracellular matrix components of the host cell. The crystal structure of Ca$^{2+}$-bound LipL32 was determined at 2.3 Å resolution. LipL32 has a novel polyD sequence of seven aspartates that forms a continuous acidic surface patch for Ca$^{2+}$ binding. A significant conformational change was observed for the Ca$^{2+}$-bound form of LipL32. Calcium binding to LipL32 was determined by isothermal titration calorimetry. The binding of fibronectin to LipL32 was observed by Stains-all CD and determined by isothermal titration calorimetry. The binding of the bound form of LipL32. Calcium binding to LipL32 was determined by isothermal titration calorimetry. The binding of fibronectin to LipL32 was observed by Stains-all CD and enzyme-linked immunosorbent assay experiments. The interaction between LipL32 and fibronectin might be associated with Ca$^{2+}$ binding. Based on the crystal structure of Ca$^{2+}$-bound LipL32 and the Stains-all results, fibronectin probably binds near the polyD region on LipL32. Ca$^{2+}$ binding to LipL32 might be important for *Leptospira* to interact with the extracellular matrix of the host cell.

Leptospirosis is the most widespread zoonosis, particularly in warm and humid climates (1–3). The clinical manifestations of leptospirosis occur when humans acquire the pathogen *Leptospira* from animals (4) via skin or gastrointestinal contact with water, food, or soil that has been contaminated with urine from infected animals. Clinical symptoms of leptospirosis vary from mild to severe, and potentially lethal forms are characterized by high fever, bleeding, and renal failure (5). The kidney is the main target of *Leptospira* in both acute and chronic infections (1, 6). Acute kidney injury as a result of tubulointerstitial nephritis is an early and primary manifestation of systemic leptospirosis (6). In chronic infections, *Leptospira* may colonize and persist in the proximal tubule, leading to carrier status, and thus may lead to chronic tubulointerstitial nephritis and fibrosis (1).

Outer membrane proteins (OMPs)2 and lipopolysaccharide located on the outer membrane are major antigens that confer immunity to *Leptospira* (7). Leptospiral OMPs are likely to have roles in host-pathogen interactions (7, 8). They elicit inflammation and lead to tubular injury through Toll-like receptor-dependent pathways. Subsequently, the nuclear transcription factor NF-κB and the mitogen-activated protein kinases are activated, leading to the differential induction of chemokines and cytokines relevant to tubular inflammation (9–11).

Bacterial pathogenesis can occur when mammalian extracellular matrix (ECM) molecules interact with bacterial cell surface proteins (12). These interactions can be used by the bacterium to adhere to tissues, evade an immune response, and enter the host. ECM components in mammalian cells are diverse and include fetuin, laminin-1, collagen, and plasma fibronectin. *Leptospira* recognizes ECM molecules and adheres to the host cell though pathogenic OMPs such as immunoglobulin-like protein (13), endostatin-like protein (14), and the lipoprotein LipL32 (10).

LipL32 is a major OMP in *Leptospira* and is highly conserved among pathogenic species. It is expressed during mammalian infection by *Leptospira* (7). LipL32 is a virulent microbial surface component that recognizes adhesive matrix molecules (15). LipL32 induces inflammation via NF-κB and mitogen-activated protein kinase pathways (4) through TLR2 (10). LipL32 adheres to the host cell by recognizing ECM molecules such as type IV collagen and plasma fibronectin (F30 and F45) (15, 16). The heparin-binding domain of F30 and the gelatin-binding domain of F45 are involved in these host cell interactions (16). Although LipL32 has been suggested to have a role in pathogenicity, a recent report indicates that LipL32 is not essential for survival or virulence (17). Thus, the role of LipL32 in *Leptospira* pathogenesis remains unclear.

