

The Novel Leptospiral Surface Adhesin Lsa20 Binds Laminin and Human Plasminogen and Is Probably Expressed during Infection[∇]

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Received 28 June 2011/Accepted 6 August 2011

Leptospirosis is an emerging infectious disease caused by pathogenic species of *Leptospira*. In this work, we report the cloning, expression, purification, and characterization of two predicted leptospiral outer membrane proteins, LIC11469 and LIC11030. The LIC11469 protein is well conserved among leptospiral strains, while LIC11030 was identified only in *Leptospira interrogans*. We confirmed by surface proteolysis of intact leptospires with proteinase K that these proteins are most likely new surface leptospiral proteins. The recombinant proteins were evaluated for their capacity to attach to extracellular matrix (ECM) components and to plasminogen. The leptospiral protein encoded by LIC11469, named Lsa20 (leptospiral surface adhesin of 20 kDa), binds to laminin and to plasminogen. The binding with both components was not detected when Lsa20 was previously denatured or blocked with anti-Lsa20 antibodies. Moreover, Lsa20 binding to laminin was also confirmed by surface plasmon resonance (SPR). Laminin competes with plasminogen for binding to Lsa20, suggesting the same ligand-binding site. Lsa20-bound plasminogen could be converted to enzymatically active plasmin, capable of cleaving plasmin substrate D-valyl-leucyl-lysine-p-nitroanilide dihydrochloride. Lsa20 was recognized by antibodies in confirmed-leptospirosis serum samples, suggesting that this protein is expressed during infection. Taken together, our results indicate that Lsa20 is a novel leptospiral adhesin that in concert with the host-derived plasmin may help the bacteria to adhere and to spread through the hosts.

Leptospirosis is a worldwide zoonotic infection of human and veterinary concern. The disease is caused by spirochetes of the genus *Leptospira*, with greater incidence in tropical and subtropical regions (16). The transmission of leptospirosis is associated with exposure of individuals near to wild or farm animals. Recently, the disease has been prevalent in cities with sanitation problems and large populations of rodent reservoirs that contaminate the environment through their urine (10). Symptoms of the disease include fever, vomiting, headache, diarrhea, and abdominal and generalized muscle pain. The severe form of leptospirosis, known as Weil's syndrome, seen in 5 to 15% of patients, is a multisystemic febrile illness, with hepatic, renal, and pulmonary involvement and a mortality rate of 5 to 40% (16). Leptospirosis represents a great economic impact in the agricultural industry because the disease affects livestock, inducing abortions, stillbirths, infertility, reduced milk production, and death (10).

Whole-genome sequencing has greatly impacted the microbial topic with the development of new large-scale technologies, such as bioinformatics. The identification of many un-

known proteins, predicted to be surface-exposed proteins, has opened possibilities for studies on protein function, leading to an understanding of the biology of the pathogen (1, 48). Genome annotation of *Leptospira interrogans* serovar Copenhageni identified more than 200 predicted outer membrane proteins (34, 35). These proteins are exposed to hosts and then are potential targets for inducing immune response during infection. In addition, it is possible that some of these membrane proteins mediate the initial adhesion to host cells, as has been reported by several researchers (5, 7, 13, 14, 22, 23, 26, 29, 37, 41, 50, 55). After adherence, pathogens have to surmount host tissue barriers in order to reach blood circulation and organs. Proteolytic activity as exerted by plasmin, a serine protease of broad substrate spectrum, may help bacteria to disseminate through the host. We have reported that leptospires bind plasminogen (PLG) at their surface and that plasmin can be generated in the presence of activator (56). More recently, we have identified eight leptospiral proteins as plasminogen receptors (54).

In the present study, we focused on two hypothetical proteins of unknown function, encoded by the genes LIC11469 and LIC11030 and predicted to be outer membrane proteins. The genes were cloned and the proteins expressed using *Escherichia coli* as a host system. The recombinant (r) proteins were purified and their ability to mediate attachment to various extracellular matrix (ECM) and biological components was

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[∇] Published ahead of print on 15 August 2011.

evaluated. We report that one of them, Lsa20, is a novel surface-exposed adhesin that binds to laminin and to plasminogen and is probably expressed during infection and may thus participate in the pathogenesis of *Leptospira*.

MATERIALS AND METHODS

ECM and biological components. Macromolecules, including the control protein fetuin, were purchased from Sigma Chemical Co. (St. Louis, MO). Laminin-1 and collagen type IV were derived from the basement membrane of Engelbreth-Holm-Swarm mouse sarcoma; cellular fibronectin was derived from human foreskin fibroblasts; plasma fibronectin, human complement serum, and fibrinogen were isolated from human plasma; and collagen type I was isolated from rat tail. ECM gel is composed primarily of laminin, collagen type IV, heparan sulfate proteoglycan, and entactin. Native plasminogen, purified from human plasma, was purchased from Merck. Human factor H was from Calbiochem.

***Leptospira* strains and serum samples.** The strains employed are pathogenic, high-passage-number cultures: *L. interrogans* serovar Canicola strain Hound Utrecht IV, *L. interrogans* serovar Pomona strain Pomona, *L. interrogans* serovar Copenhageni strain M 20, *L. interrogans* serovar Icterohaemorrhagiae strain RGA, *L. interrogans* serovar Hardjo strain Hardjoprajitno, *L. borgpetersenii* serovar Castellonis strain Castellon 3, *L. borgpetersenii* serovar Whitcomb strain Whitcomb, *L. borgpetersenii* serovar Cynoptery strain 3522C, *L. borgpetersenii* serovar Grippotyphosa strain Moskva V, *L. noguchii* serovar Panama strain CZ 214, *L. santarosai* serovar Shermani strain 1342 K, and the nonpathogenic *L. biflexa* serovar Patoc strain Patoc. Strains were cultured at 28°C under aerobic conditions in liquid EMJH medium (Difco) with 10% rabbit serum, enriched with L-asparagine (0.015% [wt/vol]), sodium pyruvate (0.001% [wt/vol]), calcium chloride (0.001% [wt/vol]), magnesium chloride (0.001% [wt/vol]), peptone (0.03% [wt/vol]), and meat extract (0.02% [wt/vol]) (51). *Leptospira* cultures are maintained in Faculdade de Medicina Veterinária e Zootecnia, USP, São Paulo, SP, Brazil. Confirmed-leptospirosis serum samples were from the Instituto Adolfo Lutz collection, São Paulo, Brazil.

MAT. The microscopic agglutination test (MAT) was performed according to the procedure described in reference 16. In brief, an array of 22 serovars of *Leptospira* spp. as antigens were employed: Australis, Autumnalis, Bataviae, Canicola, Castellonis, Celledoni, Copenhageni, Cynoptery, Djasiman, Grippotyphosa, Hardjo, Hebdomadis, Icterohaemorrhagiae, Javanica, Panama, Patoc, Pomona, Pyrogenes, Sejroe, Shermani, Tarassovi, and Wolffi. All the strains were maintained in EMJH liquid medium (Difco) at 29°C. A laboratory-confirmed case of leptospirosis was defined by the demonstration of a 4-fold microagglutination titer rise between paired serum samples. The probable predominant serovar was considered to be the one with the highest dilution that could cause 50% of agglutination. MAT was considered negative when the titer was below 100.

Characterization of CDSs *in silico*. The rationale for predicted coding sequence (CDS) LIC11469 and LIC11030 selection was mostly based on cellular localization; the prediction was performed by use of the PSORT program (33) (<http://psort.nibb.ac.jp>). The SMART (47) (<http://smart.embl-heidelberg.de/>) and PfAM (17) (<http://www.sanger.ac.uk/Software/Pfam/>) web servers were used to search for predicted functional and structural domains. The presence of lipobox putative sequence was evaluated by use of the LipoP program (24) (<http://www.cbs.dtu.dk/services/LipoP/>).

