Intermediate and C-terminal regions of leptospiral adhesin Lsa66 are responsible for binding with plasminogen and extracellular matrix components

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Leptospirosis, a worldwide zoonotic infection, is an important human and veterinary health problem. We have previously identified a leptospiral multipurpose adhesin, Lsa66, capable of binding extracellular matrix (ECM) components and plasminogen (PLG). In this work, we report the cloning, expression, purification and characterization of three fragments derived from the full-length Lsa66: N-terminal, intermediate and C-terminal regions. We employed Escherichia coli BL21-SI as expression cells. The recombinant fragments tagged with N-terminal His6 were purified by metal-charged chromatography to major protein bands that were recognized by anti-His-tag mAbs. The recombinant fragments were evaluated for their capacity to attach to ECM components and to PLG. The intermediate region bound to laminin, plasma fibronectin and PLG. Laminin also bound to the C-terminal region. Antibodies in leptospirosis-positive serum samples recognized Lsa66, being the immune epitopes located at the N-terminal and intermediate fragments. The data confirm that Lsa66 is expressed during infection and that this protein might have a role in bacterial infection.

INTRODUCTION

Leptospirosis is a widespread infectious disease caused by pathogenic species of Leptospira. The transmission of leptospirosis is associated with exposure of individuals in contact with wild or farm animals. The disease has been prevalent in cities with sanitation problems and a large population of rodent reservoirs that contaminate the environment through their urine (Faine et al., 1999). Symptoms vary from general flu-like to the most severe form of leptospirosis, known as Weil’s syndrome, with hepatic, renal and pulmonary involvement, and a mortality rate of 5–40% (Faine et al., 1999). Leptospirosis also has a great economic impact in the agricultural industry because the disease affects livestock, inducing abortions, stillbirths, infertility, reduced milk production and death (Faine et al., 1999).

The genome sequences of different leptospiral species and serovars are now available (Bulach et al., 2006; Chou et al., 2012; Nascimento et al., 2004a, b; Picardeau et al., 2008; Ren et al., 2003; Ricaldi et al., 2012). Genome annotation, together with bioinformatics tools, has facilitated the identification of many unknown, putative surface-exposed proteins, opening possibilities for studies on protein function. It is possible that a number of these membrane proteins also mediate the initial adhesion to host cells (Barbosa et al., 2006; Choy et al., 2007; Merien et al., 2000). Indeed, several adhesins of Leptospira have been identified (Atzingen et al., 2008, 2009; Carvalho et al., 2009; Domingos et al., 2012; Fernandes et al., 2012; Hauk et al., 2008; Hoke
et al., 2008; Lima et al., 2013; Lin et al., 2009a, b; Longhi et al., 2009; Mendes et al., 2011; Oliveira et al., 2010, 2011; Pinne et al., 2010, 2012; Souza et al., 2012; Stevenson et al., 2007; Vieira et al., 2010a). In addition, plasmogen (PLG) bound to some adhesins can be converted to plasmin, which could help the bacteria to overcome tissue barriers and dissemination (Domingos et al., 2012; Fernandes et al., 2012; Mendes et al., 2011; Oliveira et al., 2011; Souza et al., 2012; Verma et al., 2010; Vieira et al., 2010b). Lsa66 is one of those multipurpose adhesins that bind laminin, plasma fibronectin and PLG, and is recognized by antibodies in leptospirosis-positive serum samples (Oliveira et al., 2011). This protein was also identified in leptospiral outer-membrane microarrays as fibronectin-binding protein (Pinne et al., 2012).

In this work, we report the cloning, protein expression and purification of three fragments generated from the full-length Lsa66 in an attempt to identify which region of the protein was responsible for the reactivity observed with the full-length construct. We decided to produce three constructs, the N-terminal, C-terminal and intermediate region, aiming to map the entire protein. The purified recombinant fragments were evaluated for their ability to bind extracellular matrix (ECM) components, PLG and leptospirosis-positive serum samples. Our data showed that antibodies present in leptospirosis sera recognized the N-terminal and intermediate regions, the central fragment also bound plasma fibronectin and laminin, and the C-terminal region was reactive only with laminin. The characterization of these interactions is presented.

