Essential Calcium-binding Cluster of *Leptospira* LipL32 Protein for Inflammatory Responses through the Toll-like Receptor 2 Pathway

Received for publication, September 11, 2012, and in revised form, March 12, 2013. Published, JBC Papers in Press, March 12, 2013, DOI 10.1074/jbc.M112.418699

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**Background:** LipL32 induces a renal cell inflammatory response through the TLR2-signaling pathway.

**Results:** Ca²⁺-binding LipL32 mutants showed attenuated TLR2-mediated inflammatory responses.

**Conclusion:** The Ca²⁺-binding cluster of LipL32 is essential in regulating its interaction with TLR2 for subsequent inflammatory response induction.

**Significance:** This investigation provides significant evidence for crucial roles of the Ca²⁺-binding cluster of LipL32 for pathogenesis via association with TLR2.

Leptospirosis is one of the most prevalent zoonotic diseases caused by the pathogenic *Leptospira* worldwide. LipL32, a 32-kDa lipoprotein, is the most abundant protein on the outer membrane of *Leptospira* and has an atypical poly(Asp) motif (161DDDDGDGGDD168). The x-ray crystallographic structure of LipL32 revealed that the calcium-binding cluster of LipL32 includes several essential residues Asp132, Thr133, Asp164, Asp165, and Tyr178. The goals of this study were to determine possible roles of the Ca²⁺-binding cluster for the interaction of LipL32 and Toll-like receptor 2 (TLR2) in induced inflammatory responses of human kidney cells. Site-directed mutagenesis was employed to individually mutate Ca²⁺-binding residues of LipL32 to Ala, and their effects subsequently were observed. These mutations abolished primarily the structural integrity of the calcium-binding cluster in LipL32. The binding assay and atomic force microscopy analysis further demonstrated the decreased binding capability of LipL32 mutants to TLR2. Inflammatory responses induced by LipL32 variants, as determined by TLR2 pathway intermediates hCXCL8/IL-8, hCCL2/MCP-1, hMMP7, and hTNF-α, were also lessened. In conclusion, the calcium-binding cluster of LipL32 plays essential roles in presumably sustaining LipL32 conformation for its proper association with TLR2 to elicit inflammatory responses in human renal cells.

Leptospirosis is one of the most prevalent zoonotic diseases caused by the pathogenic spirochete *Leptospira*, particularly in warm and humid regions worldwide (1). The disease is generally transmitted through contact with urine of carrier hosts into the water or soil, causing infection on humans via skin or gastrointestinal routes (2). Clinical symptoms in humans include high fever, jaundice, and renal failure (3). The human organ that is the main target of *Leptospira* is the kidney in both acute and chronic infections (4, 5).

A line of evidence has shown that pretreatment of kidney epithelial cells with outer membrane proteins from *Leptospira* triggered significant expression of tubulointerstitial nephritis-related genes (6, 7). Surface-exposed antigens, due to their location, are likely involved in primary host-pathogen interactions, adhesion, and/or invasion (8). These interactions are followed by bacterial adhesion to tissues, immune responses, and eventually bacteria entering into the hosts. *Leptospira* surface components implicated in virulence include lipopolysaccharides (LPS) (9), major outer membrane lipoprotein 32 (LipL32) (10), Lig (*Leptospira* immunoglobulin-like) proteins (11), Len (*Leptospira* endostatin-like) proteins (12), and Loa22 (*Leptospira* OmpA-like lipoprotein) (13).

LipL32 is highly conserved and abundant in pathogenic species, but it is absent in the nonpathogenic saprophytic *Leptospira biflexa* (14). LipL32 has a lipid modification at its Cys20 residue (14) and a signal peptide tag at the N terminus (5). LipL32 is also known as Hap-1 for its possible participation in hemolysis through sphingomyelinase SphH (15). In addition, the crystal structure of LipL32 from *Leptospira* has been resolved, and a potential Ca²⁺-binding site was identified and postulated to interact with several extracellular matrix components (16–19). LipL32 exists as a jelly roll fold structure, in which calcium binding is hypothesized to be crucial in structural maintainability and thermal stability (16, 17).
Role of Calcium-binding Cluster in LipL32

Innate immunity is the first line of defense against bacterial infection in vertebrates, of which Toll-like receptors (TLRs) are major members. Eleven TLRs have been discovered in mammals, participating in various intracellular signaling pathways that ultimately induce expression of inflammatory cytokines, chemokines, adhesion molecules, and co-stimulatory proteins (20). TLRs recognize numerous microbial components that act as virulence factors (21, 22) and contain several leucine-rich repeats and a Toll/IL-1 receptor domain (22). TLR2 heterodimerizes with TLR1 or -6 to interact with ligands. LipL32 was found to initiate the signaling cascade by interacting with TLR2 but not with TLR4 (23, 24).

