Developmental and Comparative Immunology 88 (2018) 94–103

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/devcompimm

An ML protein from the silkworm *Bombyx mori* may function as a key accessory protein for lipopolysaccharide signaling

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ARTICLE INFO

Keywords:
BmEsr16
Lipopolysaccharide
Antibacterial
MD-2
Bombyx mori

ABSTRACT

Lipopolysaccharide (LPS) is a common component of the outermost cell wall in Gram-negative bacteria. In mammals, LPS serves as an endotoxin that can be recognized by a receptor complex of TLR4 (Toll-like receptor 4) and MD-2 (myeloid differentiation-2) and subsequently induce a strong immune response to signal the release of tumor necrosis factor (TNF). In *Drosophila melanogaster*, no receptors for LPS have been identified, and LPS cannot activate immune responses. Here, we report a protein, BmEsr16, which contains an ML (MD-2-related lipid-recognition) domain, may function as an LPS receptor in the silkworm *Bombyx mori*. We showed that antibacterial activity in the hemolymph of *B. mori* larvae was induced by *Escherichia coli*, peptidoglycan (PGN) and LPS and that the expression of antimicrobial peptide genes was also induced by LPS. Furthermore, both the expression of BmEsr16 mRNA in the fat body and the expression of BmEsr16 protein in the hemolymph were induced by LPS. Recombinant BmEsr16 bound to LPS and lipid A, as well as to PGN, lipoteichoic acid, but not to laminarin or mannan. More importantly, LPS-induced immune responses in the hemolymph of *B. mori* larvae were blocked when the endogenous BmEsr16 protein was neutralized by polyclonal antibody specific to BmEsr16. Our results suggest that BmEsr16 may function as a key accessory protein for LPS signaling in *B. mori*.

1. Introduction

Lipopolysaccharide (LPS) is a common component of the cell wall located in the outermost layer of Gram-negative bacteria (Raetz and Whitfield, 2002). In mammals, LPS acts as an endotoxin that induces a strong immune response and triggers the release of tumor necrosis factor (TNF), which can lead to toxic shock (Palsson-McDermott and O'Neill, 2004). Studies in *Drosophila melanogaster* have demonstrated that LPS initiates neither the Toll pathway nor the IMD (immune deficiency) pathway, suggesting that the innate immune system does not recognize LPS (Kaneko et al., 2004). However, in *Bombyx mori*, LPS can induce the expression of the antibacterial peptide genes *attacin* and *cecropin B1*, as well as lysozyme in the body fat (Tanaka et al., 2009), and *attacin*, *lebocin* and *moricin* can be strongly induced by LPS in *Manduca sexta* (Rao and Yu, 2010).

The Toll-like receptor 4 (TLR4) signaling pathway in humans is well known to be activated by LPS. LPS-binding protein (LBP) recognizes LPS and delivers it to CD14, which assists in the translocation of the LPS molecule to myeloid differentiation-2 (MD-2), another important accessory protein in TLR4-LPS signaling (Mancek-Keber and Jerala, 2006; Wright et al., 1990; Yu et al., 1997). This MD-2-LPS complex then binds to TLR4, which has a prototypical organization of N-terminal extracellular leucine-rich repeats (LRRs) and a C-terminal intracellular Toll/interleukin-1 receptor (TIR) domain separated by a single transmembrane-spanning domain (Leulier and Lemaitre, 2008). The intracellular TIR domain forms a BB ring, which is the key site of interaction between TIR domains (Vyncke et al., 2016). This rearrangement of TLR4 contributes to the recruitment of downstream adapter proteins that transduce the signaling to promote the expression of a large number of pro-inflammatory mediators, such as TNF, interleukin-1 (IL-1) and IL-6 (Saitoh et al., 2004; West et al., 2006).