Cloning, expression, and purification of recombinant proteins. Amplification of the LIC11469 and LIC11030 CDSs was performed by the PCR from *L. interrogans* serovar Copenhageni strain M20 genomic DNA using the following primer pairs: (forward [F]) 5'-CTCGACCAATTTCTTCGATCCAAATC and (reverse [R]) 5'-AAGCTTTCAATCTCTACTGCGACCC for LIC11469, and (F) 5'-CTCGAGTGTACAACGAAAAAGAGGT and (R) 5'-AAGCTTTTGA GTTGAAGGATTTGGA for LIC11030. Both gene sequences were amplified without the signal peptide tag. Gel-purified PCR fragments (Illustra GFX PCR DNA and Gel band purification kit; GE Healthcare) were cloned into the *E. coli* expression vector pAE (44) at XhoI and HindIII restriction sites. The construct was verified by DNA sequencing on an ABI Prism 3730_L sequencer (Seq-Wright, Houston, TX) with appropriate T7 promoter-specific primers (5'-TAAT ACGACTACTATAGGG and 5'-CAGCAGCCAACACTCAGTTCCT). *E. coli* BL21-SI (9) and *E. coli* BL21(DE3) Star pLysS host cells were transformed with the plasmids pAE-LIC11469 and pAE-LIC11030, respectively. Protein expression was achieved by inoculating 8 ml of a culture grown overnight in 200 ml of Luria-Bertani (LB) medium without NaCl containing 100 µg/ml ampicillin for *E. coli* BL21-SI cells, or LB medium containing 100 µg/ml ampicillin and 34 µg/ml

chloramphenicol for *E. coli* BL21(DE3) Star pLysS cells. The cultures were grown with continuous shaking at 30°C until an optical density at 600 nm (OD₆₀₀) of 0.6 and then induced for 3 h under constant agitation at 30°C in the presence of 300 mM NaCl or 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Both proteins were expressed in insoluble form, as inclusion bodies. The cells were harvested by centrifugation, and the bacterial pellet was resuspended in sonication buffer (20 mM Tris-HCl [pH 8.0], 200 mM NaCl, 200 mg/ml lysozyme, 2 mM phenylmethylsulfonyl fluoride [PMSF], and 1% Triton X-100) and lysed on ice with the aid of an ultrasonic cell disruptor (Sonifier 450; Branson). The bacterial lysate was centrifuged at 3,000 × g for 15 min at 4°C. The pellets were resuspended in buffer containing 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 8 M urea, and 1 mM β-mercaptoethanol in the case of rLIC11030 or 6 M guanidine, 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 5 mM imidazol in the case of Lsa20. The proteins were purified through Ni²⁺-charged beads in a chelating fast-flow chromatographic column (GE Healthcare). The rLIC11030 was refolded by 500× dilution with 50 mM Tris-HCl, pH 8.0, and 500 mM NaCl before chromatographic purification. The Lsa20 was refolded on-column by gradually removing urea (6 to 0 M). The efficiency of the purification was evaluated by 15% SDS-PAGE. Fractions containing the recombinant proteins were extensively dialyzed against phosphate-buffered saline (PBS) containing 0.1% glycine.

Antiserum production against recombinant proteins. The recombinant proteins were adsorbed in 10% (vol/vol) of Alhydrogel [2% Al(OH)₃, Brenntag Biosector, Denmark], used as the adjuvant. Ten micrograms of Lsa20 or rLIC11030 was subcutaneously administered in female BALB/c mice (4 to 6 weeks old). Three subsequent booster injections were given at 2-week intervals with the same protein preparation. Negative control mice were injected with PBS. Two weeks after each immunization, the mice were bled from the retro-orbital plexus, and the sera were analyzed by enzyme-linked immunosorbent assay (ELISA) for determination of antibody titers. Prior to experiments, anti-recombinant protein sera were adsorbed to a suspension of *E. coli* to suppress the reactivity of anti-*E. coli* antibodies (18). Male Golden Syrian hamsters (6 to 8 weeks old) were immunized subcutaneously with 50 µg of recombinant protein adsorbed in 10% Alhydrogel, which was used as the adjuvant. Two subsequent booster injections were given at 2-week intervals with the same preparation of recombinant protein (50 µg). Two weeks after each immunization, hamsters were bled by cardiac puncture and the sera were analyzed by ELISA for the determination of antibody titers. All animal studies were approved by the Ethics Committee of the Instituto Butantan, São Paulo, SP, Brazil.

ELISA for detection of human antibodies. Human IgG antibodies against Lsa20 or rLIC11030 were detected by ELISA as previously described (36). In brief, samples (negative and positive MAT sera from 40 confirmed-leptospirosis patients) were diluted 1:200 and evaluated for total IgG by using goat horseradish peroxidase (HRP)-conjugated anti-human IgG antibodies (1:5,000; Sigma). Cutoff values were set at 3 standard deviations above the mean OD₄₉₂ of sera from 10 healthy individuals, unexposed to leptospirosis, from the city of São Paulo, Brazil.

Protein conservation among leptospiral strains. Bacterial cultures of *Leptospira* spp. were harvested by centrifugation, washed three times with PBS containing 5 mM MgCl₂, and resuspended in PBS. The purified recombinant proteins, or the protein extracts from leptospire, were loaded into 15% SDS-PAGE gels, and the proteins transferred to nitrocellulose membrane (Hybond ECL; GE Healthcare) in semidry equipment (GE Healthcare) and then analyzed by immunoblotting (see below).

Immunoblotting analysis. The membranes were blocked overnight at 4°C with 10% nonfat dry in PBS containing 0.05% Tween 20 (PBS-T). After three washings with PBS-T, the membranes were incubated with mouse anti-Lsa20 (1:100), mouse anti-rLIC11030 (1:100), or mouse monoclonal anti-polyhistidine (1:3,000; Sigma) for 2 h at room temperature. After washing, the membranes were incubated with goat HRP-conjugated anti-mouse IgG (1:5,000; Sigma) for 1 h at room temperature. The membrane was then washed and the reactivity was revealed with ECL reagent kit chemiluminescence substrate (GE Healthcare) and subsequent exposure to X-ray film (T Mat G/RA film; Kodak, Rochester, NY).

PK accessibility assay. The enzymatic digestion was performed as described by Pinne and Haake (41) with some modification. In brief, 5 ml of *L. interrogans* serovar Copenhageni strain M20 culture grown at 28°C was harvested by centrifugation at 8,000 × g for 10 min at room temperature and gently washed with PBS containing 5 mM MgCl₂. Leptospire were washed and resuspended in proteolysis buffer (10 mM Tris-HCl, pH 8.0, 5 mM CaCl₂) with 25 µg/ml of proteinase K (PK; Sigma). As a negative control, leptospire were incubated with proteolysis buffer without PK. Both aliquots were incubated for 1 to 6 h at 37°C before the addition of 2 mM PMSF to stop PK activity. The suspensions were

subsequently pelleted by centrifugation at $11,500 \times g$ for 5 min, washed twice with PBS-5 mM $MgCl_2$, and resuspended in PBS for immunoblotting analysis using antibodies against Lsa20 and rLIC11030, as described above. Band intensities were evaluated using gel quantifier image analysis (GelQuant), version 2.7.0, in a MiniBIS Pro gel documentation system (DNR Imaging Systems, Ltd.).

Binding of recombinant proteins to ECM, plasminogen, complement, and factor H components. Protein attachment to individual macromolecules of the extracellular matrix was analyzed according to a previously published protocol (7) with some modifications. Briefly, 96-well plates (Costar High Binding; Corning) were coated with 1 μg of laminin, collagen type I, collagen type IV, cellular fibronectin, plasma fibronectin, fibrinogen, ECM mix gel, human plasminogen, factor H, complement mix, or bovine serum albumin (BSA; negative control) and fetuin (highly glycosylated attachment-negative control protein) in 100 μl of PBS for 3 h at 37°C. The wells were washed three times with PBS-T and then blocked with 200 μl of 10% (wt/vol) nonfat dry milk (overnight at 4°C). One microgram of each recombinant protein was added per well in 100 μl of PBS, and protein was allowed to attach to the different substrates for 2 h at 37°C. After washing six times with PBS-T, bound Lsa20 or rLIC11030 was detected by adding mouse antirecombinant proteins (1:1,000) in 100 μl of PBS. Incubation proceeded for 1 h at 37°C. After three washings with PBS-T, 100 μl of a 1:5,000 dilution of HRP-conjugated goat anti-mouse IgG (Sigma) 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) in citrate phosphate buffer (pH 5.0) plus 1 μl /ml H_2O_2 was added (100 μl per well). The reaction proceeded for 10 min and was interrupted by the addition of 50 μl of 4N H_2SO_4 . The absorbance at 492 nm was determined in a microplate reader (TP-reader; Thermo). For statistical analyses, the binding of recombinant proteins to ECM macromolecules was compared to its binding to fetuin by Student's two-tailed *t* test.

