RESEARCH LETTER

A novel leptospiral protein increases ICAM-1 and E-selectin expression in human umbilical vein endothelial cells

Monica L. Vieira[1,2], Lina P. D'Atri[3], Mirta Schattner[3], Alejandra M. Habarta[4], Angela S. Barbosa[1], Zenaide M. de Morais[5], Silvio A. Vasconcellos[5], Patricia A.E. Abreu[1], Ricardo M. Gómez[4] & Ana L.T.O. Nascimento[1,2]

[1]Centro de Biotecnologia, Instituto Butantan, Avenida Vital Brazil, São Paulo, SP, Brazil; [2]Interunidades em Biotecnologia, Instituto de Ciências Biomédicas, USP, São Paulo, Brazil; [3]Instituto de Investigaciones Hematológicas, Academia Nacional de Medicina, CONICET, Buenos Aires, Argentina; [4]Instituto de Bioquímica y Biología Molecular, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina; and [5]Laboratório de Zoonoses Bacterianas do VPS, Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, São Paulo, Brazil

Correspondence: Ana L.T.O. Nascimento, Centro de Biotecnologia, Instituto Butantan, Avenida Vital Brazil, 1500, 05503-900, São Paulo, SP, Brazil. Tel.: +5511 37220019; fax: +5511 37261505;
e-mail: tabet@butantan.gov.br

Received 29 May 2007; accepted 24 August 2007.
First published online October 2007.

DOI:10.1111/j.1574-6968.2007.00924.x

Editor: Stephen Smith

Keywords
Leptospira interrogans; recombinant protein; ICAM-1; E-selectin.

Abstract

It has been reported previously that activation of vascular endothelium by outer membrane proteins of the spirochetes *Borrelia* sp. and *Treponema* sp. resulted in enhanced expression of endothelial cell adhesion molecules. To investigate the role of leptospiral proteins in this process, a predicted lipoprotein encoded by the gene LIC10365 was selected, which belongs to a paralogous family that presents a domain of unknown function, DUF1565. The LIC10365 gene was cloned and the protein expressed in *Escherichia coli* C43 (DE3) strain using the vector pAE. The recombinant protein tagged with N-terminal hexahistidine was purified by metal-charged chromatography and was used to assess its ability to activate cultured human umbilical vein endothelial cells. The rLIC10365 activated endothelium in such a manner that E-selectin and intercellular adhesion molecule 1 (ICAM-1) became upregulated in a dose-dependent fashion. The LIC10365-encoded protein was identified *in vivo* in the renal tubules of animal during experimental infection with *Leptospira interrogans*. Collectively, these results implicate the LIC10365-coding protein of *L. interrogans* as a potential effector molecule in the promotion of a host inflammatory response. This is the first report of a leptospiral protein capable of up-regulating the expression of endothelial cell adhesion molecules ICAM-1 and E-selectin.

Introduction

The spirochete *Leptospira interrogans* is a highly invasive extracellular pathogen that causes leptospirosis, one of the most widespread zoonoses of human and veterinary concern, especially in tropical regions (Faine *et al.*, 1999; Levett, 2001; Vinetz, 2001; Haake *et al.*, 2002; Bharti *et al.*, 2003). Despite its importance, aspects of pathogenesis and the invasion processes by which the leptospires infect the hosts and initiate tissue colonization are poorly characterized.

A wide range of mammalian cell molecules can function as receptors for bacteria–host cell adhesion (Finlay & Cossart, 1997; Kerr, 1999; Boyle & Finlay, 2003). Surface proteins are thought to be the first contact by which the microorganisms interact and adhere to host tissues. This binding activity seems to be an essential first stage in the establishment of infection of several bacterial pathogens. Cell adhesion molecules (CAMs) are surface receptors present in eukaryotic cells that mediate cell–cell or cell–extracellular matrix interactions. Many pathogenic bacteria invade the host tissues through their ability to bind to CAMs (Finlay & Cossart, 1997; Boyle & Finlay, 2003).

In addition to lipopolysacharides, the activation of the vascular endothelium by spirochaetal proteins resulting in enhanced expression of adhesion receptors has been described. Studies with the pathogenic spirochaetes *Borrelia* sp. and *Treponema* sp., reported the stimulation of the vascular endothelium *in vitro* that lead to the upregulation of CAMs (Sellati *et al.*, 1995, 1996; Lee *et al.*, 2000, 2005; Shamaei-Tousi *et al.*, 2000). The genome sequencing of *L. interrogans* serovar Copenhageni revealed 263 predicted potential cell-surface-exposed membrane proteins

(Nascimento *et al.*, 2004a, b), but to date only a few have been characterized at the molecular level. These unknown gene products are potential candidates for mediating the interaction to host cells, triggering the colonization of the tissues, being an important first step in the infection process.