In this study, site-directed mutagenesis was used to generate Ca$^{2+}$-binding mutants of LipL32; with these, the role of the Ca$^{2+}$-binding cluster in LipL32 was investigated. The involvement of the Ca$^{2+}$-binding cluster in the LipL32-TLR2 association was further demonstrated. Moreover, LipL32 variants attenuated inflammatory responses in human renal cells. Taken together, the calcium-binding cluster is crucial for the interaction between LipL32 and TLR2, which then triggers the signaling cascade of inflammatory responses.

EXPERIMENTAL PROCEDURES

**DNA Construction and Mutagenesis**—The lipl32 gene (782 bp) was cloned from pathogenic *Leptospira shermani* genomic DNA with *Pfu*-Turbo DNA polymerase (Stratagene, La Jolla, CA) according to previous investigations (10, 23). The gene was amplified by PCR and inserted into a pRSET-c vector (Invitrogen). Mutant derivatives were generated from wild type lipl32 DNA by the QuickChangeTM site-directed mutagenesis (25). The primers used in this study are listed in supplemental Table S1. TLR2(49–538) and TLR2(589–784) DNA template (InvivoGen, San Diego) with the primers listed in supplemental Table S1 were subsequently transformed into *Escherichia coli*, and variants were verified by DNA sequencing.

The TLR2 cDNA fragments were cloned by using *Pfu*-Turbo DNA polymerase (Stratagene) from a commercially purchased template (InvivoGen, San Diego) with the primers listed in supplemental Table S1. TLR2(49–538) and TLR2(589–784) DNA was inserted into their respective pRSET-c vectors (Invitrogen), and resultant plasmids were confirmed by DNA sequencing.

**Expression and Purification of LipL32 and TLR2**—The DNA constructs of lipl32, its variants, and TLR2 fragments were transformed into the expression host cell *E. coli* BL21 (DE3) pLys (Novagen, Madison, WI). His$_6$-LipL32, its variants, and His$_6$-TLR2 fragments were grown in Luria broth (LB) medium with 100 μg/ml ampicillin at 37 °C to an absorbance at 600 nm ($A_{600}$) of ~6.0. Isopropyl 1-thio-β-galactopyranoside (500 μM) was subsequently added to induce gene expression for an additional 4 h at 37 °C. *E. coli* cells were harvested by centrifugation at 4,000 × g for 15 min and sonicated in PBS. The cell debris was discarded after centrifugation at 14,000 × g for 30 min, and the supernatant was absorbed to Ni$_2^+$-nitrilotriacetic acid-agarose resin (Qiagen, Valencia, CA) for affinity chromatography purification (26). LipL32, its variants, and the TLR2 fragment proteins were eluted with 250 mM imidazole and stored at −80 °C for further use. Imidazole was removed by dialysis before assays were conducted. To validate the inflammatory effects of LipL32, the protein was subjected to polymyxin (Invitrogen), heat, and protease K (Invitrogen) treatments, respectively, as described previously (9). To remove the His$_6$ tag, recombinant LipL32 was incubated with 0.2 mg/ml enterokinase (enteropeptidase EC 3.4.21.9; Invitrogen) at 37 °C for 16 h (27).

**Bioinformatics Analysis**—Sequence alignment of LipL32 proteins (residues 129–183) from *L. shermani* (gi: 269914333), *Pseudoalteromonas tunicate* (gi: 88860771), *Moritella* sp. (gi: 149911212), and unidentified *Eubacterium* SCB49 (gi: 149370508) was performed by using Clustal_W (28). Crystal structures were selected from the Protein Data Bank (PDB), and the PDB numbers for this protein are 2WFK (Ca$^{2+}$-bound) and 3FRK (Ca$^{2+}$-free). The Ca$^{2+}$-coordinated residues were Asp$_{132}$, Thr$_{133}$, Asp$_{164}$, Asp$_{165}$, and Tyr$_{178}$.

