FEBS Letters 583 (2009) 1381–1385

FEBS Letters

journal homepage: www.FEBSLetters.org

Leptospiral TlyC is an extracellular matrix-binding protein and does not present hemolysin activity

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ARTICLE INFO

Article history:
Received 23 January 2009
Revised 10 March 2009
Accepted 21 March 2009
Available online 27 March 2009

Edited by Michael Ibba

Keywords:
TlyB
TlyC
Hemolysis
Extracellular matrix
Leptospira

ABSTRACT

The role of TlyA, TlyB and TlyC proteins in the biology of *Leptospira* is still uncertain. Although these proteins have been considered as putative hemolysins, we demonstrate that leptospiral recombinant TlyB and TlyC do not possess hemolytic activity. However, further experiments showed that TlyC is a surface-exposed protein that seems to bind to laminin, collagen IV and fibronectin. The expression of both proteins was detected both in vitro and in vivo. Our findings suggest that TlyB and TlyC are not directly involved in hemolysis, and that TlyC may contribute to *Leptospira* binding to extracellular matrix (ECM) during host infection.

1. Introduction

Leptospirosis, the disease caused by *Leptospira*, has a mortality rate as high as 18%, especially in developing countries [1]. This disease of great medical and economic importance also occurs in temperate and developed countries, mainly associated with occupational and recreational activities [2].

The sequencing of five *Leptospira* genomes [3] allowed identification of several loci coding for putative factors presumed to play a role in pathogenesis. However, many of the proteins encoded by these loci have not yet been studied or completely explored. Actually, in most cases, their putative activities remain to be confirmed.

The Tly proteins (TlyA, TlyB and TlyC) have been described as hemolysins in *Brachyspira hyodysenteriae*, and are considered important virulent factors contributing to the disease caused by this spirochete [4]. However, the purified beta-hemolytic protein of *B. hyodysenteriae* was not found to be related to Tly proteins [5]. On the other hand, Zhang et al. [6] reported that crude extracts of *Escherichia coli* expressing the *Leptospira* orthologs of TlyA and TlyC (called HlyC by these authors) were able to produce clear hemolytic zones in sheep blood agar plates, whereas TlyB was not tested. To clarify their function, we cloned, expressed and purified *Leptospira* TlyB and TlyC proteins. The recombinant proteins were tested for their hemolytic potential and for the ability to bind to extracellular matrix (ECM) components. Moreover, the expression of both proteins in *Leptospira* was evaluated in vitro and in vivo.

2. Materials and methods

2.1. Leptospira strains and culture

Leptospira strains were obtained from the Laboratório de Zoonoses Bacterianas, FMVZ, Universidade de São Paulo. They were

Abbreviation: ECM, extracellular matrix

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0014-5793/$36.00
doi:10.1016/j.febslet.2009.03.050

cultured as described [7]. Virulence of *Leptospira interrogans* serovar Pomona strain Fromm was maintained by iterative passages in Golden Syrian hamsters.

2.2. Cloning, expression, purification of recombinant proteins and antisera production

The *tly* genes were amplified from DNA of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, using the primers described in Supplementary Table 1. The expected molecular mass of the recombinant and native proteins are also shown in Supplementary Table 1. The coding DNA fragment for TlyC excludes the sequence predicted to be a transmembrane region (amino acids 1–161); thus, the truncated recombinant protein was called rTlyC162-444. The cloning, expression and purification of the recombinant proteins were achieved essentially as described [8]. Antisera against rTlyB and rTlyC162-444 were produced as described [8], and were high-titered (Supplementary Fig. 1).

2.3. Hemolytic activity assay

A reaction mix (100 μl) containing 5% (vol/vol) sheep erythrocyte suspension and 20 mM $MgCl_2$ in PBS was incubated with 8 μg of each recombinant protein for 90 min at 37 °C. Following centrifugation, hemolysis was determined by reading the absorbance at 420 nm. As a positive control, 0.8 μg of sphingomyelinase C from *Staphylococcus aureus* (Sigma) was used, whereas a non-hemolytic recombinant protein from *Schistosoma mansoni* (Sm14) was used as a negative control [9]. All experiments were performed in triplicate and repeated three times. The hemolytic assay on sheep agar plates was performed as described [6].