CD14 is a glycoprotein (50 kDa) expressed either on the surface of

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https://doi.org/10.1016/j.dci.2018.07.012
Received 15 June 2018; Received in revised form 11 July 2018; Accepted 12 July 2018

myelomonocytic cells as a glycosylphosphatidylinositol-anchored molecule (membrane CD14, mCD14) or as a soluble molecule (sCD14) in circulation (Miyake et al., 1990). It was shown that CD14 may not be essential for the LPS response but may play a role in its amplification (Miyake, 2004). TLR4 is essential for LPS signaling, and an *in vitro* study indicated that MD-2 is also indispensable for LPS recognition (Shimazu et al., 1999). Proteins of the MD-2-related lipid-recognition (ML) family are widely distributed in plants, animals and fungi. It has been suggested that ML family members may participate in lipid metabolism and host immune responses to pathogenic components such as LPS, as well as to facilitate other cellular functions involved in lipid recognition (Inohara and Nunez, 2002).

The ML family proteins are ~150 residues long and show no homology with non-lipid transfer proteins, and they contain a putative secretion signal peptide and two pairs of conserved cysteine residues (Inohara and Nunez, 2002). It has been reported that MsML-1 in the tobacco hornworm *M. sexta*, a secreted glycoprotein in the larval hemolymph, may function as a key accessory protein for LPS signaling against Gram-negative bacterial infection (Ao et al., 2008). It is therefore necessary to investigate whether ML proteins in other insects also play a critical role in the LPS activation of antimicrobial peptides (AMPs).

Our bioinformatics analysis of the *B. mori* gene database highlighted three ML proteins, BmML-1 (GenBank ID: NP_001040454.1), BmPP (GenBank ID: NP_001037199.1) and BmEsr16 (GenBank ID: NP_001093080.1). Among them, BmEsr16 (*Bombyx mori* ecdysteroid-regulated 16 kDa protein) is a 16 kDa protein regulated by ecdysone. Studies in *M. sexta* show that the expression level of *MsEsr16* is significantly up-regulated during the metamorphosis (Meszaros and Morton, 1996). BmPP (*Bombyx mori* promoting protein) was found in the hemolymph to promote viral replication during nuclear polyhedrosis infection of *B. mori* and it binds to glucan (Kanaya and Kobayashi, 2000; Miyake et al., 2005), but BmML-1 has not been reported so far. In this paper, we showed that the antibacterial activity in *B. mori* larval hemolymph and AMP genes in *B. mori* larvae were activated by *Escherichia coli* and its cell wall components LPS and peptidoglycan (PGN), both mRNA and protein levels of BmEsr16, but not BmML-1, were induced by LPS challenge in the larvae. More recombinant BmEsr16 protein bound to smooth and rough forms of LPS and lipid A than to PGN or lipoteichoic acid (LTA). Importantly, only the LPS-induced but not *E. coli*- or PGN-induced antibacterial activity in the hemolymph and AMP gene expression in *B. mori* larvae were blocked when endogenous BmEsr16 protein was neutralized by a polyclonal antibody specific to BmEsr16, further confirming that BmEsr16 may function as a key accessory protein for LPS signaling in *B. mori*.

2. Materials and methods

2.1. Insects, bacteria and bacterial components

Bombyx mori (Dazao) larvae were obtained from the Institute of Sericulture and Agricultural Products Processing, Guangdong Academy of Agricultural Sciences and were reared on mulberry leaves at 25–28 °C. *Escherichia coli* K12D31 and *Staphylococcus aureus* were maintained in our laboratory, and ultrapure PGN-K12 and LPS-K12 from *E. coli* K12 were purchased from InvivoGen (CA, USA).

2.2. Antibacterial activity assays

On the third day of the fifth instar, each *B. mori* larva was injected with 4 μl of sterilized water, heat-killed *E. coli* K12D31 (10^5 cells/larva), ultrapure PGN-K12 (1 μg/larva), or LPS-K12 (1 μg/larva) (Cheng et al., 2008; Ooi et al., 2002). The fat body and hemolymph were collected at 24 h after injection and stored for subsequent experiments. For antibacterial activity assays, hemolymph was collected on ice, boiled at 100 °C for 5 min, centrifuged at 4 °C for 10 min at 10,000 rpm, and the supernatant was collected and used for the subsequent assays. Each treatment was performed with three replicates.

