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Research Article

RESEARCH ARTICLE

Evaluation of two novel leptospiral proteins for their interaction with human host components

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One sentence summary: We describe a novel protein in *Leptospira interrogans* that is most probably involved in host–pathogen interactions.
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ABSTRACT

Pathogenic species of the genus *Leptospira* are the etiological agents of leptospirosis, the most widespread zoonosis. Mechanisms involved in leptospiral pathogenesis are not well understood. By data mining the genome sequences of *Leptospira interrogans* we have identified two proteins predicted to be surface exposed, LIC10821 and LIC10064. Immunofluorescence and proteinase K assays confirmed that the proteins are exposed. Reactivity of the recombinant proteins with human sera has shown that rLIC10821, but not rLIC10064, is recognized by antibodies in confirmed leptospirosis serum samples, suggesting its expression during infection. The rLIC10821 was able to bind laminin, in a dose-dependent fashion, and was called Lsa37 (leptospiral surface adhesin of 37 kDa). Studies with human plasma components demonstrated that rLIC10821 interacts with plasminogen (PLG) and fibrinogen (Fg). The binding of Lsa37 with PLG generates plasmin when PLG activator was added. Fibrin clotting reduction was observed in a thrombin-catalyzed reaction, when Fg was incubated with Lsa37, suggesting that this protein may interfere in the coagulation cascade during the disease. Although LIC10064 protein is more abundant than the corresponding Lsa37, binding activity with all the components tested was not detected. Thus, Lsa37 is a novel versatile adhesin that may mediate *Leptospira*–host interactions.

Keywords: *Leptospira*; leptospirosis; adhesion; plasmin; fibrin reduction

INTRODUCTION

Leptospirosis is a globally disseminated zoonosis caused by pathogenic species of bacteria belonging to the genus *Leptospira*. The disease is endemic in tropical and subtropical countries, mainly in developing countries, where it produces urban outbreaks during the rainy season (Bharti *et al.* 2003), while in

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temperate climates it is associated with occupational and recreational activities. Almost every mammal and marsupial worldwide has been shown to be a host to leptospires (Levett 2001; Adler *et al.* 2011), carrying the bacteria mainly in their proximal renal tubules and shedding them alive through the urine.

The human or animal infection occurs from direct contact with urine or indirectly from contaminated water or soil. The symptoms range from flu-like disease, including fever, headache, anorexia, muscle pain and constipation (Faine *et al.* 1999), to the severe form of the disease, Weil's syndrome, characterized by renal and hepatic failure, pulmonary distress and death (Faine *et al.* 1999; Bharti *et al.* 2003). The disease has a great economic impact since it can promote abortions, stillbirths, reduced milk production and death in livestock (Faine *et al.* 1999; Plank and Dean 2000). Due to its importance to veterinary and public health, it is essential to discover the molecular mechanisms of the leptospiral pathogenicity.

Genomic analysis has identified more than 250 predicted outer-membrane proteins (Nascimento *et al.* 2004a,b), which, due to their location, are thought to be involved in host–pathogen interaction. Our group has focused on exploring this set of proteins as they are potential targets of the immune response during the host infection and are involved in the interaction with host components mediating the infection. Veterinary and human vaccines are based on inactivated whole cell or membrane preparations of pathogenic leptospires; however, these vaccines do not provide cross-protective immunity against leptospiral serovars not included in the vaccine preparation, failing to provide long-term protection against infection (Bharti *et al.* 2003; Koizumi and Watanabe 2005). Proteins that are conserved among different species and serovars have the potential to fulfill these requirements.

The direct attachment of leptospires to the extracellular matrix (ECM) proteins has been described (Barbosa *et al.* 2006) and, so far, several bacterial receptors have been reported, indicating the existence of multiple adhesion molecules (Stevenson *et al.* 2007; Atzingen *et al.* 2008, 2009; Pinne, Choy and Haake 2010; Mendes *et al.* 2011; Domingos *et al.* 2012, 2015; Fernandes *et al.* 2012, 2014; Pinne, Matsunaga and Haake 2012; Souza *et al.* 2012; Siqueira *et al.* 2013; Vieira *et al.* 2014; Teixeira *et al.* 2015). It is possible that this vast repertoire is essential during the first steps of the bacterial pathogenesis (Boyle and Finlay 2003).

Our group has demonstrated that leptospires can disseminate and overcome tissues by capturing human plasminogen (PLG) (Vieira *et al.* 2009) and using soluble host activators to convert this zymogen into its active form, plasmin (PLA), the main fibrinolytic protease. Moreover, it has been shown that PLA–*Leptospira* can degrade ECM components C3b and IgG, facilitating tissue penetration and immune evasion (Vieira *et al.* 2009, 2012). In addition, we have described the ability of different leptospiral strains to interact with human fibrinogen (Fg) and partially block its conversion into fibrin (Oliveira *et al.* 2013), which may also facilitate bacterial dissemination.

In this paper, we focused our studies on a probable lipoprotein and a hypothetical protein encoded by the genes LIC10821 and LIC10064, respectively. The proteins were evaluated for their cellular location, reactivity to human leptospirosis serum samples and their ability to bind ECM components and human plasma components. The recombinant protein LIC10821 was capable of adhering to laminin and therefore named Lsa37 for leptospiral surface adhesin of 37 kDa. In addition, Lsa37 is a PLG-interacting protein with the ability to generate PLA, and a Fg receptor capable of reducing fibrin clot formation. Our findings suggest that this is a multifunctional protein that may be involved in host–*Leptospira* interaction.

MATERIAL AND METHODS

Biological components

Collagen, laminin, plasma and cellular fibronectin, elastin and the control protein bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 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; elastin was derived from human aorta, and collagen type I was isolated from rat tail. Native plasminogen, purified from human plasma, and factor H were purchased from EMD chemicals, Inc. (San Diego, CA, USA). C4bp, isolated from normal human serum, was purchased from Complement Technology, Inc. (Tyler, TX, USA).

Bacterial strains and serum samples

The non-pathogenic *Leptospira biflexa* (serovar Patoc strain Patoc 1), the pathogenic attenuated *Leptospira interrogans* (serovars Canicola strain Hond Utrecht IV, Copenhageni strain M20, Hardjo strain Hardjoprajitno, Icterohaemorrhagiae strain RGA), *Leptospira kirschneri* (serovars Cynopteri strain 3522C, Grippotyphosa strain Moskva V), *Leptospira borgpetersenii* (serovar Whitcombi strain Whitcombi), *Leptospira noguchi* (serovar Panama strain CZ214), *Leptospira santarosai* (serovar Shermani strain 1342 K) were cultured at 28°C under aerobic conditions in liquid EMJH medium containing asparagine (0.015% w/v), sodium pyruvate (0.001% w/v), calcium chloride (0.001% w/v), magnesium chloride (0.001% w/v), peptone (0.03% w/v) and meat extract (0.02% w/v) (Turner 1970). The virulent strains of *L. interrogans*, serovar Kennewicki strain Pomona Fromm and Copenhageni strain FIOCRUZ L1-130, were also employed. *Leptospira* cultures are maintained in the Faculdade de Medicina Veterinária e Zootecnia, USP, São Paulo, SP, Brazil. *Escherichia coli* DH5a, and *E. coli* BL21(DE3) Star pLysS (Studier 1991) and BL21 SI (salt-induced) were used as cloning and recombinant protein expression hosts, respectively. Leptospiral DNA extraction was performed as described previously (Oliveira *et al.* 2011).

In silico analysis of the coding sequences LIC10821 and LIC10064

Predicted coding sequences (CDSs) LIC10821 (LIC_RS04235) and LIC10064 (LIC_RS00330) were selected from *L. interrogans* serovar Copenhageni genome sequences (Nascimento *et al.* 2004a,b) based on their cellular localization prediction by PSORT, http://psort.hgc.jp/ (Nakai and Horton 1999) and CELLO, http://cello.life.nctu.edu.tw/ (Yu *et al.* 2006). The signal peptide sequence was assessed by SignalP, http://cbs.dtu.dk/services/SignalP-3.0/ (Bendtsen *et al.* 2004). The Smart, http://smart.embl-heidelberg.de/ (Schultz *et al.* 1998; Letunic, Doerks and Bork 2015), PFAM, http://pfam.xfam.org/ (Finn *et al.* 2006) and LipoP, http://www.cbs.dtu.dk/services/LipoP/ (Juncker *et al.* 2003) web servers were used to search for predicted functional and structural domains within the amino acid sequence. ClustalW2 multiple sequence alignment at http://www.ebi.ac.uk/Tools/msa/clustalw2/ (Larkin *et al.* 2007) was employed to generate the phylogram, based on the sequences available at GenBank.