2.4. Immunoelectron microscopy

Leptospires (*L. interrogans* serovar Pomona strain Fromm) were washed three times with PBS and fixed with 3% paraformaldehyde in PBS for 60 min, at room temperature. The surface exposure of TlyB, TlyC and LipL32 was then examined by an immunogold assay, essentially as described [10]. A non-immune serum was used as a negative control. Grids were examined with an electron microscope (Zeiss EM 109) at an accelerating voltage of 80 kV.

2.5. ECM-binding assays

The binding of the recombinant proteins to ECM components, the dose-response curves and the inhibition assays were performed as described [7]. The inhibition of *Leptospira* (*L. interrogans* serovar Pomona strain Fromm) binding to ECM was carried out in wells coated with 100 μg of Matrigel, and 8 μg of the recombinant Tly proteins were used. All macromolecules were purchased from Sigma Chemical Co. (St. Louis, MO). All experiments were performed in triplicate and repeated three times.

2.6. RNA extraction and reverse transcriptase-PCR (RT-PCR) analysis

Leptospires were cultured at three different temperatures (20, 29 and 37 °C). RNA extraction and RT-PCR were performed as described [11]. The amplification was performed using the oligonucleotides used for cloning, except for TlyB forward primer: 5′ CCGAATCCGATAAACTAC 3′.

2.7. Immunoblot analysis

The immunoblot was performed as described [8].

2.8. In vivo detection of TlyB and TlyC by immunohistochemistry

Hamsters were inoculated intraperitoneally with 0.5 ml of PBS containing $10^{2.5}$ bacteria (*L. interrogans* serovar Icterohaemorrhagiae), sacrificed 2 weeks post infection when animals showed symptoms (e.g., weight loss, lethargy), and their kidneys were harvested and processed for the immunohistochemical analyses, performed as described [12]. Mock infected animals were used as controls.

3. Results

3.1. Recombinant TlyB and TlyC162-444 do not possess hemolytic activity

We observed that rTlyB and rTlyC162-144 are α-helix-structured (according to circular dichroism spectra, Supplementary Fig. 2), but were not able to lyse sheep red blood cells, in contrast to the positive control *S. aureus* sphingomyelinase, that fully induced hemolysis (Fig. 1). Accordingly, crude *E. coli* extracts expressing rTlyB and rTlyC162-444 were not able to produce hemolytic zones on sheep blood agar plates (Supplementary Fig. 3). Recombinant TlyC162-444 was expressed as a truncated protein lacking the first 161 amino acids that are predicted to compose the transmembrane region of TlyC; thus, it represents the surface-exposed region of the protein, and the results and conclusions obtained for TlyC are limited to this region.

3.2. TlyC is exposed on the leptospiral surface

Further characterization of Tly proteins focusing on cellular localization was assessed by immunoelectron microscopy (Fig. 2). Twelve bacteria incubated with each serum (anti-TlyB and anti-TlyC162-444) and with control sera (a negative non-immune serum and a positive anti-LipL32) were randomly chosen for quantification of colloidal gold particles bound to the bacterial surface. Anti-TlyC162-444 and anti-LipL32 means were statistically different from the non-immune mean. The anti-LipL32 mean are relative higher than the anti-TlyC162-444, but this can be attributed to the fact that LipL32 is the major outer membrane protein of pathogenic leptospires [13].

