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Original Article

The multifaceted roles of *Leptospira* enolase

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Abstract

A previous study had demonstrated that *Leptospira* enolase is secreted extracellularly by a yet unknown mechanism and reassociates with the bacterial membrane. Surface-anchored leptospiral enolase displays plasminogen binding activity. In this work, we explored the consequences of this interaction and also assessed whether *Leptospira* enolase might display additional moonlighting functions by interacting with other host effector proteins. We first demonstrated that enolase-bound plasminogen is converted to its active form, plasmin. The protease plasmin targets human fibrinogen and vitronectin, but not the complement proteins C3b and C5. *Leptospira* enolase also acts as an immune evasion protein by interacting with the negative complement regulators C4b binding protein and factor H. Once bound to enolase, both regulators remain functional as cofactors of factor I, mediating cleavage of C4b and C3b. In conclusion, enolase may facilitate leptospiral survival and dissemination, thus contributing to bacterial virulence. The identification and characterization of moonlighting proteins is a growing field of bacterial pathogenesis, as these multifaceted proteins may represent potential future therapeutic targets to fight bacterial infections.

Keywords: Leptospira; Enolase; Plasminogen; C4b binding protein; Factor H

1. Introduction

Pathogenic *Leptospira* species may cause leptospirosis, a highly frequent tropical disease that represents an important public health problem. These spirochetes have evolved virulence strategies to colonize a variety of hosts. During infection, they express membrane proteins capable of binding to extracellular matrix molecules and host cells [1–8]. Virulent *Leptospira* strains have also devised mechanisms to overcome the host's innate immune responses. It has been shown that they can resist complement-mediated killing by recruiting soluble complement regulators such as factor H (FH), C4b binding protein (C4BP) and vitronectin. In this way, they are able to control all steps of the complement cascade [9–11]. Another interesting feature related to *Leptospira* virulence is its capacity to bind human plasminogen, which is converted to plasmin in the presence of the host's specific activators [12,13]. This key enzyme of the coagulation system targets a number of substrates, including fibrinogen, fibrin, complement proteins C3 and C5, vitronectin, osteocalcin, coagulation factors V, VIII and X, injury-induced aggregated proteins, protease-activated receptor 1 and some collagenases (reviewed in [14]).

A number of *Leptospira* surface-exposed proteins have been shown to act as adhesins, invasins or evasins (reviewed in [15]). Alongside these outer membrane proteins, the involvement of moonlighting proteins in *Leptospira* pathogenesis has been evaluated in the last few years. This fascinating class of proteins is known to perform diverse and independent functions in a single organism, being found in different subcellular locations. To date, two *Leptospira* proteins displaying moonlighting activities have been described: elongation factor-thermal unstable (Ef-Tu), described as a ligand for host

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ECM molecules, plasminogen and complement FH [16], and the metabolic enzyme enolase, shown to interact with human plasminogen [17].

Enolases are among the most abundantly expressed cytosolic proteins. They are metalloenzymes that catalyze the conversion of 2-phosphoglycerate to phosphoenolpyruvate [18]. Eubacteria and archaebacteria have a single enolase gene (reviewed in [19]). Despite the lack of signal peptides or cognate protein secretion systems, they have been found localized at the surface of a variety of eukaryotic and prokaryotic cells, where they display multiple moonlighting functions [20]. Enolase of both Gram-positive and Gram-negative bacteria has been shown to bind host plasminogen. Functional consequences of plasminogen binding by these microorganisms include dissemination and transmigration, evasion from host's innate immune responses and enhanced adhesion to host cells, as shown for *Streptococcus pneumoniae*, known to use surface-displayed enolase to interact with cell-surface-bound plasminogen [21]. Recent findings by Mori and colleagues have shown that *S. pneumoniae* α-enolase induces cell death of neutrophils by releasing neutrophil extracellular traps [22]. Moreover, pneumococcal enolase was shown to bind the negative complement regulator C4BP, thus contributing to complement evasion [23]. Several microorganisms also employ enolase as an adhesin. In *Staphylococcus aureus* α-enolase was identified as a laminin-binding protein [24], and surface-exposed α-enolases from *Streptococcus suis*, *Lactobacillus plantarum* and *Paracoccidioides brasiliensis* were characterized as fibronectin-binding proteins [25–27]. More recently, *S. gallolyticus* enolase has been shown to interact with a surface-exposed component of cytokeratin 8 (CK8) of human colonocytes, possibly contributing to bacterial adherence and infection [28]. Oral bacteria, including *S. mutans* and *S. gordonii*, also display α-enolase on the surface, and the salivary mucin MUC7 is one of the host's targets for this moonlighting protein. α-Enolase has also been shown to mediate adhesion of *Mycoplasma suis* to porcine erythrocytes, a crucial step in the life cycle of this hemotrophic bacterium [29].