Table 1. Gene locus, sequence of the primers employed for DNA amplification and restriction cloning sites.

Gene locus[a]	Primers (forward)	Primers (reverse)
L*LIC10821	**CTCGAGAACGGAGGAAATA (XhoI)	***GGTACCTCAAGGATTACAAGGT (KpnI)
L*LIC10064	**CTCGAGGGCGAAGAAGATGCAATT (XhoI)	***GGTACCTTATTTAACTCTAGTCCAAGTAGATTC (KpnI)

[a]http://aeg.lbi.ic.unicamp.br/world/lic/.

Cloning and expression of recombinant proteins in *E. coli*

The genes LIC10821 and LIC10064 without the signal peptide were amplified from the genomic DNA of *L. interrogans* serovar Copenhageni by PCR with specific primers (Table 1). The PCR amplified products were cloned into pGEM-T easy vector (Promega) and positive clones were selected by DNA restriction analysis, colony PCR and further confirmed by nucleotide sequencing analysis. After confirmation of the sequences, the DNA inserts were removed by digestion with the specific restriction enzyme (Table 1) and ligated into the protein expression pAE vector (Ramos *et al*. 2004), previously digested with the same enzymes. This plasmid includes a 6XHis sequence tag at the N-terminus of recombinant proteins. The plasmids pAE-LIC10821 and pAE-10064, confirmed by sequencing, were employed to transform BL21 (DE3) Star pLysS and BL21 (SI) expression host cells, respectively. *Escherichia coli* BL21 (DE3) Star pLysS cells containing pAE-LIC10821 were grown at 37°C in Luria-Bertani (LB) broth and *E. coli* BL21(SI) containing pAE-LIC10064 were grown at 30°C in 2x yeast–tryptone (YT) broth without NaCl, both with 100 μg ml^{-1} ampicillin. The bacterial growth was achieved by continuous shaking until an optical density of 0.6 at 600 nm (OD_{600nm}) was reached. Recombinant protein synthesis was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for BL21 (DE3) Star pLysS and 300 mM NaCl for BL21 (SI). After 3 h, the bacterial suspensions were pelleted by centrifugation and resuspended in lysis buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 100 μg ml^{-1} lysozyme, 1%Triton X-100, 2 mM phenylmethylsulfonyl fluoride, PMSF). The bacterial cell pellets were lysed on ice with the aid of a sonicator tip (ultrasonic processor; GE Healthcare Bio-Sciences). The soluble and insoluble fractions were separated by centrifugation at 12 857 *g* for 10 min at 4°C. The recombinant protein Lsa37 was recovered from the insoluble fraction and was solubilized in buffer containing 8 M urea; rLIC10064 protein was expressed in the soluble fraction. Recombinant proteins were purified from bacterial cell lysates (pellet and supernatant) by using the AKTA prime Plus (GE Healthcare). The efficiency of purification of the proteins was evaluated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing high concentrations of purified recombinant proteins were pooled and extensively dialyzed against phosphate-buffered saline (PBS).

Circular dichroism (CD) spectroscopy

Measurements were obtained by circular dichroism (CD) spectroscopy at room temperature in a Jasco J-810 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan). CD spectroscopy of the far-UV spectrum for recombinant proteins in a 10 mM sodium phosphate buffer was performed. The spectra were measured and are presented as the averages of five scans recorded from 190 to 260 nm and the residual molar ellipticity was expressed in deg cm^2 dmol^{-1}. Spectral data were analyzed with the software CAPITO (http://capito.nmr.leibniz-fli.de) for estimation of the secondary structure content.

Microscopic agglutination test

The microscopic agglutination test (MAT) was performed as previously described (Faine *et al*. 1999) using a battery of 22 serovars of *Leptospira* spp. as antigens, as previously described (Oliveira *et al*. 2008). The probable predominant serovar was considered to be the serovar with the highest dilution that could cause 50% agglutination. The MAT was considered negative when the titer was <100.

Production of polyclonal antiserum against Lsa37 and rLIC10064

Five female BALB/c mice (4–6 weeks old) were immunized subcutaneously with 10 μg of each recombinant protein. The recombinant proteins were adsorbed onto 10% (v/v) Alhydrogel (2% $Al(OH)_3$; Brenntag Biosector), used as an adjuvant. Two subsequent booster injections were given at 2 week intervals with the same preparation described before. Negative control mice were injected with PBS. Two weeks after each immunization, the mice were bled from the retro-orbital plexus and pooled sera were analyzed by ELISA for determination of antibody titer.

Identification of LIC10821 and LIC10064 CDSs among leptospiral strains

Bacterial cultures of *Leptospira* spp. were harvested by centrifugation and washed. After centrifugation, cells were resuspended in PBS, SDS-PAGE loading buffer was added, the resulting protein extracts were boiled for 10 min at 96°C and then loaded into 12% SDS-PAGE and transferred to nitrocellulose membranes (Hybond ECL; GE Healthcare) in semidry equipment. Membranes were blocked with 10% non-fat dried milk in PBS containing 0.05% Tween 20 (PBS-T) and then incubated with anti-rLIC10064 or Lsa37 (1:100) mouse polyclonal serum for 2 h at room temperature. Next, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:5000, Sigma). The protein reactivity was revealed by an ECL Western blotting analysis system (GE Healthcare).

Immunofluorescence assay

The localization of LIC10821 and LIC10064 CDS proteins by immunofluorescence assay (IFA) was performed as follows: *L. interrogans* serovar Copenhageni suspensions containing approximately 10^9 cells ml^{-1} of live leptospires were harvested at 3800 *g* for 15 min, washed twice with PBS (with 50 mM NaCl), resuspended in 200 μl of PBS with 2% paraformaldehyde for 40 min at 30°C under gentle shaking. After incubation, the leptospires were washed with PBS and incubated for 1 h at 30°C with polyclonal mouse anti-serum against Lsa37, rLIC10064 and DnaK (Haake and Matsunaga 2002) at a 1:50 dilution. Pre-immune serum was also employed. The leptospires were washed (PBS containing 1% BSA) and incubated with anti-mouse IgG antibodies conjugated to fluorescein isothiocyanate (FITC, Sigma) at a dilution of 1:50 and propidium iodide diluted 40×

(Sigma-Aldrich) for 50 min. Leptospires were then washed and resuspended in 50 μl of PBS plus 50 μl anti-fading solution (ProLong Gold, Molecular Probes) for a total volume of 100 μl. The immunofluorescence-labeled leptospires were examined using a confocal LSM 510 META immunofluorescence microscope (Zeiss, Oberkochen, Germany).

Detection of native protein in leptospiral surface by ELISA

Live leptospires were coated onto enzyme-linked immunosorbent assay (ELISA) plates (10^8 cells per well), allowed to adhere for 16 h, and then the plates were blocked with PBS containing 3% BSA. Detection of surface-exposed proteins was performed by incubation (1 h) of intact bacteria with antiserum raised in mice against each recombinant protein, followed by the HRP-conjugated anti-mouse IgG (1:5000). Controls lacking at least one component of the system were employed to verify the specificity of the reaction.

Proteinase K accessibility assay

Suspensions of 5 ml live leptospires (*L. interrogans* serovar Copenhageni strain M20, ~10^8 cells ml^{-1} per treatment) were harvested at 8000 *g* for 10 min at room temperature and washed once with PBS. Leptospires were resuspended in 5 ml proteolysis buffer (10 mM Tris/HCl, pH 8.0, 5 mM $CaCl_2$) containing 25 μg ml^{-1} proteinase K (PK) (Sigma-Aldrich). Test tubes were incubated for 0–300 min before the addition of 100 mM PMSF to stop PK activity. The suspensions were subsequently centrifuged at 11 500 *g* for 5 min, washed twice with PBS and resuspended in the same buffer for ELISA using antibodies against Lsa37 (rLIC10821), rLIC10064 and DnaK, as described below. DnaK is a cytoplasmic (Guerreiro *et al.* 2001) leptospiral protein, and was employed in our experiments as a negative control.