In the present study, the cloning, expression, purification and characterization was described of one predicted leptospiral outer-membrane protein encoded by the gene *LIC10365*, first identified by bioinformatics tools in the genome sequencing of the *L. interrogans* serovar Copenhageni. The recombinant protein (rLIC10365) was expressed in the *Escherichia coli* C43 (DE3) strain and purified by metal-affinity chromatography. The protein encoded by *LIC10365* is present *in vivo* in the renal tubules of animal during leptospiral experimental infection. Assessment of the rLIC10365 protein effects of the CAM expression of human umbilical endothelial cells (HUVECS) *in vitro* indicates that the LIC10365 recombinant protein (rLIC10365) activates the expression of the ICAM-1 and E-selectin, suggesting its involvement in the initial cell adhesion interaction.

Materials and methods

In silico selection of LIC10365

Cellular localization of the protein was predicted by the PSORT program, http://psort.nibb.ac.jp (Nakai & Kanehisa, 1991). Determination of signal peptide, lipoprotein cleavage site and transmembrane domains was based on the public web servers http://www.cbs.dtu.dk/services/SignalP, http://www.cbs.dtu.dk/services/LipoP/ and http://www.cbs.dtu.dk/services/TMHMM (Schultz *et al.*, 1998; Juncker *et al.*, 2003; Bendtsen *et al.*, 2004).

Bacteria and DNA isolation

Strains of *L. interrogans* serovars Canicola (Hond Utrechet IV), Copenhageni (M-20), Hardjo (Hardjoprajtino), Icterohaemorrhagiae (RGA), Pomona (Pomona) and *Leptospira biflexa* serovar Patoc (Patoc 1) were cultured in Faculdade de Medicina Veterinária, Universidade de São Paulo, SP, Brazil). Genomic DNA of each strain was isolated from the bacterial pellet with a guanidine-detergent lysing solution (DNAzol® Reagent, Invitrogen), according to the manufacturer's instructions.

DNA cloning

Amplification of the predicted coding sequence of LIC10365 was performed by the PCR from *L. interrogans* serovar Copenhageni genomic DNA using the primer pairs: F: GGATCCAGCGGCGAAAACAGTAACG and R: GGTAC CTCACTCGAGTTTACAAGCGTTTGGAG. The LIC10365 gene sequence was amplified without the signal peptide tag. Gel-purified PCR fragments (Concert Rapid Gel Extraction System – Life Technologies) were cloned into the *E. coli* expression vector pAE (Ramos *et al.*, 2004) at BamHI and KpnI restriction sites. The construct was verified by DNA sequencing on an ABI Prism 3730 × L sequencer (Seq-Wright, Houston, TX) with appropriate vector-specific T7 (F: TAATACGACTCACTATAGGG) and pAE (R:CAGCAGC CAACTCAGTTCCT) primers.

Expression, refolding and purification of rLIC10365

Escherichia coli C43 (DE3) cells transformed with the pAE-LIC10365 were grown overnight in Luria–Bertani at 37 °C. When the $OD_{600\,nm}$ reached 0.6, recombinant protein production was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside. The induction was maintained for 4 h at 37 °C under agitation. The cells were harvested by centrifugation, the bacterial pellets were resuspended in 30 mL lysis buffer (500 mM NaCl, 20 mM Tris-HCl, and 0.1% Triton X-100, pH 8.0) and lysed by French Pressure (Aminco). The insoluble fraction was washed three times with 30 mL of buffer (20 mM Tris-HCl, 500 mM NaCl, 1 mM β-mercaptoethanol, 1 M urea and 1% Triton X-100) before solubilization with 50 mL of buffer containing 20 mM Tris-HCl, 500 mM NaCl, 5 mM β-mercaptoethanol and 8 M urea. Protein refolding was achieved by dilution of the solubilized insoluble fraction of the lysate in 2 L of buffer containing 20 mM Tris-HCl, 500 mM NaCl and 5 mM imidazole. The solution containing the refolded protein was loaded onto Ni^{2+}-charged beads of a chelating fast-flow (GE Healthcare) chromatographic column. Contaminants were washed away with low imidazol concentrations (5, 25, 40 and 60 mM) and the recombinant protein was eluted with 1 M imidazole. Samples were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The fractions containing the eluted protein were dialyzed against phosphate-buffered saline (PBS) gradually decreasing imidazole concentrations to zero.

