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Lp95, a novel leptospiral protein that binds extracellular matrix components and activates e-selectin on endothelial cells

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Summary *Objectives:* The study of a predicted outer membrane leptospiral protein encoded by the gene *LIC12690* in mediating the adhesion process.
Methods: The gene was cloned and expressed in *Escherichia coli* BL21 (SI) strain by using the expression vector pAE. The recombinant protein tagged with N-terminal hexahistidine was purified by metal-charged chromatography and used to assess its ability to activate human umbilical vein endothelial cells (HUVECs).
Results: The recombinant leptospiral protein of 95 kDa, named Lp95, activated E-selectin in a dose-dependent fashion but not the intercellular adhesion molecule 1 (ICAM-1). In addition, we show that pathogenic and non-pathogenic *Leptospira* are both capable to stimulate endothelium E-selectin and ICAM-1, but the pathogenic *L. interrogans* serovar Copenhageni strain promotes a statistically significant higher activation than the non-pathogenic *L. biflexa* serovar Patoc ($P < 0.01$). The Lp95 was identified *in vivo* in the renal tubules of animal during experimental infection with *L. interrogans*. The whole Lp95 as well as its fragments, the C-terminal containing the domain of unknown function (DUF), the N-terminal and the central overlap regions bind laminin and fibronectin ECM molecules, being the binding stronger with the DUF containing fragment.

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Conclusion: This is the first leptospiral protein capable to mediate the adhesion to ECM components and the activation of HUVECS, thus suggesting its participation in the pathogenesis of *Leptospira*.

Introduction

Leptospirosis, an emerging infectious disease, is a worldwide zoonosis of human and veterinary concern. Caused by pathogenic spirochaetes of the genus *Leptospira*, the disease presents greater incidence in tropical and subtropical regions.[1,2] The transmission of leptospirosis has been associated with exposure of individuals in close proximity to wild or farm animals.[1,3] Lately, the disease became prevalent in cities with sanitation problems and large population of urban rodent reservoirs, which contaminate the environment through their urine.[4] Fever, chills, headache, and severe myalgias characterize the early phase of the disease. Progression to multi-organ system complications occurs in 5–15% of cases, with mortality rates of 5–40%.[1,5]

Currently available veterinarian vaccines are based on inactivated whole cell or membrane preparations of pathogenic leptospires. These types of vaccine confer protective responses through, but not exclusively, the induction of antibodies against leptospiral lipopolysaccharide.[6,7] However, these vaccines do not induce long-term protection against infection and do not provide cross-protective immunity against leptospiral serovars not included in the vaccine preparation. There is a large number of pathogenic serovars (>200) that impose a major limitation to the production of a multi-serovar vaccine and to the development of immunization protocols based on whole cell or membrane preparations.

Leptospira invasiveness is attributed to its ability to disseminate widely within the host during the early stage of infection but, the mechanisms associated with this invasion are poorly understood.[1] Due to their location, surface-associated proteins are likely to be relevant in host-pathogen interactions, hence their potential to elicit several activities, including adhesion. The interaction of pathogens with the extracellular matrix (ECM) has been well documented.[8] In the case of leptospires, few adhesion ECM-binding molecules have been characterized thus far. These include the Len protein family,[9] LigA/LigB,[10] Lsa21,[11] LipL32[12,13] and TlyC.[14] Cell adhesion molecules (CAMs) are surface receptors present in eukaryotic cells that mediate cell-cell or cell-extracellular matrix interactions.[15,16] The stimulation of CAMs was published for the pathogenic spirochaetes, *Borrelia* sp. and *Treponema* sp.[17–21] among other bacteria.[22] We have shown that the recombinant protein rLIC10365 of *L. interrogans* promoted upregulation of intercellular adhesion molecule 1 (ICAM-1) and E-selectin on HUVECS.[23] More recently, we reported that three selected coding sequences (LIC10507, LIC10508 and LIC10509), located in a region of the *L. interrogans* serovar Copenhageni genome that has a potential involvement in pathogenesis,[24] were also capable to promote stimulation of ICAM-1 and E-selectin on monolayers of HUVECS.[25] We trust that understanding the molecular mechanism of leptospiral pathogenesis should help the identification of novel vaccine candidates.

In the present work, we describe the cloning, expression, purification and characterization of one predicted leptospiral outer/inner-membrane protein encoded by the gene LIC12690, identified by bioinformatics tools in the genome sequences of the *L. interrogans* serovar Copenhageni. The recombinant protein Lp95 was expressed in *Escherichia coli* BL21 (SI) strain and purified by metal-affinity chromatography. The protein encoded by LIC12690 is present *in vivo* in the renal tubules of animal during experimental infection in guinea pig. Evaluation of Lp95 effects on CAM expression of HUVECS *in vitro* indicates that the recombinant protein activates the expression of E-selectin but not ICAM-1. In addition, the whole Lp95, its C-terminal containing DUF1554 tag and overlap region fragments bind laminin and fibronectin ECM molecules, suggesting its involvement in the cell adhesion interaction.

Materials and methods

ECM components

All macromolecules, including the control protein BSA, were purchased from Sigma Chemical Co. (St. Louis, Mo.). Laminin-1 and collagen Type IV were derived from the basement membrane of Engelbreth-Holm-Swarm mouse sarcoma, cellular fibronectin was derived from human foreskin fibroblasts, plasma fibronectin was isolated from human plasma and collagen Type I was isolated from rat tail. Antibodies used to detect ECM components were from Sigma Chemical Co. (St. Louis, Mo.), produced in rabbits against the ECM components described above.

Leptospira strains and culture conditions

The non-pathogenic *L. biflexa* (serovar Patoc strain Patoc 1) and the pathogenic *L. interrogans* (serovars Canicola strain Hond Utrechet IV, Copenhageni strains M-20 and Fiocruz L1-130, Grippotyphosa strain Moskva V, Hardjo strain Hardjoprajtino, Icterohaemorrhagiae strain RGA, Pomona strain Pomona) were cultured at 28 °C under aerobic conditions in liquid EMJH medium (Difco®) with 10% rabbit serum, enriched with L-asparagine (wt/vol: 0.015%), sodium pyruvate (wt/vol: 0.001%), calcium chloride (wt/vol: 0,001%), magnesium chloride (wt/vol: 0.001%), peptone (wt/vol:0.03%) and meat extract (wt/vol: 0.02%).[26] *Leptospira* cultures are maintained in Faculdade de Medicina Veterinária e Zootecnia, USP, São Paulo, SP, Brazil.