Dose-response curves. First, 96-well plates were coated overnight in PBS at 4°C with 100 μl of 10 μg /ml plasminogen or laminin. Plates were then blocked, and increasing concentrations of the purified recombinant proteins (0 to 10 μM) were added (100 μl /well in PBS). The assessment of bound proteins was performed by incubation for 1 h at 37°C with the antiserum raised against each protein at the dilution of 1:1,000 followed by HRP-conjugated goat anti-mouse IgG (Sigma) (1:5,000 in PBS). For the reverse assay, 100 μl of 10 μg /ml solution of each purified recombinant protein was coated in the ELISA plate, and after the block, laminin or plasminogen concentrations varying from 0 to 1.5 μg in PBS were used. The assessment of bound proteins was performed by incubation with the antiserum raised against laminin (1:1,000; Sigma) or plasminogen (1:4,000; Calbiochem), respectively. The reaction was detected with OPD as described above. The ELISA data were used to calculate the dissociation constant (K_d) according to the method described by Pathirana et al. (38) and Lin et al. (27), based on the following equation: $A = A_{max} [\text{protein}] / (K_d + [\text{protein}])$, where *A* is the absorbance at a given protein concentration, A_{max} is the maximum absorbance for the ELISA plate reader (equilibrium), $[\text{protein}]$ is the protein concentration, and K_d is the dissociation equilibrium constant for a given absorbance at a given protein concentration (ELISA data point).

Plasmin enzymatic activity assay. Ninety-six-well plates were coated overnight with 10 μg /ml recombinant proteins or BSA in PBS at 4°C. The plates were then washed once with PBS-T and blocked with PBS with 10% (wt/vol) nonfat dry milk for 2 h at 37°C. The blocking solution was discarded and 100 μl /well of 10 μg /ml human plasminogen was added followed by incubation for 2 h at 37°C. Wells were washed three times with PBS-T, and then 4 ng/well of human uPA (Sigma) was added. Subsequently, 100 μl /well of the plasmin-specific substrate *D*-valyl-leucyl-lysine-*p*-nitroanilide dihydrochloride (Sigma) were added at a final concentration of 0.4 mM in PBS. Plates were incubated overnight at 37°C, and substrate was measured by taking readings at 405 nm.

Antibody inhibition assay. Ninety-six-well plates were coated overnight at 4°C with 1 μg of Lsa20, rLIC11030, BSA, laminin, or plasminogen in 100 μl of PBS for 3 h at 37°C. The wells were washed three times with PBS-T and then blocked with 200 μl of 10% (wt/vol) nonfat dry milk (overnight at 4°C). One microgram of laminin, PLG, or recombinant protein was added per well in 100 μl of PBS, and protein was allowed to attach to the different substrates for 2 h at 37°C. In one condition, the recombinant proteins were previously denatured by incubation for 10 min at 96°C; in the other, the proteins were incubated for 1 h at 37°C with antirecombinant protein antibodies produced in hamster (1:500) prior to the next step. After washing six times with PBS-T, bound Lsa20, rLIC11030, laminin, or PLG was detected by adding mouse antirecombinant proteins (1:1,000), mouse monoclonal anti-polyhistidine (1:3,000; Sigma), or antibodies against laminin (1:1,000; Sigma) or plasminogen (1:4,000; Calbiochem) in 100 μl of PBS. Incubation proceeded for 1 h at 37°C. After three washings with PBS-T, 100 μl of a 1:5,000 dilution of HRP-conjugated goat anti-mouse (or anti-rabbit)

IgG (Sigma) in PBS was added per well for 1 h at 37°C. The detection was performed with OPD as previously described.

Surface plasmon resonance (SPR). Protein-protein interactions were assessed by surface plasmon resonance with a BIAcoreT100 system (GE). Laminin was covalently immobilized on the BIAcore CM-5 sensorchip (GE) (carboxymethylated dextran matrix) according to the manufacturer's instructions. Briefly, the CM-5 chip was activated with a 1:1 mixture of 0.4 M 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide and 0.1 M *N*-hydroxysuccinimide for 7 min. Laminin (1 mg/ml) in 10 mM sodium citrate, pH 4.5, was injected over the activated CM-5 chip at 25°C. Remaining active groups on the matrix were blocked with 1 M ethanolamine-HCl, pH 8.5. Immobilization on the CM-5 sensorchip resulted in average surface concentrations of 4.9 ng/mm². Protein solutions of Lsa20 (0, 0.312, 0.625, 1.25, 2.5, 5.0, 10.0 μM) in 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20 (pH 7.4) were injected over immobilized laminin at a flow rate of 30 μl /min for 1 min at 25°C. Individual experiments were performed a minimum of three times.

Interference of laminin (or plasminogen) with plasminogen (or laminin) binding by Lsa20. Ninety-six-well plates were coated overnight at 4°C with 100 μl of 10 μg /ml Lsa20 in PBS, washed once, and blocked with 10% (wt/vol) nonfat dry milk for 2 h at 37°C. The plates were then incubated for 2 h at 37°C with 1 μg plasminogen together with increasing concentrations of laminin (0 to 1.0 μg). After four washings, plasminogen binding was quantified by specific antibodies as described above. Similar experiments were performed to evaluate the interference of plasminogen with laminin binding to Lsa20. Ninety-six-well plates were coated overnight at 4°C with 100 μl of 10 μg /ml Lsa20 in PBS, washed once, and blocked with 10% (wt/vol) nonfat dry milk for 2 h at 37°C. The plates were then incubated for 2 h at 37°C with 1 μg laminin together with increasing concentrations of plasminogen (0 to 1.0 μg). After four washings, laminin binding was quantified by specific antibodies as described above.

Inhibition of live leptospire to bind laminin or plasminogen by Lsa20. ELISA plates were coated with laminin or plasminogen (1 μg /well). The plates were washed and blocked with 10% nonfat dry milk in PBS-T for 2 h at 37°C. The blocking solution was discarded, and the wells were incubated for 90 min at 37°C with increasing concentrations of Lsa20 (0 to 7.5 μg). After three washings, 50 μl /well of 4×10^7 live low-passage-number *L. interrogans* serovar Copenhageni strain M20 leptospire were added for 90 min at 37°C. The unbound leptospire were washed, and the quantification of bound leptospire was performed indirectly by anti-LipL32 antibodies produced in mice (1:4,000), given the fact that LipL32 is a major outer membrane leptospiral protein (20); the procedure was followed by horseradish peroxidase-conjugated anti-mouse IgG antibodies, essentially as described in the work of Barbosa et al. (7). The detection was performed with OPD as previously described.

Nucleotide sequence accession numbers. GenBank accession numbers for protein sequences LIC11469 and LIC11030 are AAS70067.1 and AAS69637.1, respectively. The protein can also be accessed by the genome nomenclature for the gene locus: LIC number (*Leptospira interrogans* serovar Copenhageni).

RESULTS

Bioinformatic analysis. We have selected predicted coding sequences based on cellular localization, since surface proteins are potential targets for mediating interactions with the host. Thus, both LIC11469 and LIC11030 CDSs were predicted to be outer membrane proteins (>79%) according to the PSORT program (33). The LipoP server predicted both CDSs to be lipoproteins with a cleavage site for signal peptidase II at amino acids 19 and 20 (LIC11469) and at 17 and 18 (LIC11030) (24). The gene LIC11469 encodes a hypothetical protein with no recognized domain by BLAST (2) or PFAM (49) analysis, while LIC11030 contains three PbH1 (parallel beta-helix repeats) sequence tags, according to the SMART program (47). BLAST analysis showed that similar sequences of both genes were identified in *L. interrogans* serovar Lai (45) but only LIC11469 sequence was found in *L. borgpetersenii* (12) with 82% identity. In the case of both sequenced strains of *L. biflexa* serovar Patoc (39), a sequence having 29% identity with the gene LIC11469 was identified.