**Spectral Measurements**—Fluorescence emission spectra were measured with a Hitachi F-7000 spectrofluorometer (Hitachi, Tokyo, Japan). The intrinsic tryptophan fluorescence of wild type LipL32 and its variants was determined at an excitation wavelength of 295 nm, and emission spectra were recorded within the 310–400 nm range.

The secondary structures of wild type LipL32 and mutated LipL32 were analyzed at 25 °C on an Aviv 202 spectropolarimeter (Aviv Biomedical, Lakewood, NJ) using a 1-mm path length cuvette. The resulting spectra were corrected to remove buffer signal and smoothed using SigmaPlot 10.0. The obtained CD spectra were reported as averages of no less than three scans for each sample.

**Thermal Stability Assay**—CD spectra were used to determine protein stability by monitoring conformational states over a temperature range (from 30 to 80 °C). $T_m$ values (temperature at which half-denaturation of the protein is observed) were calculated on the basis of changes in the CD signals at 218 nm, which were smoothed by fitting the equation of SigmaPlot 10.0.

**Stains-all Binding Assay**—The integrity of the Ca$^{2+}$-binding cluster was examined by CD spectrometry using stains-all dye, a carbocyanine probe for detecting Ca$^{2+}$-binding proteins containing mainly polyanions, such as Lig and LipL32 in this study (29, 30). Wild type LipL32 and its variants (15 μM) were mixed with dye solution (200–250 μM) in 2 mM MOPS buffer with 30% (v/v) ethylene glycol (pH 7.2) and incubated for 5 min. The CD spectra were scanned from 700 to 400 nm yielding a characteristic peak at 660 nm (I band), indicative of a functional calcium-binding cluster (29, 31).

**Cell Culture, RNA Extraction, and Real Time PCR**—HK2 cells were cultured in DMEM/Ham’s F-12 medium supplemented with 5% (v/v) FCS, 2 mM glutamine, 20 mM HEPES (pH 7.0), 0.4 μg/ml hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin, and 28.9 μM sodium selenite at 37 °C in a humidified atmosphere of 5% (v/v) CO$_2$ as described previously (32). Cells were switched to a serum-free medium before addition of LipL32 to the medium. Total RNA was extracted according to the guanidinium thiocyanate/phenol/chloroform method (Cinna/Biotex Laboratories International Inc., Friendswood, TX) (32, 33). Real time PCR was performed following the manufacturer’s instructions using an ABI Prism 7700 with SYBR
Green I as a double-stranded DNA-specific dye (PE-Applied Biosystems, Cheshire, UK). Primers of hTNF-α, hCXCL8/IL-8, hCCL2/MCP-1, and hMMP7 are listed in supplemental Table S1 and were constructed to be compatible with a single RT-PCR thermal profile (95°C for 10 min, 40 cycles at 95°C for 30 s, and 60°C for 1 min). The accumulation of the PCR product was recorded in real time (PE-Applied Biosystems). The results of the mRNA levels for the different genes are displayed as transcript levels of the analyzed genes relative to GAPDH.

**ELISA** — The LipL32 and TLR2 interaction was investigated by ELISA according to a previously reported method with minor modifications (17, 19). The ELISA plates (Nunc-Immuno Plate; Thermo Scientific, Denmark) were primarily coated with 1 μg of TLR2 protein in 100 μl of PBS and incubated for 2 h at 37°C. Wells were then washed with PBST (PBS containing 0.05% (v/v) Tween 20) and blocked with 200 μl of PBS containing 1% (w/v) BSA for 1 h at 37°C. Plates were incubated overnight at 4°C. Protein samples (LipL32, mutants, wild type LipL32 in the presence of EGTA, and wild type LipL32 without the His tag; concentration ranges from 0 to 2 μM) in PBS were added to the attached TLR2 protein for 90 min at 37°C, followed by a wash with PBST. Bound LipL32 protein was allowed to interact with 100 μl of anti-LipL32 antibody (1:5,000 dilution) for 1 h. Wells were then washed with PBST. Subsequently, 100 μl of HRP conjugated to donkey anti-rabbit IgG (1:5,000 dilution) was added to each well, and the plates were incubated for 1 h at 37°C. After washing with PBST, 3,3′,5,5′-tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was included for color development. Absorbance was measured at 450 nm in a Thermomax ELISA reader (Molecular Devices, Sunnyvale, CA). The absorbance at each data point was corrected by subtracting the value of the negative control (BSA-coated well) read. The dissociation constant ($K_d$) was calculated according to a prior protocol (34) based on Equation 1,

