



Biochemical and functional analysis of TIR domain containing protein from *Brucella melitensis*

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ARTICLE INFO

Article history:

Received 3 May 2010

Available online 13 May 2010

Keywords:

Brucella

TcpB

TIR domain

NF-κB

Phosphoinositide

Inflammation

ABSTRACT

Toll/interleukin-1 like receptors are evolutionarily conserved proteins in eukaryotes that play crucial role in pathogen recognition and innate immune responses. *Brucella* are facultative intracellular bacterial pathogens causing brucellosis in animal and human hosts. *Brucella* behave as a stealthy pathogen by evading the immune recognition or suppressing the TLR signaling cascades. *Brucella* encode a TIR domain containing protein, TcpB, which suppresses NF-κB activation as well as pro-inflammatory cytokine secretion mediated by TLR2 and TLR4 receptors. TcpB targets the TIRAP mediated pathway to suppress TLR signaling. With the objective of detailed characterization, we have over expressed and purified TcpB from *Brucella melitensis* in native condition. The purified protein exhibited lipid-binding properties and cell permeability. NF-κB inhibition property of endogenous TcpB has also been demonstrated. The data provide insight into the mechanism of action of TcpB in the intracellular niche of *Brucella*.

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1. Introduction

Innate immunity is the first level of defense against an invading pathogen and pathogen recognition is mediated by a family of transmembrane receptors called Toll/interleukin-1 like receptors (TLR) [1–3]. TLRs are characterized by an extra cellular leucine-rich repeat (LRR) domain and an intracellular Toll/IL-1 receptor-like (TIR) domain [3,4]. The TIR domain is the conserved intracellular domain of TLRs and the downstream adapter molecules MyD88, TIRAP, TRAM, TRIF, and SARM [5]. Recognition of pathogen associated molecular patterns by the LRR domain of TLRs leads to the formation of a signaling complex at the cytoplasmic face which culminates into the activation of transcription factors including NF-κB and the up-regulation of several pro-inflammatory cytokines [1,6].

Microbes have evolved diverse range of strategies to subvert the innate and adaptive immune responses [7,8]. One of those strategies involves the interference of TLR signaling pathways using microbe encoded TIR domain containing proteins. Subversion of innate immune responses by TIR proteins has been described in *Brucella*, *Salmonella*, *Escherichia coli*, and *Paracoccus* [8–11]. *Brucella* are highly infectious intracellular bacteria causing brucellosis in animal and human hosts [12]. *Brucella* replicate and survive in a variety of host cells including professional and non-professional phagocytes [13,14]. *Brucella* minimally induce pro-inflammatory cytokines in the host, which is attributed to the capacity of this pathogen to evade or suppress innate immune responses [15]. *Brucella*

encodes a TIR domain containing protein (TcpB) which efficiently suppresses TLR2 and TLR4 mediated NF-κB activation and pro-inflammatory cytokine secretion [8,10,11,16]. *Brucella melitensis* deficient in the TcpB gene presented an attenuated phenotype in IRF^{-/-} mice [11]. Recent studies have revealed that TcpB targets the TLR adaptor protein, TIRAP (also known as MAL) to inhibit TLR signaling but the exact mechanism is yet to be understood [11,16].

Large scale production and purification of TIR protein from an infectious pathogen has not been previously successful hampering the detailed biochemical and structural characterization. To facilitate the in depth analysis of TcpB, we have successfully over expressed TcpB of *B. melitensis* in *E. coli* as a maltose binding protein (MBP) fusion. We have purified the protein in large quantities in non-denaturing condition and the activity of the purified protein has been verified by biochemical assays. We have demonstrated that TcpB is a cell permeable protein and the internalized TcpB could efficiently inhibit NF-κB activation induced by TLR4 receptor.