Similar to other prokaryotic lipoproteins, LipL32 has a lipid modification at its N-terminal Cys residue (7). The deduced amino acid sequence of LipL32 encodes a 272-amino acid polypeptide (32 kDa) with a 19-amino acid signal peptide followed by a lipoprotein signal peptidase cleavage site (7). The Ca$^{2+}$-free LipL32 crystal structures from *Leptospira* have been reported recently (18, 19). In this study, the crystal structure of Ca$^{2+}$-bound LipL32 from *Leptospira shermanii* was determined, and a Ca$^{2+}$-binding site was resolved. According to the structural analysis, a possible fibronectin-binding site was predicted.
Ca\(^{2+}\)-bound LipL32

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—LipL32 was isolated from *L. shermani* as described (4, 7). LipL32 (782 bp) was amplified by PCR, and the resulting fragment was digested (Xhol/Smal), inserted into pRSETc, and transformed into *Escherichia coli* BL21(DE3)pLys cells (Invitrogen). Expression of the N-terminally His\(_6\)-tagged LipL32 protein was induced at 37 °C for 4 h using 0.1 mM isopropyl -D-thiogalactopyranoside. After harvesting, the bacterial pellet was resuspended in 100 ml of phosphate-buffered saline (PBS), pH 7.5. After cell lysis, the LipL32 protein was purified with a nickel column followed by gel filtration (Superdex 200, Amersham Biosciences) using PBS. Purified LipL32 was dialyzed against 20 mM Tris, 300 mM NaCl, and 50 mM imidazole, pH 8.0, for crystallization. Because there is no homology protein phase available for LipL32, the selenomethionine-substituted protein was prepared for phase determination by the multiwavelength anomalous dispersion (MAD) method. SeMet-LipL32 was expressed from *E. coli* strain B834 and purified by His-tagged affinity chromatography according to native LipL32.

**Crystallization and X-ray Data Collection**—Crystallization trials were carried out by the hanging-drop vapor-diffusion method (20). Both protein (1 µl) and reservoir (1 µl) solutions were mixed and equilibrated against a reservoir solution (500 µl) in Linbro plates. Initial crystallization conditions were obtained using Hampton Research crystal screen kits and then optimized to obtain diffraction-quality crystals. The concentration of LipL32 used for crystallization was 10 mg/ml. LipL32 were crystallized using 41–43% Tacsimate in 20 mM Tris, pH 7.0, 300 mM NaCl, and 50 mM imidazole. LipL32 crystals were grown at 20 °C. For phase determination, anomalous data from the SeMet-LipL32 crystals were collected at 2.3 Å on the BL13B1 beamline at the National Synchrotron Radiation Research Center (Hsinchu, Taiwan). The data were processed using HKL2000 (21). The Ca\(^{2+}\)-bound LipL32 crystals belong to the tetragonal space group P4\(_2\)_2\(_1\)2 with unit cell parameters \(a = b = 121.91\) and \(c = 206.78\ \AA\). The \(V_{\text{AV}}\) (22) was calculated as 2.49 \(\AA^3\) Da\(^{-1}\) with a solvent content of 49%, containing five molecules/asymmetric unit. The data statistics are summarized in Table 1.

**Structural Determination and Refinement**—The structural phase of LipL32 was obtained by selenomethionine MAD. The selenium sites were located with SHARP (23). The electron density map was calculated with SHARP, resulting in distinguishable protein and solvent regions. SHARP automatically built the preliminary structural model, and the entire model was completed manually with the XtalView software (24). The structure refinement was carried out by CNS (25). The structural model was further refined using REFMAC5 (26) and PHENIX (27). PROCHECK (28) was used to evaluate the stereochemistry and to assign the secondary structural elements. The Ca\(^{2+}\)-bound LipL32 crystal contained five molecules/asymmetric unit, and each protein molecule has 252 amino acid residues and 528 water molecules. The structural model was refined to an R-factor of 21.9% and \(R_\text{free}\) of 26.2% at 2.3 Å. The refinement statistics are summarized in Table 1. Coordinates have been deposited in the Protein Data Bank with accession code 2WFK.