$$A = A_{\text{max}}[\text{protein}]/(K_d[\text{protein}])$$  

(Eq. 1)

where $A$ is the absorbance at a set protein concentration; $A_{\text{max}}$ is the maximum absorbance of the ELISA plate reader (equilibrium); [protein] is the protein concentration, and $K_d$ is the dissociation equilibrium constant for a given absorbance at a set protein concentration. The binding data were analyzed via the SigmaPlot 10.0 by fitting the data to the appropriate equation (35), assuming that ligands bound to one independent site.

**Atomic Force Microscope (AFM) Tip and Mica Functionalization** — The AFM cantilever made of silicon nitride, PNP-TR (NanoWorld, Neuchâtel, Switzerland), was cleaned sequentially by sonication in a series of solvents: 2-propanol, methanol, and deionized water (5 min each). Tips were functionalized according to a previous method with minor modifications (36). At first, probes were transferred to a 0.1% (v/v) solution of 3-aminopropyltrimethoxysilane (Sigma) in toluene for 2 h. Following silanization, tips were sonicated in methanol and deionized water (5 min each), consecutively. For functionalization, AFM tips were incubated in a 1% (v/v) solution of glutaraldehyde (grade II, Sigma) in PBS for 1 h at room temperature. For anchoring proteins, the tips were rinsed with PBS to remove the remaining glutaraldehyde and subsequently incubated with proteins, including wild type LipL32, Ca$^{2+}$-binding variants, wild type LipL32 in the presence of EGTA, denatured wild type LipL32, and BSA, independently, for 1 h at room temperature. The functionalized AFM tips were rinsed three times with PBS to remove unbound proteins (36). The mica surface was modified for a better deposition of the proteins of interest according to previous reports (37, 38). In brief, the mica surface (1 cm$^2$) was functionalized with 0.1% (v/v) 3-aminopropyltri-
methoxysilane (Sigma) (AP-mica) followed then by treatment with 1% (v/v) glutaraldehyde (grade II, Sigma). The proteins (1 g) were deposited covalently on the functionalized mica surface for 2 h at room temperature. Unbound proteins were removed, and the proteins fixed on mica were utilized for later AFM measurements.

**AFM Observation**—The functionalized cantilever tips had a spring constant \( k \) in the range of 0.02–0.08 newton/m, as determined from the amplitude of their thermal vibrations. A commercial AFM (Nanoscope III, Digital Instruments, Santa Barbara, CA) with a J-type scanner was employed throughout this study. The force volume software measures a force curve at each of the 999 points during a two-dimensional scan over a sample surface. The \( X-Y \) scan size was 150 \( \mu \)m, and the \( Z \) scan distance was 5 \( \mu \)m at a rate of 1 Hz. The force applied to the protein-modified mica surface was kept below 400 pN. The distance force curves and force parameters were obtained according to the methods previously described (39). For antibody neutralization analysis, the protein-modified mica was treated with anti-TLR2 antibodies (diluted 1:1,000 in PBS) for 1 h followed by five washes with PBS to remove unbound antibodies. The antibody against TLR2 (AbTLR2) was purchased from GeneTex (Irvine, CA). For binding studies, force curves from at least 10 areas on each surface were independently selected for analysis. All measurements described above were performed with modified tips and showed repeatedly similar results.

**Ca\(^{2+}\) Background Concentration**—The base-line Ca\(^{2+}\) concentration in assay medium was \( \sim 36.2 \pm 7.5 \) nM \((n=7)\), a value much below LipL32 concentration (10 \( \mu \)M) in the solution, ensuring any effects of carried over Ca\(^{2+}\) negligible in this study. The concentration of Ca\(^{2+}\) was determined using an inductively coupled plasma mass spectrometer at National Tsing Hua University Instrument Center, National Tsing Hua University, Hsin Chu, Taiwan.