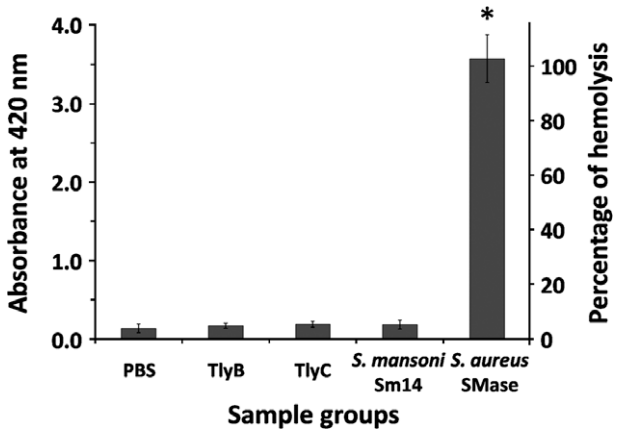

Fig. 1. Assessement of the hemolytic activity of rTlyB and rTlyC162-444 on sheep erythrocytes. Results (means ± SD) are expressed as the percentage of hemolytic activity in relation to the complete lysis of erythrocytes in distilled water. The asterisks indicate a significant difference from the control PBS at $\alpha = 0.001$ in the Tukey–Kramer test.

3.3. TlyC interacts with extracellular matrix proteins

We next investigated whether Tly proteins, notably TlyC, could establish interactions with host molecules such as ECM proteins. As depicted in Fig. 3A, rTlyC162-444 specifically binds to laminin, collagen IV and plasma fibronectin while no specific binding was detected with rTlyB. A dose-response curve shows that rTlyC162-444 has a slightly higher affinity for plasma fibronectin (Fig. 3B). Recombinant TlyC162-444 binds with high affinity to the proteolytic fragment F30 of plasma fibronectin, and only marginally to the fragment F45 (Fig. 3C). Heparin could inhibit rTlyC162-444 binding to F30 in a concentration-dependent manner (Fig. 3D). The adhesion of leptospires to Matrigel was reduced by 17% when rTlyC162-444 was used as a competitor (Supplementary Fig. 4).

3.4. TlyB and TlyC are expressed in vitro and in vivo

RT-PCR results indicate that all *tly* genes are transcribed in *L. interrogans* Pomona Fromm (Fig. 4A), even when the cultures were grown at different temperatures, but transcription of *tlyA* and *tlyC*

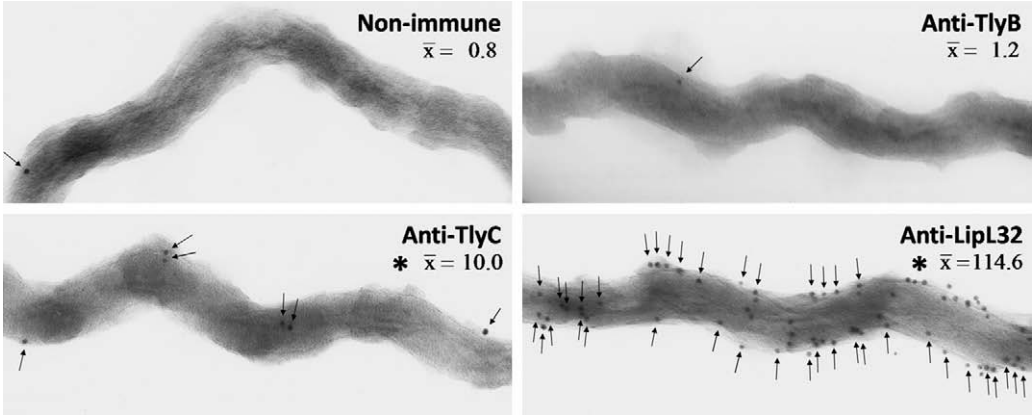

Fig. 2. Surface labeling of *Leptospira interrogans* by immunoelectron microscopy. Representative micrographs of leptospires treated with each serum are shown. Colloidal gold particles bound to surface of the leptospires are indicated by arrows. The mean number of particles detected per organism is showed for each serum. Asterisks indicate a significant difference from the non-immune control mean at $\alpha = 0.005$ in Student's two-tailed *t*-test. Original magnification: 250000×.