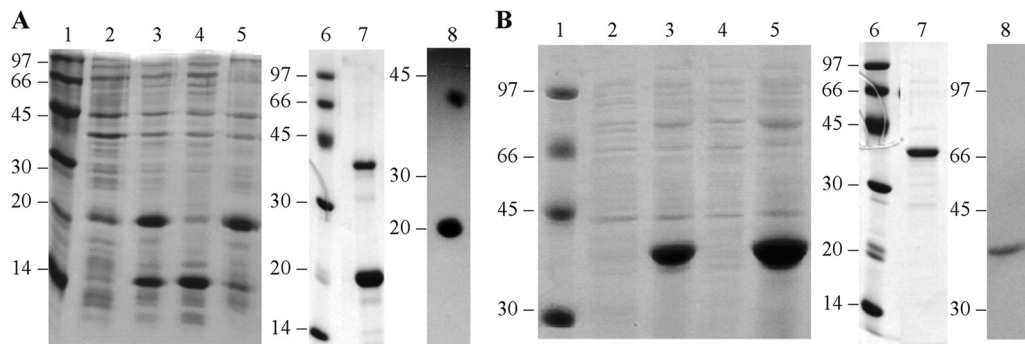


FIG. 1. Recombinant protein analysis by SDS-PAGE and Western blotting. Lsa20 (A) and rLIC11030 (B) protein expression from NaCl-induced *E. coli* BL21-SI and IPTG-induced *E. coli* BL21(DE3) Star pLysS cultures, respectively. Lanes: 1 and 6, molecular mass protein marker; 2, noninduced total bacterial extract; 3, total bacterial cell lysates after induction; 4 and 5, soluble and insoluble (pellet) fractions of the induced culture, respectively; 7, purified recombinant proteins in Coomassie blue-stained gels; 8, Western blotting of the recombinant proteins probed with anti-His tag antibodies.

Cloning, expression, and characterization of recombinant protein. Oligonucleotides for PCR amplifications (see Materials and Methods) were designed based on genome sequences excluding the signal peptides. The amplified coding sequences were cloned into an *E. coli* pAE vector (44) and the proteins were expressed as full-length proteins with a 6×His tag at the N terminus of each recombinant protein. Both proteins were expressed in the bacterial pellet, in their insoluble form, as inclusion bodies (Fig. 1A and B, lanes 5). Proteins were recovered from inclusion bodies after solubilization with 8 M urea or 6 M guanidine. The purification was performed by metal-chelating chromatography under denaturing conditions followed by refolding by gradual removal of urea. The proteins were purified by metal-chelating chromatography, and proteins were recovered with 0.5 and 1.0 M imidazol for Lsa20 and rLIC11030, respectively. Protein purification was evaluated by SDS-PAGE, as shown in Fig. 1A and B, lanes 7. The recombinant protein bands were further confirmed by Western blotting probed with anti-His tag antibodies (Fig. 1A and B, lanes 8). In the case of Lsa20, another protein band, with approximately double the molecular mass, was also copurified and detected with the anti-His tag (Fig. 1A, lanes 7 and 8) and is probably a protein dimer. The calculated 20-kDa and 37-kDa molecular masses of the recombinant proteins Lsa20 and rLIC11030 comprise the vector fusion plus the encoded amino acids.

Protein conservation among leptospiral reference strains. Protein expression and conservation among *Leptospira* strains were assessed with total protein extracts from the same pathogenic strains of *Leptospira* mentioned above and the nonpathogenic strain *L. biflexa* serovar Patoc. Cell extracts were gel fractionated, proteins were membrane transferred, and Western blotting was performed by probing the membranes with polyclonal serum from mice immunized with Lsa20 (Fig. 2A) and rLIC11030 (Fig. 2B) recombinant proteins. The serological reactivity showed conservation of LIC11469 CDS in the main serovars of *L. interrogans* and at least in one serovar of *L. borgpetersenii* out of four tested (Fig. 2A). A protein band was detected in the nonpathogenic saprophytic *L. biflexa* strain, in agreement with BLAST analysis showing a partial match with the sequence present in the sequenced strain. Protein expres-

sion of LIC11030 CDS was observed in the same three serovars of *L. interrogans* where transcripts were identified (Fig. 2B).

Cellular localization of the recombinant proteins by protease assay. We have performed localization experiments with proteinase K treatment of intact leptospires (37, 41). A proteinase K solution of a 25- μ g/ml concentration was used for both proteins, and aliquots of the reaction mixture were taken for 6 h. Samples were gel fractionated, and proteins were blotted into the membranes. Proteins were then probed with antiserum against each of them. The CDSs LIC11469 and LIC11030 were both susceptible to protease treatment, as protein bands gradually disappear over time (Fig. 3A and B). The protoplasmic cylinder marker protein GroEL (21), used as negative control for surface proteolysis, was not digested or was only mildly digested under the same conditions tested (Fig. 3C), suggesting that both coding sequences assayed are surface exposed. Densitometric readings provide information regarding the amount of remaining proteins after the addition of proteinase K over time. The percentage was calculated based on the corresponding protein amount at time zero (Fig. 3D) and shows that after 6 h approximately 60% of GroEL remained, while no LIC11469 or LIC11030 CDSs were detected.

Adhesion of Lsa20 and rLIC11030 proteins to ECM components. As Lsa20 and rLIC11030 proteins are probably surface exposed, we evaluated whether they could mediate host colonization by adhering to extracellular matrix proteins. Thus, laminin, collagen type I, collagen type IV, cellular fibronectin, plasma fibronectin, ECM gel, and the control proteins BSA and fetuin were immobilized on 96-well microdilution plates, and recombinant protein attachment was assessed by an ELISA, as previously described in the work of Barbosa et al. (7). As shown in Fig. 4A, Lsa20 protein exhibited efficient adhesiveness to laminin ($P < 0.05$), while rLIC11030 did not show significant binding to any of ECM tested, including the ECM gel. The adherence of Lsa20 to ECM gel was expected as laminin is one of the components (see Materials and Methods). No statistically significant adhesiveness was observed with Lsa20 protein when wells were coated with collagen types I and IV, plasma or cellular fibronectin, BSA, or the highly glycosylated control protein fetuin. The interaction between Lsa20 with laminin was also assessed on a quantitative basis by

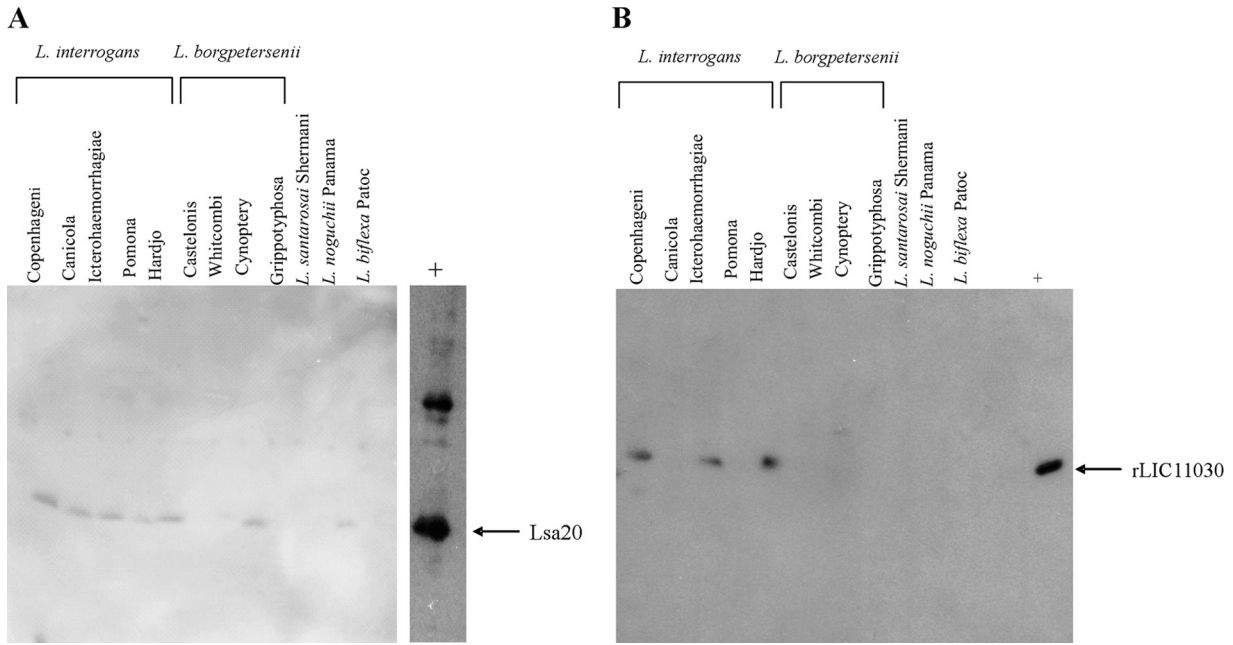


FIG. 2. Conservation of LIC11469 and LIC11030 coding sequences among leptospira strains. Whole-cell lysates, Lsa20, and rLIC11030 were separated by SDS-PAGE, transferred into membranes, and probed with antiserum against each recombinant protein followed by anti-mouse IgG conjugated to peroxidase. Reactivity was detected by ECL kit and X-ray film exposure. Western blotting of gel transferred to membrane probed with anti-Lsa20 (A) and with anti-rLIC11030 (B). On the right are lanes (+) containing the respective recombinant proteins as markers.