**RESULTS AND DISCUSSION**

**Characterization of LipL32 Calcium Binding Variants**—LipL32 contains a Ca\(^{2+}\)-binding site composed of Asp\(^{132}\), Thr\(^{133}\), Asp\(^{164}\), Asp\(^{165}\), and Tyr\(^{178}\) residues (Fig. 1A and B). Importantly, these residues are highly conserved in LipL32 from various pathogenic bacteria such as *L. shermani*, *P. tunicate*, *Moritella* sp., and unidentified *Eubacterium* SCB49 (Fig. 1C). Similar polyanion clusters for Ca\(^{2+}\) binding were also found in LigBCen2 of *Leptospira interrogans* (gi: 199584090) (30) and in ChaA Na\(^{+}/\)Ca\(^{2+}\) antiporter for *E. coli* (gi: 388477298) (40). To verify the role of the Ca\(^{2+}\)-binding cluster involved in LipL32 binding to induce inflammatory response induction in human kidney cells, site-directed
mutagenesis was used to generate five Ca\textsuperscript{2+} binding variants: D132A, T133A, D164A, D165A, and Y178A variants.

A carboxylic dye (stains-all) that interacts generally with polyanions, such as in the poly(Asp) region, was first employed as a probe to investigate the structural integrity of the Ca\textsuperscript{2+} -binding cluster (29, 30). The stains-all assay of LipL32 containing a poly(Asp) region revealed a J band peaked at 660 nm, indicating a conformational change in the structure (Fig. 2A, Table 1) (cf. Refs. 17, 29, 30). The calcium binding variants yielded a prominent decrease of J bands at 660 nm. For instance, a J band ellipticity (θ) angle value of $-67.2$ millidegrees was measured for wild type LipL32, whereas values of $-1.9$, $-2.3$, $-0.6$, $-1.2$, and $-2.3$ millidegrees were obtained for the D132A, T133A, D164A, D165A, and Y178A variants, respectively (Fig. 2A and Table 1). The results of the stains-all assay conceivably suggest structural changes in the Ca\textsuperscript{2+} -binding cluster. Furthermore, removal of Ca\textsuperscript{2+} from wild type LipL32 by EGTA decreased the ellipticity of the J band to 70% of the original level, indicating that a depletion of Ca\textsuperscript{2+} induces conformational alteration (supplemental Fig. S1A) (cf. Refs. 17, 30). Removal of the His\textsubscript{6} tag (27) from recombinant LipL32 by an enterokinase did not alter the J band value, excluding the possibility of a His\textsubscript{6}-tail artifact in data for in this study (supplemental Fig. S1A). Notably, the J band disappearance in mutants validated the hypothesis that every residue in this cluster of LipL32 is crucial for the structural integrity of this novel Ca\textsuperscript{2+} -binding protein (30).

The intrinsic fluorescence of LipL32 shows a 350-nm peak in its fluorescence emission profile (Fig. 2B, compare with Ref. 16). However, the calcium binding variants displayed a shift in the maximum wavelength, indicating a conformational change in the structure (Fig. 2B and Table 1). Furthermore, the CD spectrum of the LipL32 showed a broad trough between 220 and 200 nm (Fig. 2C and Table 1, compare with Ref. 16). The midpoint temperature ($T_m$) of protein unfolding was calculated by monitoring the change in the ellipticity value at 218 nm against the temperature scanned (Fig. 2D). As shown in Table 1, LipL32 has a midpoint of 64.4 ± 0.9 °C. Three mutants reported similar $T_m$ values to the wild type protein, but the D164A and D165A variants exhibited a significant decline in $T_m$ to 60.1 ± 0.3 and 60.7 ± 0.4 °C, respectively (Table 1). These changes suggest that the Ca\textsuperscript{2+} -binding cluster is important for stabilizing the overall structure of LipL32, in particular with the possible assistance from residues Asp\textsuperscript{164} and Asp\textsuperscript{165}.