METHODS

ECM and biological components. Macromolecules (laminin and plasma fibronectin), including the control protein BSA, were purchased from Sigma-Aldrich. Laminin-1 was derived from the basement membrane of Engelbreth-Holm-Swarm mouse sarcoma; plasma fibronectin was isolated from human plasma and native PLG, which was purified from human plasma, and was purchased from EMD Chemicals.

Leptospira strains and sera. The non-virulent Leptospira interrogans serovar Copenhageni strain M20 was cultured at 28 °C under aerobic conditions in liquid EMJH medium (Difco) with 10% rabbit serum, 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). Leptospira cultures were maintained at the Faculdade de Medicina Veterinária e Zootecnia, USP, São Paulo, Brazil. Leptospirosis-positive serum samples were from the Instituto Adolfo Lutz collection, São Paulo, Brazil.

DNA isolation and PCR analysis. Leptospira cultures were harvested by centrifugation at 15 500 g for 30 min and washed gently twice in sterile PBS. Genomic DNA was isolated from the pellets by the guanidine-detergent lysing method using DNAzol Reagent (Invitrogen), according to the manufacturer’s instructions. Primers were designed according to L. interrogans serovar Copenhageni genome sequences (GenBank accession no. AE016823) and are listed in Table 1. PCR was performed in a reaction volume of 25 µl containing 100 ng genomic DNA, 1 × PCR buffer (20 mM Tris/HCl, pH 8.4, 50 mM KCl), 2 mM MgCl2, 20 pmol of each specific primer, 200 µM of each dNTP and 2.5 U Taq DNA polymerase (Invitrogen). Cycling conditions were: 94 °C for 4 min, followed by 40 cycles at 94 °C for 50 s, 60 °C for 50 s and 72 °C for 90 s, and a final extension cycle of 7 min at 72 °C. The PCR products obtained for each corresponding fragment were cloned into pGEM-T Easy vector (Promega) and subcloned into the pAE expression vector (Ramos et al., 2004) at the restriction sites depicted in Table 1. The pAE vector allows the expression of recombinant proteins with a minimal His6-tag at the N terminus. All cloned sequences were confirmed by DNA sequencing in an ABI 3100 automatic sequencer (PE Applied Biosystems).

Expression and purification of recombinant fragments. Recombinant fragment expression was achieved in E. coli BL21-SI strain by the action of T7 DNA polymerase under the control of the osmotically induced promoter proU. E. coli BL21-SI containing recombinant plasmids was grown at 30 °C in 2YT broth without NaCl and with 100 µg ampicillin ml−1 with continuous shaking until an OD600 of 0.6–0.8 was reached. Recombinant fragment synthesis was induced by the addition of 30 mM NaCl. After 3 h, the cells were harvested by centrifugation and the bacterial pellets suspended in lysis buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 100 µg lysozyme ml−1, 1 % Triton X-100, 2 mM PMSF). The bacterial cell pellets were lysed on ice with the aid of a sonicator (Ultrasonic Processor; GE Healthcare). The insoluble fraction was washed with 20 ml buffer (20 mM Tris/HCl, pH 8.0, 500 mM NaCl, 1 M urea, 0.1 % Triton X-100) and resuspended in a buffer containing 20 mM Tris/HCl, pH 8.0, 500 mM NaCl, 10 % (v/v) glycerol and 8 M urea. The recombinant fragments were then purified through metal-chelating chromatography under denaturing conditions followed by