varying the protein concentration, as depicted in Fig. 4B. A dose-dependent and saturable binding was observed when increasing concentrations of the recombinant protein (0 to 10 μ M) were allowed to adhere to a fixed laminin concentration

(1 μ g) (Fig. 4B). No significant attachment of rLIC11030 to laminin was detected within the same protein concentration range. Binding saturation level was reached with a protein concentration of $\sim 9 \mu$ M (K_d , $1,988.00 \pm 563.49$ nM). Binding

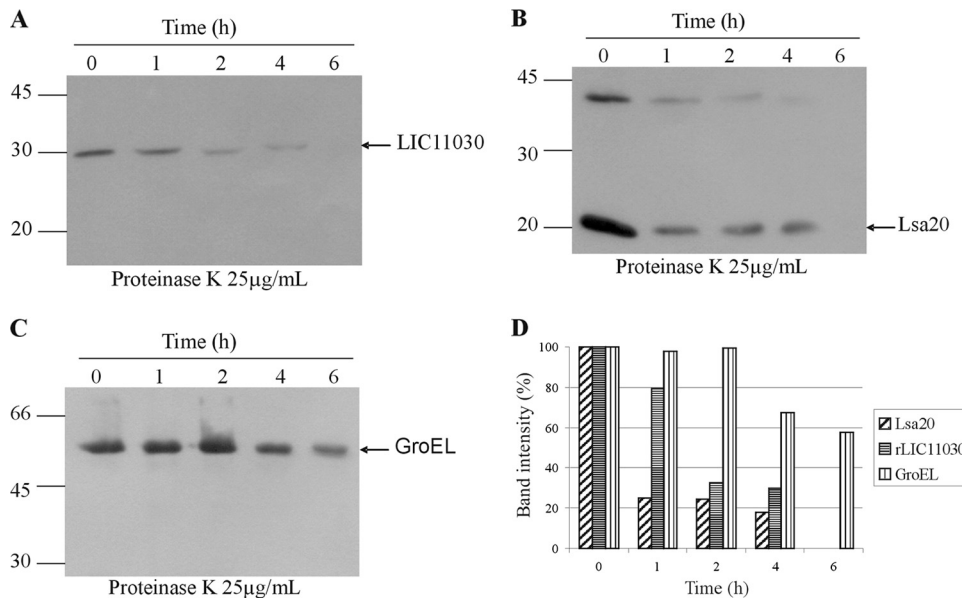


FIG. 3. Protease accessibility assay of LIC11469- and LIC11030-encoded proteins of *L. interrogans*. Viable leptospira were incubated with the indicated concentrations of proteinase K. Approximately 30 μ g of total leptospiral extract was loaded per lane and separated by SDS-PAGE. After transfer, the immobilized extracts were subjected to immunoblot analysis using Lsa20 (A)- and rLIC11030 (B)-specific antibodies. (C) Antibodies specific for the protoplasmic cylinder protein GroEL were employed. The serum dilution was 1:100. The GroEL protein (same amount of cell extract) was used as an indicator of outer membrane integrity and as an internal control for proteinase K resistance. (D) Optical densities of the protein bands were taken, and the relative percentage was based on the corresponding protein amount at time zero (no proteinase K addition).

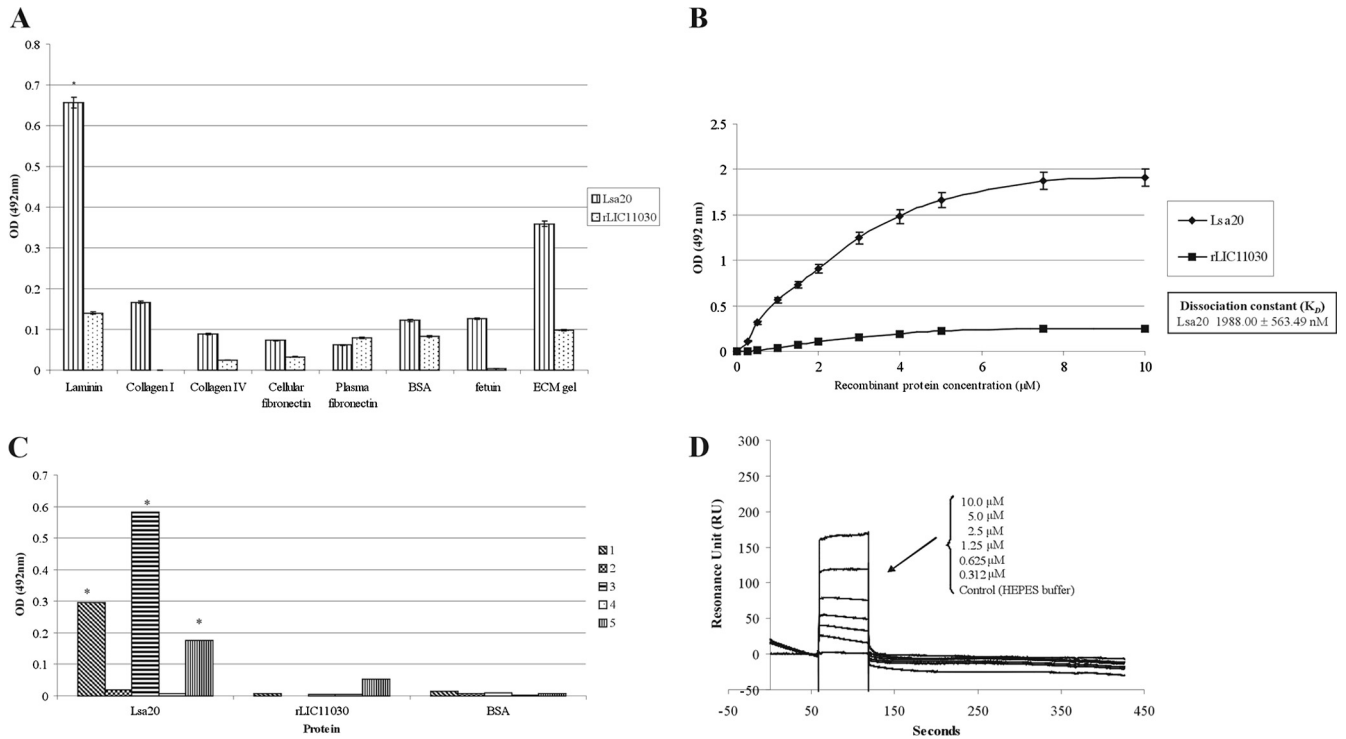


FIG. 4. Binding characteristics of Lsa20 and rLIC11030 to ECM components. (A) Wells were coated with 1 µg of laminin, collagen type I, collagen type IV, cellular fibronectin, plasma fibronectin, ECM gel, and the control proteins BSA and fetuin. One microgram of the recombinant proteins was added per well, and the binding was measured by an ELISA. Data represent the means ± standard deviations of results from three independent experiments. For statistical analyses, the attachment of recombinant proteins to the ECM components was compared to its binding to BSA as well as to fetuin by the two-tailed *t* test (*, *P* < 0.005). (B) Lsa20 dose-dependent binding experiments: each point was performed in triplicate and is expressed as the mean absorbance value at 492 nm ± standard error for each point. Protein rLIC11030 was included as the negative control. (B) The dissociation constant (*K_d*) was calculated based on ELISA data for the Lsa20 recombinant protein, which reached equilibrium at a concentration of ~9 µM. (C) Wells were coated with 1 µg of laminin, Lsa20, rLIC11030, or BSA (control). One microgram of the proteins was added per well, and the binding was measured by an ELISA. Bars: 1, coating with laminin followed by recombinant protein or BSA and incubation with anti-polyhistidine monoclonal antibodies; 2, coating with laminin followed by incubation with recombinant protein previously blocked by incubation with specific antibodies produced in hamster, and then incubation with anti-polyhistidine monoclonal antibodies; 3, coating with laminin followed by recombinant protein or BSA and incubation with specific mouse antirecombinant protein; 4, coating with laminin followed by incubation with denatured recombinant protein and incubation with specific mouse antirecombinant protein; 5, coating with recombinant protein or BSA followed by incubation with laminin and antilaminin antibodies. Data represent the means ± standard deviations of results from two independent experiments. For statistical analyses, the attachment of recombinant proteins to laminin was compared to its binding to BSA by the two-tailed *t* test (*, *P* < 0.02). (D) Analysis of the interaction of Lsa20 with laminin by using the BIAcore T100 system. Protein solutions of Lsa20 (0, 0.312, 0.625, 1.25, 2.5, 5.0, 10 µM) in 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20 (pH 7.4) were injected over immobilized laminin at a flow rate of 30 µl/min for 1 min at 25°C.