'In silico' identification and characterization of the protein

Predicted coding sequence (CDS) LIC12690 was selected from the *L. interrogans* serovar Copenhageni genome sequences[27] based on its cellular localization prediction by

PSORT program, http://psort.nibb.ac.jp,[28,29] and P-Classifier program, http://protein.bii.a-star.edu.sg/localization/gram-negative/index.html.[30] The SMART, http://smart.embl-heidelbergde,[31,32] PFAM, http://www.sanger.ac.uk/Software/Pfam,[33] and LipoP, http://www.cbs.dtu.dk/services/LipoP,[34] web servers were used to search for predicted functional and structural domains within the amino acid sequences of the selected sequences. The predicted sequence of the lipobox was evaluated by use of the SpLip program, as described by Setubal et al.[35] Sequence analysis was performed with ClustalX[36] and a tree displayed program by the Neighbour-Joining method.[37]

DNA isolation and PCR analysis

Leptospira cultures were harvested by centrifugation at 11,500*g* for 30 min and gently washed in sterile PBS twice. Genomic DNA was isolated from the pellets by guanidine-detergent lysing method using DNAzol® Reagent (Invitrogen), according to the manufacturer's instructions. A 399-bp LIC12690 DNA fragment was amplified using oligonucleotides LIC12690-F: 5′ CACCTTTTCTAACTTCGCGGACTC 3′ and LIC 12690-R: 5′ GTATAAGTTCCTGGAGACCAATTGAGAG 3′ designed according to *L. interrogans* serovar Copenhageni genome sequences (GenBank accession AE016823). PCR was performed in a reaction volume of 25 μl containing 100 ng of genomic DNA, 1× PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 2 mM $MgCl_2$, 20 pmol of each specific primer, 200 μM of each dNTP, and 2.5 U *Taq* DNA Polymerase (Invitrogen). Cycling conditions were: 94 °C, 5 min, followed by 35 cycles at 94 °C, 50 s, 62 °C, 50 s, 72 °C, 90 s, and a final extension cycle of 7 min at 72 °C. PCR amplified products were loaded on a 1% agarose gel for electrophoresis and visualization with ethidium bromide.

RNA extraction and RT-PCR analysis

For reverse transcription (RT)-PCR, total RNA was isolated by the acid guanidinium thiocyanate phenol-chloroform method using TRIzol® Reagent (Invitrogen) according to the manufacturer's recommendations. One microgram of RNA from each sample was treated with 1 U of DNAse I Amplification Grade (Invitrogen) for 15 min at room temperature. DNAse I was inactivated by the addition of 1 μl of 25 mM EDTA solution followed by an incubation at 65 °C for 10 min. DNAse-treated RNAs were reversely transcribed using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). One tenth of RT products were amplified in a 25-μl reaction mix using oligonucleotides LIC12690-F/LIC112690-R as described above. Samples quantity and integrity were verified by amplification of a 1042-bp 16S ribosomal cDNA fragment using oligomers 16S-F 5′ CAAGTCAAGCGGAGTAGCAATACTCAGC 3′ and 16S-R 5′ GATGGCAACATAAGGTGAGGGTTGC 3′.

DNA recombinant techniques

Predicted CDS LIC12690 and subfragments (N-terminus, C-terminus and Overlap) were amplified by the PCR from total *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 genomic DNA using the primer pairs: LIC12690F: 5′ CACCT TTTCTAACTTCGCGGACTC 3′ and LIC12690R: 5′ CTATTGTT CCACACAAAGAATGC 3′; N-terminusF: 5′ CTCGAGAATACAAA ATCTCAAAAC 3′ and N-terminusR: 5′ AAGCTTCTAAAGGATC TCTAAATCC 3′; C-terminusF: 5′ CTCGAGCAAAATGGAGAAGACGTTAC 3′ and C-terminusR: 5′AAGCTTGAATTGTGAC TCCTAGGAAAC 3′; OverlapF: 5′ CTCGAGTCAACCACTTCCGATTTC 3′ and OverlapR: 5′AAGCTTCTACATCAGTCTT GCACCTTC 3′. Underlined nucleotides indicate restriction sites (XhoI/HindIII). LIC12690 PCR product was cloned into pENTR-TOPO vector (Invitrogen) followed by transfer/recombination of DNA insert into the *E. coli* expression vector pDEST17 (Invitrogen) using the LR Clonase (Invitrogen). Subfragment PCR products were cloned into pGEM-T easy vector (Promega) and subcloned into the pAE expression vector.[38] Both vector pDEST17 and pAE allow the expression of recombinant proteins with a minimal 6 × His-tag at the N-terminus. All cloned sequences were confirmed by DNA sequencing with an ABI 3100 automatic sequencer (PE Applied Biosystems, Foster city, CA).

Expression and purification of recombinant protein

Protein expression was achieved in *E. coli* BL21 (SI) strain by the action of T7 DNA polymerase under control of the osmotically induced promoter *proU*.[39] *E. coli* BL21 (SI) containing recombinant plasmids were grown at 30 °C in Luria—Bertani broth without NaCl and with 100 μg/ml ampicillin with continuous shaking until an optical density at 600 nm of 0.6—0.8 was reached. Recombinant protein synthesis was induced by the addition of 300 mM NaCl. After 3 h, the cells were harvested by centrifugation, the bacterial pellets resuspended in lysis buffer (10 mM Tris—HCl, pH 8.0, 150 mM NaCl, 100 μg/ml of lysozyme, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride [PMSF]). The bacterial cell pellets were lysed on ice with the aid of a sonicator (Ultrasonic Processor; GE Healthcare). The insoluble fraction was washed five times with 20 ml of buffer (20 mM Tris—HCl, pH 8.0, 500 mM NaCl, 1 M urea and 0.1% Triton X-100) and resuspended in a buffer containing 20 mM Tris—HCl, pH 8.0, 500 mM NaCl and 8 M urea. The protein was then purified through metal chelating chromatography in a Sepharose fast flow column (GE Healthcare) and fractions were analyzed in 12% SDS-PAGE. Only Lp95 full-length was refolded by 500 × dilution with 50 mM Tris—HCl, pH 8.0, and 500 mM NaCl and purified through metal chelating chromatography in a Sepharose fast flow column (GE Healthcare). The protein was extensively dialyzed against phosphate-buffered saline (PBS), pH 7.4, 0.1% (wt/vol) glycine solution (at the proportion of 10 ml of protein per 1000 ml of buffer) with at least five changes of buffer every 4 h for 48 h.

Antiserum

Ten female BALB/c mice (4—6 weeks old) were immunized subcutaneously with 10 μg of Lp95 full-length protein. The recombinant protein was adsorbed in 10% (vol/vol) of Alhydrogel (2% $Al(OH)_3$, Brenntag Biosector, Denmark), used as adjuvant. Two subsequent booster injections were given at two-week intervals with the same preparation of 10 μg of Lp95 protein. Negative control mice were injected with PBS. One week after each immunization, the mice were bled from the retro-orbital plexus and the pooled sera

were analyzed by enzyme-linked immunosorbent assay (ELISA) for determination of antibody iters. All animal studies were approved by the Ethics Committee of the Instituto Butantan, São Paulo, SP, Brazil.

Limulus amebocyte lysate assay (LAL-test)

The chromogenic LAL assay for endotoxin activity of the protein samples was performed using the QCL-1000 kit (Bio-Whittaker Inc., Walkersville, MD), according to the manufacturer's instructions.

Endothelial cell culture

Endothelial cells were obtained from human umbilical veins (HUVECs) by collagenase (GIBCO) digestion according to the method of Jaffe et al.[40] Cells were grown to confluence in 25 cm^2 tissue culture flasks that were precoated with 1% gelatin (Sigma). The growth medium consisted of RPMI 1640 supplemented with 10% fetal bovine serum (FBS, GIBCO), 90 μg/ml heparin (GIBCO), 50 μg/ml endothelial cell growth supplement (ECGS, Sigma), 2 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma) at 37 °C in a humidified 5% CO_2 incubator. Confluent HUVECs were passaged with 0.05% trypsin + 0.02% EDTA and were routinely used between the first and third passages. Cultured cells were identified as endothelial by their morphology and von Willebrand Factor (vWF) antibody binding. The experiments with recombinant proteins were performed in the presence of 7 μg/ml polymyxin B (Sigma) to rule out lipopolysaccharide (LPS) interference.