<table>
<thead>
<tr>
<th>Lsa66 fragment</th>
<th>Sequence of primers for PCR amplification (5’→3’)</th>
<th>Recombinant fragment molecular mass (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-terminal</td>
<td>F: GGATCCGAAGCCTTTTGACCCCAAT (BamHI) R: CCATGTTAATTCTGTCATCCTACAAT (NcoI)</td>
<td>25.5</td>
</tr>
<tr>
<td>Intermediate</td>
<td>F: GGATCCGCTTCCTGTGATCATATTATTTCTCAA (BamHI) R: CCATGTTAATGCATCTTCCTAACCCGC (NcoI)</td>
<td>36.9</td>
</tr>
<tr>
<td>C-terminal</td>
<td>F: GGATCCGCTACCAACTTGAGATGGC (BamHI) R: CCATGTTAAGTGAAAGATATAAAATCGATTC (NcoI)</td>
<td>23.3</td>
</tr>
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refolding by gradual removal of urea. Fractions were analysed in 15% SDS-PAGE. The fragments were dialysed extensively against PBS, pH 7.4, 0.1% (w/v) glycine solution (at 10 ml protein per 1000 ml buffer) containing a decreasing gradient of glycerol (10–0%), pooled and stored at 4 °C.

Antiserum. Five female BALB/c mice (4–6 weeks old) were immunized subcutaneously with 10 μg recombinant fragments. The recombinant fragments were adsorbed in 10% (v/v) Alhydrogel [2% Al(OH)3; Brenntag Biosector] used as adjuvant. Two subsequent booster injections were given at 2-week intervals with the same preparation of 10 μg fragments. Negative-control mice were injected with PBS. Two weeks after each immunization, the mice were bled from the retro-orbital plexus and the pooled sera were analysed by ELISA for determination of antibody titre.

Ethics statement. All animal studies were approved by the Ethical Committee for Animal Research of Instituto Butantan, São Paulo, SP, Brazil, under protocol 798/11. The Committee in Animal Research in Instituto Butantan adopts the guidelines of the Brazilian College of Animal Experimentation.

Western blotting assay. The purified recombinant fragments were loaded into 15% 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 in PBS containing 0.05% Tween 20 (PBS-T), and then incubated with anti-N-terminal, anti-intermediate and anti-C-terminal mouse serum (1:500) or anti-His antibody (1:2000) (Sigma) in PBS-T for 1 h. The reactivity of fragments was revealed by use of the ECL chemiluminescence substrate reagent kit (GE Healthcare). The luminescence generated by the reaction was detected with the aid of a Carestream molecular imaging instrument (Equilab) connected to a Gel Logic 2200PRO imaging system.

Microscopic agglutination test (MAT). The MAT was performed according to Faine et al. (1999) as described previously (Oliveira et al., 2011).

ELISA for detection of human antibodies. Human IgG and IgM antibodies against N-terminal, intermediate and C-terminal fragments were evaluated by ELISA. We employed 18 pairs of serum samples: MAT− and the respective MAT+. As serum conversion normally occurs by day 10 of the infection, MAT− represents the early stage while MAT+ refers to the convalescent phase of the disease. Serum samples were diluted 1:200, and evaluated for total IgG using HRP-conjugated anti-human IgG and IgM antibodies, 1:5000 (Sigma). Cut-off values were set at 3 sd above the mean absorbance at 492 nm (A492) of normal human serum obtained from Sigma-Aldrich.