2. Materials and methods

2.1. Cloning and over expression of TcpB

Full-length TcpB gene was amplified from the chromosome of *B. melitensis* by PCR using TcpB.F (GAATTCATGTCTAAAGAGAAA-CAAGCC) and TcpB.R (CTGCAGTCAGATAAGGGAATGCAG) primers. The PCR amplicon was gel purified and cloned into pGEMT vector (pGEMTtcpB) and sequenced to verify the identity. TcpB gene was then released from pGEMTtcpB with BamHI/EcoRI and cloned into

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the corresponding sites of pMALc4G *E. coli* expression vector (NEB) to generate pMALTcPB. BL21 DE3 cells were transformed with pMALTcPB construct and the transformants were analyzed for MBP-TcpB expression. To perform this, *E. coli* BL21 cells harboring the pMALTcPB plasmid were grown at 37 °C and induced with IPTG to a final concentration of 0.5 mM when the OD₆₀₀ reached 0.6. After the induction, cells were grown at the same temperature for 3 h. Cells were then spun down, re-suspended in water and mixed with SDS-PAGE sample buffer (Bio-Rad) (1:1 ratio). The tubes were boiled for 5 min and a fraction of the boiled protein sample was analyzed by SDS-PAGE.

To perform the Western blotting, a fraction of the uninduced and induced protein samples were resolved on SDS-PAGE gel. Protein bands were then electroblotted to nitrocellulose membrane (Bio-Rad). The membrane was then blocked with 5% milk for 1 h and probed with monoclonal anti-MBP antibody conjugated with HRP (NEB) overnight. SuperSignal West Pico Chemiluminescent Substrate (Pierce) was used to detect the reaction and the signal was captured on X-ray film.

2.2. Purification of MBP-TcpB

Overnight grown *E. coli* BL21 cells harboring the pMALTcPB plasmid was inoculated (0.1%) into 1 L of LB medium with glucose (2 g) and ampicillin (100 µg/mL). The culture was grown at 37 °C and induced with IPTG to a final concentration of 0.5 mM when the OD₆₀₀ reached 0.6. After the induction, cells were grown at 25 °C for 5 h. Amylose affinity chromatography was employed for purification. Cells were collected by centrifugation and washed with phosphate-buffered saline. Cells were then re-suspended in sonication buffer containing 50 mM Tris-HCl [pH 8.0], 1 M NaCl, 1 mM EDTA, and 1× protease inhibitor cocktail (Pierce). Cells were sonicated and then centrifuged at 16,000g for 20 min to clarify the supernatant. The supernatant was collected and passed through a column harboring 5 mL of amylose resin (NEB). The column was then washed with the sonication buffer followed by the same buffer containing decreasing concentrations of NaCl (750, 500, 250, and 100 mM). Protein elution was performed with elution buffer containing 50 mM Tris-HCl [pH 8.0] and 30 mM maltose. The eluted protein was passed through an SP Sepharose (Sigma) column. The column was washed with 10 column volumes of 50 mM Tris-HCl [pH 8.0] and eluted with elution buffer containing 50 mM Tris-HCl and 1 M NaCl. The eluted protein was concentrated using centricon protein concentrator (Millipore) and dialyzed in a buffer containing 50 mM Tris-HCl, 100 mM NaCl, and 1 mM EDTA.

2.3. Separation of TcpB from MBP by Genenase I digestion

One hundred micrograms of MBP-TcpB fusion protein was incubated with 1 µg of Genenase I (NEB) at room temperature for 8 h. To separate the digested products, ion exchange chromatography was employed. The digested sample was diluted with column buffer (50 mM Tris-HCl [pH 8] and 1 mM EDTA) and applied to a column containing 3 mL SP Sepharose (Sigma). The column was then washed with 10 column volumes of buffer. The bound protein was eluted with column buffer containing increasing concentrations of NaCl. The fractions were collected and analyzed by SDS-PAGE. TcpB containing fractions were pooled and concentrated. Purified TcpB was then dialyzed against the buffer containing Tris-HCl [pH 8.0], 100 mM NaCl, and 50% glycerol and stored in -20 freezer as aliquots.

