Functional and immunological evaluation of two novel proteins of *Leptospira* spp.

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This work shows the production and characterization of two novel putative lipoproteins encoded by the genes LIC10645 and LIC10731 identified in the genome sequences of *Leptospira interrogans*. *In silico* conservation analysis indicated that the proteins are well conserved among pathogenic leptospiral serovars and species. Recombinant proteins were obtained in *Escherichia coli* BL21(DE3) Star pLysS strain, purified by metal-affinity chromatography, and used for characterization and immunological evaluations. Recombinant proteins were capable of eliciting a combination of humoral and cellular immune responses in animal models, and could be recognized by antibodies present in human serum samples. The recombinant proteins Lsa44 and Lsa45 were able to bind laminin, and were named Lsa44 and Lsa45 for leptospiral surface adhesins of 44 and 45 kDa, respectively. The attachment to laminin was dose-responsive with $K_D$ values of 108.21 and 250.38 nM for Lsa44 and Lsa45, respectively. Moreover, these proteins interact with plasminogen (PLG) with $K_D$ values of 53.56 and 36.80 nM, respectively. PLG bound to the recombinant proteins could be converted to plasmin (PLA) in the presence of an activator. Cellular localization assays suggested that the Lsa44 and Lsa45 were surface-exposed. These are versatile proteins capable of interacting with laminin and PLG/PLA, and hence could mediate bacterial adhesion and contribute to tissue penetration.

INTRODUCTION

Leptospirosis is a zoonosis of global importance caused by pathogenic bacterial species of the genus *Leptospira* (Bharti et al., 2003; Faine et al., 1999). In urban environments, rodents are the main host reservoirs of leptospires, shedding live bacteria through their urine (Ko et al., 1999; Vinetz et al., 1996). Humans are infected via contact with urine of wild or domestic animal carriers, either directly or indirectly through contaminated water or soil (Adler & de la Peña Moctezuma, 2010). The disease presents a broad spectrum of symptoms, including fever, vomiting, headache, diarrhoea, and abdominal and generalized muscle pain. Due to these flu-like signs, leptospirosis remains under-diagnosed. Progression to multiorgan system complications, known as Weil’s syndrome, occurs in 5–15% of cases, with mortality rates of 5–40% (Faine et al., 1999; Ko et al., 1999; Levett, 2001; Plank & Dean, 2000). Currently available commercial vaccines are based on inactivated whole-cell or membrane preparations of pathogenic leptospires, named bacterins. They confer protective responses mostly through the induction of antibodies against LPS antigens (Adler & de la Peña Moctezuma, 2010; de la Peña-Moctezuma et al., 1999). Nevertheless, 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 (Adler & de la Peña Moctezuma, 2010). Due to the large number of serovars (Bharti et al., 2003), the search for conserved and protective antigens is being pursued (Koizumi & Watanabe, 2005). Leptospiral surface-exposed...
proteins are potential targets to promote immune responses during natural infection and may also mediate pathogen-host interactions.

The adhesion of pathogens to extracellular matrix (ECM) components is considered to be essential in the initial stage of the infection (Ljungh & Wadström, 1996). Indeed, we have reported that pathogenic leptospires are capable of binding ECM macromolecules (Barbosa et al., 2006). Thus far, several proteins of *Leptospira* have been assigned as possible mediators for ECM attachment (Atzingen et al., 2008, 2009; Barbosa et al., 2006; Carvalho et al., 2009; Choy et al., 2007; Domingos et al., 2012; Fernandes et al., 2012; Hoke et al., 2008; Mendes et al., 2011; Oliveira et al., 2010, 2011; Pinne et al., 2010; Souza et al., 2012; Stevenson et al., 2007; Vieira et al., 2010a). After adherence, pathogens have to overcome host tissue barriers to reach the blood circulation and target organs. Our group has reported previously that leptospires bind plasminogen (PLG) at their surface and that proteolytic activity is achieved due to the generation of plasmin (PLA), capable of degrading laminin and fibronectin (Vieira et al., 2009; Vieira, 2012).

In the present study, we describe the cloning, expression, purification, and immunological and functional characterization of two novel predicted outer-membrane lipoproteins, encoded by the genes LIC10645 and LIC10731, identified in the genome sequences of *Leptospira interrogans* serovar Copenhageni by bioinformatics tools (Nascimento et al., 2004). The recombinant proteins were capable of adhering to laminin, and named Lsa44 (LIC10645) and Lsa45 (LIC10731) for leptospiral surface adhesin of 44 and 45 kDa, respectively. In addition, these are PLG-interacting proteins with the ability to generate PLA. Our findings suggested that Lsa44 and Lsa45 proteins may be involved in host–pathogen interactions.

**METHODS**

**Bacterial strains and serum samples.** The pathogenic, low-passage, virulent *L. interrogans* serovar Copenhageni strain FIOCRUZ L1-130 was cultured at 28 °C under aerobic and static conditions in liquid EMJH medium (Difco BD) with 10% (v/v) rabbit serum, which was enriched with l-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). Human serum samples from patients with confirmed leptospirosis were from the Serum Collection of the Instituto Adolfo Lutz, São Paulo, Brazil, and were donated for research purposes only. *Escherichia coli* DH5α and *E. coli* BL21(DE3) Star pLysS (Studier, 1991) were used as cloning and recombinant protein expression hosts, respectively. Leptospiral DNA extraction was performed as described previously (Oliveira et al., 2011).