Binding of recombinant fragments to laminin, plasma fibronectin and PLG. The binding of the recombinant fragments to laminin, plasma fibronectin and PLG was evaluated according to a previously published protocol (Barbosa et al., 2006) with some modifications. ELISA plate wells were coated with 1 μg laminin, plasma fibronectin, PLG and BSA (negative control) in 100 μl PBS for 2 h at 37 °C. The wells were washed three times with PBS-T and then blocked with 200 μl 10% (w/v) non-fat dry milk plus 1% BSA (overnight at 4 °C). Then, 1 μg of each recombinant fragment (N-terminal, intermediate and C-terminal) was added per well in 100 μl PBS and fragments were allowed to attach to the different substrates for 2 h at 37 °C. After washing six times with PBS-T, bound fragments were detected by adding an appropriate dilution of mouse antiserum in 100 μl PBS (1:1000). Dilutions of mouse antiserum against each recombinant protein were equalized to give an A492 value of 1.0. In addition, anti-polyhistidine mAbs were employed as protein-binding probes at 1:2000 dilution. Incubation proceeded for 1 h at 37 °C and, after three washes with PBS-T, 100 μl 1:5000 dilution of HRP-conjugated goat anti-mouse IgG in PBS was added per well for 1 h at 37 °C. The wells were washed three times and o-phenylenediamine (OPD; 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 was allowed to proceed for 10 min and was then interrupted by the addition of 50 μl 8 M H2SO4. A492 was determined in a microplate reader (Multiskan EX; Labsystems Uniscience). For statistical analyses, the binding of recombinant fragments to ECM macromolecules and PLG was compared with binding to BSA by Student’s two-tailed t-test.

Dose–response curves. First, 96-well plates were coated with 1 μg laminin, plasma fibronectin and PLG in 100 μl PBS for 2 h at 37 °C. Plates were then blocked and increasing concentrations of the purified recombinant fragments (0–4000 nM) were added (100 μl per well in PBS). The assessment of bound fragments was performed by incubation for 1 h at 37 °C with the antiserum raised against each fragment at the dilution of 1:1000 followed by HRP-conjugated goat anti-mouse IgG (Sigma) (1:5000 in PBS). The reaction was detected with OPD as described above.

Equilibrium dissociation constant (Kd) for the recombinant fragment binding to ECM and PLG. The data of the dose–response curves were used to calculate the Kd for a given protein concentration (ELISA data point), according to a method described previously (Lin et al., 2009a), following the equation $K_d = \frac{[\text{Protein}]}{[\text{Protein}]} - \frac{[\text{Protein}]}{[\text{Protein}]}$, where A is the absorbance at a given protein concentration, $A_{\text{max}}$ is the maximum absorbance for the ELISA plate reader (equilibrium) and [Protein] is the protein concentration.

Interaction of denatured recombinant fragments with ECM and PLG. First, ELISA plates were coated with 100 μl 10 μg laminin ml−1, plasma fibronectin or PLG, which was allowed to adhere for 2 h at 37 °C. Plates were washed three times with PBS-T and blocked with 200 μl 10% (w/v) non-fat dry milk plus 1% BSA (overnight at 4 °C). Then, 1 μg of each recombinant fragment was denatured by incubation at 96 °C for 10 min and added per well in 100 μl PBS. The recombinant fragments were allowed to attach to ECM and PLG at 37 °C for 90 min. After washing six times with PBS-T, bound recombinant fragments were detected by incubation with mouse serum raised against the respective fragments (dilutions described above) at 37 °C for 1 h. After three washes with PBS-T, 100 μl 1:5000 dilution of HRP-conjugated goat anti-mouse IgG (Sigma) in PBS was added per well for 1 h at 37 °C. The detection was performed with OPD as described above. BSA was used as negative control (data not shown). For statistical analyses, the attachment of denatured recombinant fragments to laminin, plasma fibronectin and PLG was compared to untreated fragment binding by Student’s two-tailed t-test ($^*P<0.005; **P<0.05$).

Antibody inhibition assay. Ninety-six-well plates were coated with 100 μl 10 μg ml−1 laminin, plasma fibronectin and PLG, which was allowed to adhere for 2 h at 37 °C. Plates were then blocked overnight with 200 μl 10% (w/v) non-fat dry milk plus 1% BSA. Prior to the next step, the recombinant fragments were incubated for 1 h at 37 °C with the respective antibodies diluted 1:200 in 100 μl PBS. After incubation, each recombinant protein was added at 1 μg per well in 100 μl PBS, and allowed to attach to laminin, plasma fibronectin and PLG for 90 min at 37 °C. After washing six times with PBS-T, bound recombinant fragments were detected by adding HRP-conjugated mouse anti-polyhistidine mAb (Sigma) diluted 1:2000. Incubation proceeded for 1 h at 37 °C. The detection was performed with OPD as described above. BSA was used as negative control (data not shown).
For statistical analyses, the attachment of blocked recombinant fragments to ECM and PLG was compared to the binding with the untreated proteins by Student’s two-tailed t-test (*$P<0.005$; **$P<0.05$).