**Isothermal Titration Calorimetry (ITC)**—Reaction enthalpy measurements were carried out with a VP-ITC calorimeter (MicroCal Inc.) at 30 °C. The Ca\(^{2+}\) in the LipL32 sample was removed by EGTA (5 mM), which was incubated at room temperature for 30 min. The sample was examined by inductively coupled plasma atomic emission spectrometry. The LipL32 protein sample (1.5 ml, 120 \(\mu\)M) was then titrated against CaCl\(_2\) (3 mM) with stirring at 300 rpm. CaCl\(_2\) was added to the protein sample with 30 injections. Each injection of 10 \(\mu\)l of CaCl\(_2\) was delivered over 20 s with 300-s spacing between injections to achieve equilibrium. Data were analyzed using MicroCal ITC software and fitted to an independent binding model.

**Stains-all Binding Assay**—The binding of Ca\(^{2+}\) and fibronectin F30 to LipL32 was monitored by CD spectra using Stains-all dye. F30 (Sigma) is a human fibronectin fragment (30 kDa) containing a heparin-binding domain. The Stains-all binding assay was carried out as described (29). The CD spectra (Aviv 62DS) were recorded under N\(_2\) atmosphere at 37 °C in a 0.1-cm path length quartz cell for far- and near-UV CD spectra. The Ca\(^{2+}\) ions were removed from LipL32 by EGTA (5 mM) and examined by inductively coupled plasma atomic emission spectrometry (data not shown). LipL32 (15 \(\mu\)M) with and without EGTA treatment was mixed with Stains-all dye solution (200–250 \(\mu\)M) in 2 mM MOPS, pH 7.2, containing 30% ethylene glycol and incubated for 5 min at 37 °C. There was no extra calcium added.
in 400 to 700 nm. To monitor the fibronectin-LipL32 interaction, F30 (5 and 20 μg) was added to the LipL32-Stains-all complex in the presence or absence of Ca²⁺, and CD spectra were recorded.

**Enzyme-linked Immunosorbent Assay (ELISA)**—The interaction between LipL32 and F30 was analyzed by ELISA according to a published protocol (30). The ELISA plates (Nunc-Immuno Plate MaxiSorp surface) were first coated with F30 (1 μg) in PBS (100 μl) and incubated for 2 h at 37 °C. The wells were washed with PBST (PBS and 0.05% Tween 20) three times and then blocked with bovine serum albumin (1%, 200 μl) for 1 h at 37 °C, and the plates were incubated overnight at 4 °C. Protein samples (LipL32 or LipL32 treated with EGTA; 0–60 μmol) in PBS were added to the attached F30 fragment for 90 min at 37 °C and then washed six times with PBST. The bound LipL32 protein was detected by adding rabbit anti-LipL32 serum (100 μl) at 1:9,000 dilution) was added and incubated for 1 h at 37 °C, after washing three times with PBST, 3,3′,5,5′-tetramethylbenzidine and H₂O₂ single solution (Invitrogen) was added for color development. The reaction was allowed to proceed for 15 min and then terminated by adding H₂SO₄ (50 μl, 2 μl). There was no extra calcium added in the buffer used for ELISA. The absorbance at 450 nm was measured in a ThermoMax microplate reader (Molecular Devices). Three independent experiments were carried out. The mean absorbance ± S.D. of three independent data sets were calculated. The absorbance at each data point was corrected by subtracting a background reading generated with a bovine serum albumin-coated well. The binding data were analyzed using SigmaPlot by fitting the data to the appropriate equation (31) assuming that ligands bound to one independent site.

**RESULTS**

**LipL32**—The sequence and expression of LipL32 are highly conserved among pathogenic *Leptospira* species. However, there is no homologous LipL32 protein found outside the *Leptospira* species. The amino acid sequence of LipL32 is shown in Fig. 1. LipL32 has a unique polyaspartate (polyD) region that contains a cluster of seven aspartate residues in a span of eight amino acids, 142DDDDDGDD 149 (Fig. 1). This continuous Asp sequence pattern is not typically found in other protein sequences except for a stretch of sequential aspartate residues at the N terminus of LipL36 (32). In contrast, the LipL32 polyD region is located in the middle of the protein and is highly conserved among LipL32 proteins from the *Leptospira* superfamilies.