Antisera against rLIC10365

Ten female BALB/c mice (18–22 g) were immunized subcutaneously with 10 μg of recombinant LIC10365 with 10% (v/v) of Alhydrogel (2% $Al(OH)_3$, Biosector, Denmark) as an adjuvant. Two subsequent booster injections were given at 2-week intervals with the same protein preparation. One week after each immunization, the mice were bled from the retro-orbital plexus and the pooled sera were analyzed by an enzyme-linked immunosorbent assay (ELISA) for determination of antibody titers. The control group was inoculated with PBS and an adjuvant.

Western blotting

This was performed as described by Neves *et al.* (2004). Intended for detection of recombinant protein by antibodies present in the serum of a mouse immunized with rLIC10365, the membrane was washed with PBS-T and incubated with mouse anti-rLIC10365 (1 : 5000) in 5% nonfat dry milk–PBS-T. This serum was previously treated with a suspension of *E. coli* C43 (DE3) cell lysate/5% nonfat dry milk for 1 h. After the washings, the membrane was incubated with an anti-mouse IgG (HRP)-conjugate (1 : 5000) in 5% nonfat dry milk–PBS-T for 1 h. The bands were revealed with ECL reagents (GE Healthcare).

RNA extraction and reverse transcriptase (RT)-PCR analysis

For RT-PCR, total RNA was isolated from leptospires cultured as mentioned previously by the acid guanidinium thiocyanate phenol–chloroform method using TRIzol® Reagent (Invitrogen) according to the manufacturer's recommendations. One microgram of RNA from each sample was treated with 1 U of DNAse I (Invitrogen) for 15 min at room temperature. DNAse I was inactivated by the addition of 1 μL of 25 mM EDTA solution, followed by an incubation at 65 °C for 10 min. DNAse-treated RNAs were reversely transcribed using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). The PCR reactions and cycling conditions were the same as those described for LIC10365 cloning. All samples were tested with and without reverse transcriptase to rule out genomic DNA contamination. PCR-amplified products were loaded onto 1% agarose gels for electrophoresis and visualized by ethidium bromide staining.

In vivo detection of LIC 10365 by immunohistochemistry

In order to detect LIC10365-encoded protein in kidney tissues, three guinea-pigs of 180 g were inoculated intraperitoneally with 0.5 mL of PBS (pH 7,2) containing $10^{2.5}$ bacteria (*L. interrogans* serovar Copenhageni), killed 2 weeks post infection (p.i.) when the animals presented symptoms, e.g. weight loss, lethargy (Faine *et al.*, 1999), and their kidneys were harvested and processed for routine histology. Mock-infected animals were used as controls. After rehydratation, the tissue Pro-Bond Plus slides were heated three times for 5 min in a 10 mM citrate buffer in a microwave oven. The sections were then cooled and immersed in 3% H_2O_2 for 15 min to inhibit endogenous peroxidase activity. To block nonspecific antigen sites, sections were incubated with PBS with 5% of normal goat serum for 20 min at room temperature. Then, slides were incubated with the above-described primary polyclonal murine antiserum diluted 1 : 100 for 1 h at room temperature or with an anti-LipL32 murine serum (Gamberini *et al.*, 2005), as positive control. After several washes with PBS, specimens were incubated with a secondary antibody (goat anti-mouse immunoglobulin) conjugated to a peroxide-labeled dextran polymer (DAKO EnVision) for 20 min at room temperature and again washed with PBS. A diaminobenzidine/hydrogen peroxidase substrate was incubated for 2–10 min to reach the appropriate intensity and slides were rinsed with distilled water to stop the staining reaction. Immunostained sections were counterstained with hematoxylin for 1 min, bathed under tap water, rinsed with distilled water and dehydrated in increasing ethanol concentrations followed by xylene (each treatment, 5 min). Finally, the slides were mounted with a coverslip in a nonaqueous permanent mounting medium and observed in a Leica photomicroscope.