2.4. Analysis of PIP binding property of purified MBP-TcpB fusion protein

Phosphoinositide phosphate (PIP) binding of refolded MBP-TcpB fusion protein was performed as described [11]. PIP strips

(Echelon) were blocked with blocking buffer (10 mM Tris [pH 8.0], 150 mM NaCl, 0.1% Tween 20, and 0.1% ovalbumin) for 1 h and then probed with 500 ng of purified MBP-TcpB fusion protein for 3 h at 25 °C in the presence of anti-MBP monoclonal antibody conjugated with HRP (NEB). The bound protein was detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce). To perform liposome pull-down assays, 20 µl of 1 mM PolyPIPosomes (Echelon) were mixed with 1 µg of MBP-TcpB fusion protein in binding buffer (50 mM Tris [pH 8.0], 150 mM NaCl, and 0.05% Nonidet P-40) and rotated for 30 min at 25 °C. The liposomes were washed three times with the binding buffer and analyzed by SDS-PAGE. The bound protein was detected by Western blotting.

2.5. Analysis of cell permeable property of MBP-TcpB

RAW 264.7 cells (1×10^5) were seeded into 12-well plates and allowed to adhere overnight. The medium (RPMI) was then replaced with fresh medium (0.5 mL) and increasing concentrations (5, 10, 30, and 50 µg/mL) of purified MBP-TcpB or MBP alone were added. The plates were incubated for 8 h at 37 °C. The culture medium was then aspirated out and the wells were washed five times with PBS. Cells were then treated with 1× trypsin-EDTA for 5 min. Adhered cells were scraped off and the cell suspension was transferred to 1 mL tubes and centrifuged to collect the cells. Cells were lysed with M-PER mammalian protein extraction reagent (Pierce). Cell lysates were clarified by centrifugation and mixed with SDS-PAGE sample buffer. A fraction of the samples were resolved on SDS-PAGE gel, and the presence of MBP-TcpB was detected by Western analysis using anti-MBP antibody conjugated with HRP (NEB).

To analyze the inhibition of IκBα degradation, RAW cells were incubated with various concentrations (10, 30, and 50 µg/mL) of purified MBP-TcpB for 8 h. Cells were then induced with 100 ng/mL of *E. coli* lipopolysaccharide (Sigma) overnight. After induction, cells were washed with PBS and lysed with M-PER mammalian protein extraction reagent (Pierce). Clarified lysates were subjected to SDS-PAGE and Western blotting. After blocking with 5% milk the membrane was probed with anti-IκBα (Cell Signaling Technology) and HRP conjugated anti-mouse IgG (Pierce). The membrane was

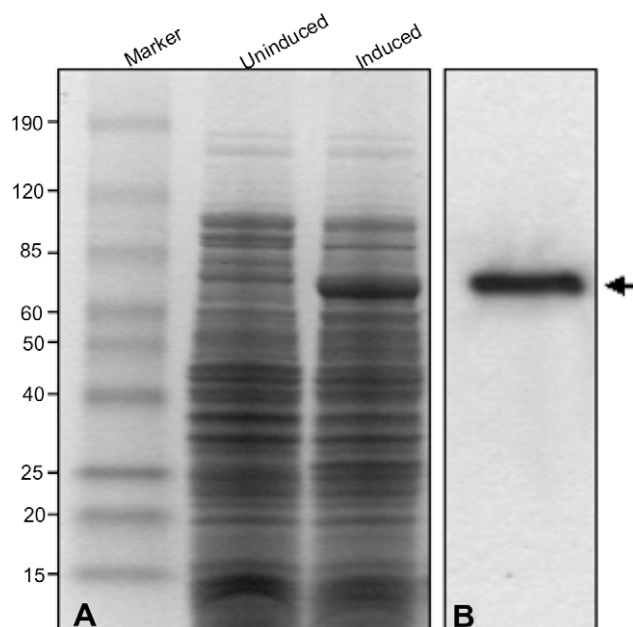


Fig. 1. Over expression of MB-TcpB. (A) Uninduced and induced *E. coli* (BL21) cells harboring pMALTcPB construct and (B) confirmation of the over expressed MBP-TcpB by Western analysis using anti-MBP antibody.

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