of Lsa20 to laminin was also detected when anti-polyhistidine monoclonal antibodies were used to probe Lsa20 attached to laminin (Fig. 4C, bar 1) instead of mouse polyclonal antibodies against Lsa20 (Fig. 4C, bar 3), or the inverse situation, protein-coated well bound to laminin and probed with antilaminin antibodies (Fig. 4C, bar 5). No binding to laminin was detected when Lsa20 was previously denatured (Fig. 4C, bar 4) or blocked with anti-Lsa20 produced in hamsters (Fig. 4C, bar 2). No binding was detected when the same set of conditions was applied with rLIC11030 and BSA. Interaction of Lsa20 with laminin was also determined using the BIAcore T100 system, with protein concentration range from 0 to 10 µM that was injected over immobilized laminin (Fig. 4D).

Recombinant leptospiral protein Lsa20 binds human plasminogen. In our previous work we have shown that leptospires bind plasminogen on their surface (56) and we have also described several proteins that probably are plasminogen recep-

tors (54). We thus decided to evaluate if the selected surface-exposed proteins, Lsa20 and rLIC11030, can also adhere to human plasminogen *in vitro*. In addition, we investigated whether the recombinant proteins can also bind factor H, fibrinogen components and human complement, as previously reported for other recombinant proteins (8, 28, 40, 53). Our data show that Lsa20 binds human plasminogen (*P* < 0.05), while very low reactivity was detected with rLIC11030 and the control protein BSA (Fig. 5A). None of the proteins interacted significantly with factor H, human complement or fibrinogen (Fig. 5A). The binding between Lsa20 and plasminogen is dose dependent and saturable when increasing concentrations of the recombinant protein Lsa20 (0 to 10 µM) were allowed to react with a fixed (1 µg) plasminogen concentration (*K_d*, 509.13 ± 77.47 nM) (Fig. 5B). No significant attachment of rLIC11030 to plasminogen was detected within the same protein concentration range. Binding of Lsa20 to plasminogen was

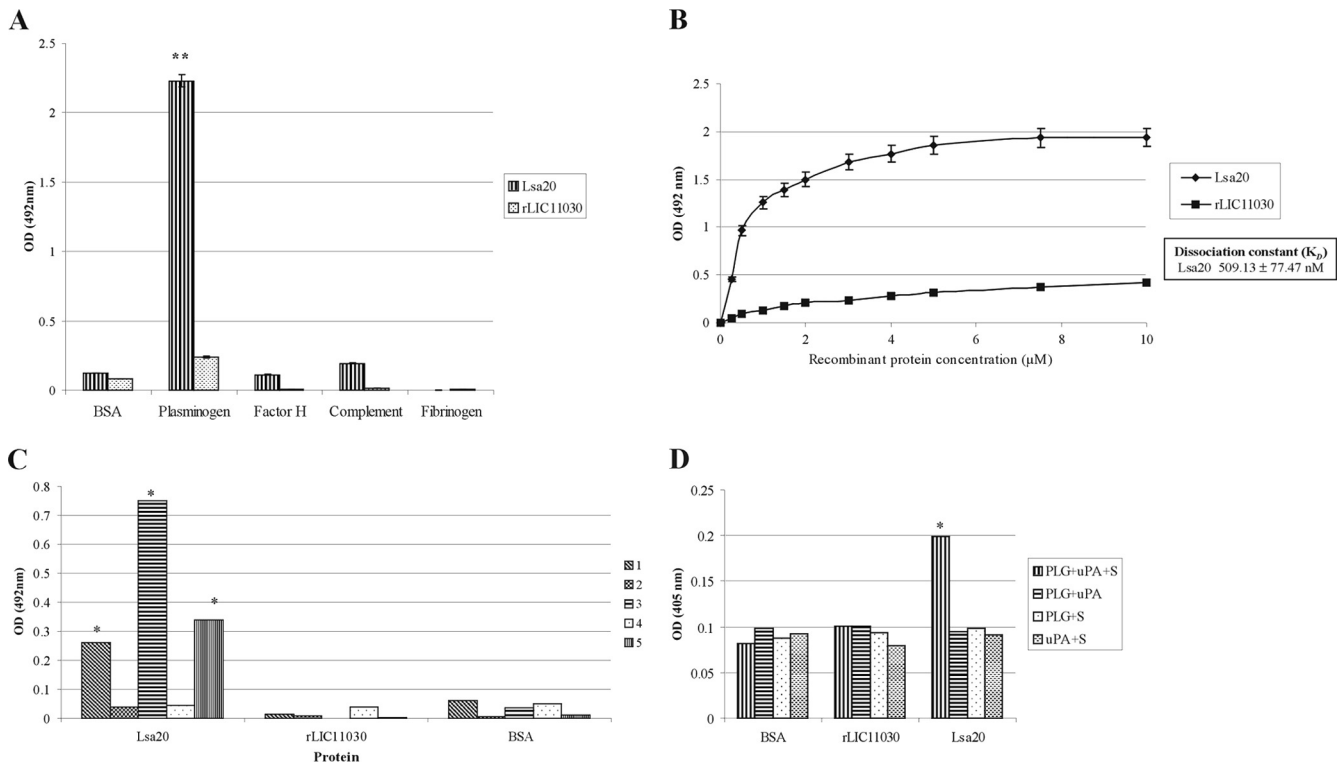


FIG. 5. Recombinant protein Lsa20 binds human plasminogen and generates enzymatically active plasmin. (A) Components were coated into ELISA plates (10 µg/ml) and allowed to interact with 1 µg per well of the recombinant proteins Lsa20 and rLIC11030. The binding was detected by specific serum antirecombinant proteins. Bars represent the mean of absorbance at 492 nm ± standard deviation of three replicates for each protein and are representative of three independent experiments. For statistical analyses, the binding of Lsa20 to human plasminogen was compared to its binding to BSA by a two-tailed *t* test (**, *P* < 0.0005). (B) Plasminogen (10 µg/ml) was immobilized into 96-well ELISA plates, and 0 to 10 µM each recombinant protein was added for interaction. Protein rLIC11030 was included as the negative control. The binding was detected using antiserum raised in mice against the recombinant proteins in appropriate dilutions (1:1,000 for Lsa20) followed by horseradish peroxidase-conjugated anti-mouse IgG. Data represent the mean absorbance values ± standard deviation of three replicates for each experimental group. (B) The dissociation constant (*K_d*) was calculated based on ELISA data for Lsa20 recombinant protein when the equilibrium concentration was reached. (C) Wells were coated with 1 µg of PLG, Lsa20, rLIC11030, or BSA (control). One microgram of the proteins was added per well, and the binding was measured by an ELISA. Bars: 1, coating with PLG followed by recombinant protein or BSA and incubation with anti-polyhistidine monoclonal antibodies; 2, coating with PLG followed by incubation with recombinant protein previously blocked by incubation with specific antibodies produced in hamster, and then incubation with anti-polyhistidine monoclonal antibodies; 3, coating with PLG followed by recombinant protein or BSA and incubation with specific mouse antirecombinant protein; 4, coating with PLG followed by denatured recombinant protein and incubation with specific mouse antirecombinant protein; 5, coating with recombinant protein or BSA followed by incubation with PLG and anti-PLG antibody. Data represent the means ± standard deviations of results from two independent experiments. For statistical analyses, the attachment of recombinant proteins to laminin was compared to its binding to BSA by the two-tailed *t* test (*, *P* < 0.02). (D) Plasmin generation by plasminogen bound to recombinant protein Lsa20 was assayed by modified ELISA for immobilized proteins receiving treatment with PLG plus uPA plus specific plasmin substrate (PLG+uPA+S) or control treatments lacking one of the three components (PLG+uPA, PLG+S, uPA+S). BSA was employed as the negative control. Bars represent mean absorbance at 405 nm, as a measure of relative substrate degradation ± standard deviation of four replicates for each experimental group, and are representative of two independent experiments. Statistically significant binding in comparison to the negative control (BSA) is depicted: *, *P* < 0.003.