### TABLE 1

Spectral characteristics of calcium binding variants

| Sample | Ellipticity $(-\theta_{660})$ | $\alpha$-Helix | $\beta$-Sheet | $F_{\text{max}}$ | $T_m$ |
|--------|-----------------|--------------|--------------|-------------|------|
|        | millidegree     | %            | %            | A.U.        | °C   |
| WT     | 67.2            | 10.5         | 29.1         | 891.7       | 64.4 ± 0.9 |
| D132A  | 1.9             | 15.4         | 26.4         | 842.5       | 65.5 ± 0.5  |
| T133A  | 2.3             | 10.5         | 29.1         | 935.0       | 63.2 ± 0.7  |
| D164A  | 0.6             | 15.4         | 26.4         | 812.5       | 60.1 ± 0.3  |
| D165A  | 1.2             | 10.5         | 29.1         | 899.1       | 60.7 ± 0.4  |
| Y178A  | 2.3             | 10.5         | 29.1         | 926.6       | 62.0 ± 0.5  |

### TABLE 2

Interaction between LipL32 and TLR2 as determined by ELISA and AFM

| Sample | $K_d$ ($K_m$) | $pN$ (%) | $Ab_{TLR2}$ | $F_{\text{max}}$ |
|--------|--------------|----------|-------------|-----------------|
| WT     | 0.13 ± 0.01  | 46.0 ± 1.1000 | 26.7 ± 0.5  | 100.0 |
| D132A  | 0.19 ± 0.04  | 29.4 ± 1.4 (63.9) | 25.1 ± 0.9  | 94.0 |
| T133A  | 0.41 ± 0.07  | 39.3 ± 2.9 (85.4) | 24.2 ± 0.7  | 90.6 |
| D164A  | 0.13 ± 0.01  | 54.9 ± 3.5 (119.3) | 20.3 ± 0.5  | 76.0 |
| D165A  | 0.22 ± 0.03  | 30.9 ± 2.2 (67.1) | 22.4 ± 0.1  | 83.9 |
| Y178A  | 0.20 ± 0.04  | 24.2 ± 1.7 (52.6) | 17.8 ± 0.3  | 66.7 |
| WT/EGTA| 0.30 ± 0.09  | 30.3 ± 1.3 (65.9) | ND          |      |
| BSA    | ND            | 23.3 ± 1.8 (50.7) | ND          |      |
| Tip only| ND           | 33.3 ± 0.8 (72.4) | ND          |      |

### FIGURE 3

Interaction of LipL32 and TLR2 as determined by ELISA. A, schematic presentation of TLR2 protein. TLR2 (784 residues) is composed of leucine-rich repeats (LRR), transmembrane (TM), and Toll/IL-1 receptor (TIR) domains. Two TLR2 fragments, TLR2(49–538) and TLR2(589–784), were prepared according to methods described under “Experimental Procedures.” B, LipL32 binding to TLR2(49–538) and TLR2(589–784) with BSA as a negative control. C, binding of TLR2(49–538). The binding capability of TLR2(49–538) was examined by using synthetic lipopeptide Pam\textsubscript{3}CSK\textsubscript{4} and lipoteichoic acid (LTA), respectively; BSA was used as blank. The concentrations of LipL32, Pam\textsubscript{3}CSK\textsubscript{4}, lipoteichoic acid, and TLR2 fragments were all 2 μM.

### Binding of TLR2 to LipL32 as Monitored by ELISA

Two TLR2 fragments, TLR2(49–538) and TLR2(589–784) (Fig. 3A), were generated for investigating the interaction between LipL32 and TLR2 (cf. 32). Fig. 3B depicts that LipL32 can bind to TLR2(49–538), but not to TLR2(589–784), indicating that the binding domain is indeed located in the leucine-rich repeat domain of TLR2. Several TLR2 ligands such as synthetic lipopeptide Pam\textsubscript{3}CSK\textsubscript{4} and lipoteichoic acid were also used to verify the feasibility of the binding assay for the binding of LipL32.
Role of Calcium-binding Cluster in LipL32

Figure A: AFM images of LipL32 showing纳米-scale structures.

Figure B: Cartoon representation of TLR2.

Figure C: Schematic diagram showing the interaction of LipL32 with TLR2.

Figure D: Diagram illustrating the effect of anti-TLR2 antibody on the interaction.