Limulus amebocyte lysate assay (LAL test)

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

Endothelial cell culture

Endothelial cells were obtained from HUVECs by collagenase (GIBCO) digestion according to the method of Jaffe *et al.* (1973). Cells were grown to confluence in 25 cm^2 tissue culture flasks that were precoated with 1% gelatin (Sigma). The growth medium consisted of RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (GIBCO), 90 $\mu g\,mL^{-1}$ heparin (GIBCO), 50 $\mu g\,mL^{-1}$ endothelial cell growth supplement (ECGS) (Sigma), 2 mM sodium pyruvate, 2 mM L-glutamine, 100 $U\,mL^{-1}$ penicillin (Sigma) and 100 $\mu g\,mL^{-1}$ streptomycin (Sigma) at 37 °C in a humidified 5% CO_2 incubator. Confluent HUVECs were passaged with 0.05% trypsin +0.02% EDTA and were routinely used between the first and third passages. Cultured cells were identified as being endothelial by their morphology and von Willebrand Factor (vWF) antibody binding. All experiments were performed in the presence of 7 $\mu g\,mL^{-1}$ polymyxin B (Sigma) to rule out lipopolysaccharide interference.

Expression of E-selectin and ICAM-1

HUVECs were incubated with LIC10365 at the indicated concentrations for 1 h in RPMI-1640 medium. The protein was removed by washing, and endothelial cells were further incubated in RPMI 1640 with 10% FBS. ICAM-1 expression was assessed after 12 h. HUVECs were detached by treatment with a 0.25% trypsin and 0.02% EDTA solution and incubated in the dark, at 4 °C for 15 min, with PE-mouse

anti-human CD54 (clone HA58, BD Pharmingen). For E-selectin expression, cells were harvested after 4 h and stained with a primary anti-CD62E monoclonal antibody (MoAb) (clone 1.2B6 Immunotech) and a secondary FITC-conjugated fragment $F(ab')_2$ anti-mouse IgG (Immunotech). For nonspecific binding, anti-CD54 or anti-CD62E were replaced by a correspondent concentration of irrelevant isotype-matched IgG1. After labeling, the cells were washed, fixed with 1% paraformaldehyde and analyzed by flow cytometry in a FACScan cytofluorimeter (Becton Dickinson, Mountain View, CA). Appropriate settings of forward and side scatter gates were used to examine 10 000 cells per experiment. The percentage of positive cells was determined by the thresholds set using isotypic controls.

All animal studies were approved by the Ethics Committee of the Faculty of Exact Sciences, University of La Plata, Argentina, and of the Instituto Butantan, Sao Paulo, Brazil.

Results

Bioinformatic analysis of the LIC10365 sequence

The gene encoding LIC10365 was identified in the chromosome I by analysis of the probable ORFs present in the genome of *L. interrogans* serovar Copenhageni (Nascimento *et al.*, 2004a, b). The gene consists of 1080 base pairs, encoding a protein of 359 amino acid residues. LIC10365 is predicted to be an outer-membrane lipoprotein, based on the search for sequence motifs by the servers Lipo-P (Juncker *et al.*, 2003), TMHMM (Schultz *et al.*, 1998) and PSORT (Nakai & Kanehisa, 1991). According to the LipoP predictor, there is one signal cleavage type II at amino acids 16–17 and two signal cleavages type I, at 22–23 and 27–28 positions, being the best score prediction at amino acids 16–17 SpII site (Juncker *et al.*, 2003). BLAST analysis showed that LIC10365 is part of a paralogous family that includes LIC11207 (50% identity, 65% similarity), LIC10821 (48% identity, 64% similarity), LIC10774 (38% identity, 58% similarity) and LIC11030 (35% identity, 48% similarity) (Altschul *et al.*, 1997; Schäffer *et al.*, 2001). In addition, this predicted protein family has in common a domain of unknown function (DUF 1565) according to the PFAM server (Finn *et al.*, 2006). The theoretical pI (isoelectric point) and molecular mass (M) of the cleaved mature polypeptide are 5.62 and 36400.41 Da, respectively. Identical and similar predicted coding sequences of LIC10365 were identified in *L. interrogans* serovar Lai (Ren *et al.*, 2003) and in *Leptospira borgpetersenii* serovar Hardjo–bovis strain JB197 (Bulach *et al.*, 2006) genome sequences.

Protein expression, purification and Western blotting

The rLIC10365 protein was expressed using the *E. coli* C43 (DE3) strain. This strain, initially described by Miroux & Walker (1996), is reported to grow successfully to high cell densities and express membrane and globular proteins without toxic effects to *E. coli*. The expected recombinant protein, of *c.* 37.7 kDa, was expressed in the induced culture (Fig. 1a), although a small protein leak was visible under noninduced conditions. The rLIC10365 protein was expressed in an insoluble form, as inclusion bodies, and had to be solubilized and refolded before loading onto a Ni^{2+}-charged chromatographic column. The purified recombinant protein was recovered from the column with 1 M imidazole as a major protein band, as shown in Fig. 1b. The reactivity of the recombinant protein with mouse anti-rLIC10365 serum showed a major reactive band corresponding to the rLIC10365 (Fig. 1c and d) either of its purified form or of induced bacterial cell lysate. The lower molecular mass protein bands seen in Fig. 1d are probably due to some protein degradation.