also detected when anti-polyhistidine monoclonal antibodies were used to probe Lsa20 attached to plasminogen (Fig. 5C, bar 1) instead of mouse polyclonal antibodies against Lsa20 (Fig. 5C, bar 3), or the inverse situation, protein-coated well bound to plasminogen and probed with anti-plasminogen antibodies (Fig. 5C, bar 5). No binding to plasminogen was detected when Lsa20 was previously denatured (Fig. 5C, bar 4) or blocked with anti-Lsa20 produced in hamsters (Fig. 5C, bar 2). No binding was detected when the same set of conditions was applied with rLIC11030 and BSA.

Plasmin generation from plasminogen-bound Lsa20. Enzymatically active plasmin is generated by plasminogen bound to the surface of *L. interrogans* when its activator is present (56).

To assess whether the plasminogen bound to Lsa20 generates proteolytic activity, as previously reported with other recombinant proteins (52, 54), a microplate was coated with Lsa20, blocked, and then incubated with plasminogen. Unbound plasminogen was washed away, and the uPA (urokinase)-type plasminogen activator together with a plasmin-specific chromogenic substrate was added. The reaction was carried out overnight and the plasmin activity was evaluated by measuring the cleavage of the plasmin-specific substrate D-valyl-leucyl-Lysine-*p*-nitroanilide dihydrochloride at 405 nm. The plasminogen captured by Lsa20 protein could be converted into plasmin, as indirectly demonstrated by specific proteolytic activity exerted by plasmin (Fig. 5D). The negative controls lacking

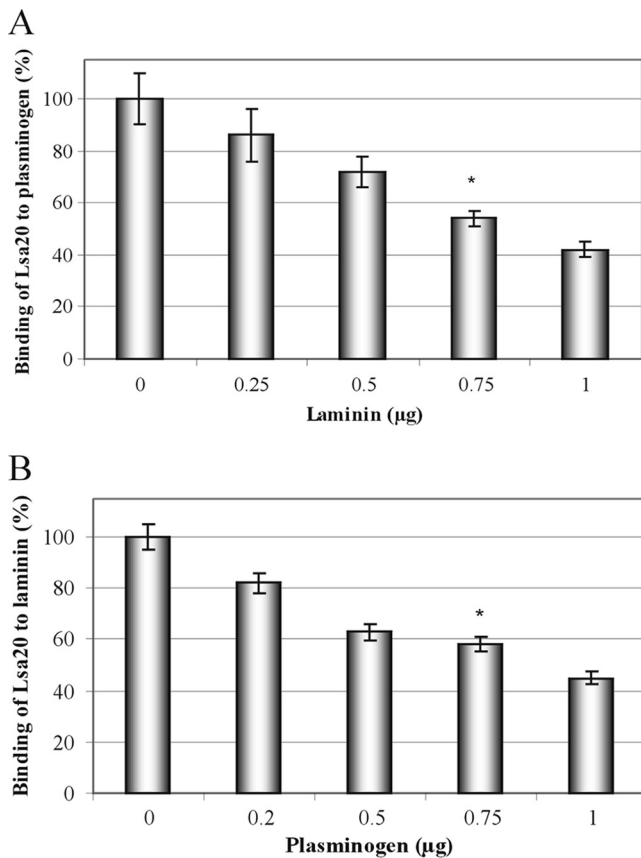


FIG. 6. Laminin and plasminogen compete for the binding to Lsa20. (A) The effect of laminin on the binding of plasminogen (10 µg/ml) to immobilized Lsa20 (10 µg/ml) was assessed with the addition of increasing concentrations of laminin (0 to 1.00 µM). (B) The effect of plasminogen on the binding of laminin (10 µg/ml) to immobilized Lsa20 (10 µg/ml) was assessed with the addition of increasing concentrations of plasminogen (0 to 1.00 µM). The detection of Lsa20-bound plasminogen (A) or Lsa20-bound laminin (B) was performed by use of specific antibodies. Bars represent the mean absorbance values \pm standard deviation of four replicates for each condition and are representative of two independent experiments. Results of statistically significant interference with binding in comparison with the positive control (no addition of laminin or plasminogen) are shown: *, $P < 0.05$.

plasminogen, uPA or the chromogenic substrate showed no enzymatic activity.

Laminin and plasminogen compete for the binding with Lsa20. As both components exhibit adhesiveness to Lsa20, we have evaluated whether laminin or plasminogen competes for the same binding site to Lsa20 or interferes somehow in the interaction. We performed the binding assay in the presence of increasing concentrations of laminin (0 to 1.0 µg) or plasminogen (0 to 1.0 µg). The results show that both laminin (Fig. 6A) and plasminogen (Fig. 6B) interfere with the binding to Lsa20 in a dose-dependent fashion. The addition of laminin decreased the plasminogen binding to Lsa20 that reached significance with laminin at 0.75 µg ($P < 0.05$) (Fig. 6A). Similar behavior was observed when an increasing concentration of plasminogen was added to the laminin Lsa20 reaction mixture (Fig. 6B), suggesting that both molecules compete for the same protein binding site.

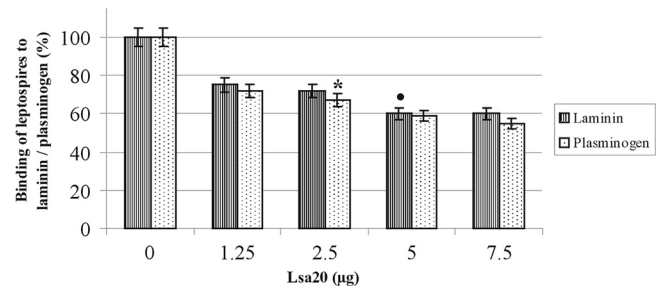


FIG. 7. Inhibition of *L. interrogans* attachment to laminin or to plasminogen by Lsa20. Laminin or plasminogen (1 µg/well) was adsorbed to microtiter plates followed by incubation with increasing concentrations of Lsa20 (0 to 7.50 µg) for 90 min at 37°C. Live leptospire (100 µl/well of 4×10^7 *L. interrogans* serovar Copenhageni strain M20 leptospire) were added for 90 min at 37°C. The unbound leptospire were washed, and the quantification of bound leptospire was performed indirectly by anti-LipL32 antibodies produced in mice (1: 4,000 dilution) followed by horseradish peroxidase-conjugated anti-mouse IgG antibodies. Each point represents the mean absorbance value at 492 nm \pm standard deviation of three replicates. Data are representative of two independent experiments. *, $P < 0.009$; ●, $P < 0.005$.

Inhibition of *L. interrogans* attachment to laminin or to plasminogen by Lsa20. It has been previously shown that the recombinant proteins Lsa24/(LfhA/LenA) (7), Lsa63 (55), and OmpL37 (40) exhibited an inhibitory effect on the binding of leptospire to ECM macromolecules. We thus carried out similar experiments to evaluate the effect of Lsa20 on leptospiral adherence to laminin or plasminogen by employing an ELISA in function of protein concentration (0 to 7.5 µg). The results show that the addition of an increasing concentration of Lsa20 reduced the leptospiral binding to laminin and to plasminogen molecules in a dose-dependent manner (Fig. 7). A reduction in the number of leptospire adhering to plasminogen was significant with 2.5 µg of Lsa20 ($P < 0.05$), while with binding to laminin, significance was reached with 5.0 µg protein ($P < 0.05$). The experiment was performed in triplicate and Fig. 7 shows one representative data of two independent experiments.