Bacterial growth curve experiment: *E. coli* K12D31 and *S. aureus* were inoculated on LB solid plates overnight at 37 °C, and a single colony was then inoculated in 3–5 ml of LB liquid medium at 37 °C and incubated overnight at 220 rpm. The cultured bacteria were then inoculated in fresh LB medium at 37 °C and incubated at 250 rpm until the OD_{600} was approximately 0.6. The bacteria from the logarithmic growth phase were added to 96-well plates at 80 μl/well, and the heat-treated hemolymph supernatant from different treatments above was added to the 96-well plates at 20 μl/well to obtain a final volume of 100 μl/well. The 96-well plates were then incubated at 37 °C, and the OD_{570} was measured every hour with the microplate reader.

Inhibition zone experiment: The agarose medium was melted by heating and then cooled to approximately 50 °C. *E. coli* K12D31 and *S. aureus* (OD_{600} = 0.3) were added to the agarose medium at one part in a thousand, mixed well and poured into petri dishes. The medium was perforated at a pore size of ~3 mm on the petri dishes with a punch after being completely solidified. Next, 20 μl of heat-treated hemolymph supernatant sample was added to each well until it had completely diffused within 1 h at room temperature. The petri dishes were incubated overnight in a 37 °C incubator, and finally, the diameter of the inhibition zone was observed.

2.3. RNA extraction and quantitative real-time PCR (RT-qPCR)

The total RNA was isolated from the fat body of day 3 fifth instar *B. mori* larvae 24 h after the injection of bacteria or bacterial components (LPS and PGN) by using TRIzol Reagent (TaKaRa, Dalian, China), and the first strand cDNA was synthesized by using the PrimeScriptTM RT Reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). Quantitative real-time polymerase chain reaction was performed in SsoFast™ EvaGreen® Supermix (Bio-Rad, USA). The 20 μl reaction mixture included 2 μl of cDNA template, 0.8 μl of each primer (10 μM), 10 μl of SYBR®Green Mix, and 6.4 μl of H_2O and was placed in 96-well plates. PCR was performed with the following program: 95 °C for 30 s, then 40 cycles of 95 °C for 5 s and 60 °C for 30 s, followed by a dissociation curve. The Ribosomal protein 49 (*rp49*) gene was used for normalization of the cDNA templates. The primer sequences were BmML-1F: 5′-TGA GTA AGG CGG TCC AGT T -3′ and BmML-1R: 5′-TCC GTC TAC ACC AAT AAA AGG-3’ (for *BmML-1*); Esr16-F: 5′-GTG AGC GGA TGC GAA GAG T-3′ and Esr16-R: 5′-GGG AAC GGT ATC GGG ATG T-3’ (for *BmEsr16*); and rp49-F: 5′-CAG GCG GTT CAA GGG TCA ATA C-3′ and rp49-R: 5′-TGC TGG GCT CTT TCC ACG A-3’ (for *rp49* gene), respectively. The experimental results were recorded and analyzed by a CFX96 quantitative PCR machine. The comparative threshold cycle (CT) method was used to calculate the fold changes in gene expression (Pfaffl, 2001), and 3 independent biological replicates were performed and analyzed by ANOVA.

2.4. Construction of HEK293 cell line stably expressing BmMLs and purification of BmMLs

The cDNA fragment was generated by PCR with the primers 5′-ATG TTT GAA ACG AGC GC-3′ and 5′-AGA AAT CTT AAC AGG TAC-3’ (for *BmML-1*) and with 5′-ATG TTG TTT TTC ATC ACT GC-3′ and 5′-GAC CAG TTT AGC GTT TAT AAG-3’ (for *BmEsr16*). The PCR products were digested and homologously recombined to the NHE I digested lentivirus vector pCPPT-IRES-GFP. The recombinant vectors were used to transfect the HEK293 cells and the cell lines stably expressing BmML-1 and BmEsr16 were obtained by flow cytometer. As the two proteins are secreted proteins, large volumes (100 mL) of cell culture media were collected for purification of BmEsr16 and BmML-1 using ANTI-FLAG®M2 Magnetic Beads (Sigma-Aldrich, MO, USA) following the manufacturer's instructions. Finally, the purified BmML proteins were