The crystal structure of Ca²⁺-bound LipL32 was determined by MAD at 2.3 Å. Ca²⁺-bound LipL32 consists of an N-terminal part, a central domain, and a C-terminal segment with an overall dimension of 40 × 50 × 60 Å (Fig. 2A). An extra strong electron density (3.0σ in the F₂ − F₁ map) observed around the polyD region was attributed to a Ca²⁺ ion based on binding distance and coordination. Because there was no calcium added during protein isolation, the Ca²⁺ ion was most likely incorporated into LipL32 endogenously. The protein sample was examined by inductively coupled plasma atomic emission spectrometry. The Ca²⁺ ion is trapped in the negatively charged surface formed by the cluster of aspartate residues, 142DDDDDGDD 149, of which Asp 145 and Asp 146 were directly involved in Ca²⁺ binding. The Ca²⁺ ion is coordinated by Asp 113 and Thr 114 (in loop α1β7) and by Asp 145, Asp 146, and Tyr 159 (in loop β8β9) with an octahedral geometry (Fig. 2B).

**LipL32 is a Ca²⁺-binding Protein**—Calcium binding to LipL32 was examined by ITC in solution. The calcium ions that were bound to the LipL32 proteins in vivo during isolation were removed by EGTA prior to the titration experiment. The binding appeared to be an exothermic reaction with enthalpy, ΔH = (−1.21 × 10⁹) ± (3.38 × 10⁸) kcal/mol, and entropy, ΔS = −18.84 kcal/mol. Fitting to one set of site macroscopic model data, the Kd of Ca²⁺ binding to LipL32 was calculated to be (6.07 × 10⁸) ± (3.32 × 10⁸) M⁻¹, and the Kd was 16.46 μM with a N value of 1.01 ± 0.02 (Fig. 2C).

The ability of Stains-all to interact with polyanions provides a useful indicator to understand the environment of acidic residues, such as the polyD region in LipL32 (29). Hence, the Stains-all binding assay was used to inspect the calcium-binding protein in solution. Stains-all can occupy the Ca²⁺-binding site on proteins and can mimic the Ca²⁺-binding environment. In CD spectra, there is an intense biphasic peak in the 600–680 nm region (called J bands) that corresponds to the Stains-all signal. In the presence of Stains-all dye, LipL32 revealed a strong J band at 660 nm. This signal was diminished significantly upon adding excess Ca²⁺ (data not shown). This indicates the Ca²⁺-binding nature of LipL32 and the similarity of its Ca²⁺-binding site to a β-crystalline conformation (33, 34).
FIGURE 2. Ca\(^{2+}\)-bound LipL32. A, the overall structure of Ca\(^{2+}\)-bound LipL32. A ribbon drawing of four \(\alpha\)-helices (yellow, labeled \(\alpha1\)–\(\alpha4\)) and 12 \(\beta\)-strands (green, labeled \(\beta1\)–\(\beta12\)) is shown. B, the Ca\(^{2+}\)-binding site of LipL32. The Ca\(^{2+}\)-coordinated residues Asp\(^{113}\), Thr\(^{114}\), Asp\(^{145}\), Asp\(^{146}\), and Tyr\(^{159}\) are shown by bonds and sticks and are colored according to Corey-Pauling-Koltun models. The hydrogen bonds are shown as dashed lines. The Ca\(^{2+}\) ion is shown as a yellow-colored sphere. C, the calcium binding affinity for LipL32 determined by ITC. Data for heat change obtained by calcium titration are shown in the upper panel. Data after peak integration are shown in the lower panel, and the solid line represents the best fits to a single-site binding model.
Several types of Ca\textsuperscript{2+}-binding motifs in bacterial proteins have been identified (35), such as the EF-hand motif (36), leukotoxin (37), and the hemolysin-type Ca\textsuperscript{2+}-binding domain (38). These proteins bind Ca\textsuperscript{2+} through oxygen atoms supplied by numerous glutamate/aspartate side chains similar to the polyD region (142DDDDDDGDGG149) identified in LipL32. In addition, an EHDSDSDDD motif responsible for Ca\textsuperscript{2+} binding has been identified in the E. coli ChaA Na\textsuperscript{+}/Ca\textsuperscript{2+} antiporter (39). This type of continuous acidic sequence for Ca\textsuperscript{2+} binding has also been found in eukaryotic Ca\textsuperscript{2+}-binding proteins (calsequestrin and calreticulin) (39–41), histidine-rich Ca\textsuperscript{2+}-binding protein (42), and the canine cardiac sarcolemmal Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (43).