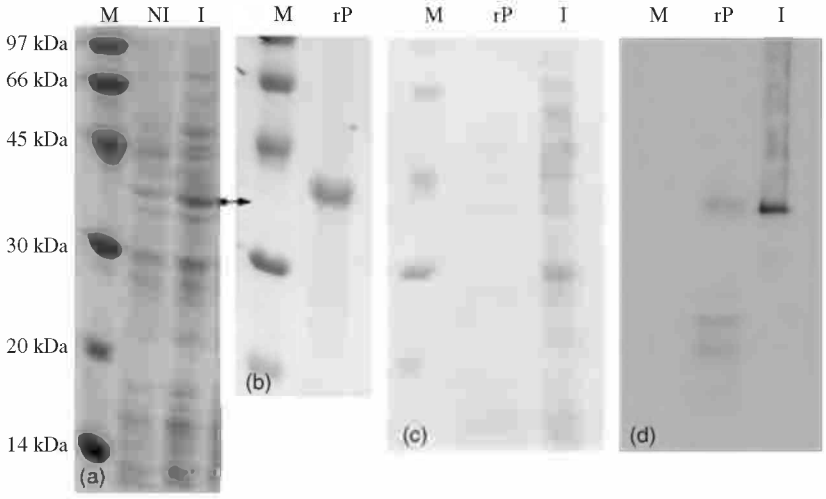

Fig. 1. Expression, purification and Western blotting of the rLIC10365. (a) Protein expression of *Escherichia coli* C43(DE3) strain transformed with pAE-LIC1065 by 12% SDS-PAGE. (b) Analysis of rLIC10365 protein after elution from Ni^{2+} – charged beads with 1 M imidazole. (c) Blotted proteins in membrane stained with Ponceau S. (d) Reactive protein bands probed with a mouse serum (1 : 1000) against rLIC10365. NI and I, noninduced and induced total bacterial extract, respectively. rP, purified recombinant protein; M, low molecular mass protein marker.

Gene and protein conservation among *Leptospira* strains

The conservation of the *LIC10365* gene in five pathogenic strains and in one saprophytic nonpathogenic strain of *Leptospira* was examined by PCR with the same pair of primers used previously for gene amplification. The band corresponding to 1038-bp DNA fragment of *LIC10365* was amplified by PCR in three strains belonging to the pathogenic species of *L. interrogans* (serovars Copenhageni, Hardjo and Icterohaemorraghie). No amplification product was detected in the strains belonging to the serovars Canicola, Pomona or in the nonpathogenic strain Patoc 1 (*L. biflexa* serovar Patoc) (Fig. 2a). The expression of *LIC10365* by *in vitro* cultured leptospires was evaluated by PCR amplification of reversely transcribed total RNA. The results obtained revealed the presence of *LIC10365* transcripts in all the three strains mentioned above (Fig. 2b). DNA contamination was discarded as no amplification was observed in the absence of reverse transcriptase (Fig. 2c). The integrity of the samples was verified by amplification of a 1042-bp 16S ribosomal cDNA fragment (not shown).

Immunohistochemistry evaluation with serum anti-rLIC10365

Kidney tissues from guinea-pigs obtained at 14 days p.i. showed well conserved cortical and medullary architecture. A few glomeruli were shrunken or contracted; likewise some tubules contained proteinaceous material mixed with erythrocytes. An early mixed-cell infiltrate was occasionally noted near larger vessels. *Leptospira interrogans* antigens located at the renal tubular lumen stained positive with the rLIC10365 antiserum (Fig. 3b). In contrast, little LIC10365 antigen was observed at the sites of interstitial inflammatory cell infiltrates. Compared with LipL32, a major antigen expressed in leptospires (Haake *et al.*, 2000; Nally *et al.*, 2007) and used as a positive control of the infection, staining of the LIC10365 protein was less intense (Fig. 3c). In negative control experiments, the rLIC10365 antiserum did not show reactivity to normal guinea-pig kidney sections (Fig. 3a).