**In silico sequence analysis.** Conservation of the coding sequences LIC10645 (GenBank accession no. YP_000629.1) and LIC10731 (GenBank accession no. YP_000713.1) was assessed using Clustal 2.1 multiple-sequence alignment, http://www.ebi.ac.uk/Tools/msa/clustalw2/ (Larkin et al., 2007). Cellular localization prediction was performed by using PSORT and CELLO web servers, http://psort.hgc.jp/form.html (Nakai & Kanehisa, 1991) and http://cello.life.nctu.edu.tw/ (Yu et al., 2004), respectively. The presence of a putative lipobox sequence was evaluated by using LipoP, http://www.cbs.dtu.dk/services/LipoP/ (Junker et al., 2003). The signal peptide sequence was assessed by SignalP, http://www.cbs.dtu.dk/services/SignalP-3.0/ (Bendtsen et al., 2004).

**Cloning, expression and purification of Lsa44 and Lsa45.** The amplification of LIC10645 and LIC10731 was performed by PCR with *L. interrogans* serovar Copenhageni strain FIOCRUZ L1-130 genomic DNA using specific primers (Table 1). The gene sequence was amplified without the signal peptide tag. The PCR fragments of 1212 bp (LIC10645) and 1164 bp (LIC10731) were ligated into the *E. coli* expression vector pAE (Ramos et al., 2004) at the restriction sites presented in Table 1. This expression vector allows the inclusion of a His epitope at the N-terminus of the recombinant proteins. Plasmids pAE-LIC10645 and pAE-LIC10731 were used to transform *E. coli* BL21(DE3) Star pLysS. The expression of the recombinant proteins was induced by 1 mM IPTG for 3 h under constant agitation at 37 °C in the presence of 50 µg ampicillin ml⁻¹ and 34 µg chloramphenicol ml⁻¹. The cells were harvested by centrifugation, and the resulting bacterial pellet was resuspended in sonication buffer [20 mM Tris/HCl (pH 8.0), 200 mM NaCl, 200 µg lysozyme ml⁻¹, 2 mM PMSF and 1% Triton X-100] and lysed on ice with the aid of a sonicator tip (ultrasonic processor; GE Healthcare Bio-Sciences). After centrifugation (12 000 g for 10 min), the insoluble fraction was resuspended in a buffer containing 20 mM Tris/HCl (pH 8.0), 500 mM NaCl, 8 M urea and 1 mM β-mercaptoethanol. The proteins were purified through Ni²⁺-charged chelating fast-flow chromatographic columns (GE Healthcare) as described previously (Fernandes et al., 2012). The efficiency of the purification was evaluated by 12% SDS-PAGE. Fractions containing the recombinant proteins were pooled and extensively dialyzed against buffer containing 500 mM NaCl, 20 mM Tris/HCl (pH 8) and 0.1% (w/v) glycine for 24 h. The final yield of the process to obtain recombinant proteins was 22.3 and 4.7 mg (1 induced bacterial culture)⁻¹ for Lsa44 and Lsa45, respectively.

**Circular dichroism (CD) spectroscopy.** Purified recombinant proteins were dialyzed against sodium phosphate buffer (pH 7.4) and CD spectroscopy measurements were performed as described previously (Oliveira et al., 2011). The residual molar ellipticity was expressed in degree cm² dmol⁻¹. Spectrum data were evaluated with k2d3 software (http://ogi.ca/projects/k2d3/) that calculates the secondary structure content from the ellipticity experimental data (Louis-Jeune et al., 2012).

**Antiserum production against recombinant protein and isotype determination.** BALB/c mice (4–6 weeks old) were immunized subcutaneously with 10 µg of the recombinant proteins mixed with 10% (v/v) Alhydrogel [2% Al(OH)₃; Brenntag Biosector] as an adjuvant as described previously (Fernandes et al., 2012). Negative-control mice were injected with PBS mixed with adjuvant. Two weeks after each immunization, the mice were bled from the retro-orbital plexus, and the resulting pooled sera analysed by ELISA for the determination of antibody titres and concentration. First, anti-recombinant proteins sera were adsorbed into a suspension containing *E. coli* lysed cells to avoid reactivity with anti-*E. coli* antibodies (Gruber & Zingales, 1995). For isotype determinations, total IgG, IgG1 and IgG2a serum levels were measured by the incubation of the pooled mouse sera with the recombinant proteins (250 ng per well), followed by incubation with horseradish peroxidase (HRP)-conjugated anti-mouse total IgG (for total IgG determination) or goat anti-mouse IgG1 or IgG2a (1:2000 dilution) followed by incubation with HRP-conjugated anti-goat IgG (1:10 000) (for isotype determination). The wells were washed and 3,3',5,5'-tetramethylbenzidine (TMB; 1 mg ml⁻¹) in citrate phosphate buffer (pH 5.0) plus 1 µl H₂O₂ ml⁻¹ was added (100 µl per well). The reaction proceeded for 10 min and was interrupted by the addition of 50 µl of 8 M H₃SO₄. Readings were...
The protein reactivity was revealed by ECL reagent kit (GE conjugated anti-His tag antibodies (1:10 000; Sigma) were also used. After washing, the membranes were incubated with HRP-conjugated anti-(1:800) mouse polyclonal serum for 2 h at room temperature. After washing six times with PBS-T (PBS containing 0.05% Tween 20), bound proteins were detected by the addition of an appropriate dilution of mouse antiserum that resulted in an A492 value of 1 in previous titrations in 100 μl PBS (1:800 for both proteins). Incubation proceeded for 1 h at 37 °C and after three washes with PBS-T, 100 μl of a 1:5000 dilution of HRP-conjugated goat anti-mouse IgG in PBS was added per well followed by 1 h incubation at 37 °C. The wells were washed three times and the reactivity was detected with substrate OPD as described above. For statistical analyses, the binding of the recombinant proteins to ECM macromolecules and serum components was compared with their binding to gelatin negative control by using Student’s two-tailed t-test. Binding was also confirmed by using HRP-conjugated anti-His mAbs previously titrated against the recombinant protein and used at a dilution that generates an A492 value of approximately 1.