Expression of E-selectin and ICAM-1

HUVECs were incubated with 100 μl of leptospires (10^5) in bacteria culture medium (mock) or purified Lp95 at the indicated concentrations for 1 h in RPMI-1640 medium. As longer incubation times with the live bacteria induced HUVECs lysis, leptospires or purified Lp95 were removed by washing, and endothelial cells were further incubated in RPMI 1640 with 10% FBS. E-selectin and ICAM-1 expression were assessed after 4 or 12 h, respectively. The ratio of bacteria to endothelial cells was 5:1 and during the 1 h incubation, leptospiral mobility could be observed. Under these conditions LPS (from *E. coli* 0111:B4, Sigma L-4391), used as positive control, was capable to trigger ICAM-1 and E-selectin expression in almost 50 and 80% of HUVECs, respectively. Lp95 (25 μg/ml) was pre-treated with proteinase K (PK, 500 ng/ml) to evaluate residual protein effect on CAM induction. PK treatment was also performed with tumor necrosis factor—alpha (TNF-α, 10 ng/ml), included in the experiment as a cytokine positive control, and with LPS (1 μg/ml). The induction was measured before and after treatment with the enzyme. The leptospires or the protein were removed by washing, and endothelial cells were further incubated in RPMI 1640 with 10% FBS. HUVECs were detached by treatment with a 0.25% trypsin and 0.02% EDTA solution and incubated in the dark, at 4 °C for 15 min, with PBS containing 10% FBS (in order to inactivated trypsin) and PE-mouse anti-human CD54 (clone HA58, BD Pharmingen) in the case of ICAM-1. For E-selectin expression, cells were harvested after 4 h, combined with a primary anti-CD62E monoclonal antibody (MoAb, clone 1.2B6, Immunotech) and a secondary FITC-conjugated fragment F $(ab')_2$ anti-mouse IgG (Immunotech). For non-specific binding, anti-CD54 or anti-CD62E were replaced by a correspondent concentration of irrelevant isotype-matched IgG1. After labeling, cells were washed, fixed with 1% paraformaldehyde and analyzed by flow cytometry in a FACScan cytofluorimeter (Becton Dickinson, Mountain View, CA). Appropriate settings of forward and side scatter gates were used to examine 10,000 cells per experiment. The percentage of positive cells was determined by the thresholds set using isotypic controls.

"*In vivo*" detection of LIC12960 protein by immunohistochemistry

In order to detect LIC12960 protein in kidney tissues, three guinea pigs of 180 g were inoculated intraperitoneally with 0.5 ml of PBS (pH 7.2) containing $10^{2.5}$ *L. interrogans* serovar Copenhageni Fiocruz L1-130 sacrificed two weeks post infection (p.i.) when animals presented symptoms, e.g., weight loss, lethargy, and their kidneys were harvested and processed for routine histology. Mock-infected animals were used as controls. After rehydratation, the tissue Pro-Bond Plus slides were heated three times for 5 min in a 10 mM citrate buffer in a microwave oven. The sections were then cooled and immersed in 3% H_2O_2 for 15 min to inhibit endogenous peroxidase activity. To block non-specific antigen sites, sections were incubated with PBS with 5% of normal goat serum for 20 min at room temperature. Then, slides were incubated with the above described primary polyclonal murine antiserum diluted 1:100 for 1 h at room temperature. After several washes with PBS, specimens were incubated with secondary antibody (goat anti-mouse immunoglobulin) conjugated to peroxide labeled dextran polymer (EnVision, DAKO) for 20 min at room temperature and again washed with PBS. Diaminobenzidine/hydrogen peroxidase substrate was incubated for 2—10 min to reach the appropriate intensity and slides were rinsed with distilled water to stop the staining reaction. Immunostained sections were counterstained with hematoxylin for 1 min, bathed under tap water, rinsed with distilled water and dehydrated in increasing ethanol concentrations followed by xylene (each treatment, 5 min). Finally, the slides were mounted with a coverslip in a nonaqueous permanent mounting medium and observed in a Leica photomicroscope. All animal studies were approved by the Ethics Committee of the Faculty of Exact Sciences, University of La Plata, Argentina.

ECM ligand-binding assays

Aliquots (1 μg) of each recombinant protein and negative control protein BSA were subjected to SDS-PAGE and then transferred to nitrocellulose membranes. Membranes were blocked with 5% BSA in PBS-T (0.05% Tween 20), then incubated for 1 h at room temperature with either murine laminin, human fibronectin (plasma, 70 kDa proteolytic fragment and cellular), calf collagen type I or human collagen type IV at different concentrations in PBS-T. Following extensive washing with PBS-T, membranes were incubated

for 1 h with rabbit polyclonal antibodies specific for either ECM component (diluted 1:1000). Finally, the membranes were washed with PBS-T and incubated for 1 h with anti-rabbit horseradish peroxide-conjugated (diluted 1:5000, Sigma). Bound antibodies were detected using ECL reagent kit chemiluminescence substrate (GE Healthcare). Metaperiodate treatment was performed by diluting 1 μg/ml of laminin in 50 mM sodium acetate buffer, pH 5.0, with 200 mM sodium metaperiodate just before the use on ligand-binding assay.

Factor H ligand affinity blot assay

Interactions between Lp95 and purified human factor H were examined according to protocol previously described by Verma et al.[41] Aliquots (1 μg) of each recombinant protein and negative control protein BSA were subjected to SDS-PAGE, and then transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in PBS-T (0.05% Tween 20), then incubated for 1 h at room temperature with purified human factor H (0.5 μg/ml; Calbiochem) in PBS-T. Following extensive washing with PBS-T, membranes were incubated for 1 h with goat polyclonal anti-factor H antibody (diluted 1:10,000, Calbiochem). Finally, the membranes were washed with PBS-T and incubated for 1 h with anti-goat horseradish peroxide-conjugated (diluted 1:20,000, Sigma). Bound antibodies were detected using ECL reagent kit chemiluminescence substrate (GE Healthcare).

Statistical analysis

All results are expressed as means ± SEM. Student's paired *t* test was used to determine the significance of differences between means, and *p* lower than 0.05 was considered as statistically significant.

Nucleotide sequence accession numbers

The accession number for public data base for protein sequence LIC12690 is YP_002611. The protein can also be accessed by the genome nomenclature for the gene locus, LIC number (*Leptospira interrogans* Copenhageni).

Results

Bioinformatic analysis of the LIC12690 sequence

The gene encoding LIC12690 was identified in the chromosome I by analysis of the probable ORFs present in the genome of *L. interrogans* serovar Copenhageni.[24,27] LIC12690 is predicted to be an outer/inner membrane protein (>70%), based on the search for sequence motifs by the servers PSORT[29] and P-Classifier.[30] The LipoP server predicted LIC12690 CDS to be a lipoprotein with a cleavage site for signal peptidase II at amino acids 16–17[34] corroborating with the result obtained on SpLip program.[35] BLAST analysis showed that LIC12690 has 17–23% of identity with Leptospiral endostatin-like proteins.[9] In addition, this predicted protein family has in common a domain of unknown function (DUF1554) according to the PFAM server.[33] Similar predicted CDS LIC12690 were identified in *L. interrogans* serovar Lai (99% identity with LA0962)[42] but is absent in both *L. borgpetersenii*[43] and *L. biflexa*[44] genome sequences.