**Metaperiodate treatment of laminin and plasma fibronectin.** Microtitre wells were coated with 1 µg laminin or plasma fibronectin in 50 mM sodium acetate buffer, pH 5.0, and incubated for 16 h at 4 °C. Wells were washed three times with the same buffer and ECM components were treated with sodium metaperiodate (0–100 mM) in the same buffer for 15 min at 4 °C in the dark. After three washes with 50 mM sodium acetate buffer, wells were blocked with 200 µl 1% BSA for 1 h at 37 °C. Binding of recombinant fragments (1 µg per 100 µl in PBS per well) to metaperiodate-treated ECM components was evaluated as described before with non-treated ECM.

### RESULTS

**Expression and purification of Lsa66 fragments**

We generated three fragments of the Lsa66 full-length protein, originally cloned from aa 30–590: the N-terminal portion from aa 30 to 219, the intermediate or central region from aa 170 to 483 and the C-terminal fragment from aa 420 to 590, containing the OmpA-like domain from aa 504 to 583 (Fig. 1a). The selected regions were amplified by PCR from genomic *Leptospira* DNA. The amplified fragments were cloned into the pAE vector and proteins were expressed with a His6-tag at the N terminus, using *E. coli* BL21-SI as host. The three fragments were

**Fig. 1.** (a) Schematic representation of Lsa66. The diagram shows the signal peptide (aa 1–29), the OmpA-like domain (aa 504–583), the N-terminal fragment (aa 30–219), the intermediate fragment (aa 170–483) and the C-terminal fragment (aa 420–590). (b) SDS-PAGE. Coomassie blue-stained gel depicting the purified recombinant fragments (2 µg). MM, molecular mass standard proteins. (c, d) Western blotting analysis. The recombinant fragments were membrane blotted and proteins were probed with anti-His-tag mAbs (c) and the corresponding homologue antiserum raised in mice (d).
expressed in their insoluble form, as inclusion bodies, recovered by urea, purified by metal-chelating chromatography under denaturing conditions and refolded by gradual removal of urea. An aliquot of each protein was taken and analysed by SDS-PAGE (Fig. 1b). The stained gel showed protein bands of ~26, 37 and 23 kDa, corresponding to the N-terminal, intermediate and C-terminal regions, respectively (Fig. 1b). The recombinant fragments were confirmed by Western blotting with anti-His-tag mAbs and with polyclonal antibodies raised in mice against each fragment. The data are depicted in Fig. 1(c, d, respectively) and showed that anti-His-tag antibodies recognized all recombinant

**Fig. 2.** Recognition of Lsa66 fragments by IgG antibodies in leptospirosis serum samples. Positive sera responders were detected by ELISA with each recombinant fragment and serum samples from leptospirosis in both the early (MAT–) and convalescent (MAT+) phases of the disease. The reactivity was evaluated as total IgG antibodies. The cut-off values are depicted as horizontal bars and were defined as the mean + 3 SD obtained for commercial sera from healthy individuals. Data for the (a) N-terminal, (b) intermediate and (c) C-terminal fragments, respectively.
fragments (Fig. 1c), while blotted recombinant fragments were recognized by the respective homologue antiserum (Fig. 1d).