**Conformational Changes Triggered by Ca\textsuperscript{2+} Binding**—The structural superimposition of Ca\textsuperscript{2+}-free LipL32 (Protein Data Bank code ZZZ8) (18) and Ca\textsuperscript{2+}-bound LipL32 is shown in Fig. 3A. The β-sandwich architecture is similar between both structures; however, the overall structures reveal a root mean square deviation of 3.3 Å for α-carbon atoms. Based on the structural comparison between Ca\textsuperscript{2+}-bound and Ca\textsuperscript{2+}-free LipL32, several critical conformational changes were observed around loops α1β7, β8β9, and β9β10 as a result of Ca\textsuperscript{2+} binding (Fig. 3A). In Ca\textsuperscript{2+}-bound LipL32, a huge conformational change appears from β8 to loop β8β9, where the polyD region is (142DDDDDDGDGG149), for Ca\textsuperscript{2+} binding. Loop β8β9 is tug up to a β-structure (Fig. 3A). Ca\textsuperscript{2+} is trapped in loop β8β9, and the conformation of loop β8β9 is affected by Ca\textsuperscript{2+} binding (Fig. 2B). However, Lys\textsuperscript{140}–Asp\textsuperscript{146} are missing in the Ca\textsuperscript{2+}-free LipL32 structures (18, 19). Furthermore, Hauk et al. (19) suggested that the thermal stability of LipL32 could be increased in the presence of Ca\textsuperscript{2+} by a CD experiment. The stability of Ca\textsuperscript{2+}-bound LipL32 might be due to interactions between Ca\textsuperscript{2+} and loop β8β9 around the Ca\textsuperscript{2+}-binding site.

In Ca\textsuperscript{2+}-bound LipL32, loop β8β9 is further stabilized by a stacking interaction between His\textsuperscript{156} and Trp\textsuperscript{111} on loop α1β7 (Fig. 3B), and this stacking interaction was not found in Ca\textsuperscript{2+}-free LipL32 (18). In addition, there is a conformational change around loop α1β7, particularly in 111WFTDW115 (Fig. 3B). Trp\textsuperscript{111} is buried in the β-sandwich hydrophobic core in Ca\textsuperscript{2+}-free LipL32. In contrast, the tryptophan ring of Trp\textsuperscript{111} is flipped and stacked with His\textsuperscript{156} in Ca\textsuperscript{2+}-bound LipL32 (Fig. 3B). The tryptophan fluorescence results indicate that the conformation of Trp\textsuperscript{111} is quenched in Ca\textsuperscript{2+}-bound LipL32 (data not shown). In Ca\textsuperscript{2+}-free LipL32, Phe\textsuperscript{112} is exposed to solvent; however, it is buried inside of the protein, with no further interaction in Ca\textsuperscript{2+}-bound LipL32 (Fig. 3B). In Ca\textsuperscript{2+}-free LipL32, Trp\textsuperscript{115} is exposed to solvent; nevertheless, it is stabilized by forming a hydrogen bond with Asp\textsuperscript{146} in Ca\textsuperscript{2+}-bound LipL32 (Fig. 2B).

A significant conformational change also occurs in loop β9β10 with an 18-Å difference between Ca\textsuperscript{2+}-free and Ca\textsuperscript{2+}-bound LipL32 (Fig. 3A). Loop β9β10 is highly exposed and quite flexible with a high B-factor. In Ca\textsuperscript{2+}-bound LipL32, loop β9β10 participates in the crystallographic dimer interactions. In contrast, in Ca\textsuperscript{2+}-free LipL32, loop β9β10 is exposed to the solvent with no interacting partner.