Sequence comparison between LIC12690 and reported leptospiral adhesins

To investigate whether LIC12690 protein shared a sequence similarity or a common domain with other previously identified leptospiral ECM-binding proteins[9–13,45]; we proceeded with a sequence analysis using Clustal X program and a tree-display NJ plot.[36,37] The calculated tree derived from sequence alignment is depicted in Fig. 1 and clearly shows that LIC12690 CDS is unrelated to all adhesins previous described.

Distribution and expression of *LIC12690* gene among *Leptospira* strains

The presence of *LIC12690* gene in six pathogenic strains and in one saprophytic strain of *Leptospira* was examined by PCR with a pair of primers designed according to *L. interrogans* serovar Copenhageni genome sequences. A 399-bp DNA fragment of LIC12690 was amplified by PCR in four strains belonging to the pathogenic species of *L. interrogans* (serovars Canicola, Copenhageni, Hardjo and Icterohaemorraghie)

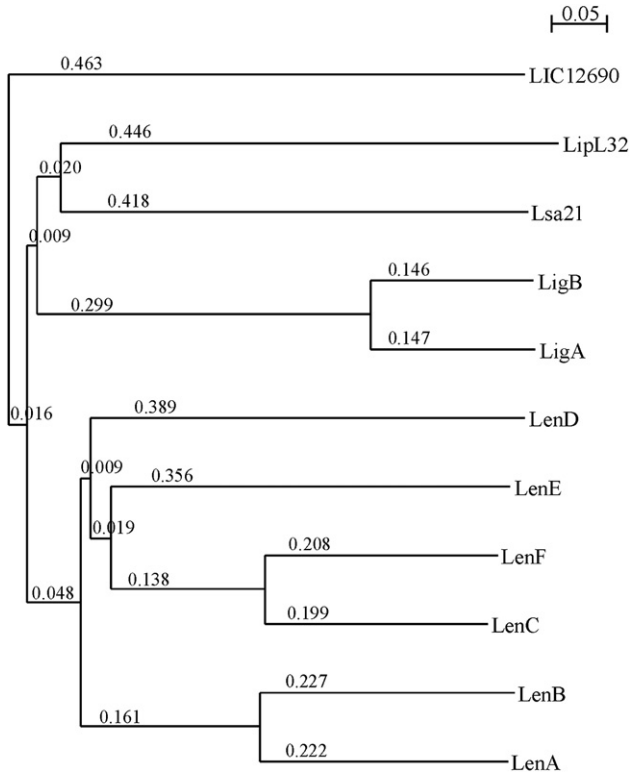

Figure 1 Sequence comparison between LIC12690 encoded protein and leptospiral proteins. Unrooted phylogenetic tree of predicted amino acid sequences of the LIC12690, LipL32, Lsa21, LigA/LigB and Len family proteins. The tree was generated by Clustal X program and displayed by NJ plot. Branch lengths are depicted.

(Fig. 2A). No amplification product was detected in the strains belonging to the serovars Grippotyphosa, Pomona or in the non-pathogenic strain Patoc 1 (*L. biflexa* serovar Patoc) (Fig. 2A). 16S DNA amplification was performed to attest template integrity (Fig. 2A). The expression of LIC12690 by *in vitro* cultured leptospires was evaluated by PCR amplification of reversely transcribed total RNA. The results obtained revealed the presence of LIC12690 transcripts in all the four strains mentioned above (Fig. 2B). DNA contamination was discarded as no amplification was observed in the absence of reverse transcriptase. The integrity of total RNA used in RT-PCR experiment was assured by the presence of a 1042-bp 16S ribosomal cDNA fragment in all samples (Fig. 2B).

Cloning, expression and purification of recombinant protein

The *LIC12690* gene was amplified, without the signal peptide sequence, and the DNA insert cloned and expressed as a full-length protein in *E. coli*. Recombinant protein was expressed with 6 × His-tag at the N-terminal, purified by metal chelating chromatography and, an aliquot of each step of the process was analyzed through SDS-PAGE (Fig. 3). The expected protein band of 94.5 kDa is shown in NaCl-*E. coli* BL21 (SI)-induced culture and in the cell pellet, as inclusion bodies (Fig. 3, lane 3 and 5, respectively). Although the Lp95 presented low expression level, it was consistently recovered from the column in the absence of urea (Fig. 3, lane 6).

Endothelial expression of E-selectin and ICAM-1

To determine whether endothelial cells were activated by exposure to Lp95, cultures of HUVECs were treated with the indicated concentrations of Lp95 and the surface levels of E-selectin and ICAM-1 were evaluated by fluorescence-activated cell sorter (FACS) analysis. The results obtained show that the basal expression of E-selectin molecules was markedly increased after treatment with Lp95 in a concentration dependent manner (Fig. 4A, white bars). In contrast, ICAM-1 expression was not modified by Lp95 (data not shown). Although the LPS content of the sample was estimated to be 1.1×10^{-4} μg/mL (8.8×10^{-6} μg LPS per 1 μg of Lp95) we ruled out that upregulation of E-selectin was associated to LPS contamination since the induction of CAM molecules on HUVECS was evaluated in the presence of polymyxin B (7 μg/mL) that effectively blocked E-selectin expression triggered by LPS (Fig. 4A, gray bars). Besides, another recombinant protein, rLIC12906 containing higher LPS levels than Lp95 (0.88 μg/mL) was not able to enhance E-selectin expression (Fig. 4A, black bar).

Although we have evaluated the induction of CAM molecules on HUVECS by several recombinant leptospiral proteins,[23,25] the effect promoted by whole live *Leptospira* sp. was never assessed. Fig. 4B shows that the pathogenic *L. interrogans* and the non-pathogenic *L. biflexa* promoted the induction of both, ICAM-1 and E-selectin, although statistically significant higher levels of CAMs were achieved by the pathogenic strain ($P < 0.01$). Higher activation level of CAMs was achieved by both strains when compared to mock control ($P < 0.01$) (Fig. 4B, both graphs).