Recent published data have shown that enolase from *Leptospira interrogans* is secreted into the extracellular medium and then reassociates with the bacterial surface by interacting with outer membrane proteins [17]. Anti-enolase antibodies recognize intact, non-permeabilized leptospires, thus indicating that the protein is indeed surface-exposed. As shown for many other bacteria, membrane-anchored leptospiral enolase displays plasminogen binding activity [17].

Given the role of enolase in bacterial pathogenesis, we aimed to further characterize this multifunctional protein in *Leptospira*. In the present work, the functional consequences of plasminogen binding to leptospiral enolase were assessed, and the interaction of this particular protein with other host molecules was evaluated. Here we demonstrate that plasminogen bound to enolase is converted to plasmin, which in turn degrades natural substrates, including fibrinogen and vitronectin. Moreover, leptospiral enolase binds the complement regulators FH and C4BP. Once bound to enolase, both FH and C4BP remain functional, acting as cofactors for factor I (FI) in the cleavage of C3b and C4b, respectively. Taken together, our data suggest that enolase may contribute to leptospiral pathogenesis.

2. Materials and methods

2.1. Purified proteins, sera and antibodies

Fibrinogen (F3879), plasminogen (P7999), vitronectin (V8379), urokinase plasminogen activator (uPA) (U4010), plasminogen activator inhibitor-1 (PAI-1) (A8111) and the chromogenic substrate D-valyl-leucyl-lysine-ρ-nitroanilide dihydrochloride (V7127) were acquired from Sigma–Aldrich. Normal human sera (NHS), human FH, human C4BP, C3b, C4b and FI were purchased from Complement Technology. Goat anti-human FH and mouse anti-human C4BP were purchased from Quidel and goat anti-human C3 or C4 polyclonal antibodies were purchased from Complement Technology. All secondary peroxidase-conjugated antibodies were acquired from Sigma–Aldrich.

2.2. Cloning, expression and purification of enolase

The *L. interrogans* open reading frame LIC11954 encoding enolase was amplified by PCR from *L. interrogans* genomic DNA (L1-130) using primers F: 5′-CTCGAGTCTCATCACTCTCAAATTCAG-3′ and R: 5′-GCGAAGCTTTTATAAATTATAAAAAGTTTCCC-3′. PCR fragments were cloned into the pGEM-T Easy vector (Promega) and transformed into *Escherichia coli* DH5α. Following digestion with restriction enzymes *Xho*I and *Hind*III, fragments were subcloned into the pAE vector for expression of the recombinant protein with an N-terminal 6XHis tag [30]. The constructs were verified by DNA sequencing with appropriate vector-specific primers. Expression of the recombinant protein in mid-log-phase cultures of *E. coli* BL21 (DE3) was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37 °C. After 3 h of incubation, cells were harvested by centrifugation and the bacterial cell pellet was resuspended in a solution containing 20 mM sodium phosphate (pH 7.4) and 100 mM NaCl and lysed in a sonicator. The his-tagged protein was purified using the AKTA Purifier 10 system (GE Healthcare). The suspension was loaded onto a Ni^{2+}-charged chelating Sepharose HisTrap HP (GE Healthcare). Contaminants were washed away with a solution containing 20 mM sodium phosphate (pH 7.4), 500 mM NaCl and 20 mM imidazole. The recombinant protein was then eluted with a solution containing 20 mM sodium phosphate (pH 7.4), 500 mM NaCl and increasing amounts of imidazole from 20 to 500 mM. The protein was extensively dialyzed against phosphate-buffered saline (PBS) at 4 °C for 48 h. Purified protein samples were analyzed by 12% SDS–PAGE.

2.3. Plasminogen activation

Microtiter plate wells were coated with recombinant proteins (10 μg/mL) or with BSA and blocked with 3% BSA diluted in PBS. Plasminogen (20 μg/mL) was added and