Furthermore, in Ca\textsuperscript{2+}-free LipL32, two hydrogen bonds, Asp\textsuperscript{113}–Asn\textsuperscript{157} and Arg\textsuperscript{155}–Tyr\textsuperscript{198}, might be used to close up the binding pocket and prevent Ca\textsuperscript{2+} binding (Fig. 3B). Conversely, in Ca\textsuperscript{2+}-bound LipL32, Asp\textsuperscript{113} is shifted to interact directly with Ca\textsuperscript{2+}, and Tyr\textsuperscript{198} is rotated and exposed to the solvent to prohibit hydrogen bonding with Arg\textsuperscript{155}. Therefore, both the Asp\textsuperscript{113}–Asn\textsuperscript{157} and Arg\textsuperscript{155}–Tyr\textsuperscript{198} hydrogen bonds were abolished, resulting in a huge conformational change in loop β8β9 of Ca\textsuperscript{2+}-bound LipL32. Consequently, the Ca\textsuperscript{2+}-binding channel is opened up, and Ca\textsuperscript{2+} binding takes place in Ca\textsuperscript{2+}-bound LipL32.

The putative Ca\textsuperscript{2+}-binding sites in Ca\textsuperscript{2+}-free LipL32 were proposed by Hauk et al. (19). One is located at an acidic loop between α3 and β9, which is similar to the Ca\textsuperscript{2+}-binding site determined in Ca\textsuperscript{2+}-bound LipL32, around the polyD region and loop β8β9. However, the other possible Ca\textsuperscript{2+}-binding site, located at a hydrophobic pocket around β3, β4, and β5, is not observed in Ca\textsuperscript{2+}-bound LipL32.

Interactions between LipL32 and collagen have been reported (16). The possible collagen-binding site in LipL32 is composed of Leu\textsuperscript{53}, Val\textsuperscript{54}, Tyr\textsuperscript{62}, Trp\textsuperscript{115}, Arg\textsuperscript{117}, Tyr\textsuperscript{151}, and Tyr\textsuperscript{198}, forming a hydrophobic binding surface (18). Based on structural comparison between Ca\textsuperscript{2+}-free and Ca\textsuperscript{2+}-bound LipL32, Tyr\textsuperscript{151} of Ca\textsuperscript{2+}-bound LipL32 is distant from the possible collagen-binding domain because there is a huge conformational change in loop β8β9 due to Ca\textsuperscript{2+} binding (Fig. 3C). In addition, Trp\textsuperscript{115} and Tyr\textsuperscript{198} reveal different orientations due to Ca\textsuperscript{2+} binding (Fig. 3C). Both residues are close to the Ca\textsuperscript{2+}-binding site in Ca\textsuperscript{2+}-bound LipL32, and their conformation might be affected by Ca\textsuperscript{2+} binding (Fig. 2B). The Ca\textsuperscript{2+} binding of LipL32 may relate to the collagen interaction of LipL32.

**Ca\textsuperscript{2+} Modulates Fibronectin Binding to LipL32**—Interactions between LipL32 and plasma fibronectin have been reported (16). The binding of LipL32 to F30 and the inhibition of this binding by heparin have been detected (16). Heparin is an anionic molecule of glycosaminoglycan with three negatively charged sulfate groups. Interactions between LipL32 and F30 were monitored by Stains-all dye and ELISA, and the correlation between this interaction and Ca\textsuperscript{2+} binding was determined.