Reactivity of Lsa20 and rLIC11030 with serum samples from individuals with leptospirosis. Serum samples from individuals with confirmed leptospirosis were used to test recognition of Lsa20 and rLIC11030. We performed ELISA and employed 40 paired samples of the early (MAT-negative) and convalescent (MAT-positive) phases of the disease. The recombinant protein Lsa20 was recognized by IgG antibodies in both phases of the disease, early and convalescent (Fig. 8), while very low or no reactivity was detected with rLIC11030 (data not shown). A total of 25% responders presented IgG antibodies against Lsa20 in the early phase of the disease, while 50% presented IgG antibodies in the convalescent phase (Fig. 8). These data suggest that Lsa20 protein is expressed during leptospiral infection.

DISCUSSION

One feature of leptospiral infection is the rapid dissemination inside the host and colonization of renal tubules (16). The capacity of the leptospire to adhere to laminin, cellular fi-

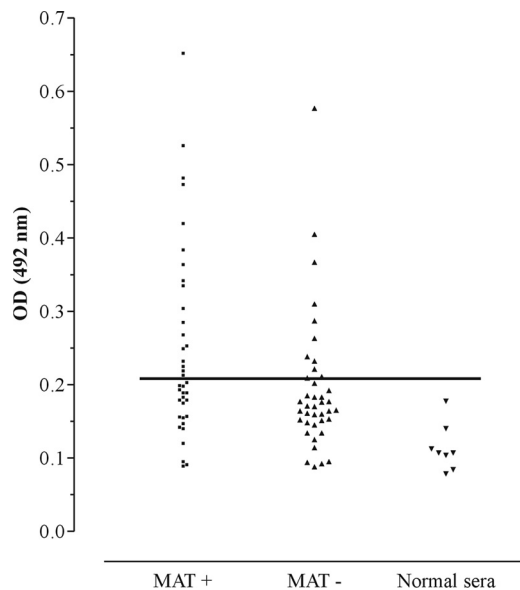


FIG. 8. Recognition of recombinant protein Lsa20 by IgG antibodies of individuals diagnosed with leptospirosis. Positive sera (responders) were determined by ELISA with the recombinant protein and serum samples from patients in both phases of the disease. The reactivity was evaluated as IgG antibodies. Serum was considered MAT positive or MAT negative if agglutination was detected when the sera were tested for their reactivities with isolates of the 22 serovars (see Materials and Methods). The cutoff values are defined as the mean plus 3 standard deviations obtained for sera from five healthy individuals.

bronectin, plasma fibronectin, and collagen types I and IV has been shown (7), and thus far several leptospiral adhesin ECM-binding proteins have been identified. These include the 36-kDa fibronectin-binding protein (31), LfhA/Lsa24 (7, 53), LigA and LigB proteins (14, 25), Len-family proteins (50), Lsa21 (5), LipL32 (22, 23), Lsa27 (29), Lp95 (6), TlyC (13), LipL53 (37), Lsa63 (55), and OmpL37 (40). After adherence, the bacteria have to overcome the barriers imposed by epithelial tissues and extracellular matrices. The acquisition of host-derived plasmin by leptospiral receptor-bound plasminogen can degrade fibronectin, which may help bacterial dissemination (56).

Two putative leptospiral outer membrane proteins of unknown function were selected for our studies, LIC11469 and LIC11030. We report that one of them is a novel outer membrane protein of *Leptospira*, named Lsa20, which binds strongly to laminin, a constituent of the basement membrane underlying both the epithelium and the endothelium, and to plasminogen generating enzymatically active plasmin. The LIC11469 and LIC11030 CDSs were cloned and expressed in *E. coli* as 20- and 37-kDa full-length recombinant proteins, respectively, having 6×His tags at the N termini. The purified proteins exhibited a single major band by SDS-PAGE and were recognized by anti-His tag antibodies and homolog sera from mice immunized with each recombinant protein. The protein band of approximately 40 kDa is probably Lsa20 protein dimer because it was recognized by both antibodies employed. The coding sequence LIC11469 (Lsa20) was conserved in several serovars of *L. interrogans* and other pathogenic species tested,

unlike the LIC11030 coding sequence, which appears to be restricted to *L. interrogans*. None of the coding sequences were detected in the nonpathogenic *L. biflexa* strain.

We have performed proteolysis studies on intact *Leptospira* cells to determine whether the predicted outer proteins in our studies are indeed located on the bacterial surface. Proteinase has been previously employed with other spirochetes (11, 15, 43) and bacteria (32) to evaluate the surface exposure of proteins. Pinne and Haake (41) and Oliveira et al. (37) have used proteinase K to assess outer membrane proteins in *Leptospira*. Proteolysis of the predicted CDSs LIC11469 and LIC11030 showed that both leptospiral proteins are sensitive to proteinase K cleavage, contrasting with the cytoplasmic protein GroEL, which showed only a slight digestion, suggesting their surface localization. Interestingly, the native protein encoded by the LIC11469 gene, like the Lsa20 recombinant protein, also appears to exhibit both monomer and dimer forms.

Lsa20 is probably a surface-exposed protein. It is therefore possible that it may have a role in the attachment to host tissues. Indeed, a dose-dependent specific and saturable binding of Lsa20 to immobilized laminin was observed (K_d , $1,988.00 \pm 563.49$ nM), fulfilling the properties of receptor-ligand interaction. No binding was detected when Lsa20 was subjected to denaturing conditions or previously blocked with hamster anti-Lsa20 serum. Moreover, binding of Lsa20 to laminin was directly detected by SPR analysis. However, the affinity of Lsa20 to laminin is lower compared to the K_d (410 ± 81 nM) obtained with OmpL37 and the same ECM component (40). Of all the ECM components tested (Fig. 4A), attachment to laminin suggests that this protein is the major ECM target for Lsa20, a feature shared with other leptospiral laminin-binding proteins (7, 29, 50).

Plasminogen, a key component of the host fibrinolytic system, is the zymogen form of plasmin, a serine protease that has the ability to degrade a broad spectrum of substrates, including fibrin clots, connective tissue, and components of extracellular matrices (3, 4, 42). We have reported that *Leptospira* spp. bind plasminogen at their surface, generating plasmin when urokinase activator is available (56). We have also identified proteins that act as leptospiral plasminogen receptors (54). Lsa20 also exhibits plasminogen-binding activity. No attachment of Lsa20 to plasminogen was detected when the protein was previously denatured or blocked by anti-Lsa20 produced in hamsters. The binding is dose dependent on protein concentration and reached saturation around $9 \mu\text{M}$ Lsa20 (K_d , 509.13 ± 77.47 nM). This binding affinity is lower than that seen for other recombinant proteins in our laboratory (54). As shown with other protein plasminogen receptors (52, 54), plasmin is actively generated in the presence of urokinase activator. Similar to LenA (LfhA/Lsa24), which binds laminin and plasminogen (7, 52), there is a competition for the binding of both ligands to Lsa20. However, in contrast with LenA (53), no binding activity of Lsa20 was detected with factor H.

The presence of Lsa20 inhibited the binding of intact *L. interrogans* to immobilized laminin or plasminogen. The inhibitory effect exerted by the recombinant protein was moderate, although statistically significant with both ligands, and can be explained by the existence of additional *L. interrogans* binding proteins contributing to the leptospiral attachment to laminin and to plasminogen. Similar data were obtained with Lsa24,

Lsa63, LigA/LigB, and OmpL37 leptospiral proteins (7, 14, 40, 55).

In conclusion, we have identified a novel leptospiral protein, Lsa20, as a laminin-binding protein and as a receptor for plasminogen. We have characterized both protein-ligand interactions and show that Lsa20-bound plasminogen generates enzymatically active plasmin in the presence of urokinase activator. Lsa20 is recognized by antibodies in serum samples of individuals diagnosed with leptospirosis, suggesting that the protein is expressed during infection. This protein was previously genome annotated as a putative outer membrane protein of *L. interrogans* with unknown function (35). We show now that the protein encoded by LIC11469 is indeed a surface-exposed protein with dual adherence and proteolytic binding activities that may have roles in leptospiral pathogenesis.

ACKNOWLEDGMENTS

We are deeply indebted to Alexsander Seixas de Souza (Departamento de Parasitologia, Instituto Butantan, São Paulo, Brazil) for use of Confocal facilities and helpful discussion.

This work was supported by FAPESP, CNPq, and Fundação Butantan, Brazil; R.S.M. and M.V.A. have fellowships from FAPESP.

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Editor: J. B. Bliska