<table>
<thead>
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<th>Gene locus*</th>
<th>Given name</th>
<th>Genome annotation</th>
<th>Primer sequence (restriction site underlined)</th>
<th>Molecular mass (kDa)†</th>
</tr>
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<td>Lsa44</td>
<td>Probable lipoprotein</td>
<td>F: 5′-CTCGAGATTCGAATCTGTTAAAAAGA-3′ Xhol \hspace{1cm} R: 5′-AGCTTTTACGACGCTGTAGCTG-3′ HindIII</td>
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<td>LIC10731</td>
<td>Lsa45</td>
<td>Probable lipoprotein</td>
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</table>

†Molecular mass calculated from the amino acid sequences of leptospiral protein plus amino acids encoded by the pAE vector.

taken at 492 nm with a microplate reader (Multiskan EX; Thermo Fisher Scientific). The A492 values exhibited by different dilutions of mouse serum were compared with a curve generated by coating the plates with different concentrations of mouse total IgG, IgG1 or IgG2a (Southern Biotech).

**Limulus amoebocyte lysate (LAL) assay.** The chromogenic LAL assay for endotoxin activity of the proteins was performed using the QCL-1000 assay kit (Lonza Walkersville) according to the manufacturer’s instructions. The endotoxin contents of Lsa44 and Lsa45 samples were estimated by this test to be 0.8 and 0.6 μg ml⁻¹, respectively.

**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.

**Immunoblotting assay.** The purified recombinant proteins were loaded onto 12% SDS-PAGE gels and electro-transferred onto nitrocellulose membranes (Hybond ECL; GE Healthcare) on semidyir equipment. Membranes were probed with anti-Lsa44 or anti-Lsa45 (1:800) mouse polyclonal serum for 2 h at room temperature. After washing, the membranes were incubated with HRP-conjugated antimouse IgG (1:5000; Sigma) in PBS for 1 h. Monoclonal HRP-conjugated anti-His tag 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).

**Lymphoproliferation assay and cytokine production.** At the end of the immunization protocols, BALB/c mice were sacrificed, their spleens were aseptically removed, and cells were cultured for the lymphoproliferation assay and cytokine production essentially as described in Fernandes et al. (2012).

**ELISA for detection of human antibodies.** Human IgG antibodies against Lsa44 and Lsa45 were detected by ELISA as described previously (Oliveira et al., 2008). Cut-off values were set at 3 SD above the mean A492 obtained from commercial normal human sera against the recombinant proteins (Sigma and Complement Technology). Comparison of frequency of responders between the proteins was analysed statistically using the χ² test.

**Proteinase K accessibility assay.** The proteinase K accessibility assay was performed according to Domingos et al. (2012). We included a blank control containing all of the reaction mixture except antibodies against the proteins. The reactivity was detected with substrate OPD as described above. For statistical analyses, the A492 values obtained at different treatments were compared against the treatment at ‘time=0 h’ at which none of the proteins tested were yet degraded (maximal signal).

**Binding of recombinant proteins to ECM and serum components.** Protein attachment to individual macromolecules of ECM (all from Sigma) and serum components (EMD Chemicals) was analysed according to a previously reported protocol (Atzingen et al., 2008). Briefly, ELISA plates (Costar High Binding; Corning) were coated with 1 μg each component or the negative controls BSA, gelatin and fetuin in 100 μl PBS for 3 h at 37 °C, and then blocked overnight at 4 °C. One microgram of each recombinant protein was added per well in 100 μl PBS and proteins were allowed to attach to the different components for 2 h at 37 °C. After washing six times with PBS-T (PBS containing 0.05% Tween 20), bound proteins were detected by the addition of an appropriate dilution of mouse antiserum that resulted in an A492 value of 1 in previous titrations in 100 μl PBS (1:800 for both proteins). Incubation proceeded for 1 h at 37 °C and after three washes with PBS-T, 100 μl of a 1:5000 dilution of HRP-conjugated goat anti-mouse IgG in PBS was added per well followed by 1 h incubation at 37 °C. The wells were washed three times and the reactivity was detected with substrate OPD as described above. For statistical analyses, the binding of the recombinant proteins to ECM macromolecules and serum components was compared with their binding to gelatin negative control by using Student’s two-tailed t-test. Binding was also confirmed by using HRP-conjugated anti-His mAbs previously titrated against the recombinant protein and used at a dilution that generates an A492 value of approximately 1.