Immunoblotting assay

The purified recombinant proteins were loaded onto 12% SDS-PAGE and transferred to nitrocellulose membranes (Hybond ECL; GE Healthcare) in semi-dry equipment. Membranes were blocked with 10% non-fat dried milk and 1% BSA containing 0.05% Tween 20 (PBS-T) and probed with anti-Lsa37 or anti-rLIC10064 (1:1000) mouse polyclonal serum for 2 h at room temperature. After washing, the membranes were incubated with HRP-conjugated anti-mouse IgG (1:5000; Sigma) in PBS for 1 h. HRP-conjugated anti-His tag monoclonal antibodies (1:10 000; Sigma) were also used. The protein reactivity was revealed by an ECL reagent kit (GE Healthcare), as described previously (Fernandes *et al.* 2012).

ELISA for detection of human antibodies

Human IgG antibodies against Lsa37 and rLIC10064 were detected by ELISA as described previously (Oliveira *et al.* 2008). Cut-off values were set at 3 SD above the mean OD_{492nm} obtained from commercial normal human sera against the recombinant proteins (Sigma and Complement Technology).

Binding of Lsa37 and rLIC10064 to ECM and plasma components

Attachment of recombinant proteins to individual ECM macromolecules and plasma components was analyzed according to a previously published protocol (Teixeira *et al.* 2015), with some modifications. Briefly, 96-well plates were coated with 1 μg laminin, collagen type I, collagen type IV, cellular and plasma fibronectin, elastin, human PLG, factor H, fibrinogen, C4BP and BSA (negative control) in 100 μl PBS for 16 h at 4°C. The wells were washed three times with PBS-T and then blocked with 200 μl PBS-T containing 10% (w/v) non-fat dried milk for 1 h at 37°C. One microgram of each recombinant protein in 100 μl PBS was added to each well, and protein was allowed to attach to different substrates for 2 h at 37°C. After washing six times with PBS-T, bound recombinant proteins were detected by adding an appropriate dilution of mouse antiserum in 100 μl PBS. Dilutions of mouse antiserum against each recombinant protein were equalized based on the OD_{492nm} value of 1.0 in titration experiments. After three washings with PBS-T, 100 μl 1:5000-diluted HRP-conjugated rabbit anti-mouse IgG (Sigma-Aldrich) in PBS was added per well for 1 h at 37°C. In addition, anti-His monoclonal antibodies were employed as protein-binding probes at 1:5000 dilution. The wells were washed three times and *o*-phenylenediamine (OPD; Sigma-Aldrich) (1 mg ml^{-1}) in citrate phosphate buffer (pH 5.0) plus 1 μl ml^{-1} H_2O_2 was added (100 μl well^{-1}). The reaction was allowed to proceed for 15 min then interrupted by the addition of 50 μl 2 M H_2SO_4. Readings were taken at 492 nm in a microplate reader (Multiskan EX; Thermo Fisher). For statistical analyses, the binding of recombinant protein to ECM macromolecules and plasma components was compared with its binding to BSA using Student's two tailed t-test, and the *P* value was given in comparison with BSA, used as negative control.

Dose–response curves

First, 96-well plates were coated with 1 μg laminin, PLG and fibrinogen in 100 μl PBS for 2 h at 37°C. Plates were then blocked and increasing concentrations of the purified Lsa37 (0–2.7 μM) were added (100 μl well^{-1}, in PBS). The assessment of bound protein was performed by incubation for 1 h at 37°C with the antiserum raised against the protein in PBS. The reaction was detected with OPD as described above.

Characterization of protein binding to PLG

To determine the role of lysine residues in the binding of Lsa37 to PLG, recombinant protein was added to the component-coated wells with the lysine analog 6-aminocaproic acid (ACA; Sigma-Aldrich) with increasing concentrations ranging from 0 to 1 M. The detection of bound recombinant protein was performed as described above.

PLA enzymatic activity assay

ELISA plates (96-well) were coated overnight with 10 μg ml^{-1} recombinant proteins or BSA (negative control) in 100 μl PBS at 4°C. The plates were washed once with PBS-T and blocked for 2 h at 37°C with PBS containing 10% (w/v) 10 μg ml^{-1} non-fat dried milk. The blocking solution was discarded and the plate washed three times with PBS-T; 10 μg ml^{-1} human PLG was added per well (100 μl), and incubation continued for 2 h at 37°C. Wells were washed three times with PBS-T, and then 4 ng human urokinase-type PLG activator (uPA; Sigma-Aldrich) was added per well. Subsequently, 100 μl of the PLA-specific substrate D-valyl-leucyl-lysine-*p*-nitroanilide dihydrochloride (Sigma-Aldrich) was added to a final concentration

of 0.4 mM in PBS. Plates were incubated overnight, and substrate degradation was measured by taking readings at 405 nm.

Antibody inhibition assay

Ninety-six-well plates were coated with 100 μl of a 10 μg ml^{-1} laminin, PLG and fibrinogen solution, which were allowed to interact for 2 h at 37°C. Plates were then blocked overnight. Before the next step, the recombinant protein was co-incubated for 1 h at 37°C with anti-Lsa37 antibodies (1:100) in 100 μl PBS. After incubation, 1 μg of antibody-blocked recombinant protein in 100 μl PBS was added to each well to interact with components for 2 h at 37°C. The plate was washed six times with PBS-T, bound recombinant protein was detected using monoclonal HRP-conjugated mouse anti-His (Sigma) diluted 1:5000 for 1 h at 37°C. Detection was performed with OPD, as previously described. For statistical analysis, the percentage of recombinant protein binding was compared with its binding with untreated protein (100% binding) by the ANOVA method.

Statistical analysis

All results are expressed as the mean ± standard deviation (SD). Student's paired t-test was used to determine the significance of differences between means and $P < 0.05$ was considered statistically significant. ANOVA was used for multiple comparisons, followed by the Tukey post-test. Three or two independent experiments were performed, each one in triplicate.

Ethics statement

All animal studies were approved by the Ethical Committee for Animal Research of the Instituto Butantan, Brazil, under protocol no. 798/11. The Committee for Animal Research in Instituto Butantan adopts the guidelines of the Brazilian College of Animal Experimentation. Human serum samples from patients with confirmed leptospirosis were obtained from the Serum Collection of Instituto Adolfo Lutz, São Paulo, Brazil, and were donated to be used for research purposes only. The Ethical Committee for Human Research of ICB/Universidade de Sao Paulo rules that this work does not involve procedures regulated by CONEP/Brazil no. 466/2012, and official approval was, therefore, not required.

RESULTS

In silico analysis of coding sequences

We selected two CDSs from the genome of *L. interrogans* serovar Copenhageni for further studies and characterization. The protein coded by the gene LIC10821 has been annotated as a probable lipoprotein, comprising an N-terminus sequence recognized by the SpII (signal peptidase) machinery, responsible for the covalent attachment of a fatty acid at the cysteine N-terminus of the proteins (Juncker *et al.* 2003). The predicted cleavage site for this protein is at amino acid 17–18. The protein encoded by the LIC10064 gene is annotated as a hypothetical protein, with a predicted cleavage site, in this case SpI, located between amino acids 26 and 27. Both CELLO and PSORT softwares predict the location of LIC10821 and LIC10064 proteins at the outer membrane and at the periplasmic space of the bacteria, respectively. Domains of unknown function (DUFs) were found within the proteins according to PFAM and SMART webserver analysis, DUF1565 and DUF2147 for LIC10821 and LIC10064, respectively. Multiple sequence alignment was performed with ClustalW2 (Larkin *et al.* 2007), comparing LIC10821 (Fig. 1A) and LIC10064 (Fig. 1B) with the sequences available in GenBank. The depicted phylograms show a high level of conservation among serovars of *L. interrogans* and other pathogenic species for both sequences. LIC10064 also has a low level of similarity with the sequences present in saprophyte strains, which are organized in a more distant branch (Fig. 1B). Western blotting assay confirms the high level of conservation of this protein (LIC10064) among different species and serovars of *Leptospira* (Fig. 1C). Interestingly, this protein was detected in the virulent strain employed, *L. interrogans* FIOCRUZ L1-130, and it is absent in the saprophyte *L. biflexa*, suggesting that it might be important for virulence. We were not able to detect the native LIC10821 in leptospiral protein extracts by western blotting, possibly due to its low copy number in the bacteria (Malmström *et al.* 2009).