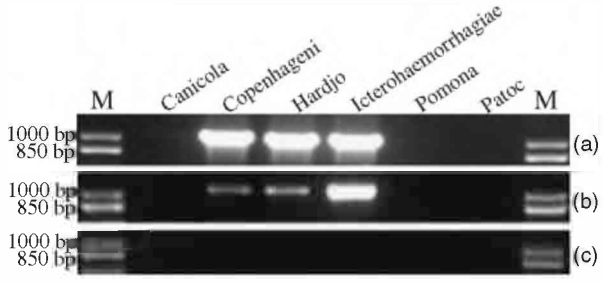

Fig. 2. DNA conservation and expression among leptospiral strains. (a) Genomic DNA from *Leptospira biflexa* Patoc and from five serovars of *Leptospira interrogans* pathogenic species were subjected to PCR analysis with LIC10365-specific primers. The expected size of the PCR product is 1038 bp. (b) RT-PCR analysis of LIC10365 performed with the same primer pairs mentioned above. (c) Reverse transcriptase omitted. M, molecular weight markers.

Endothelial expression of E-selectin and ICAM-1

To determine whether endothelial cells were activated by exposure to rLIC10365, cultures of HUVECs were treated with the indicated concentrations of rLIC10365 and the surface levels of E-selectin and ICAM-1 were evaluated by fluorescence-activated cell sorter analysis. These molecules have been shown to be upregulated on activation of the endothelium by the pathogenic spirochaetes *Borrelia* sp. (Sellati *et al.*, 1995, 1996; Shamaei-Tousi *et al.*, 2000) and *Treponema* sp. (Lee *et al.*, 2000, 2005). The results show that the basal expression of both CAMs was markedly increased after treatment with rLIC10365 in a concentration-dependent manner (Fig. 4a and b). In order to exclude the participation of lipopolysacharides in the activation process, the lipopolysacharide content of the sample was estimated to be 1.2×10^{-2} μg mL^{-1} by the LAL assay. The induction of ICAM-1 on HUVECS by lipopolysaccharides was evaluated in the absence and in the presence of 7 μg mL^{-1} of polymyxin B (Fig. 4c). As a control, we included another recombinant protein, rLIC10793, that although it had similar lipopolysacharides content to rLIC10365, did not enhance ICAM-1 expression. From this experiment, it is clear that the concentration of polymyxin was sufficient to inactivate the lipopolysacharides content in the protein samples, thus ruling out lipopolysacharides interference on the induction of CAM by rLIC10365.

Discussion

Several surface membrane proteins have been predicted during genome annotation of *L. interrogans* serovar Copenhageni (Nascimento *et al.*, 2004a), some of which are hypothetical proteins of unknown function but, due to their putative location, are believed to play a role in host–pathogen interactions. To date, four extracellular matrix (ECM) leptospiral adhesins have been published: a 36-kDa fibronectin-binding protein of unknown identity isolated from the outer sheath of a virulent variant of pathogenic leptospires (Merien *et al.*, 2000), a 24-kDa laminin-binding protein named Lsa24 (Barbosa *et al.*, 2006)/LfhA (Verma *et al.*, 2006) and LigA, LigB proteins (Choy *et al.*, 2007). Although binding protein receptors of CAM have been described for the pathogenic spirochetes *Borrelia* sp. and *Treponema* sp. (Sellati *et al.*, 1995, 1996; Lee *et al.*, 2000, 2005), to date, no leptospiral protein has been identified as a CAM inducer.