**Kₐ values for the binding of recombinant proteins to ECM and serum components.** ELISA plates were coated with 1 μg ECM or serum components and were allowed to adhere for 3 h at 37 °C. Plates were then blocked overnight, and increasing concentrations of each purified recombinant protein was added (100 μl per well in PBS) and incubated for 2 h at 37 °C. The assay of bound protein was performed with polyclonal antiserum raised in mice against each protein. The ELISA data were used to calculate the equilibrium dissociation constant (Kₐ), according to a method described elsewhere (Lin et al., 2009), following the equation $Kₐ=K_{max}[protein]/A$–[protein], where $A$ is the absorbance at a given protein concentration, $K_{max}$ is the maximum absorbance for the ELISA plate reader (equilibrium), [protein] is the protein concentration and $Kₐ$ is the equilibrium dissociation constant for a given protein concentration (ELISA data point). When the maximum absorbance was not achieved, we used a linear regression for estimating the dissociation constant.

**Time course of interaction of recombinant proteins to ECM and serum components.** We performed a binding assay, as described above, but varying the time of interaction, ranging from 5 min to 2 h (considered the maximal binding). The assessment of bound protein was performed with polyclonal antiserum as already described. The
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Statistical analysis. All results are expressed as the mean ± SD. Student’s paired t-test was used to determine the significance of differences between means and P < 0.05 was considered statistically significant. Three or two independent experiments were performed, each one in triplicate.

RESULTS

Bioinformatics analysis

The selected coding sequences LIC10645 and LIC10731 have been annotated in the genome as probable lipoproteins, due to the presence of an amino acid sequence that is recognized by the enzyme signal peptidase II, responsible for the covalent attachment of a fatty acid at the cysteine N-terminus of the proteins (Juncer et al., 2003). The predicted cleavage sites for LIC10645 and LIC10731 were at aa 28–29 and 31–32, respectively (Fig. 1a). CELLO predicts LIC10731 and LIC10645 to be located at the outer membrane and as extracellular, whereas PSORT predicts LIC10731 to be located at the periplasmic space/outer membrane and LIC10645 to be an outer-membrane protein.

Multiple sequence alignment was performed with Clustal 2.1 (Larkin et al., 2007), comparing LIC10645 (Fig. 1b) and LIC10731 (Fig. 1c) with the sequences available in GenBank. The depicted phylogram shows a high level of conservation among serovars of L. interrogans for both sequences. LIC10645 has similarity with other pathogenic species (Fig. 1a) and also in one of saprophytic species (Leptospira kneyti). LIC10731 shows similarity/proximity with the sequences in pathogenic strains and a low level of similarity with the sequences present in intermediate-pathogenic strains (Matthias et al., 2008), which are organized in a more distant branch (Fig. 1b).

Cloning, expression and purification of the recombinant proteins

LIC10645 and LIC10731 were amplified without the signal peptide and cloned into pAE vector. Induction of expression of the recombinant proteins Lsa44 and Lsa45 was performed in the host strain E. coli BL21(DE3) Star pLysS. The proteins were expressed in their insoluble form, as inclusion bodies (Fig. 2a, lane 5, for Lsa44; Fig. 2b, lane 5, for Lsa45). Attempts to obtain the proteins in their soluble form were unsuccessful. Proteins were recovered from inclusion bodies after 8 M urea solubilization and purified by metal-chelating chromatography after protein refolding. Samples were analysed by SDS-PAGE (Fig. 2a, lane 6, for Lsa44; Fig. 2b, lane 6, for Lsa45). The recombinant proteins bands were confirmed by Western blots probed with polyclonal antiserum raised in mice against each protein (Fig. 2c, lanes 1 and 3, for Lsa44 and Lsa45, respectively) and with His tag mAb (Fig. 2c, lanes 5 and 6, for Lsa44 and Lsa45, respectively). The calculated molecular masses of 43.8 kDa for Lsa44 and 44.7 kDa for Lsa45 include the vector fusion plus the encoded amino acid sequence. The structural integrity of each purified protein was assessed by CD spectroscopy in order to evaluate the secondary structure.
content of proteins after the refolding process (Fig. 1d). Analysis of the spectra data by K2D3 (Louis-Jeune et al., 2012) showed a mixture of secondary structures: 28.7 % α-helix and 15.1 % β-strand for Lsa44; 10.7 % α-helix and 32 % β-strand for Lsa45. The data showed that the proteins were suitable for further studies.