Cloning, expression and purification of the recombinant proteins

LIC10821 and LIC10064 genes were amplified without the signal peptide and cloned into pAE vector (Ramos *et al.* 2004). Induction of expression of the recombinant proteins Lsa37 (rLIC10821) and rLIC10064 was performed in the host strains *E. coli* BL21 (DE3) Star pLysS and BL21 SI, respectively. The protein rLIC10064 was expressed in its soluble form and Lsa37 in its insoluble form, as inclusion bodies (not shown). Attempts to obtain this protein in its soluble form were unsuccessful. Protein recovered from inclusion bodies after 8 M urea solubilization (Lsa37) and from the supernatant (rLIC10064) was purified by metal-chelating chromatography. The recombinant protein bands were confirmed by western blotting analysis when the membranes were probed with His tag monoclonal antibodies (lane 1 in Fig. 2A and B, for Lsa37 and rLIC10064, respectively), and with polyclonal antiserum raised in mice against each protein (lane 3 in Fig. 2A and B, for Lsa37 and rLIC10064, respectively). For the anti-His detection, recombinant protein Lsa63 was employed as a positive control (lane 2 in Fig. 2A and B). The calculated molecular masses of 36.9 kDa for Lsa37 and 17.8 kDa for rLIC10064 include the vector fusion plus the coding amino acid sequence. The structural integrity of each purified protein was assessed by CD spectroscopy in order to evaluate their secondary structure content (Fig. 2C and D). Analysis of the spectra data by the CAPITO program (Wiedemann, Bellstedt and Gorlach 2013) showed a predominance of β-strand for both proteins (41% for Lsa37 and 44% for rLIC10064). The data indicate that the proteins were suitable for further studies.

Assessment of cellular location of native proteins LIC10821 and LIC10064 by IFA

In order to assess whether the chosen CDSs are located at the bacterial surface, we set out to analyze the protein location by using immunofluorescence microscopy. DnaK, a cytoplasmic protein, was used as negative control. Leptospires were visualized by propidium iodide staining (Fig. 3, PI), followed by protein detection with the corresponding polyclonal antiserum, raised in mice against each recombinant protein, in the presence of anti-mouse IgG antibodies conjugated to FITC. Green fluorescence could be observed for LIC10821 and more prominently for LIC10064, but not with DnaK the negative control, or when pre-immune serum was used. These assays also confirm the

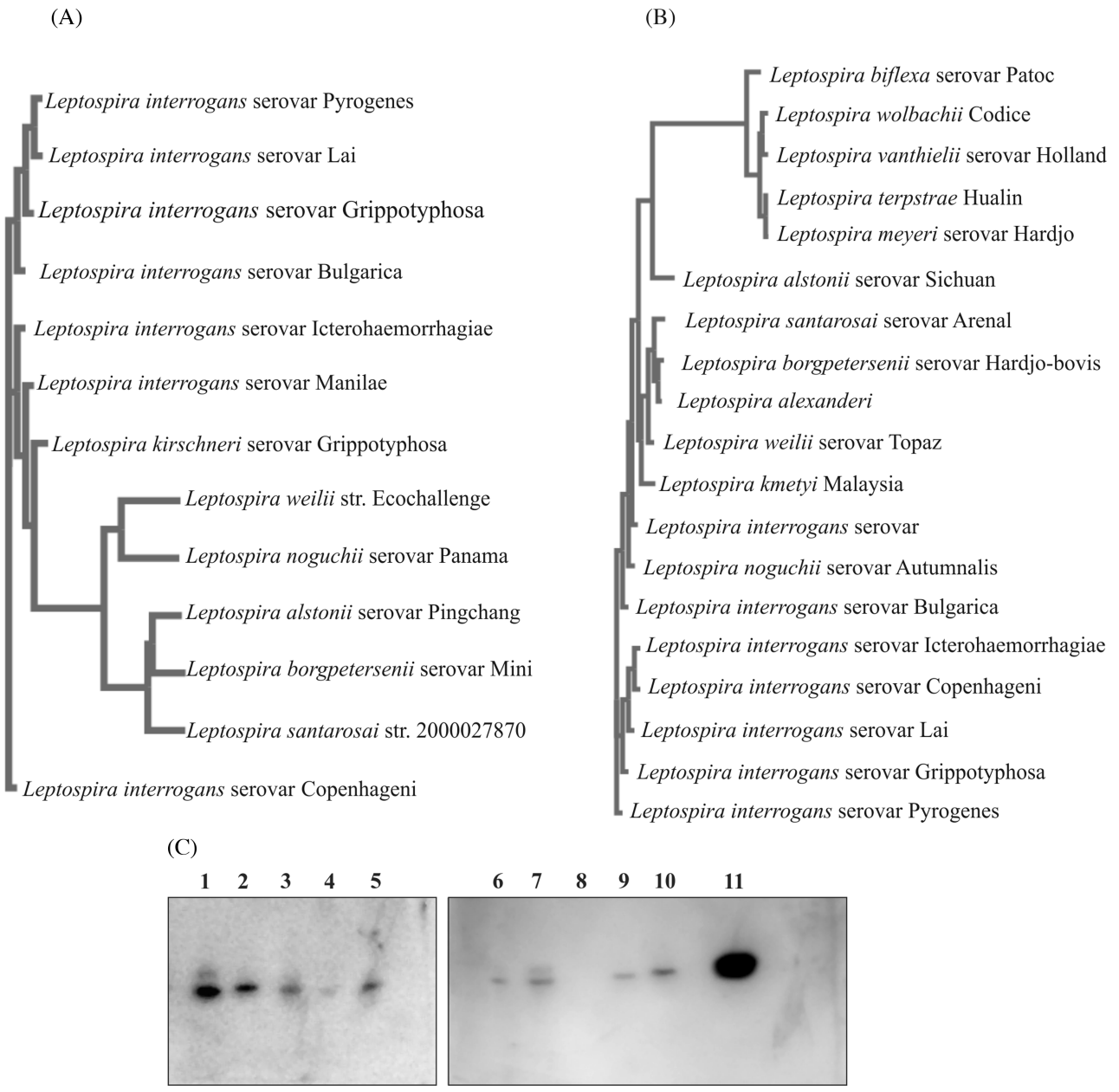

Figure 1. LIC10821 and LIC10064 sequence conservation among *Leptospira* spp. BLAST analysis was performed among sequences of amino acids available in GenBank database and leptospiral sequences were employed to perform Clustal Omega multiple sequences alignments. The resulting phylograms show the high level of sequence conservation for (**A**) LIC10821 and (**B**) LIC10064. (**C**) Western blot employing anti-LIC10064 antibodies was performed against different leptospiral species and serovars. Lanes: 1, *L. interrogans* serovar Copenhageni strain M20; 2, *L. kirshnery* serovar Grippothyphosa; 3, *L. kirshnery* serovar Cynoptery; 4, *L. borgepetersenii* serovar Whitcombi; 5, *L. interrogans* serovar Canicola; 6, *L. interrogans* serovar Hardjo; 7, *L interrogans* serovar Shermani; 8, *L. biflexa* serovar Patoc; 9, virulent *L. interrogans* serovar Copenhageni strain L1-130; 10, virulent *L. interrogans* serovar Kennewick strain Pomona Fromm. Lane 11 refers to the positive control rLIC10064.

presence of both proteins in *Leptospira* strains, most probably located at the cell surface. The difference in fluorescence intensity was expected, since the protein LIC10064 is more abundant in the bacteria, as determined by quantitative proteomics (Malmström *et al.* 2009).