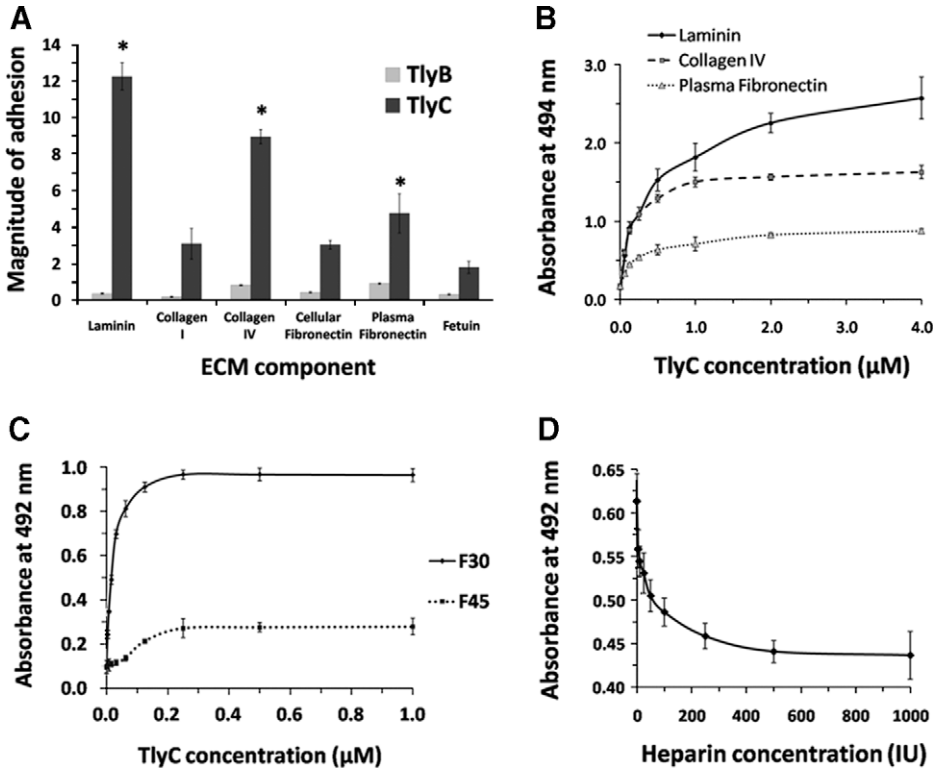

Fig. 3. (A) rTlyB and rTlyC162-444 binding to ECM components. The magnitude of adhesion represents n-fold greater binding relative to the average binding to BSA. The asterisks indicate a significant difference from the control fetuin at $\alpha = 0.001$ in the Tukey–Kramer test. (B) Binding of rTlyC162-444 to laminin, collagen IV and plasma fibronectin as a function of protein concentration. Apparent K_ds of rTlyC binding to ECMs are 184 ± 13 nM for laminin, 105 ± 1 nM for collagen IV and 89 ± 7 nM for plasma fibronectin. (C) Binding of rTlyC162-444 to proteolytic fragments of plasma fibronectin (F30 and F45) as a function of recombinant protein concentration. (D) Binding of rTlyC162-444 to F30 was assayed in the presence of increasing amounts of heparin. For (B–D) each point represents mean ± SD.

was more evident when *Leptospira* were cultured at higher temperatures. TlyB and TlyC proteins were also detected in crude extracts of several *Leptospira* strains grown at 29 °C (Fig. 4B). Curiously, TlyB but not TlyC was identified in the saprophyte *Leptospira biflexa* serovar Patoc, although it harbors both genes [5]. Compared to the recombinant protein, native TlyC has a higher molecular mass, as expected. Furthermore, the expression of TlyB and TlyC was detected in vivo, during *Leptospira* infection in hamsters (Fig. 4C). LipL32 expression was also detected (positive control, not shown), but uninfected tissue did not show reactivity with any serum (Fig. 4C).