**Reactivity of recombinant fragments with sera from confirmed cases of leptospirosis**

As antibodies present in confirmed cases of leptospirosis recognize Lsa66 (Oliveira et al., 2011), we decided to evaluate which portion of the protein was responsible for this reactivity. Thus, N-terminal, intermediate and C-terminal fragments were set to react with antibodies present in serum samples of early (MAT−) and convalescent (MAT+) phases of leptospirosis. We performed ELISA and employed 18 paired samples from both phases of the disease. N-terminal and intermediate fragments exhibited reactivity for MAT− and MAT+ (Fig. 2a, b), while C-terminal fragments showed no reactivity with samples of both phases of the disease (Fig. 2c), suggesting that this region of Lsa66 is not exposed during infection. At the early phase of the disease, 28 % of responders presented IgG antibodies against the N-terminal fragment, whilst the number increased to 39 % at the convalescent phase. However, the intermediate fragment showed 28 and 66.7 % reactivity for MAT− and MAT+, respectively.

**Evaluation of the interaction of Lsa66 recombinant fragments with laminin, plasma fibronectin and PLG**

Lsa66 is an OmpA-like protein with ECM- and PLG-binding properties. Thus, we set out to investigate which portion of Lsa66 was involved in these interactions. Immobilized laminin, plasma fibronectin, PLG and the control protein BSA were incubated with Lsa66 and the recombinant fragments: N-terminal, intermediate and C-terminal fragments. The reaction was probed with polyclonal antibodies against each fragment and showed that the intermediate fragment interacted with laminin, plasma fibronectin and PLG, while the C-terminal fragment only bound to laminin (Fig. 3a). No binding was observed with the N-terminal fragment with any tested component. Similar results were achieved when binding was performed using anti-His-tag mAbs (Fig. 3b). The binding of Lsa66, and the intermediate and C-terminal fragments to laminin, and the interaction of Lsa66 and the intermediate fragment to plasma fibronectin and to PLG, were also assessed after submitting them to denaturing conditions at 96 °C for 10 min and after blocking the proteins with the corresponding antibody. The adhesion of heat-denatured proteins to laminin was almost totally abolished in the case of Lsa66, and the intermediate and C-terminal fragments

![Fig. 3. Binding of recombinant protein Lsa66 and fragments to ECM components and to PLG. Microtitre plates were coated with 1 \( \mu \)g laminin, plasma fibronectin and PLG. BSA was included as control. Lsa66, and the N-terminal, intermediate and C-terminal fragments were added at 1 \( \mu \)g per well and the binding was measured by ELISA. (a) Fragment binding was detected by polyclonal antibodies against each recombinant fragment. (b) Fragment binding was evaluated by anti-polyhistidine mAbs. Data represent the mean ± s.d from three independent experiments. For statistical analyses, the attachment of recombinant fragments to the ECM macromolecules and PLG was compared with binding to BSA by Student’s two-tailed \( t \)-test (*\( P \leq 0.0005 \); **\( P \leq 0.005 \)).](image-url)
Characterization of the interaction of recombinant fragments with laminin, plasma fibronectin and PLG

Binding between the intermediate and C-terminal fragments with laminin was evaluated on a quantitative basis as depicted in Fig. 5(a). A dose-dependent and saturable binding was observed when increasing concentrations of the recombinant fragments (0–2000 nM) were allowed to adhere to a fixed laminin concentration (1 µg). Based on the ELISA data, the calculated $K_D$ values for the recombinant fragments with laminin were $51.10 \pm 5.50$ and $75.32 \pm 14.50$ nM for the intermediate and C-terminal fragments, respectively. Likewise, the interaction of the intermediate fragment with plasma fibronectin and PLG was also assessed by keeping the plasma fibronectin and PLG concentration constant (1 µg) and changing the intermediate fragment concentration (0–4000 nM), as shown in Fig. 5(b, c, respectively). The calculated $K_D$ values for the intermediate fragment with plasma fibronectin and PLG were $166.30 \pm 41.50$ and $744.70 \pm 177.50$ nM, respectively.