Cellular location assessment by ELISA and PK proteolysis

Leptospires immobilized onto ELISA plates were incubated with polyclonal antiserum against each recombinant protein, followed by the HRP-conjugated secondary antibodies. Controls lacking leptospires or the primary antibody were employed. As depicted in Fig. 3B, a significant signal was obtained when the full system was compared to the controls lacking at least one of the components, suggesting the recognition of the native proteins in the surface of intact bacteria. After PK proteolysis of intact leptospires, detection of native proteins was performed as above. Both proteins had similar patterns of decreasing signal, statistically significant after 1 h incubation for LIC10821 and 3 h for LIC10064 (Fig. 3C). Only a slight signal was detected with antiserum against DnaK, an abundant cytoplasmic marker, used as a negative control (Guerreiro *et al.* 2001), and no significant

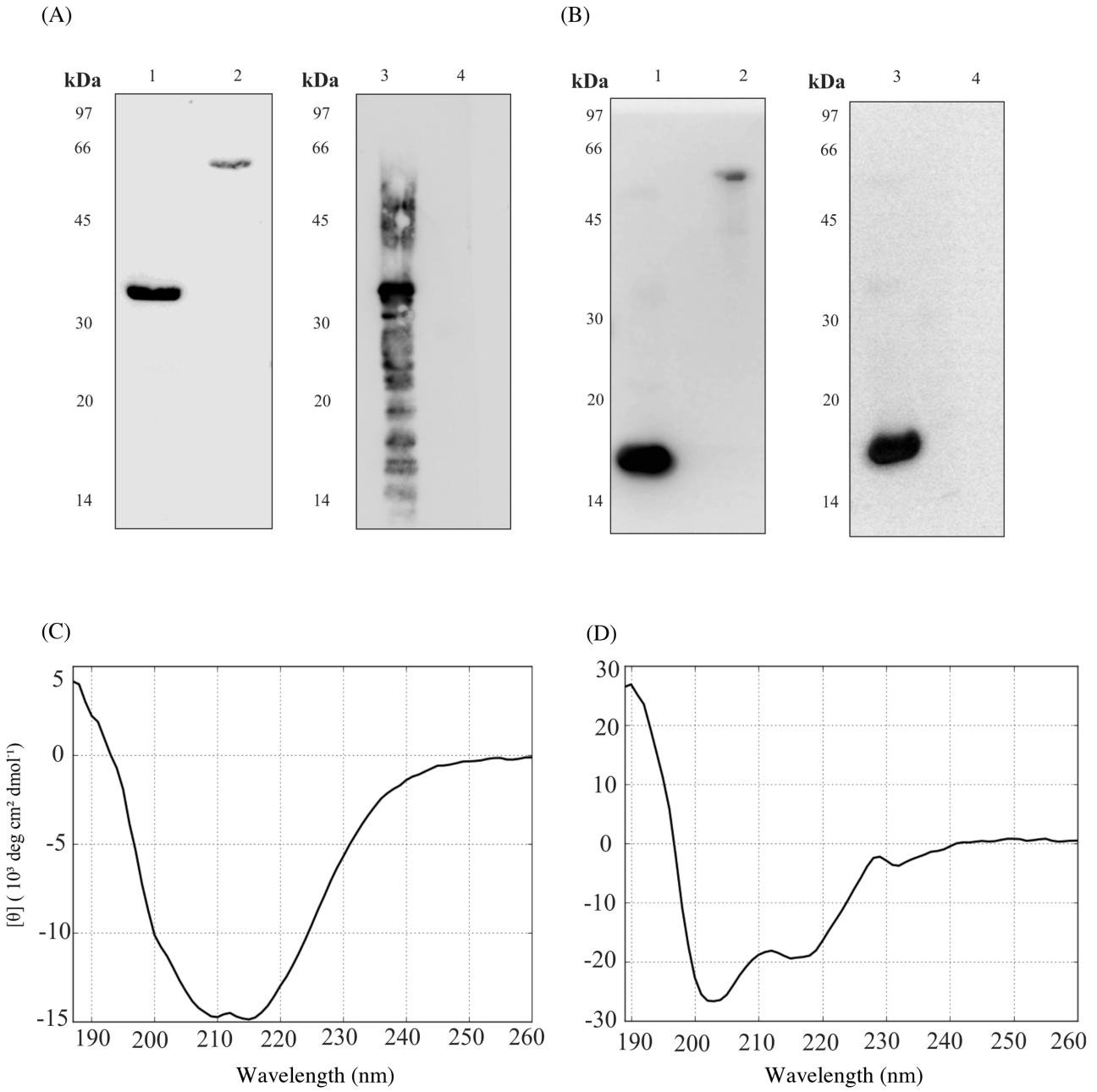

Figure 2. Western blotting of recombinant proteins and evaluation of their secondary structure. Western blotting of Lsa37 (rLIC10821) (**A**) or rLIC10064 (**B**) probed with anti-His tag monoclonal antibodies (lanes 1, in A and B) or with the respective polyclonal antiserum (lanes 3, in A and B). Lanes 2 and 4 refers to the control Lsa63 incubated either with anti-His or anti-LIC, respectively. Secondary structure evaluation obtained by circular dichroism spectra of the recombinant proteins Lsa37 (**C**) and rLIC1064 (**D**). The far-UV CD spectra are presented as an average of five scans.

signal decrease was observed, confirming the integrity of the bacteria. The data from intact bacteria ELISA and proteinase K digestion stongly suggest that both proteins are surface exposed.

Detection of human antibodies against the recombinant proteins

Aiming to verify whether the native proteins are expressed during infection, we evaluated the reactivity of the recombinant proteins against a set of confirmed leptospirosis human serum samples, measuring total IgG antibodies. We employed samples at the early (MAT–) and convalescent (MAT+) phases of the disease, in comparison to the reactivity of commercial normal human serum samples, employed for cut-off calculation (see Methods). The protein Lsa37 was recognized by antibodies in both phases of the disease, 56% and 55% for MAT+ and MAT–, respectively, (Fig. 4), while very low reactivity was detected with rLIC10064, 6% and 13% for MAT+ and MAT–, respectively. The results suggest that although the protein corresponding to rLIC10064 is present in higher copy number than the counterpart of Lsa37 in *Leptospira*, the latter seems to be much more immunogenic, and capable of inducing an immune response in humans.