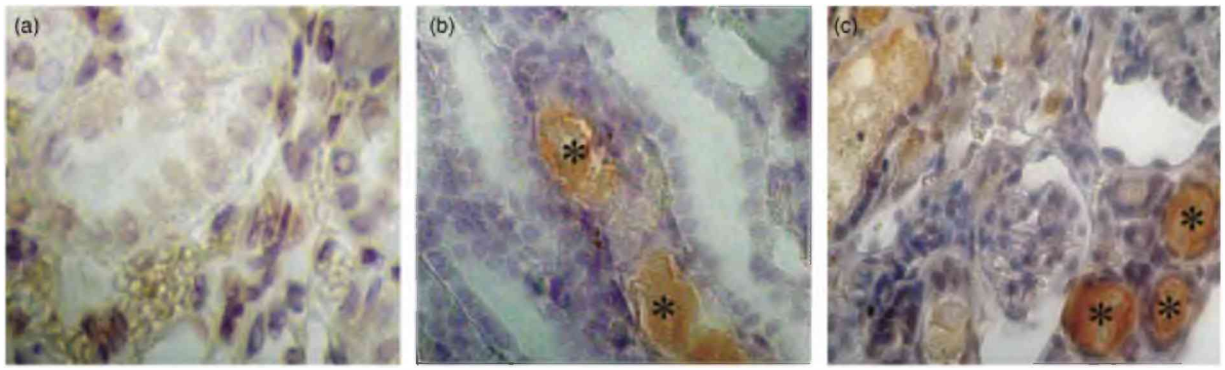

Fig. 3. Immunohistochemistry of the kidney. Tissue obtained at 14 days postinfection with virulent *Leptospira interrogans* after haematoxylin counterstaining. Antigen (*) was detected on leptospires within the renal tubular lumen with both rLIC10365 (b) and LipL32 (c) antiserum. No staining was observed with LIC10365 antiserum in the kidney from uninfected animal (a). A: × 250; B and C: × 400.

The selected LIC10365 coding sequence is part of a paralogous family that comprises LIC11207, LIC10821, LIC10774 and LIC11030. Additionally, this protein family shares a domain of unknown function, DUF 1565, that is found in several bacteria and in the archaeon *Methanosarcina acetivorans* (Altschul *et al.*, 1997; Schäffer *et al.*, 2001; Finn *et al.*, 2006). The best score prediction for lipoprotein signal peptide (SPaseII) was obtained at the 16–17th position by the LipoP method (Juncker *et al.*, 2003). For the recombinant prokaryotic expression, plasmid pAE-LIC10365 was designed in a manner to exclude the signal peptide region and to express an N-terminal 6X His-tag that allows rapid medium-scale purification by metal affinity chromatography. The expression of rLIC10365 was achieved using *E. coli* C43(DE3) cells, that is a BL21(DE3)-mutated strain (Miroux & Walker, 1996). Highly hydrophobic proteins, such as globular or membrane ones, frequently exhibit a toxic effect that can cause plasmid loss and instability, or even cell death. The strain used was reported to overcome the toxicity by raising the plasmidial stability, promoting higher protein expression in inclusion bodies, or by locating overexpressed protein in the membrane compartment (Miroux & Walker, 1996). The recombinant protein rLIC10365 was expressed at the expected size of *c.* 37.7 kDa, mainly in inclusion bodies. Despite the presence of contaminants, the purified protein exhibited a single main band in SDS-PAGE.

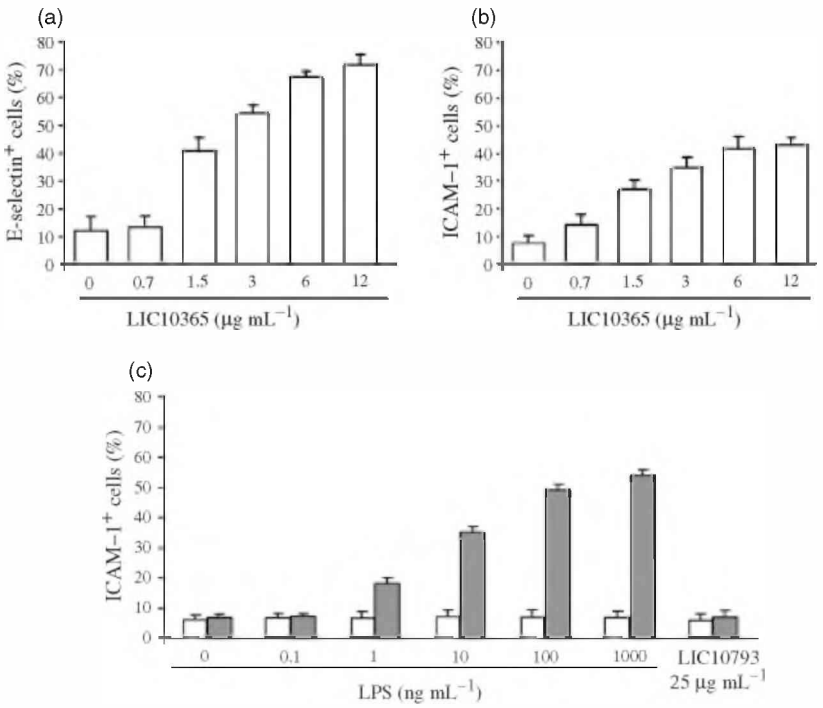

Fig. 4. rLIC10365-induced surface E-selectin and ICAM-1 expression on HUVECs. Confluent monolayers of HUVECs were stimulated for 1 h with rLIC10365, rLIC10793 or lipopolysacharides at the indicated concentrations with (white bars) or without (gray bars) 7 μg mL^{-1} of polymyxin B. Then medium was removed and cells were cultured with RPMI-1640 supplemented with 10% FBS. (a) E-selectin expression was evaluated 4 h poststimulation. (b–c) ICAM-1 expression was evaluated 12 h poststimulation. Data shown are mean ± SEM of four independent experiments.