**Immunological characterization of recombinant proteins**

The proteins were used to immunize mice and the induced antibodies analysed by ELISA. As depicted in Fig. 3(a), low levels of IgG1 isotype were detected for both proteins, whilst IgG2a antibodies against Lsa44 and Lsa45 were not detected.
No antibody levels were found in the control PBS-injected group (not shown). The recombinant proteins were able to elicit lymphoproliferation of cultured cells of immunized animals (Fig. 3b). A high proliferation level was obtained when cells were treated with concanavalin A (Con A), employed as a positive control (not shown). The addition of Lsa44 and Lsa45 to lymphocytes from animals that had not been primed with the recombinant proteins (PBS control) produced negligible levels of proliferation (data not shown). Supernatants of cultured spleen cells from Lsa44 and Lsa45 immunized mice were analysed for the presence of the cytokines IL-10, IL-4, IFN-γ and TNF-α, chosen to discriminate cellular Th1 (IFN-γ and TNF-α) and humoral Th2 (IL-10 and IL-4) immune responses (Reed et al., 2009; Storni et al., 2005). Stimulation with Lsa44 promoted significant secretion of IFN-γ ($P<0.0001$), whilst in the case of IL-10 the secretion was similar to that obtained with cells from animals that were not primed with protein (PBS injected), only stimulated during cell culture (Fig. 3c, d). Lsa45 elicited an induction of IFN-γ, TNF-α and IL-10 cytokines, with statistically significant values when compared with immunized but not stimulated animal cells ($P<0.01$, $P<0.01$ and $P<0.001$ for IFN-γ, TNF-α and IL-10, respectively). The IL-4 level induced by this protein was similar to that observed in the control group, i.e. animals injected with PBS but stimulated with Lsa45 (Fig. 3d). To rule out the participation of *E. coli* endotoxin, which has immunostimulatory properties, we evaluated the content of LPS by the LAL assay, estimated to be 0.8 and 0.6 μg ml$^{-1}$ for Lsa44 and Lsa45, respectively. This value falls in the range of the values obtained with recombinant proteins in our previous work, from
1.2 \times 10^{-4} \text{ to } 0.88 \mu g \text{ ml}^{-1}, \text{ which did not interfere with the upregulation of cell adhesion molecules induced by recombinant proteins on human umbilical vein endothelial cells (Gomez et al., 2008). Moreover, the immune response induced by Lsa44 and Lsa45 is different, the induction being higher with the protein (Lsa45) with lower endotoxin content.}

**Detection of antibodies in confirmed leptospirosis human samples**

To investigate whether Lsa44 and Lsa45 are expressed by leptospires during infection and able to promote an immune response in the host, we evaluated the reactivity of the recombinant proteins Lsa44 and Lsa45 with antibodies in confirmed leptospirosis human serum samples. We employed 30 paired samples at the early (MAT–) and convalescent (MAT+) phases of the disease, in comparison with the reactivity of commercial normal human serum samples employed for cut-off calculation (see Methods). Antibodies in human leptospirosis samples recognized both recombinant proteins at the convalescent phase (MAT+) (Fig. 4), with 87 and 36% responders for Lsa44 and Lsa45, respectively. The reactivity of the proteins with serum samples at the early phase of the disease (MAT–) was 67 and 10% for Lsa44 and Lsa45, respectively (Fig. 4). The difference between the protein responders in leptospirosis serum samples was statistically significant in

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**Fig. 3.** Evaluation of immune response elicited by recombinant proteins in the mouse model. (a) Evaluation of total circulating IgG antibodies and subclasses in sera from BALB/c mice immunized with Lsa44 or Lsa45. Absorbance values were obtained from different pooled serum dilutions, and compared against a concentration curve of total IgG, IgG1 and IgG2a. (b) Analysis of lymphocyte proliferation response using spleens of mice immunized with recombinant proteins. Cells were cultured, followed by a pulse with medium alone, Con A (5 \mu g \text{ ml}^{-1}) or the immunizing recombinant protein (5 \mu g \text{ ml}^{-1}). Lymphocytes stimulated with medium or Con A were used as negative and positive (not shown) controls, respectively. The proliferative response was measured by a colorimetric (bromodeoxyuridine) ELISA. The data represent the mean \pm SD A_{492} values of three determinations of two independent experiments. For statistical analysis, A_{492} values for the recombinant protein-immunized group treated with the recombinant protein were compared with those for the group treated with medium alone by the two-tailed t-test (*P<0.05 and **P<0.01). Spleen cells were isolated and cultured in 24-well tissue culture plates, each well containing 5\times10^6 cells. (c, d) After 48 h, cell-free culture supernatants were collected, and the level of cytokines, T_{h}1 IFN-\gamma and TNF-\alpha (c) or T_{h}2 IL-10 and IL-4 (d), was measured by ELISA. For statistical analysis, concentration values for the recombinant protein-immunized group stimulated with the recombinant protein were compared with those treated with medium alone by the two-tailed t-test (**P<0.01, ***P<0.001 and ****P<0.0001).
both phases, MAT+ and MAT− (P<0.0001). The results suggest that both proteins are expressed during natural infection, and that the immunogenic epitopes are maintained after purification and refolding of the recombinant proteins.

**Cellular localization of Lsa44 and Lsa45**

Assessment of the cellular localization of the selected proteins on the bacteria was performed by proteinase K accessibility assay. After proteolysis of intact leptospires, detection of native proteins was performed by incubation with antiserum raised in mice against each recombinant protein followed by incubation with HRP-conjugated anti-mouse IgG. Lsa44 and Lsa45 had similar patterns of decreasing signal, statistically significant after 1 h incubation (Fig. 5). Only basal signal was detected when antiserum against DnaK, an abundant cytoplasmic marker, was used as a negative control (Haake & Matsunaga, 2002), confirming the integrity of bacteria. The data from proteinase K digestion suggested that both proteins are surface exposed.