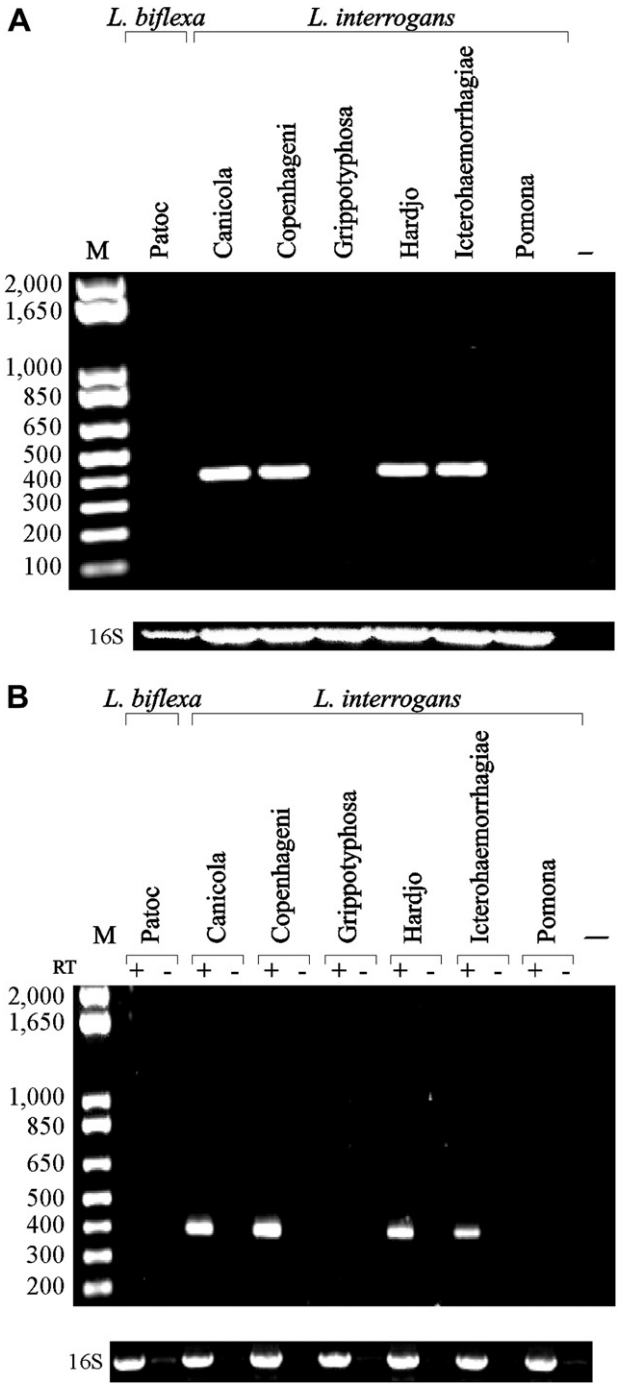

Figure 2 Distribution and expression of *LIC12690* gene in saprophytic and pathogenic leptospires. (A) Genomic DNA from *L. biflexa* Patoc and from six serovars belonging to the pathogenic species *L. interrogans* was subjected to PCR analysis with LIC12690 specific primers designed according to *L. interrogans* serovar Copenhageni genome sequences. Amplification of 16S DNA shows template integrity. The expected size of the PCR product is 399 bp. No DNA was added to the negative control reaction (−). (B) RT-PCR analysis of LIC12690 transcripts in high-passage *L. interrogans* strains. Reactions were performed with the same primer pairs mentioned above. Samples quantity and integrity were verified by amplification of a 1.042-bp 16S ribosomal cDNA fragment. RT+: reverse transcriptase present; RT−: reverse transcriptase omitted; M: molecular mass markers (pb).

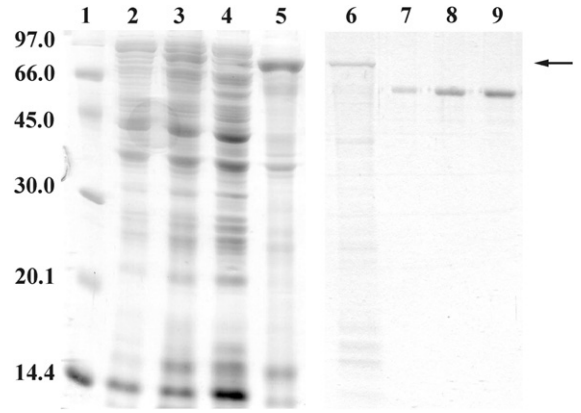

Figure 3 Analysis of Lp95 from NaCl-induced *E. coli* Bl21-SI by SDS-12% PAGE. Lane 1, molecular mass protein marker (in kDa); lane 2, non-induced culture; lane 3, induced culture; lanes 4 and 5, supernatant and inclusion body pellet after bacterial cell lysis and centrifugation, respectively; lane 6, purified protein eluted from Ni^{+2}—charged chelating sepharose column with 1 M imidazole; lanes 7—9, BSA 250 ng, 500 ng and 750 ng, used to estimate protein concentration. Protein bands were visualized by Coomassie blue staining. Positions of molecular mass standards are indicated to the left (in kDa).

Treatment of Lp95 (25 μg/ml) with proteinase K (500 ng/ml), prior to CAM measurements, abolished E-selectin upregulation induction by the recombinant protein (Fig. 5A). TNF-α treatment with PK produced similar result (Fig. 5B) but, as expected, did not modify the upregulation of E-selectin promoted by LPS (Fig. 5C).

Immunohistochemistry evaluation with serum anti-Lp95

To evaluate whether the LIC12690 encoded protein is expressed during infection, kidney tissues obtained after 14 days p.i. of experimentally infected guinea pigs were probed with a serum raised against Lp95. The kidney tissues showed well conserved cortical and medullary architecture. A few glomeruli were shrunken or contracted; likewise some tubules contained proteinaceous material mixed with erythrocytes. An early mixed-cell infiltrate was occasionally noted near larger vessels. The use of Lp95 antiserum revealed the presence of *L. interrogans* antigens located at the renal tubular lumen (Fig. 6A). In addition, some LIC12690 antigen was also observed at the sites of interstitial inflammatory cell infiltrates (Fig. 6B). In negative control experiments, the Lp95 antiserum did not show reactivity to normal guinea-pig kidney sections (Fig. 6C).

Cloning, expression and purification of Lp95 fragments

Due to difficulties associated with Lp95 solubility, we decided to generate three fragments corresponding to the N-terminal, C-terminal and overlap portions of the *LIC12690* gene (Fig. 7A). The DNA fragments were amplified and the inserts cloned into *E. coli* expression vector, pAE.[38] Recombinant proteins were expressed with 6 × His-tag at the N-terminal, purified by metal chelating chromatography. The recombinant protein fragments were consistently recovered from the column as single major band indicating that most of the contaminants had been removed (Fig. 7B) but, the three portions, as the whole protein, tend to sediment in PBS.

Ligand immunoblot analyses

A previous study indicated that all Len proteins, but Len A, bind laminin and fibronectin.[9] We thus examined whether the DUF1554 (*len*-motif) contribute to the adhesion of the Lp95 and which fragments were involved in the binding. We have employed blotting instead of ELISA based assay due to the poor solubility of the protein. The Lp95 and each protein fragment were mixed up with SDS buffer, subjected onto SDS-PAGE and transferred to nitrocellulose membranes. Blotted proteins were stained Ponceau S (Fig. 8A and C) then examined for their ability to bind soluble laminin, fibronectin (plasma and cellular), collagen type I and IV. Our data with affinity blotting shows that Lp95 (Fig. 8, lane 2), C-terminal fragment (Fig. 8, lane 4), overlap fragment (Fig. 8, lane 5) bind laminin (I), plasma fibronectin (II) fibronectin proteolytic fragment of 70 kDa (III) and cellular fibronectin (IV); the N-terminal fragment (Fig. 8, lane 3) binds laminin (I) and cellular fibronectin (IV). The binding with all the ECM components was dependent on protein fragment concentration ranging from 0.5 to 2.0 μg. ECM-binding protein Lsa21 was used as positive control[11] while rLIC10494, previously shown to be non reactive with ECM components[45] was added as a negative control. The assays with proteolytic fragments of fibronectin indicated that the 70 kDa fragment could account for part of the binding by the Lp95 and its fragments observed with intact fibronectin (Fig. 8 II and III, lanes 2—5). No ECM binding was detected with BSA protein (Fig. 8, lane 6), employed as nonspecific binding control, although a weak band appeared when the highest laminin concentration (2.0 μM) was employed. No significant binding of Lp95 and fragments to collagen type I or type IV were detected (data not shown). The treatment with the oxidizing agent metaperiodate abolished practically all the binding of Lp95 to laminin (not shown). The ability of Lp95 to bind factor H was also analyzed by ligand affinity blot and revealed that the recombinant protein did not bound factor H because the control protein BSA exhibited the same affinity (data not shown).