Stains-all is a convenient probe to study Ca\textsuperscript{2+} binding and to examine the protein–protein interactions of a Ca\textsuperscript{2+}-binding protein (29, 33, 44). In the presence of Stains-all dye, Ca\textsuperscript{2+}-bound LipL32 revealed a J band (Fig. 4A). When F30 was added to LipL32, F30 competed with Stains-all for the Ca\textsuperscript{2+}-binding site, and the Stains-all signal J band was diminished. The major change in J band ellipticity appeared to be F30 concentration-dependent (Fig. 4A). Therefore, the F30-binding site might be in close proximity to the Ca\textsuperscript{2+}-binding site on LipL32. When Ca\textsuperscript{2+} was depleted by EGTA, Stains-all dye occupied most of the Ca\textsuperscript{2+}-binding sites of LipL32, resulting in a stronger J band (Fig. 4B) than that in Ca\textsuperscript{2+}-bound LipL32 (Fig. 4A). However, there was a minor change in the J band ellipticity when F30 was added in the absence of Ca\textsuperscript{2+} (Fig. 4B). This indicates that the amount of F30 binding to Ca\textsuperscript{2+}-free LipL32 was decreased. We suggest that the binding affinity of F30 for Ca\textsuperscript{2+}-free LipL32 is weaker than that for Ca\textsuperscript{2+}-bound LipL32.

In a dose-dependent ELISA experiment, the interaction between LipL32 and fibronectin was detected by binding of
the F30 fragment. For Ca\(^{2+}\)-bound LipL32, an interaction between LipL32 and F30 was observed. However, for Ca\(^{2+}\)-free LipL32, this interaction was decreased. The $K_d$ values for F30 binding to Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free LipL32 were determined to be 0.29 $\pm$ 0.01 M ($p < 0.0001$, $R^2 = 0.99$) (Fig. 4C) and 1.15 $\pm$ 0.06 M ($p < 0.0001$, $R^2 = 0.99$) (Fig. 4D), respectively. The binding affinity of F30 for Ca\(^{2+}\)-bound LipL32 is stronger than that for Ca\(^{2+}\)-free LipL32. Consequently, the calcium binding of LipL32 promotes the interaction of LipL32 and F30.
DISCUSSION

The crystal structure of Ca\textsuperscript{2+}/H\textsubscript{11001}-bound LipL32 was determined. Ca\textsuperscript{2+} was bound on an acidic surface patch formed by the polyD sequence. Upon Ca\textsuperscript{2+} binding, a significant conformational change was induced around the Ca\textsuperscript{2+}-binding cleft at loops 8 and 9. The K\textsubscript{d} for calcium binding to LipL32 was determined by ITC. Our work demonstrates that LipL32 is a novel Ca\textsuperscript{2+}-binding protein. The interaction of LipL32 with fibronectin (F30) was observed by Stains-all assay, and the binding affinity was determined by ELISA. The results indicate that the Ca\textsuperscript{2+} ion facilitates fibronectin binding to LipL32. On the basis of the crystal structure of Ca\textsuperscript{2+}-bound LipL32 and the assay results in solution, we suggest that the Ca\textsuperscript{2+}-binding site and the fibronectin-interacting region of LipL32 might be in close proximity. Calcium ion not only stabilizes the conformation of LipL32 but also enhances the binding affinity of LipL32 for fibronectin. The functional role of calcium binding has been reported; the host-pathogen infections caused by *Leptospira* are affected by calcium ion (45, 46). Therefore, the Ca\textsuperscript{2+} binding of LipL32 might play an essential role in *Leptospira* infections.

The OMPs of *Leptospira* elicit inflammation through the Toll-like receptor-dependent pathway. LipL32 is one of the most abounded OMPs in *Leptospira*. TLR2 is the major inflammation target of LipL32. The interaction of LipL32 with F30, an ECM on the outer membrane of the host cell, might bring LipL32 and TLR2 together and induce inflammation in the host. Consequently, LipL32 could be used as a target protein to implement the inflammation induced by *Leptospira* in clinical studies.

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REFERENCES

1. Farr, R. W. (1995) *Clin. Infect. Dis.* 21, 1–6
2. Biegel, E., and Mortensen, H. (1995) *Ugeskr. Laeger* 157, 153–157
3. Bharti, A. R., Nally, J. E., Ricaldi, J. N., Matthias, M. A., Diaz, M. M., Lovett, M. A., Levett, P. N., Gilman, R. H., Willig, M. R., Gotuzzo, E., and Vinetz,