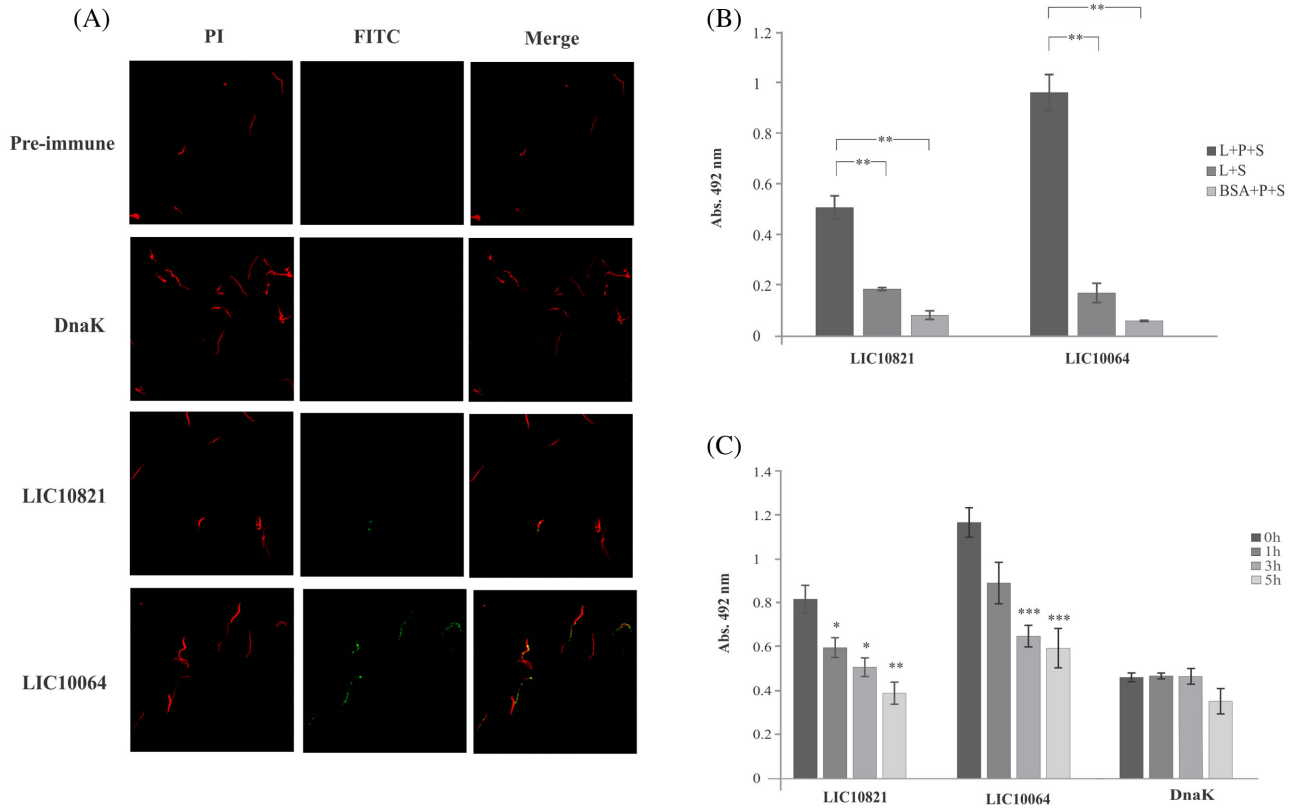

Figure 3. Cellular localization of native proteins in *Leptospira*. (**A**) *Leptospira interrogans* serovar Copenhageni strain M20 were fixed with paraformaldehyde and polyclonal anti-Lsa37 and anti-rLIC10064 were used to identify surface-exposed protein; serum against DnaK was used as a marker for non-exposed, cytoplasmic protein. Pre-immune serum was used as a negative control. FITC-conjugated secondary antibodies were used to reveal the surface-bound antibodies. Leptospires were identified by propidium iodide (PI) staining of the DNA. Co-localization is shown in the merged images. (**B**) Leptospires were immobilized onto ELISA plates and incubated with polyclonal antibody against each recombinant protein, followed by the HRP-conjugated secondary antibody, S. The controls were employed lacking one of the components of the system (leptospires, L, or primary antibody, P). For statistical analyses we compared the groups using ANOVA (**$P < 0.01$), with the Tukey post-test. (**C**) Cellular localization of native proteins Lsa37 and rLIC10064 by proteinase K accessibility assay. Viable leptospires were incubated with 25 μg ml^{-1} proteinase K at the indicated times. The suspensions were recovered, washed, resuspended in PBS and coated onto a microplate. Mice antisera against Lsa37, rLIC1064 and DnaK were added and the reaction detected with HRP-conjugated anti-mouse IgG. Blanks were run in parallel, in which antisera against the proteins were omitted. Bars represent mean ± standard deviation at 492 nm of three replicates for each protein and are representative of three independent experiments. For statistical analyses, the signal was compared between 0 h and other times of treatment with proteinase K by the two-tailed t-test (*$P < 0.1$, **$P < 0.01$, ***$P < 0.001$).

Lsa37 is a laminin-binding protein

We decided to evaluate whether the recombinant proteins could mediate host colonization by interacting with the ECM components. Thus, laminin, elastin, collagen type I, collagen type IV, cellular fibronectin and the control protein BSA were immobilized onto 96-well microdilution plates, and recombinant protein attachment to the components was assessed by ELISA, as described previously (Fernandes *et al.* 2014), using antiserum against each recombinant protein. As shown in Fig. 5A, Lsa37 displayed a significant binding to laminin when compared to the negative control ($P < 0.01$) and this interaction was further confirmed using anti-His monoclonal antibodies (Fig. 5B) ($P < 0.001$). No adhesion to the components tested was observed for rLIC10064. Blockage of immunogenic epitopes of Lsa37 caused a reduction of approximately 20% when compared to the maximal protein binding to laminin (Fig. 5C). This suggests that some epitopes are located next to or within the interaction domains. A dose-dependent curve was observed for this interaction (Fig. 5D); however, no saturation was achieved for the protein concentrations assayed.

Lsa37 interaction with plasma components

It has been previously demonstrated that *Leptospira* interact with several host plasma components enabling the bacteria to overcome host tissues barriers (Vieira *et al.* 2009), facilitating immune evasion (Barbosa *et al.* 2009; Vieira *et al.* 2011; Castiblanco-Valencia *et al.* 2012) and decreasing clot formation (Oliveira *et al.* 2013). Thus, we decided to assess whether the recombinant proteins could participate in any of these processes by binding to plasma components PLG, plasma fibronectin, factor H, fibrinogen and C4BP. Components were coated onto ELISA plates and allowed to interact with the recombinant proteins. The results show that Lsa37 attaches to PLG ($P < 0.05$) and fibrinogen ($P < 0.01$) when the reaction was probed with polyclonal antibodies (Fig. 6A). The data were confirmed when the bindings were detected with anti-His antibodies (Fig. 6B). No reactivity was detected with the other plasma components tested or with rLIC10064. The interaction of Lsa37 with both components was dose dependent (Fig. 6C and D), and in the case of PLG, binding saturation was achieved with 675 nM protein, with a dissociation constant (K_D) of 110.2 ± 22.4 nM. The involvement of Lsa37

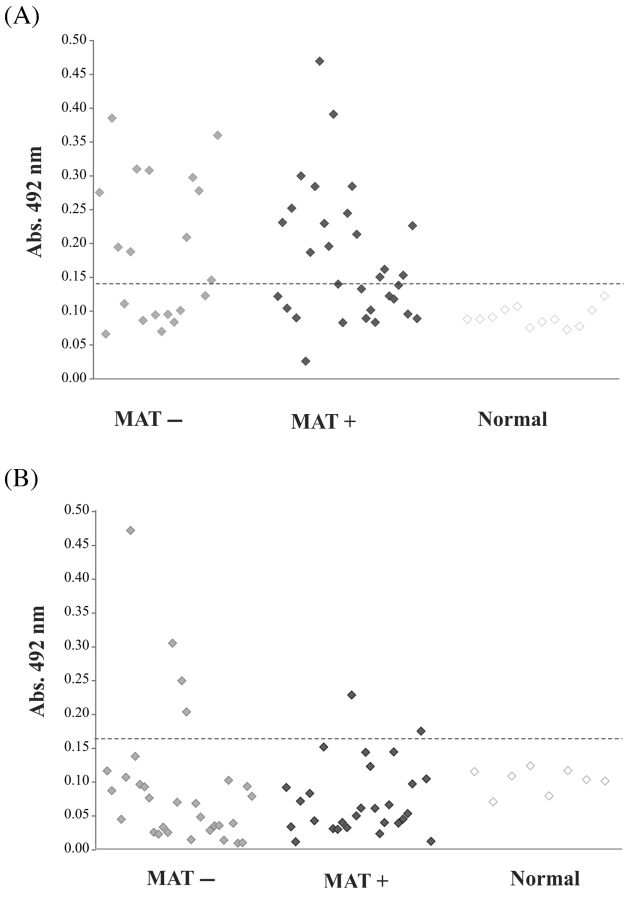

Figure 4. Detection of antibodies against recombinant proteins in human leptospirosis sera. Reactivity of recombinant proteins Lsa37 (**A**) and rLIC10064 (**B**) was assessed in human leptospirosis in comparison with normal serum samples. The cutoff values (dashed lines) were defined as the mean plus 3 SD obtained with normal human sera. The number of responders was 55% (MAT–) and 56% (MAT+) for Lsa37; 13% (MAT–) and 6% (MAT+) for rLIC10064.

immune epitopes in the interactions with PLG and fibrinogen is shown by the decrease in binding when the protein was previously incubated with mice anti-Lsa37 serum (Fig. 6E). A less pronounced decrease was observed when the protein was incubated with mice pre-immune serum, employed as a control, suggesting non-specific bindings.

Lsa37 interaction with fibrinogen causes a reduction of fibrin formation

Leptospires are able to interact with human fibrinogen and to reduce the formation of a fibrin clot in a thrombin-catalyzed reaction (Choy *et al.* 2007; Oliveira *et al.* 2013). Some bacterial Fg-binding proteins have already been identified that lead to a partial reduction in fibrin clotting (Lin *et al.* 2011; Oliveira *et al.* 2013). In order to evaluate the consequence of Lsa37 interaction with Fg, this component was incubated with increasing concentrations of recombinant protein (0–1350 nM). At the highest concentration employed, Lsa37 bound to Fg could significantly reduce the fibrin clot formation ($P < 0.01$) to approximately 40% (Fig. 6F).

Characterization of Lsa37 binding to PLG and PLA formation

It is well known that PLG kringle domains frequently mediate interactions with lysine residues of the bacterial receptors (Lahteenmaki, Kuusela and Korhonen 2001). Moreover, it has been previously shown that these domains participate in the binding of PLG to intact live *L. interrogans* serovar Copenhageni strain L1-130 cells (Vieira *et al.* 2009). Based on these findings, the participation of lysine residues in the binding of Lsa37 to PLG was evaluated by the addition of increasing concentrations of the lysine analog ACA on the reaction mixture. As illustrated in Fig. 6G, when 100 mM ACA was added to the reaction mixture, the binding of the protein to PLG was statistically reduced ($^{**}P < 0.01$), suggesting the participation of these domains in the interaction.