Influence of carbohydrate moieties on the binding of Lsa66 and recombinant fragments to laminin and plasma fibronectin

To analyse the involvement of the carbohydrate moieties in the interaction of laminin with Lsa66, and the intermediate and C-terminal fragments, as well as in the interaction of plasma fibronectin with Lsa66 and the intermediate fragment, laminin and plasma fibronectin were oxidized by increasing concentrations of sodium metaperiodate (5–100 mM) for 15 min at 4 °C. The oxidation effect is shown in Fig. 6. No reduction in the interaction of oxidized laminin was observed with Lsa66, and the intermediate and C-terminal fragments (Fig. 6a), whereas the reduction in the interaction of oxidized plasma fibronectin with Lsa66 and the intermediate fragment was dose-dependent and statistically significant when compared with non-treated wells (0 nM) (Fig. 6b) ($*P<0.01; **P<0.001$). These results indicated the importance of sugar residues in the interaction of plasma fibronectin with Lsa66 and the intermediate fragment.

Reactive regions of Lsa66 protein

The intermediate fragment was generated with an overlap region from aa 170 to 219 of the N-terminal region and with an intersection from aa 420 to 483 of the C-terminal region (Fig. 1a). The results with leptospirosis serum samples showed major reactivity with the intermediate region, minor reactivity with the N-terminal region and no reactivity with the C-terminal region. Moreover, our data showed that both the intermediate and C-terminal fragments were capable of binding laminin (Figs 4a and 5a), antibody blocking, respectively, and at $20 \pm 8.25$ and $40 \pm 5.25$ % for heat denaturing, respectively (Fig. 4c).
while only the intermediate fragment had the ability to interact with plasma fibronectin and PLG (Figs 4b, c and 5b, c). Based on these results, we proposed the scheme depicted in Fig. 7.

**DISCUSSION**

We have identified a protein with an OmpA-like domain at the C terminus (OmpA C-like) in *Leptospira* spp., encoded by the LIC10258 gene, that we called Lsa66 (Oliveira et al., 2011). We have reported that this protein is expressed on the surface of bacteria and is capable of interacting with laminin, plasma fibronectin and PLG. Moreover, Lsa66 was reactive with leptospirosis serum samples at the convalescent phase (MAT+). Recently, this protein has been identified as human plasma fibronectin ligand in leptospiral outer-membrane protein microarray studies (Pinne et al., 2012). OmpA outer-membrane proteins of *E. coli* and other enterobacteria have been shown to have adhesin/invasin activities (Smith et al., 2007).

Loa22 was the first protein identified in pathogenic *Leptospira* having a C-terminal OmpA consensus (Koizumi & Watanabe, 2003). This protein was further reported to be essential for leptospiral virulence (Ristow et al., 2007) and to promote inflammatory responses in cultured rat renal cells (Zhang et al., 2010). OmpA-like proteins of leptospires have been described as vaccine candidates for leptospirosis (Yan et al., 2010). Thus, it is possible that OmpA-like proteins have a function in leptospiral pathogenesis. In this work, we report the cloning, expression and purification of the Lsa66

![Fig. 5. Characterization of recombinant fragment binding to ECM components and to PLG. To evaluate the specificity of the interaction of the N-terminal, intermediate and C-terminal fragments to ECM and PLG, plates were coated with 1 μg laminin (a), plasma fibronectin (b) or PLG (c) and each recombinant fragment was added at 0–4000 nM for interaction. The binding was detected using antiserum raised in mice against each fragment (1:1000) followed by HRP-conjugated anti-mouse IgG (1:5000). Data represent the mean ± SD absorbance values of three replicates for each experimental group. The results are representative of two independent experiments. K_D was calculated based on ELISA data for the recombinant fragments that had reached the equilibrium concentration.](image-url)
fragments in order to evaluate which portion of the protein was reactive with positive serum samples of leptospirosis and involved in the binding with the ECM and PLG.