Graphs showing force distribution at different conditions: Tip only, WT, D112A, B6A, T133A, D144A, Y178A, R185A, T185A, R185A.
to TLR2(49–538) (Fig. 3C) (32). To further investigate the role of the Ca$^{2+}$-binding cluster in the interaction of LipL32 with TLR2, the binding efficiency of LipL32 and its variants to TLR2(49–538) was measured (Table 2). A $K_d$ value of 0.13 ± 0.01 $\mu$M was calculated for the binding of wild type LipL32 to TLR2(49–538). The $K_d$ value for interaction between LipL32 without the His$_6$ tag and TLR2(49–538) was similar to that of LipL32 with the His$_6$ tag. However, in the presence of EGTA, the $K_d$ value of wild type LipL32 to TLR2(49–538) increased to 0.30 ± 0.09 $\mu$M compared with the 0.13 ± 0.01 $\mu$M value in the absence of EGTA, demonstrating that removal of calcium diminished the binding of LipL32 to TLR2(49–538). Moreover, the $K_d$ values for the binding of the LipL32 variants to TLR2(49–538) were relatively increased in comparison with wild type LipL32. The D164A variant was an exception in that it retained a similar binding affinity, suggesting that mutations in the Ca$^{2+}$-binding cluster lessened the binding of LipL32 to TLR2(49–538) (Table 2). It is possible that the D164A variant, deprived of Ca$^{2+}$, could induce a conformational state that still exhibits binding capacity to TLR2. Notwithstanding, it is conceivable that Ca$^{2+}$ binding of LipL32 is crucial for maintaining its structural integrity to bind TLR2, resulting consequently in inflammatory responses of human kidney cells (see below).

**Binding of TLR2 to LipL32 as Monitored by AFM**—AFM is a powerful tool for investigating the forces between individual ligands and receptors as well as for protein-protein interactions. To determine the role of the Ca$^{2+}$-binding cluster in the LipL32 and TLR2 interaction, TLR2 was first deposited through covalent modification on the mica surface as shown in Fig. 4A. The tip modified with BSA served as negative control, and the tip without any modification served as mock control (Fig. 4B). The force-distance curve was determined to generate an interaction force of 46.0 ± 1.1 pN between the TLR2(49–538) molecule and the modified LipL32 on the tip (Fig. 4C and Table 2, compare with Ref. 23). To confirm the protein-protein interaction, TLR2(49–538) was first denatured at 100 °C for 30 min before it was fixed on the mica surface (supplemental Fig. S2A). For reciprocally denatured TLR2 and LipL32, interaction forces declined (23.3 ± 1.1 and 30.2 ± 1.4 pN) relative to control, illustrating the requirement of a proper conformation of TLR2(49–538) for its association to LipL32 (supplemental Fig. S2, B and C). Moreover, in the presence of EGTA, the interaction force decreased to 30.3 ± 1.3 pN (supplemental Fig. S2D and Table 2), indicating that the removal of Ca$^{2+}$ presumably resulted in a conformational change that abolishes the binding of LipL32 to TLR2(49–538). Furthermore, most calcium binding variants showed obvious decreases to, for instance, 29.4 ± 1.4 pN (63.9% of the wild type) for the D132A variant, 39.3 ± 2.9 pN (85.4% of the wild type) for the T133A variant, 30.9 ± 2.2 pN (67.1% of the wild type) for the D165A variant, and 24.2 ± 1.7 pN (52.6% of the wild type) for the Y178A variant, in rupture force for their binding to TLR2(49–538), except D164A variant slightly increased the interaction force (54.9 ± 3.5 pN, 119.3% of the wild type; Fig. 4C and Table 2). The evidence above demonstrates as well that the residues of the Ca$^{2+}$-binding cluster are essential for a proper conformation of LipL32 and for its binding to TLR2(49–538). The antibody neutralization technique was further used to confirm the protein-protein interaction of TLR2 and LipL32 (23, 33). The AFM tip was first modified by LipL32, and then an anti-TLR2 antibody (Ab$_{TLR2}$) was utilized for covering TLR2 on the mica surface. The interaction force of LipL32 at the tip and TLR2-Ab$_{TLR2}$ on mica consequently decreased to 26.7 ± 0.5 pN (Fig. 4D and Table 2). Similarly, calcium binding variants exhibited a declined interaction force to <25 pN when studied by this method. Ab$_{TLR2}$ neutralization effects confirmed an interaction between LipL32 and TLR2 (cf. 23). Thus, ELISA and AFM demonstrated that calcium binding variants diminished binding of LipL32 to TLR2.