4. Discussion

Controversy about the function of Tly proteins is apparent in the report describing that crude extracts of *E. coli* expressing the *Brachyspira* Tly proteins display hemolytic activity in blood agar plates [4], although the hemolysin purified from *Brachyspira* was not related to the sequence of Tly proteins [5]. Indeed, Hsu et al. [5] pointed out that *E. coli* can express cryptic hemolytic proteins, and that the expression of a heterologous gene can induce a hemolytic phenotype in the bacteria by the activation of the transcription of cryptic hemolytic genes, a phenomenon already reported in studies of the *Salmonela* SlyA protein [14]. In *Leptospira,* the only report on hemolytic activity of Tlys was also based on extracts of *E. coli* expressing the recombinant proteins [6]. Here, we tested if TlyB and TlyC are truly hemolysins, through the use of recombinant proteins (TlyA could not be expressed using the system described here).

We observed that purified recombinant TlyB and TlyC162-444 proteins were not able to lyse sheep erythrocytes, and that crude extracts of *E. coli* expressing both proteins did not produce hemolytic zones on sheep blood agar plates. Our results suggest that neither TlyB nor TlyC possess hemolytic activity, in contrast to those previously reported by Zhang et al. [6], in which TlyC (or HlyC) was claimed to be a hemolysin. However, as we used a truncated recombinant protein representing solely the surface-exposed portion of TlyC, we can not rule out the possibility that an intact full length protein may be required for proper TlyC activity. Alternatively, TlyC may need a co-factor or may act indirectly as a hemolysin. With regard to Zhang's results, it is possible that *E. coli* expressing TlyC may have also expressed other proteins, such as the cryptic hemolysin SheA [5], conferring a non-specific hemolytic phenotype, although this was not observed in our conditions (Supplementary Fig. 3).

Since the putative activity of Tly proteins has not been confirmed, we investigated novel aspects of these proteins. LipL32, the major outer membrane protein characterized on the *Leptospira* surface [13], was previously associated with hemolytic features [15], but has recently been shown to have an additional function related to ECM binding [16].

As leptospiral TlyC protein is exposed on the bacterial surface (Fig. 2), we wondered whether it could interact with host components, particularly with ECM proteins. We observed that rTlyC162-444 interacts with laminin, collagen IV and plasma fibronectin with high affinity (Fig. 3). Recombinant TlyC162-444 binds to laminin with a slightly lower affinity (K_d = 184 nM) when compared to LenB (K_d = 118 nM), the member of the Len family of laminin-binding *Leptospira* proteins showing the lower K_d [17]. The apparent K_d calculated for rTlyC162-444-fibronectin binding (89 nM) was similar to the K_d calculated for the fragments of other ECM-binding *Leptospira* proteins, LigA and LigB (ranging from 69 to 132 nM) [18]. Recombinant TlyC162-444 binds to the heparin-binding domain of fibronectin (fragment F30), which is presumed to be the major acceptor site for bacterial binding [19], and this interaction could be partially inhibited by heparin (Fig. 3D). Furthermore,