Leptospires present a vast repertoire of ECM-binding proteins, which most probably mediate the adhesion of the bacteria to host (Vieira et al., 2014). It has been reported that the interaction of Leptospira spp. and PLG with the generation of plasmin on the bacterial surface leads to laminin and fibronectin degradation – a mechanism that could aid bacterial penetration (Vieira et al., 2009, 2012).

Indeed, this proteolytic system has been described for several invasive pathogens (Siemens et al., 2011; Stie et al., 2009), including the spirochaetes Borrelia and Treponema (Coleman et al., 1995; Fenno et al., 2000). Several leptospiral proteins have been described with ECM- and PLG-binding capacities, including Lsa66 (Domingos et al., 2012; Fernandes et al., 2012; Mendes et al., 2011; Oliveira et al., 2011; Souza et al., 2012; Verma et al., 2010b).

The intermediate and C-terminal fragments of Lsa66 are involved in the binding with laminin, whereas plasma
fibronectin and PLG interact only with the intermediate region. The reason why Lsa66 seems to be less efficient in binding the ECM might be due to the N-terminal fragment that is not reactive with the ECM. The $K_D$ values for the binding of laminin with the intermediate and C-terminal regions are of the same order of magnitude as that obtained with Lsa66, 55.4 ± 15.9 nM, and the same ligand (Oliveira et al., 2011). The C-terminal reactive site most probably does not encompass the OmpA-like domain because Loa22 protein does not bind laminin (Barbosa et al., 2006). Moreover, it is possible that the binding of laminin with the C terminus occurs at the region where there is an overlap between the intermediate and C-terminal regions (aa 420–503) (Fig. 7). The calculated $K_D$ for the binding of plasma fibronectin and the intermediate fragment is also similar to that obtained with the full-length protein (290.8 ± 11.8 nM) (Oliveira et al., 2011). Indeed, fibronectin binding seems to be fully contained within the intermediate region of Lsa66. However, the $K_D$ for the binding of PLG and the central region is one order of magnitude higher than that calculated with Lsa66 (744.7 ± 177.5 vs 68.8 ± 25.2 nM) (Oliveira et al., 2011). These results suggest that in the case of reaction with PLG, the protein conformation is important while with laminin and plasma fibronectin, only linear epitopes are crucial for the binding. We have shown that Lsa66 was able to inhibit L. interrogans attachment to the ECM and PLG, but the inhibition was moderate, particularly with the ECM (Oliveira et al., 2011). For this reason, we may anticipate that Lsa66 fragments will promote inhibition of leptospirosis interactions with these components.

Lsa66 has shown positive reactivity with serum samples from patients diagnosed with leptospirosis. In the present study, we show that the N-terminal and intermediate fragments are involved in antibody recognition, with 39 and 67% of responders being MAT+, respectively, and 28% of responders being MAT− for both fragments. Our data suggest that the regions encompassing the N-terminal and intermediate of Lsa66 are more sensitive than MAT, and could be further explored as novel antigens to diagnose leptospirosis. In addition, the N-terminal region that interacts with antibodies might belong to the region where there is an intersection with the intermediate segment. The fact that there is no contribution of the C-terminal region to antibody recognition suggests that this region, which comprises the OmpA-like domain, has no immune epitopes. This may explain why 60% of the binding remained when laminin was set to react with the C-terminal fragment previously blocked with antiserum (Fig. 4a).

CONCLUSIONS

We have identified ECM-, PLG- and antibody-reactive regions of the adhesin Lsa66. The entire protein reacted with leptospirosis-positive serum samples, but showed only poor protection in an animal model (Atzingen et al., 2012). It is possible that, similar to what happened with patient serum samples where two fragments presented a higher percentage of reactivity than the entire Lsa66, these fragments could have a better performance in animal protection assays. Mapping these epitopes should expand our understanding of the Leptospira–host interaction at the molecular level and help identify immune targets needed to fight leptospirosis.

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