This main band was recognized by antibodies present in mouse serum raised against rLIC10365 as a major protein.

The gene *LIC10365* was found to be conserved among three of the five pathogenic leptospiral strains analyzed and absent in one nonpathogenic strain. Although further confirmation will be required, the absence of some genes in certain serovars belonging to *L. interrogans* is not new. Genes that are exclusive to serovars Copenhageni or Lai have been documented previously (Nascimento *et al.*, 2004a). The *LIC10365* gene is present in serovar Lai of *L. interrogans* (Ren *et al.*, 2003) and has a similar counterpart in *L. borgpetersenii* serovar Hardjo–bovis (Bulach *et al.*, 2006). *Leptospira interrogans* and *L. borgpetersenii* pathogenic species combined encompass 140 strains and 125 diverse antigenic brand referred to as serovars (Brenner *et al.*, 1999). As expected, *LIC10365* transcripts were only detected in the strains previously shown to harbor the gene, indicating mRNA expression under *in vitro* conditions.

In fact, the results indicate that the *LIC10365*-encoded protein is expressed both during cultivation and during infection. This is relevant because some proteins found in organisms grown under culture conditions are not expressed during infection. For example, LipL36 is expressed at high levels by cultivated *Leptospira kirschneri* but is not detectable either within infected hamster kidneys by immunohistochemistry or by sera from hamsters infected with a host-derived organism (Haake *et al.*, 1998; Barnett *et al.*, 1999). In contrast, LipL32 is detectable both by immunohistochemistry and by immunoblot (Haake *et al.*, 2000). The immunohistochemistry data presented here show that *LIC10365*-encoded protein, such as OmpL1, LipL41 and LipL32, among others (Barnett *et al.*, 1999), is expressed by *L. interrogans* within the renal tubules of experimentally infected guinea-pigs (Fig. 3).

Upregulation of ICAM-1 and E-selectin expression was observed after the treatment of cultured endothelial cells with rLIC10365. The low lipopolysacharide content ($10^{-2}\,\mu g\,mL^{-1}$) of the protein sample, in addition to the treatment with the antibiotic polymyxin B, excluded any possibility of residual *E. coli* lipopolysacharides interference with the results. In addition, the rLIC10793 protein that has a similar lipopolysacharide content of the rLIC10365 failed to induce CAM expression. The upregulation of CAMs induced by the recombinant protein and the detection of the leptospiral protein in experimentally infected animals are difficult to compare in a timely way. As a new hypothetical protein, the goal was to show that this protein is expressed by the leptospires during infection. In a previous study by Sellati *et al.* (1996) only the lipidated form of the recombinant protein OspA of *Borrelia burgdoferi* was capable of promoting upregulation of CAMs. In the present case the increase of ICAM-1 and E-selectin expression was induced by the nonlipidated protein in HUVECS, suggesting that in the present experimental model, the lipid moiety is not involved in this interaction.

These findings indicate that rLIC10365 triggers endothelial cell activation by promoting the expression of ICAM-1 and E-selectin, which are essential adhesion molecules for cell–cell and cell–extracellular matrix interactions, and for neutrophil recruitment and migration through endothelial cells. It is widely known that many pathogenic bacteria infect host tissues through their ability to bind to CAMs by exploiting these receptors as a means of uptake. Based on these assertions and the fact that rLIC10365 is expressed during infection, it is possible that the protein encoded by the gene *LIC10365* may play an important role during pathogenesis by eliciting expression of the host cell receptors. However, upregulation of these adhesion molecules *in vivo* remains to be ascertained.

This is the first report of a leptospiral protein that is capable of up-regulating the expression of endothelial cell adhesion molecules ICAM-1 and E-selectin, thus providing a possible mechanism for some of the inflammatory features related to the endothelial damage observed in leptospirosis.

Acknowledgements

This work was supported by FAPESP, CNPq and Fundação Butantan, Brazil, CABBIO 5102/04 and PICT 13768, Argentina. M.L.V. has an MSc fellowship from FAPESP (Brazil). L.P.D. and A.M.H. have a CONICET (Argentina) fellowship. The authors thank Jim Hesson for critically reading the manuscript.

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