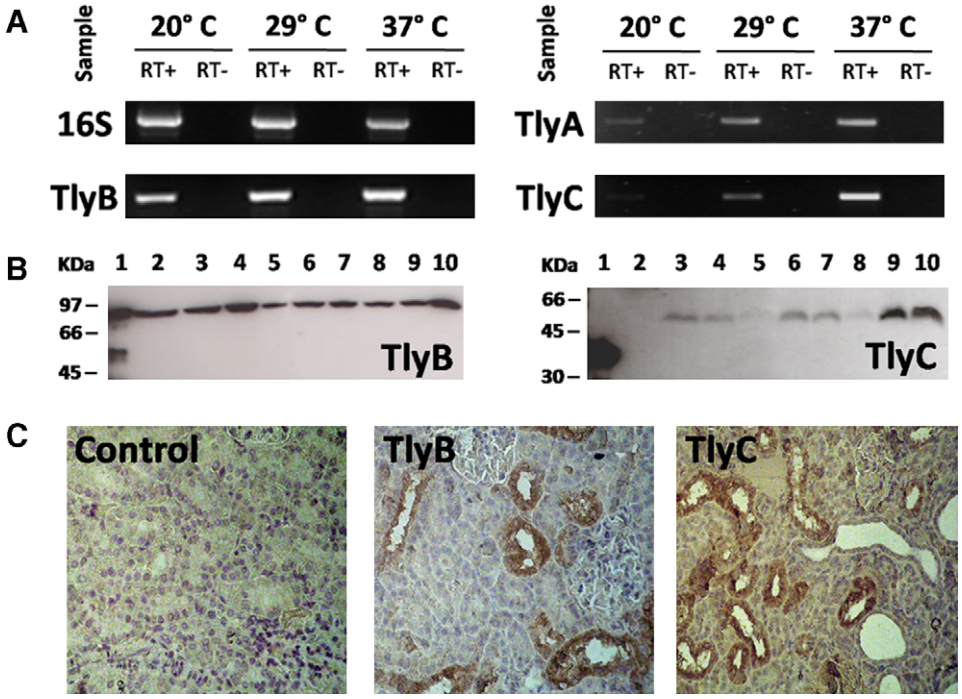

Fig. 4. (A) RT-PCR analysis of *tlyA*, *tlyB* and *tlyC* transcripts in leptospires. Samples quantity and integrity were verified by amplification of a 16S ribosomal cDNA fragment. RT+: reverse transcriptase present; RT−: reverse transcriptase omitted. (B) Expression of the native proteins TlyB and TlyC in a panel of several *Leptospira* serovar extracts. Lanes (serovar and strain are, respectively, shown after leptospire species): 1 = Recombinant protein; 2 = *L. bifexa* Patoc PatocI; 3 = *L. interrogans* Copenhageni M20; 4 = *L. interrogans* Pomona Pomona; 5 = *L. interrogans* Pomona Fromm; 6 = *L. interrogans* Hardjo Hardjoprajitno; 7 = *L. interrogans* Autumnalis Akiyami A; 8 = *L. interrogans* Pyrogenes Salinem; 9 = *L. interrogans* Canicola Hond-UtrechtIV; 10 = *L. kirshneri* Grippothyphosa MoskvaV. (C) In vivo detection by immunohistochemistry of TlyB and TlyC proteins in renal tubules of hamsters infected with leptospires. Original magnification: 250×.

Leptospira adhesion to Matrigel was partially inhibited by a competitive assay with rTlyC162-444 (Supplementary Fig. 4). This inhibition (17%) was similar to that obtained by Barbosa et al. [7] using LenA/Lsa24 (~17% inhibition of *Leptospira* binding to laminin), but smaller than that obtained with LigA (39% inhibition of *Leptospira* binding to fibronectin) [18].

Interestingly, *tlyC* transcripts were detected at 37 °C (Fig. 4A), which strongly suggests that the protein is expressed during infection. Indeed, anti-TlyC162-444 serum labeled renal tubules of hamsters infected with leptospires (Fig. 4C), and anti-TlyC162-444 antibodies were detected in leptospirosis patients (Supplementary Fig. 5), which further supports that this protein is expressed and presented to the immune system during infection. However, both rTlyB and rTlyC162-444 were not protective against leptospirosis in a hamster model (data not shown).

In conclusion, our results suggest that TlyB and TlyC do not exert a direct hemolytic activity in vitro, in contrast to previous reports on TlyC. Our data indicate that TlyC is a surface-exposed protein that interacts with major ECM components. Therefore, it may play a role in *Leptospira* attachment to host during infection and colonization of target tissues, contributing to the pathogenesis of the disease.

Acknowledgments

We express our gratitude to Felipe Santos and to Débora Silva for their technical assistance. We thank Dr. Albert Leyva for English editing of the manuscript. This work was supported by grants from FAPESP, CNPq, and Fundação Butantan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.03.050.

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