Our group has reported that PLG bound to leptospiral proteins can be activated to PLA by host activators (Vieira *et al.* 2009). To assess whether this is the case for Lsa37-bound PLG, ELISA plates were coated with the recombinant protein, followed by incubation with PLG. The uPA-type PLG activator was added together with a plasmin-specific chromogenic substrate. The PLG bound to the protein could be converted into PLA, as demonstrated indirectly by the specific proteolytic activity (Fig. 6H). No cleavage of the chromogenic substrate was observed in controls lacking at least one of the components of the reaction. rLIC10064 protein was also employed as a negative control.

DISCUSSION

Pathogens possess a vast repertoire of proteins involved in the initial steps of binding to host components and tissues. The ECM constitutes a protein complex, whose composition and structural organization influences numerous biological processes (Patti and Höök 1994). As a universal constituent of animal tissues, the ECM can also serve for the attachment of colonizing micro-organisms (Patti *et al.* 1994; Ljungh and Wadström 1996). The complete genome sequencing of several bacterial species has offered an amazingly high number of different adhesion encoding genes, suggesting many paths to bacterial attachment (Brzuszkiewicz *et al.* 2006; Kline *et al.* 2009). In *Yersinia* spp., a group of proteins mediate pathogen–host interactions acting at different stages of infection, complementing each other, by binding a variety of host components (Mikula, Kolodziejczyk and Goldman 2012). Adhesins have been reported to promote immune responses against many pathogens in animal models, and are potential targets to induce protection in hosts (Kline *et al.* 2009).

To date, several leptospiral ECM-binding recombinant proteins have been published (Vieira *et al.* 2014). These are putative adhesins thought to participate in the initial steps of the colonization process of pathogenic *Leptospira*. Our group is devoted to exploring the genome sequences of *L. interrogans* and searching for possible proteins that could mediate host–pathogen interactions (Vieira *et al.* 2014; Fernandes *et al.* 2015). In this work, we characterized two novel proteins, encoded by the genes LIC10821 and LIC10064, annotated as lipoprotein and hypothetical protein, of unknown function. Cellular localization experiments consistently indicate that both proteins are surface exposed and thus are at the interface for the interaction with host components. Contrary to the data obtained with the recombinant protein rLIC10064, which showed no binding activity to the ECM components tested, rLIC10821 could bind to laminin in a dose-dependent manner, and was thus named Lsa37. As

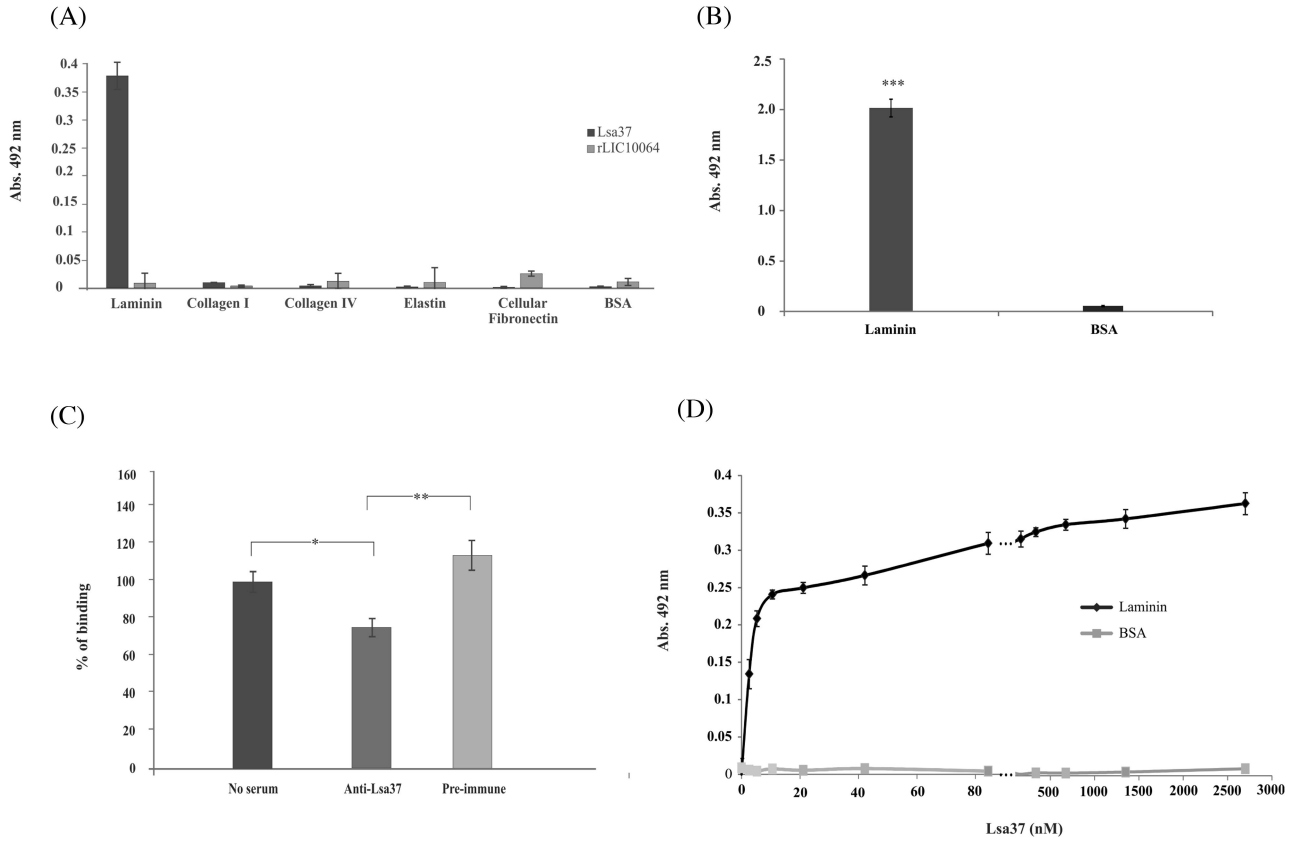

Figure 5. Interaction of Lsa37 and rLIC10064 with ECM components. (**A**) Wells were coated with 1 μg of ECM macromolecules and the control proteins BSA. One microgram of Lsa37 or rLIC10064 was added per well and attachment was evaluated by ELISA. Binding to laminin was confirmed by employing anti-His monoclonal antibodies (**B**). For statistical analyses, the binding of recombinant protein to the ECM components was compared to its binding to BSA by the two-tailed t-test (***$P < 0.001$). (**C**) Effect of mouse polyclonal anti-Lsa37 sera upon the binding of the corresponding protein with laminin, as compared with the binding in the absence of antibodies. Pre-immune serum was used as control. For statistical analyses, ANOVA was performed (*$P < 0.1$, **$P < 0.01$). (**D**) Effect of increasing concentration of Lsa37 on the binding to a constant laminin concentration was assayed. Each point represents values determined in triplicate and data are expressed as the mean absorbance value at 492 nm. BSA was used as a negative control.

reported for many pathogens, the multitude of putative adhesins expressed by leptospires is most probably related to the bacterial invasion tactics, and we may anticipate the necessity for a multicomponent vaccine against leptospirosis.

After adherence, the bacteria have to overcome the barriers imposed by tissues and the ECM. The PLG activation/fibrinolytic system with generation of plasmin was described for virus, parasites and bacteria (Klempner *et al.* 1996; Fenno *et al.* 2000; LeBouder *et al.* 2008; Rojas *et al.* 2008; Funk *et al.* 2016; Gründel *et al.* 2016). *Borrelia burgdorferi* and *Borrelia hermsii* bind human Lys-plasminogen specifically and generate PLA using the host PLG activator. Plasmin-coated *Borrelia* exhibited an increased ability to penetrate endothelial cell monolayers when compared to untreated controls (Coleman *et al.* 1995). ECM degradation by PLA-bound to *Borrelia* was also demonstrated (Coleman, Roemer and Benach 1999). Furthermore, PLG was shown to be required for efficient dissemination of *B. burgdorferi* in ticks (Coleman *et al.* 1997). *Treponema denticola*, the etiologic agent of periodontitis, also bind PLG (Fenno *et al.* 2000). Leptospires are able to capture the PLG from host plasma and subvert its machinery to convert the surface-bound PLG to its active form, PLA, due to the lack of endogenous activators (Vieira *et al.* 2009). PLA-coated leptospires are capable of degrading ECM components such as laminin and fibronectin (Vieira *et al.* 2009). These activities help the bacteria through tissue penetration (Vieira *et al.* 2013). To date, several spirochetal PLG-binding proteins have been described (Vieira *et al.* 2012). Lsa37 showed dose-dependent, saturable and high affinity binding to PLG, which could be converted to PLA in the presence of an activator. Furthermore, PLA bound to leptospiral receptors is able to degrade immune mediators such as C3b and IgG (Vieira *et al.* 2011), and this property might contribute to the ability of bacteria to avoid complement killing, opsonization and phagocytosis.