Discussion

Predicted outer membrane proteins have been identified during genome annotation of pathogenic *L. interrogans* serovars Lai[42] and Copenhageni[27] and *L. borgpertsenii*.[43] The majority is assigned as hypothetical proteins of unknown function but, due to their putative location, it is believed to play a role in the host—pathogen interactions. It is well known that pathogen adhesion to and colonization of host tissue is an early and critical event in the infection process. To date, several extracellular matrix (ECM) leptospiral adhesins have been published: a 36-kDa fibronectin-binding

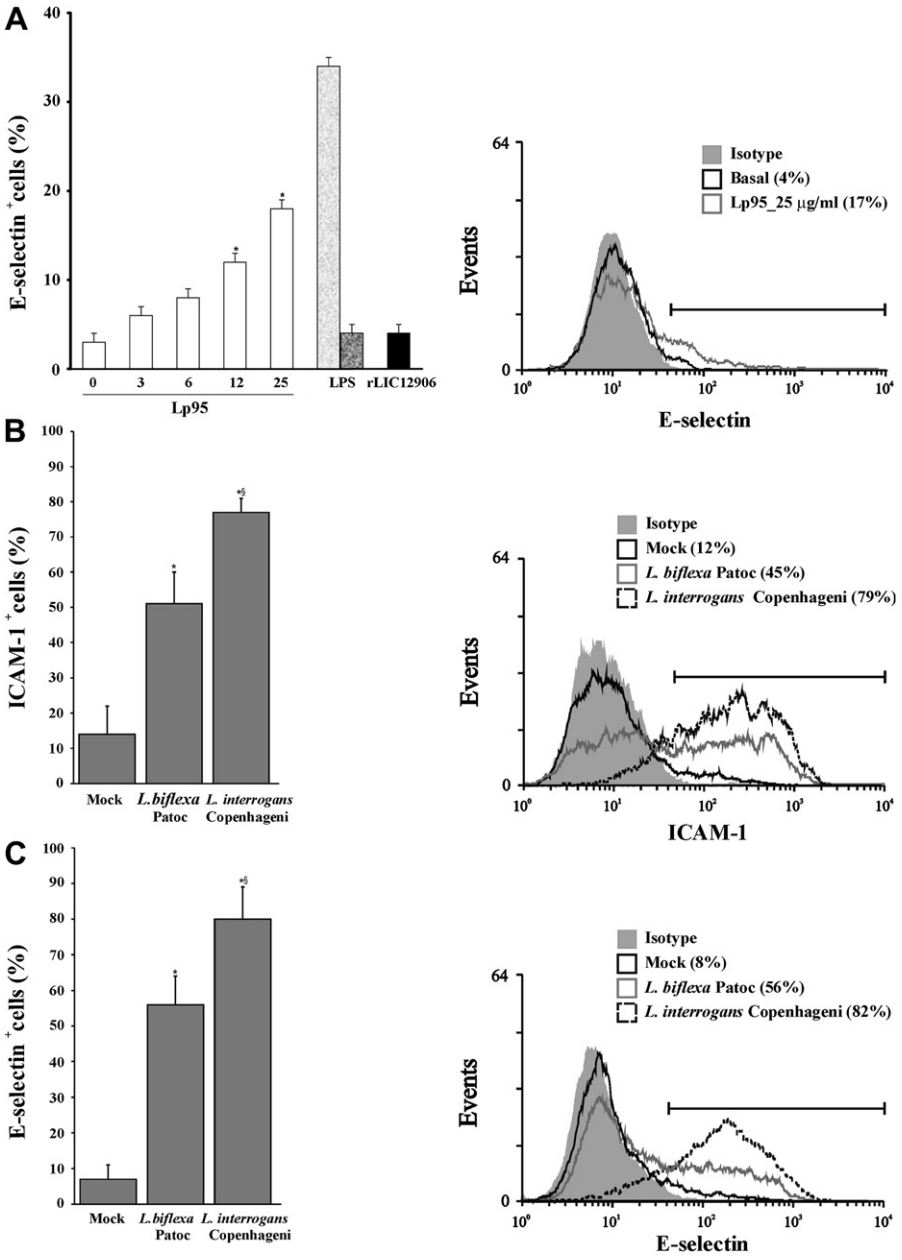

Figure 4 Induction of E-selectin expression on HUVECs by Lp95 or whole *Leptospira*. Left panels: (A) confluent monolayers of HUVECs treated with polymyxin (7 μg/mL) were stimulated for 1 h with the indicated concentrations (μg/mL) of Lp95 (white bars) or rLIC12906 (50 μg/ml) (black bar) as negative control. LPS (1 μg ml^{-1}) with or without polymyxin B (7 μg ml^{-1}) (dark and light gray, respectively) was used as a positive control. Then medium was removed and cells were further cultured for 4 h with RPMI-1640 supplemented with 10% FBS. Data shown are mean ± SEM of three independent experiments $^{*}P < 0.01$ vs basal. (B) Induction of ICAM-1 and E-selectin by mock (only media), 10^5 *L. biflexa* serovar Patoc strain Patoc 1 or *L. interrogans* serovar Copenhageni strain Fiocruz L1-130. $^{*}P < 0.01$ vs mock and $^{\S}P < 0.01$ vs non-pathogenic leptospires. Right panels show the original flow cytometry histograms for A, B and C experiment. Data shown are mean ± SEM of three independent experiments.

protein of unknown identity isolated from the outer sheath of a virulent variant of pathogenic leptospires,[46] a Len family protein[9] including LenA, formely named LfhA/Lsa24,[41,45] LigA/LigB proteins,[10] Lsa21[11] and LipL32.[12,13] Protein activation of endothelial CAMs' has been described for the pathogenic spirochetes *Borrelia* sp. and *Treponema* sp.[17,18,20,21] and for the pathogenic *L. interrogans*.[23,25]

In this work, we report for the first time a *L. interrogans* protein, genome annotated as hypothetical, that is capable to bind laminin and fibronectin ECM components and to activate the endothelial CAM, E-selectin. The protein encoded by the *LIC12690* gene is predicted to be an outer/inner membrane[29] and to be a lipoprotein.[34] The protein encoded by LIC12690 has a sequence tag named DUF1554 that is present in the Len family protein.[9] However, contrasting with the Len proteins that divide a common branch inside their family, the CDS LIC12690 appears alone on its branch. The LIC12690 encoded transcript is