**Adhesion of Lsa44 and Lsa45 to ECM components**

We decided to evaluate whether the recombinant proteins could mediate host colonization by interacting with the ECM. Thus, laminin, collagen type I, collagen type IV, cellular fibronectin, plasma fibronectin, elastin, and the control proteins gelatin, BSA and fetuin were immobilized on 96-well microdilution plates, and recombinant protein attachment was assessed by ELISA as described previously (Atzingen et al., 2008), using antiserum against the recombinant proteins. As shown in Fig. 6(a), both recombinant proteins displayed statistically significant adhesiveness to laminin (P<0.001) and this interaction was confirmed using anti-His mAbs (Fig. 6b) (P<0.0001 for Lsa44; P<0.01 for Lsa45). Antiserum against each recombinant protein was capable of reducing their interaction with laminin in a dose-dependent manner (Fig. 6c), whilst almost no effect was observed with pre-immune or non-immune serum. This suggests that some epitopes are located next to or within the interaction domains. No effect on the binding of the proteins with laminin was observed when the proteins were heat-denatured (Fig. 6d) compared with the untreated proteins.
suggested that these interactions do not depend on the protein structures. Interactions with laminin were also assessed on a quantitative basis. Increasing recombinant protein concentrations were allowed to react to a fixed immobilized laminin concentration (Fig. 6e). In the case of Lsa44, a binding saturation level was achieved with a protein concentration of 1 μM, with $K_D$ values of 108.21 ± 43.28 nM. For Lsa45, saturation was not reached at the protein concentration range employed. We thus decided to obtain this value mathematically using linear regression and the calculated $K_D$ was 250.38 nM. The time course of binding reaction was also analysed. The results showed that both recombinant proteins displayed low adsiveness at the beginning of the reaction, which gradually increased, reaching 100% of the binding after 2 h (Fig. 6f).

**Lsa44 and Lsa45 bind to human PLG**

In our previous work, we showed that leptospires bind PLG on their surface and we also described several proteins that are probably PLG receptors (Vieira et al., 2010b). Thus, the recombinant proteins were assayed for their ability to adhere to human PLG in vitro. In addition, we investigated whether the recombinant proteins could also bind other serum components, such as factor H, human complement, fibrinogen and C4BP. Our data showed that both proteins bound to human PLG ($P<0.01$ for Lsa44; $P<0.0001$ for Lsa45), whilst no reactivity was detected with the other serum components and the control proteins (Fig. 7a). This binding was also confirmed by using anti-His mAbs ($P<0.01$) (Fig. 7b). It is well known that PLG kringle domains frequently mediate interactions with lysine residues of the bacterial receptors (Lahteenmäki et al., 2001). These domains were shown previously to participate in the binding of PLG and intact live *L. interrogans* serovar Copenhageni strain L1-130 cells, because the derivative and analogue of lysine, ACA, almost totally inhibited binding (Vieira et al., 2009). Based on these findings, the participation of lysine residues in the binding of the recombinant proteins was evaluated by the addition of ACA during interaction. As illustrated in Fig. 7(c), when 2 mM ACA was added to the reaction mixture, the binding of the protein to PLG was reduced drastically ($P<0.01$), suggesting strongly the participation of these domains in the recombinant protein interaction with PLG. We further evaluated the interaction of PLG with the recombinant proteins by pre-incubating these proteins with several dilutions of mouse anti-recombinant protein polyclonal serum prior to the adhesion reaction. An inhibitory effect on the interaction with PLG was observed for both Lsa44 and Lsa45, which was dependent on the serum dilution (Fig. 7d), suggesting that the binding domains are next to or within the immunogenic epitopes. Loss of protein structure by thermal denaturation reduced 25.6 and 13.4% of the interaction with PLG ($P<0.05$ and **$P<0.01$**). The interactions with PLG were also evaluated on a quantitative basis. Increasing protein concentration was allowed to react with a fixed immobilized PLG concentration (Fig. 7g). Binding saturation level was reached with 1 μM for both proteins, with $K_D$ values of 53.56 ± 18.49 and 36.80 ± 20.39 nM for Lsa44 and Lsa45, respectively.

**PLA generation from bound PLG**

It was demonstrated previously that enzymically active PLA is generated by PLG bound to the surface of *L. interrogans* when its activator is present (Vieira et al., 2009). To assess whether the PLG bound to the recombinant proteins generates proteolytic activity, as reported for other
Fig. 6. Interaction of Lsa44 and Lsa45 with ECM components. (a) Wells were coated with 1 μg laminin, collagen type I, collagen type IV, cellular fibronectin, plasma fibronectin, elastin, and the control proteins gelatin, BSA and fetuin. One microgram of the recombinant protein was added per well and binding was measured by ELISA. Data represent mean ± sd from three independent experiments. For statistical analyses, the attachment of the recombinant proteins to ECM components was compared with their binding to gelatin by the two-tailed t-test, (**P < 0.001). (b) Binding activity was confirmed by employing anti-His tag mAbs and measured by ELISA. Data represent mean ± sd from three independent experiments. For statistical analyses, the attachment of the recombinant protein was compared with its binding to gelatin by the two-tailed t-test (**P < 0.01 and ****P < 0.001). (c) Effect of pre-immune and mouse polyclonal anti-recombinant protein serum dilutions upon the binding of Lsa44 and Lsa45 with laminin compared with the binding in the absence of antibodies (**P < 0.01). (d) Effect of heat-inactivated recombinant protein upon binding to laminin. Results are expressed as a percentage of binding, which was generated from A492 values. (e) Protein concentration dose–response binding to laminin. Each point represents values determined in triplicate and data are expressed as the mean ± sd A492. Gelatin was included as a negative control. (f) Time course for the interaction of recombinant proteins to laminin. A binding assay was performed as described in (a), except that it was measured as (continued)
incubation with PBS-injected mice serum (serum was added to the proteins could be converted into PLA, as demonstrated indirectly by the specific proteolytic activity (Fig. 7h). Control reaction mixtures lacking PLG, uPA or the chromogenic substrate showed no significant enzymic activity.