The formation of a fibrin clot at the endothelium injury site is a strategy employed by the host to prevent pathogen dissemination (Houston *et al.* 2012). The reduction of fibrin clot formation shown with leptospiral binding to fibrinogen may facilitate bacterial dissemination, in concert with fibrinogen degradation via the PLG/PLA system (Oliveira *et al.* 2013). The protein Lsa37 binds to fibrinogen and this interaction was shown to hamper the fibrin clot formation; hence, this protein could contribute to bacterial dissemination via reduction of clot formation.

Taken together, our results indicate that both proteins encoded by the genes LIC10821 and LIC10064 are surface exposed. These proteins were previously genome-annotated as hypothetical proteins of unknown function and, although the leptospiral LIC10064 protein is present in higher copy number per bacterium than LIC10821, our studies did not reveal any possible function for this protein. The corresponding bacterial Lsa37 is expressed during natural infection, as demonstrated by the presence of

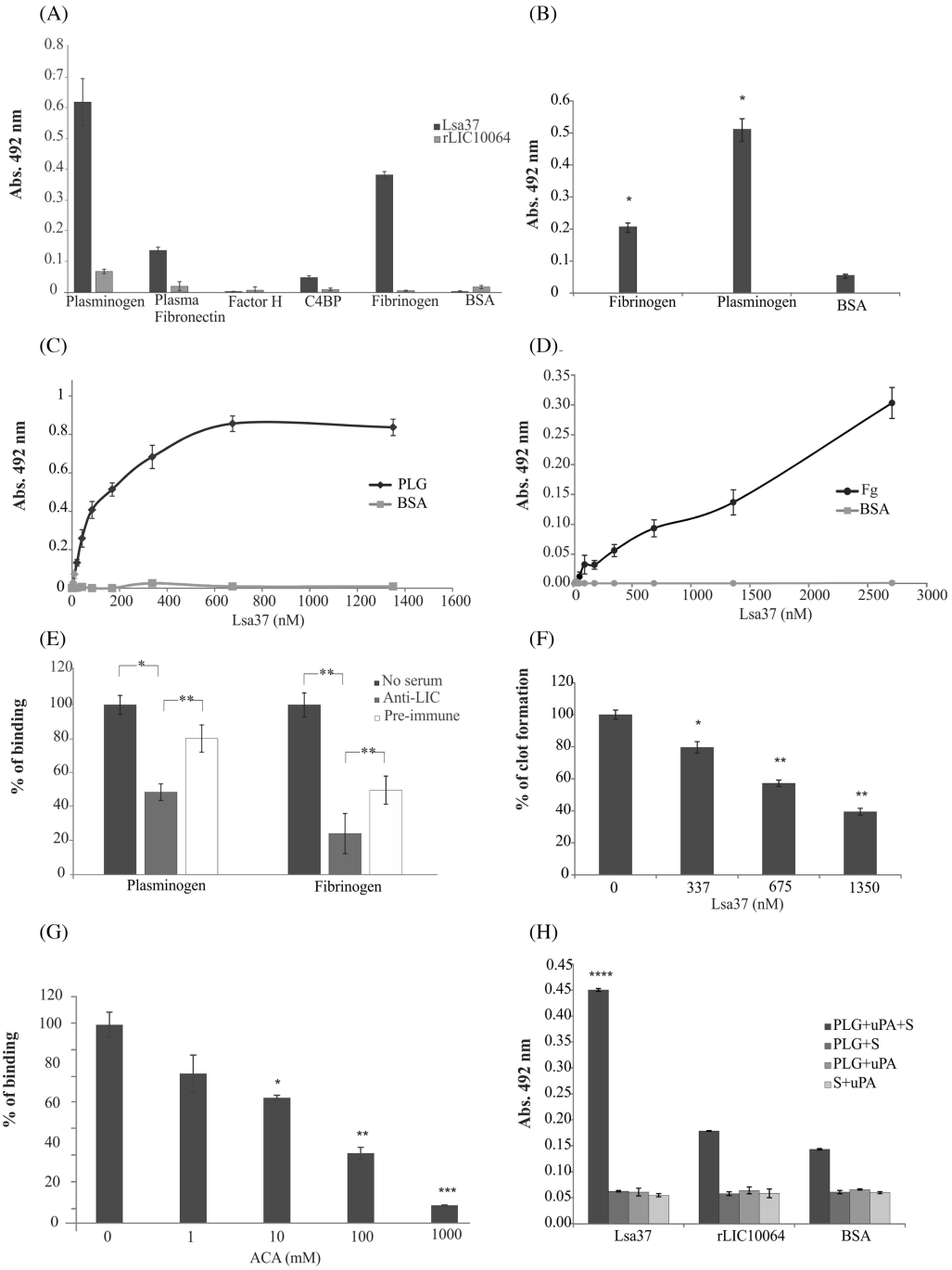

Figure 6. Assessment of Lsa37 and rLIC10064 binding to plasma components. (**A**) Wells were coated with 1 μg of each plasma component and the control protein BSA. One microgram of Lsa37 and rLIC10064 were added per well and binding was measured by ELISA. (**B**) Binding to plasminogen and fibrinogen was confirmed by employing anti-His monoclonal antibodies. All data represent mean ± the standard deviation from three independent experiments. For statistical analyses, the binding of recombinant proteins to the plasma components was compared to its binding to BSA by using a two-tailed t-test ($^{*}P < 0.1$). Dose-dependent binding of Lsa37 to plasminogen (**C**) and fibrinogen (**D**) was performed. Each point represents values determined in triplicate and data are expressed as the mean absorbance value at 492 nm. BSA was used as a negative control. The dissociation constant (K_D) was calculated based on ELISA data for Lsa37 that reached equilibrium. (**E**) Effect of mouse polyclonal anti-Lsa37 serum upon the binding of the corresponding protein with plasminogen and fibrinogen was compared with the binding in the absence of antibodies. For statistical analyses, we compared the groups using ANOVA ($^{*}P < 0.1$, $^{**}P < 0.01$). (**F**) Inhibition of fibrin clot formation by Lsa37. Fibrinogen (1 mg ml^{-1}) was pre-incubated with different protein concentrations and then thrombin was added. The positive control of the reaction, considered 100% of fibrin clot formation, employed fibrinogen plus thrombin while in the negative control thrombin was omitted. For statistical analyses, treatments were compared to the positive control by two-tailed t-test ($^{*}P < 0.1$, $^{**}P < 0.01$). (**G**) The role of lysine residues in protein–PLG interaction was assessed by ELISA and co-incubation with ACA. For statistical analyses, the binding of the Lsa37 in the presence of ACA was compared with the binding to PLG without ACA by the two-tailed t-test ($^{*}P < 0.1$, $^{**}P < 0.01$, $^{***}P < 0.001$). (**H**) PLA generation by PLG bound to Lsa37 was measured indirectly by the cleavage of PLA-specific substrate. Controls lacking at least one component were used; BSA and rLIC10064 were employed as control proteins. Bars represent mean absorbance at 405 nm (OD_{405nm}) as a measure of relative substrate cleavage ± the standard deviation of three replicates; one representative, of two independent experiments, is shown. Statistically significant differences were observed relative to control BSA ($^{***}P < 0.001$). S, substrate; uPA, urokinase-type PLG activator.

antibodies in human leptospirosis serum samples. The recombinant protein Lsa37 (rLIC10821) was characterized as multifunctional, binding to laminin, PLG and Fg, and we may assign possible functions to this protein of bacterial attachment, tissue invasion and immune evasion strategies.

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