**Inflammatory Response Induced by the Outer Membrane Protein LipL32**—Recognition of bacterial components by host TLRs initiates signaling cascades that stimulate nuclear transcription factor κB (NF-κB) and mitogen-activated protein kinases (MAPKs) and induces chemokines and cytokines (20, 24). The expression levels of hCXCL8/IL8, hCCL2/MCP-1, hMMP7, and hTNF-α (6) were measured to investigate the role of the Ca$^{2+}$-binding cluster in LipL32 (Fig. 5 and Table 3). The leptospiral outer membrane protein extraction solubilized by using Triton X-114 was employed as the positive control, and PBS alone served as mock control. Twenty four hours after adding LipL32 proteins (including wild type and LipL32 mutants) to HK2 cells, hCXCL8/IL8, hCCL2/MCP-1, hMMP7, and hTNF-α mRNA levels significantly changed as compared with the wild type. To exclude LPS contamination from the bacterial extract, the LPS chelating agent polymyxin (10 μg/ml) was included (Fig. 5A; compare with Ref. 9). Following treatment by polymyxin-containing beads, eluted LipL32 retained relatively elevated inflammatory effects on HK2 cells, demonstrating that the responses indeed stemmed from the specific interaction of LipL32 with TLR2 (Fig. 5A). Additionally, heat denaturation (100 °C, 30 min) and protease K digestion (20 μg/ml at 63 °C for 18 h) of LipL32 independently promoted the disappearance of signals, indicating explicitly the involvement of the LipL32 in inflammatory responses (Fig. 5A) (9). Moreover, concentration dependence of inflammatory responses by LipL32 was scrutinized (Fig. 5B). The optimal dose for all inflammatory responses was ~2.5 μg/ml LipL32 (cf. supplemental Fig. S3). These results unambiguously confirm that the inflammatory responses of HK2 cells came directly from...
LipL32, presumably through its interaction with TLR2 (cf. Ref. 23). Furthermore, expression of hCXCL8/IL8 was decreased to 57.9, 47.4, 47.4, 57.9, and 63.2% of the wild type upon incubation with the D132A, T133A, D164A, D165A, and Y178A mutants, respectively (Fig. 5C). Likewise, hMMP-7, hCCL2/MCP-1, and hTNF-α expression showed a decrease upon treatment with the calcium binding variants, similar to their effects on the hCXCL8/IL8 (Fig. 5, D–F). The reduced levels of
The binding of Ca$^{2+}$ to LipL32 optimizes LipL32 to TLR2 binding conformation that seems responsible for critical inflammatory responses. However, failure of the D164A mutant to diminish LipL32 binding to TLR2 may be attributed to a preservation of confirmation that keeps TLR2 binding. Nevertheless, claims made about the D164A mutant require further experimental justification.

Recent reports indicate that calcium is not necessary for LipL32 binding to host extracellular matrix proteins, e.g. fibronectin, collagen type IV, and plasminogen (19), despite the evidence of the crystal structure showing calcium bound in a conserved calcium-binding cluster (16, 17). This study demonstrated diminished interaction of calcium binding variants with TLR2 using ELISA and AFM. The calcium binding variants of LipL32 decreased the inflammatory response of human renal cells compared with the wild type LipL32. Addition of Ca$^{2+}$ and EGTA both increased the $K_d$ value of the LipL32 and TLR2 interaction (Table 2 and supplemental Table S2). EGTA and Ca$^{2+}$ also elicited a fluorescence emission profile for wild type LipL32, above background levels of Ca$^{2+}$ (supplemental Fig. S1, B and E). LipL32 protein could natively exist in a transient mixture of Ca$^{2+}$-bound (closed state, Fig. 1A) and Ca$^{2+}$-free (open state, Fig. 1B) conformations. Because Ca$^{2+}$ binding involves a large conformational change of the calcium-binding cluster, the specific LipL32 conformation that favorably interacts with TLR2 should be distinctly identified. Nevertheless, this work verifies an effect of mutations to the calcium-binding cluster on the immune response and provides details of how these residues are involved. Notwithstanding, more experiments are required to elucidate the mechanism of LipL32 binding to TLR2 that results in severe damage to the kidney. In conclusion, this work reveals that the calcium-binding cluster of LipL32 is essential for the stabilization of its overall structure to bind TLR2 and the elicitation of the subsequent inflammatory responses in human kidney cells.

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