Antiserum against each recombinant protein partially inhibited leptospiral binding to laminin and PLG

The role of native proteins on leptospiral binding to laminin and PLG was assessed by soluble-phase interaction assay. Leptospires were pre-incubated with antiserum raised against the recombinant proteins or controls with no serum and with serum of PBS-injected mice and then allowed to interact in solution with the components. Supernatants were used to coat ELISA plates and absorbance values were compared against a curve of known masses. Antiserum anti-Lsa44 and anti-Lsa45 could partially and significantly inhibit leptospire adhesion to laminin when compared with the control in which no serum was added (P<0.01) and also in comparison with incubation with PBS-injected mice serum (P<0.01) (Fig. 8). A statistically significant reduction was observed on the binding of leptospires to PLG when the cells were treated previously with PBS-injected mice serum, probably due to the presence of PLG in the serum. When pretreated with antibodies against Lsa44 and Lsa45, a significant reduction of the binding of leptospires to PLG was seen when compared with the control mice serum (P<0.01) (Fig. 8). These results suggest that antibodies against both Lsa44 and Lsa45 can recognize their counterpart proteins on the leptospiral surface, and that these proteins mediate leptospiral binding to laminin and PLG.

DISCUSSION

It has been reported that cell-mediated immunity is required to protect fully against bovine leptospirosis, stimulated by IFN-γ release (Naiman et al., 2002). CD8+ T-cells against leptospiral protein LigA were found in peripheral blood mononuclear cells of leptospirosis patients (Guo et al., 2010). A recent work published by Hartwig et al. (2013) evaluated the protective effect of LemA protein by a DNA vaccine containing the lemA gene; they observed that this vaccine preparation could confer protective, but not sterilizing, immunity. However, no antibodies against LemA recombinant protein were found. The authors speculated on the role of a cellular immune response as the mechanism of protection.

We have studied two sequences predicted to encode outermembrane lipoproteins, LIC10645 and LIC10731. These sequences have a high degree of conservation among distinct serovars of L. interrogans and were also present in other pathogenic species. The sequences were cloned, and the expressed recombinant proteins, Lsa44 and Lsa45, were able to promote a combination of humoral and cellular immune response in mice, as denoted by the high concentration of antibodies found in animal sera, induction of cellular lymphoproliferation (particularly by Lsa45) and increased cytokine levels. These results might be explained in part by the effects exerted by the aluminium salt employed as an adjuvant, which is known to stimulate both T\(_h1\) and T\(_h2\) immune responses (Marrack et al., 2009). Vernel-Pauillac & Merien (2006) demonstrated that pathogenic leptospires could elicit T\(_h1\) responses together with anti-LPS antibodies in an animal model. Moreover, T\(_h1\) and T\(_h2\) immune responses were involved in the protective mechanism elicited by LigA DNA vaccine in an animal model (Faisal et al., 2008).