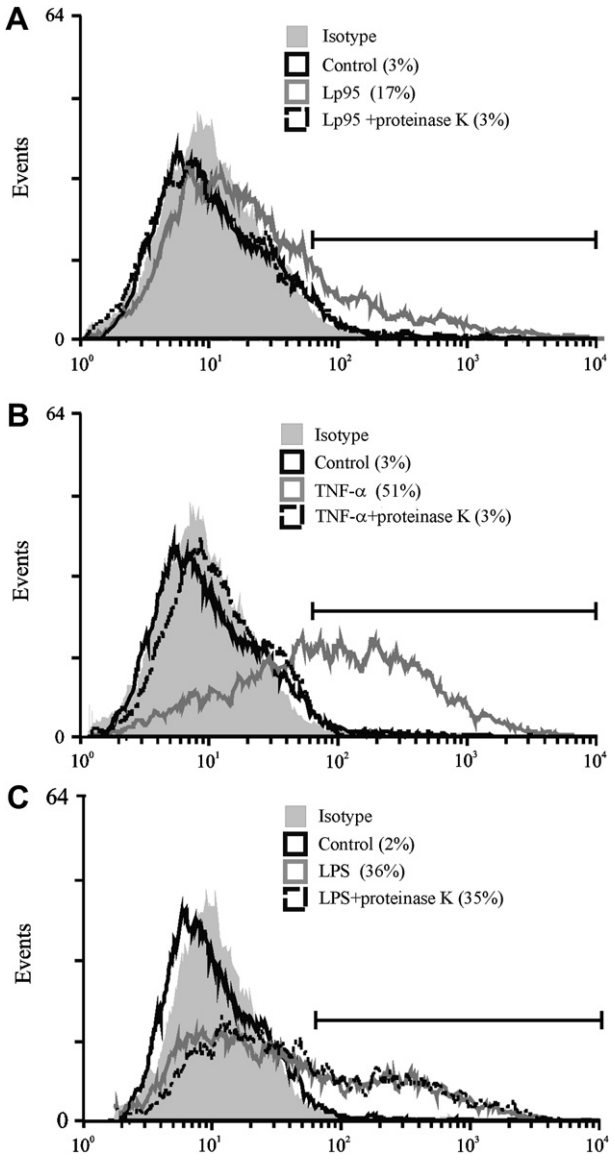

Figure 5 Effect of PK upon induction of E-selectin expression on HUVECs by Lp95. Flow cytometry histograms showing the effect of treatment of Lip95 (25 μg/ml) (A), TNF-α (10 ng/ml) (B) and LPS (1 μg/ml) (C) with proteinase K (500 ng/ml) prior to E-selectin induction on HUVECs.

conserved among important pathogenic serovars of *L. interrogans* such as Canicola, Hardjo and Icterohaemorrhagiae but absent in serovars Pomona, Grippotyphosa and in the non-pathogenic *L. biflexa*. The LIC12690 encoded protein was expressed in *E. coli* as a 95-kDa full-length recombinant protein, named Lp95. Although the purified protein exhibited a single major band in SDS-PAGE, the Lp95 tend to sediment in solution. Nevertheless, activation of endothelial cellular adhesion molecule E-selectin was clearly demonstrated. Furthermore, treatment of the protein Lp95 with proteinase K abolished E-selectin induction on HUVECs. Contrary to the previous reported proteins rLIC10365[23] and rLIC10507, rLIC10508, rLIC10509,[25] the Lp95 did not promote upregulation of ICAM-1.

Sellati et al.[17] have shown that only the lipidated form of the recombinant protein OspA of *B. burgdoferi* was capable to promote upregulation of CAMs. Heterologous expression of lipoproteins in *E. coli* does not result in the expression of acylated recombinant protein and a plasmid vector containing the appropriate signal sequences is required.[47] Nonetheless, we have previously demonstrated that *E. coli* expressed recombinant proteins of *Leptospira* were capable to induce the increase of CAMs in HUVECS.[23,25] These data suggested that in our experimental model the lipid moiety was not involved in this interaction and we believe that this should also be the case for Lip95 protein.

Leptospiral immunity following infection is generally, but not exclusively, mediated by antibody against leptospiral LPS.[7] Constitutively expressed on endothelial cells only at low levels, the expression of CAMs is upregulated in response to cytokines, LPS, and bacterial lipoproteins.[48,49] Although CAMs stimulation has been shown for the pathogenic spirochetes *Borrelia and Treponema*,[17–21] it has never been evaluated for whole *Leptospira* spp. Our studies show that both pathogenic and non-pathogenic *Leptospira* promote upregulation of ICAM-1 and E-selectin on HUVECs. This was expected because LPS is a stronger inducer of CAMs. However, the pathogenic *L. interrogans* promoted a greater activation than the non-pathogenic *L. biflexa* suggesting the presence of other factors in the pathogenic strains. It is worth mentioning that either the Lp95 or the protein coding sequences previously reported are all absent in the saprophytic *L. biflexa* strain.[23,25] The LIC12690 encoded protein antigen was identified both at

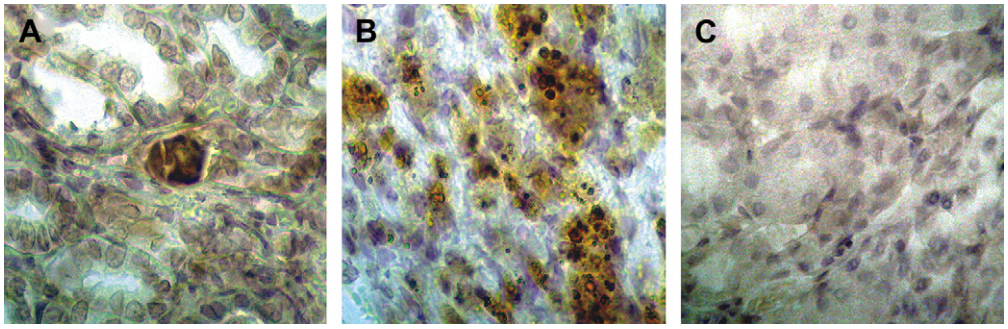

Figure 6 Immunohistochemistry. Kidney tissues obtained from guinea pigs 14 days after infection with *L. interrogans* virulent strain Fiocruz L1-130, were probed with serum raised against Lp95. (A) Antigen Lp95 recognition on leptospires within the renal tubular lumen, (B) and in interstitial inflammatory cell infiltrates. (C) No reactivity was observed when the antiserum was employed in kidney from uninfected animals. Slides were counterstaining with hematoxylin. Magnification: ×450.

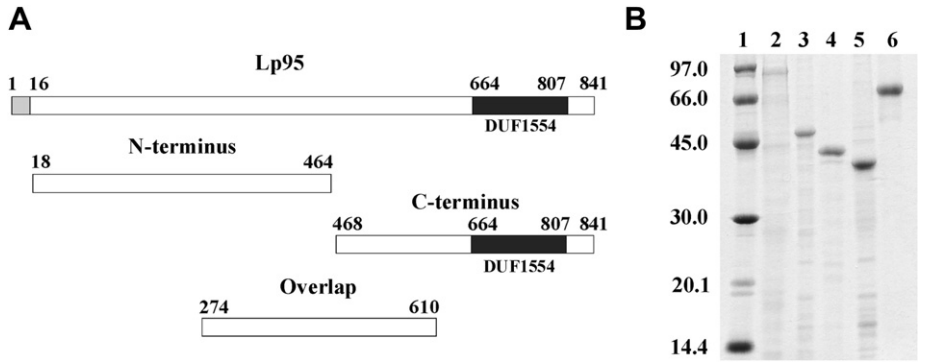

Figure 7 Schematic representation of Lp95. (A) Shows the signal peptide (amino acids 1—16), the DUF1554 domain (amino acids 664—807), N-terminal domain (amino acids 18—464), C-terminal domain (amino acids 468—841), and the overlap domain (amino acids 274—610). (B) Analysis of recombinant protein purification: SDS-12% PAGE of purified protein eluted from Ni^{+2}—charged chelating Sepharose column with 1 M imidazole. Lane 1, molecular mass protein marker (in kDa); lane 2, Lp95 full-length (94.5 kDa); lane 3, N-terminal fragment (51.9 kDa); lane 4, C-terminal fragment (43.5 kDa); lane 5, overlap fragment (39.7 kDa); lane 6, BSA. Protein bands were visualized by Coomassie blue staining. Positions of molecular mass standards are indicated to the left (in kDa).