It is well known that pathogen adhesion to and colonization of host tissue is an initial and critical event in the infection process. The ECM constitutes a protein complex, whose composition and structural organization influence numerous biological processes, such as adhesion, migration, proliferation or differentiation of eukaryotic cells (Patti et al., 1994). As a ubiquitous constituent of animal tissues, the ECM can also serve for the attachment of colonizing micro-organisms (Ljungh & Wadström, 1996). Among spirochaetes, Borrelia species have been shown to express a 47 kDa outer-membrane protein (BBK32) that confers attachment to fibronectin (Probert & Johnson, 1998) and two surface lipoproteins (DbpA and DbpB) that interact with decorin, a dermatan sulfate proteoglycan associated with collagen fibres (Guo et al., 1998). Treponema pallidum, the causative agent of syphilis, also interacts with the ECM via Tp0155 and Tp0483 outer-surface proteins to fibronectin (Cameron et al., 2004) and Tp0751 bound to a variety of laminin isoforms (Cameron, 2003; Cameron et al., 2005). We have reported previously the adhesion of leptospires to ECM components (Barbosa et al., 2006) and, thus far, various leptospiral ECM-binding proteins have been identified (Atzinger et al., 2009; Choy et al., 2007; Domingos et al., 2012; Hoke et al., 2008; Mendes et al., 2011; Oliveira et al., 2010, 2011; Pinne et al., 2010; Stevenson et al., 2007; Vieira et al., 2010a). Lsa44 and Lsa45 are exclusive laminin-binding proteins similar to other leptospiral adhesins described previously (Mendes...
Fig. 7. Binding of Lsa44 and Lsa45 to PLG and PLA generation. (a) Wells were coated with 1 μg PLG, factor H, complement mix, fibrinogen, C4BP, and the control proteins gelatin, BSA and fetuin. One microgram of the recombinant protein was added per well and binding was measured by ELISA. Data represent mean ± SD from three independent experiments. For statistical analyses, the attachment of the recombinant protein to the serum components was compared with its binding against gelatin by the two-tailed t-test (**P<0.01 and ****P<0.0001). (b) Binding activity was confirmed by employing anti-His mAbs and measured by ELISA. Data represent mean ± SD from three independent experiments. For statistical analyses, the attachment of the recombinant protein was compared with its binding to gelatin by the two-tailed t-test (**P<0.01). (c) Binding of recombinant proteins to PLG was carried out in the presence or absence (no inhibition) of the lysine analogue ACA. Bound proteins were detected by antiserum raised in mice. Bars represent mean ± SD A₄₀₂ of triplicate determinations and are representative of two independent experiments. For statistical analyses, the attachment of the recombinant protein in the presence of ACA was compared with the binding to PLG without ACA (no inhibition) by the two-tailed t-test (***P<0.01 and ****P<0.0001). (d) Effect of pre-immune and mouse polyclonal anti-recombinant protein serum dilution upon binding with PLG compared with the binding in the absence of antibodies (**P<0.01 and ***P<0.001). (e) Effect of heat-denatured recombinant proteins upon their binding to PLG. Results are expressed as a percentage of binding, which was generated from A₄₀₂ values, comparing the binding of untreated protein to PLG, by the two-tailed t-test (*P<0.05). (f) Time course of interaction of recombinant proteins to PLG. A binding assay was performed as described in (a), except that the interaction was measured as a function of the time. For statistical analysis, binding obtained at different times of incubation was compared with maximal binding (120 min incubation) by the two-tailed t-test (*P<0.05, **P<0.01 and ***P<0.001). (g) Lsa44 and Lsa45 dose-dependent binding to PLG. Each point represents data determined in triplicate and results are expressed as mean ± SD A₄₀₂ for each point. Gelatin was included as a negative control. (h) PLA generation by PLG bound to recombinant proteins was measured indirectly by the cleavage of the specific PLA substrate using a modified ELISA. The immobilized recombinant proteins received the following treatment: PLG, uPA and the specific PLA substrate (PLG+uPA+S) or controls lacking one of the three components. BSA was (continued)
et al., 2011), but distinct from other adhesins that have a broader spectrum of ligand binding (Atzingen et al., 2008; Carvalho et al., 2009; Fernandes et al., 2012; Hoke et al., 2008; Oliveira et al., 2011; Pinne et al., 2010; Souza et al., 2012). After adherence, the bacteria have to surmount the barriers imposed by tissues and the ECM. The acquisition of host-derived PLA by leptospiral receptor-bound PLG can degrade fibronectin and laminin that may aid bacterial propagation (Vieira et al., 2009; Vieira, 2012). By data mining the available genome sequences of *L. interrogans* (Nascimento et al., 2004), we have identified novel hypothetical proteins, several of them described as PLG-binding proteins (Domingos et al., 2012; Oliveira et al., 2011; Souza et al., 2012; Vieira et al., 2010b). Lsa44 and Lsa45 proteins are also able to bind PLG that is converted to functional PLA in the presence of uPA. The generation of enzymically active PLA on the leptospiral surface leads to a decrease of C3b and IgG deposition, and consequently opsonization and phagocytosis (Vieira et al., 2011). PLA generation on the surface of the zoonotic pathogen *Streptococcus canis* has been shown to enhance bacterial survival by decreasing phagocytic killing (Fulde et al., 2013). Moreover, PLA generation in *Leptospira* was shown to enhance the migration activity of bacteria through human umbilical vein endothelial cell monolayers and to promote transcriptional upregulation of matrix metalloprotease-9, thus increasing the protease power of leptospires (Vieira et al., 2013). The fact that only a partial reduction was achieved when leptospires were pretreated with antibodies against each protein can be explained by the presence of several laminin-binding proteins in *Leptospira* (Vieira et al., 2013).

Lsa44 and Lsa45 share serum recognition properties with the adhesins described elsewhere, LipL32 (Flannery et al., 2001; Xu et al., 2008), LigA and LigB proteins (Palaniappan et al., 2004; Srimanote et al., 2008), Lsa27 (Longhi et al., 2009), Lsa63 (Vieira et al., 2010a), Lsa66 (Oliveira et al., 2011), and OmpL37 (Pinne et al., 2010), which have shown positive reactivity with serum samples from patients diagnosed with leptospirosis. Protein recognition by serum...
of confirmed leptospirosis samples together with the immunofluorescence and proteinase K accessibility data suggest that these proteins are probably surface exposed.

In conclusion, we report the characterization of two proteins of *Leptospira* annotated in the genome as possible lipoproteins of unknown function. The proteins are conserved among different pathogenic leptosporal serovars, and Lsa45 was able to elicit T α1 and T α2 immune response in mice. However, the humoral immune response induced by this protein in humans is low, with 36 and 10 % of responders in the convalescent and early phase, respectively. An opposite behaviour was observed with Lsa44 that produced a lower immune response in mice, but was recognized by 87 and 67 % of the serum samples in the convalescent and early phase, respectively. In any event, these two adhesins are probably expressed during infection, and could participate in adhesion by interacting with laminin, and in tissue invasion and dissemination by generating proteolytic activity via bound PLA on the bacterial surface.

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