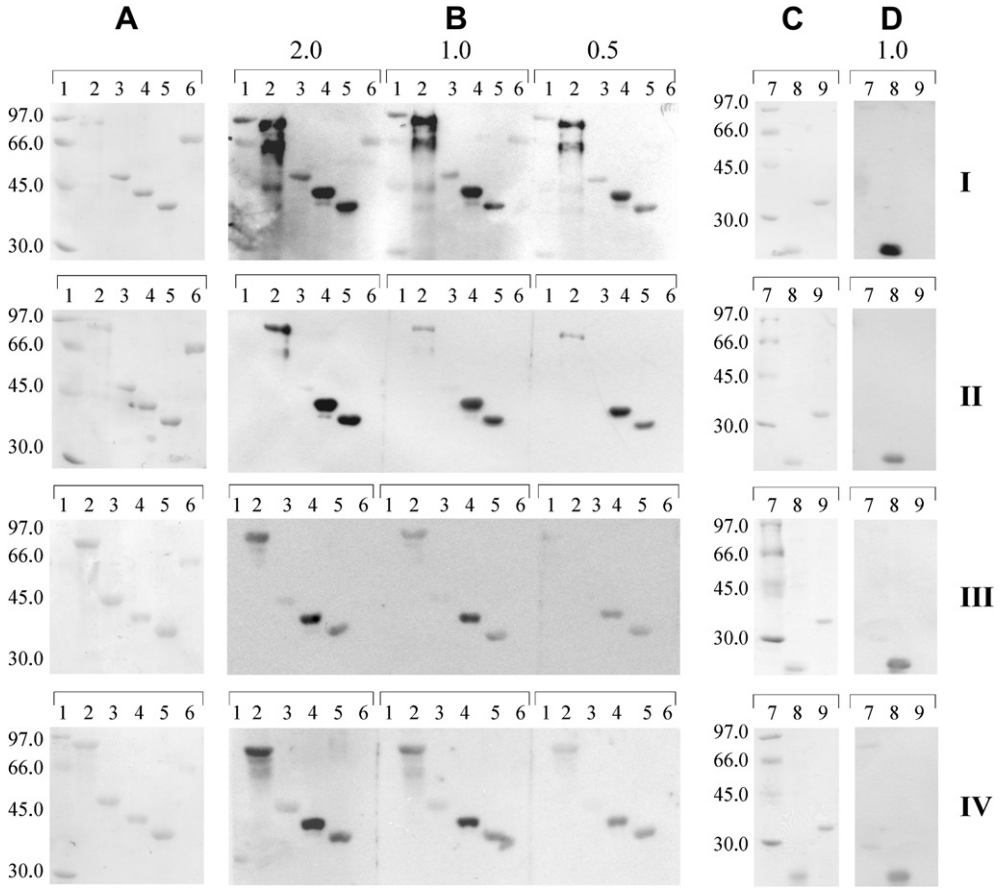

Figure 8 Ligand affinity blot analyses of Lp95 with purified extracellular matrix components. In (A and C), protein bands were visualized by Ponceau staining. In (B and D) nitrocellulose membranes containing immobilized recombinant protein were incubated with ECM components and the binding was revealed with specific antibodies. (I) laminin, (II) plasma fibronectin, (III) 70 kDa proteolytic fragment of plasma fibronectin, (IV) cellular fibronectin. ECM concentrations ranged from 2 μg to 0.5 μg. Lane 1, molecular weight protein marker (in kDa); lane 2, Lp95 full-length (94.5 kDa); lane 3, N-terminal fragment (51.9 kDa); lane 4, C-terminal fragment (43.5 kDa); lane 5, overlap fragment (39.7 kDa); lane 6, BSA (negative control), lane 7, Lsa21, lane 8, rLIC10494. Positions 1 and 7 of molecular mass standards are indicated to the left (in kDa).

the tubules lumen and associated with inflammatory cell infiltrates of kidney obtained from experimentally infected guinea pigs, suggesting the expression of the protein during animal infection.

CAMs have been reported to be used by several cells and extracellular molecules in host defense against infection. Nevertheless, CAMs are also employed by several microorganisms, including protozoa, fungi, bacteria and viruses, during their pathogenesis.[22] Selectins, integrins, ICAM-1 and VCAM were stimulated by *Staphylococcus aureus* and their role suggested being mitogenic with massive cytokine release.[50] In the case of *Mycobacterium tuberculosis*, activation of complement receptor 3 (CR3) and integrin CD11b/CD18 was related to CR3 phagocytosis without oxygen burst.[51] Adherence and pathogenesis of attaching and effacing (A/E) lesion was the assigned role of enteropathogenic *E. coli* (EPEC) activation of integrins,[52] while for *Neisseria* spp. the induction of heparan sulphate proteoglycan was related to invasion of Chang epithelial cells.[53]

The Lp95 mediates the attachment to laminin and to both plasma and cellular fibronectin. The participation of His-tag in mediating this binding was ruled out by the inclusion of the recombinant His tagged-LIC10494 protein, previously shown to be non-adhesive to ECM molecules.[45] Even though a faint protein band can be observed with control BSA and the highest laminin concentration, the fact that it disappeared when laminin concentration decreased while the binding can still be seen with the whole Lp95 protein and its fragments suggest that protein adhesion to BSA was probably nonspecific. Differences in ligand binding were clear among the Lp95 fragments: the three portions bind laminin but C-terminal containing DUF1554 displayed appreciably greater affinity for laminin and cellular fibronectin; as for plasma fibronectin only the C-terminal and the overlap region fragments exhibited binding affinity. Similar to Lsa24[45] and Lsa21,[11] chemical oxidation of laminin carbohydrate moieties by sodium metaperiodate caused significant reduction in the binding activity of Lp95 and its fragments, thus indicating the sugar moiety involvement in interactions between this recombinant protein and ECM macromolecules. Interestingly, although Len A and Len B proteins were capable to bind host complement factor H,[9,41] no binding was exhibited when Lp95 was assayed with this factor (data not shown).

It was suggested that leptospiral attachment to ECM correlates with virulence, since virulent lines of *L. interrogans* attached to ECM more effectively than intermediate virulent and avirulent lines of the same strains.[54] The extracellular matrix of mammals is composed of two main classes of macromolecules: glycosaminoglycans (GAGs), usually found covalently linked to proteins in the form of proteoglycans, and fibrous proteins with both structural and adhesive functions such as collagens, elastin, fibronectin, and laminin. Besides serving as a scaffold to stabilize the physical structure of tissues, the ECM plays an important role in regulating eukaryotic cell adhesion, differentiation, migration, proliferation, shape and function.[8]

In conclusion, we describe Lp95 as a novel leptospiral protein that exhibits extracellular matrix-binding properties. In addition, this protein possesses the ability to stimulate at least one endothelial CAMs' and its presence has been detected in cellular infiltrates of kidney tissues during the experimental infection of animals with *Leptospira*. Taken together our results suggest that Lp95 may mediate attachment to host tissues thus contributing to the infectious process.

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