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Identification and function of cAMP response element binding protein in Oak silkworm *Antheraea pernyi*

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ABSTRACT

Cyclic AMP response element binding (CREB) proteins participate in the regulation of many biological processes. However, little is known about their role in immune regulation in the Oak silkworm (*Antheraea pernyi*). In this study, a CREB gene was identified in *A. pernyi* and its role in immune regulation was investigated. ApCREB shares conserved signature motifs with other CREB proteins, and includes a typical leucine zipper domain, specific DNA-binding site, nuclear localisation signal (NLS) and cAMP-dependent protein kinase phosphorylation site. Recombinant ApCREB was expressed in *Escherichia coli* and used to raise rabbit anti-ApCREB polyclonal antibodies. *ApCREB* mRNA was detected in all examined tissues, with maximum expression in the midgut and integument. Following exposure to four pathogenic microorganisms (*Beauveria bassiana, Escherichia coli, Micrococcus luteus,* and Antheraea pernyi nuclear polyhedrosis virus), expression of *ApCREB* was up-regulated by *B. bassiana*, *E. coli* and ApNPV, down-regulated by *M. luteus*. RNA interference of *ApCREB* affected mRNA expression levels of antimicrobial peptide genes attacin-1, cecropin B, defensin-1, gloverin, and lebocin-2. These findings demonstrate that *ApCREB* is a CREB homologue that may be involved in innate immunity in *A. pernyi.*

1. Introduction

Cyclic AMP (cAMP) responsive element binding (CREB) protein is one of the best-characterised stimulus-inducible transcription factors belonging to the large family of basic leucine zipper (bZIP) domain proteins (Yin et al., 1994). Typical CREB proteins consist of a kinase-induced domain (KID) and two glutamine-rich domains, which are essential for transcription activity and signal-induced activation, respectively (Steven and Seliger, 2016). CREB proteins can be phosphorylated at Ser 133 on the KID by various kinases including protein kinase A (PKA), protein kinase C (PKC), calmodulin kinases (CaMKs), and pp90 ribosomal protein S6 kinase (pp90 RSK) (Shaywitz and Greenberg, 1999; Yang et al., 2013). Enhanced PKA activity triggers hemolymph antibacterial activity as well as an increase in lysozyme in the fat body of insects infected with exogenous pathogens (Cytrynska et al., 2006). PKC deficiency leads to macrophage activation defects in mice and failure to clear bacterial infections (Tan and Parker, 2003). Antiviral protein kinase R (PKR) inhibits the key actin-modifying protein gelsolin to control fundamental innate immunity and counteract viral entry into cells (Irving et al., 2012). Phosphorylated CREB (pCREB) interacts with its coactivator protein, CREB-binding protein (CBP), and the CREB/CBP complex then recruits transcriptional cofactors at the CRE site to initiate CREB-dependent gene transcription (Gerritsen et al., 1997; Naqvi et al., 2014; Sheppard et al., 1999).

Numerous studies in mammals demonstrated that CREB is involved in multiple biological processes, including NF-κB pathway signalling, macrophage survival, cell proliferation, differentiation, learning, long-term memory, and immune regulation (Mayr and Montminy, 2001; Wen et al., 2010; Yin et al., 1995a). Emerging studies in invertebrates also showed that CREB and CREB variants have many physiological functions (Poels and Broeck, 2004). For example, in mussel hemocytes, CREB participates in immune responses (Canesi et al., 2006), and in honeybee brains, regulation of CREB-dependent genes differs with age (Gehring et al., 2016). In Lymnaea neurons, CREB functions as a key player in behavioural plasticity (Sadamoto et al., 2004). However, little is known about the immune regulation of CREB in insects.

The Oak silkworm (*Antheraea pernyi*) is an important economic Lepidopteran insect, and a better understanding of its immune mechanisms could improve sericulture of this species. In the present study, a CREB homologue from *A. pernyi* was identified, and its expression pattern and activation mechanism were analysed. The results suggest ApCREB might play a role in the immune response to pathogenic microorganisms.

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2. Materials and methods

2.1. Insects and treatments

A. pernyi larvae were reared on fresh Oak leaves under a 12 h:12 h light:dark cycle at 23 ± 1 °C and 60 ± 10% relative humidity (Zhang et al., 2015). At the third day of the fifth instar stage, larvae were divided into five groups, and each group was injected with *Escherichia coli*, *Beauveria bassiana*, *Micrococcus luteus*, or Antheraea pernyi nuclear polyhedrosis virus (ApNPV). Phosphate-buffered saline (PBS) was injected as a control. Fat body tissue was frozen in liquid nitrogen and stored at −80 °C until needed for RNA or protein extraction. All experiments were performed a minimum of three times to ensure reproducibility.

2.2. Quantitative real-time PCR analysis

Larvae at the third day of the fifth instar stage were injected into the body with *E. coli Trans1-T1* (10 μl, 109 cfu/ml), *M. luteus* (10 μl, 0.5 mg/ml), *B. bassiana* (blastopores, 10 μl, 109 cfu/ml) or ApNPV (10 μl, 109 PIBs/ml). Expression profiles of *ApCREB* in the fat body were determined at 1.5, 3, 6, 12, 24, and 48 h after microorganism challenge using quantitative RT-PCR. *ApCREB* expression levels after PBS injection were used as controls. Larvae were dissected, samples removed, total RNA was extracted using TRIzol reagent (Takara, Dalian, China), and reverse-transcribed into cDNA using PrimeScript RT Master Mix (Takara, Dalian, China) according to the manufacturer's instructions. Generated cDNA was diluted to a final concentration of 150 ng/μl for use as template. A total volume of 20 μl, containing 10 μl 2× SYBR Premix Ex *Taq*II (Tli RNase Plus; Takara), 7 μl RNase-free H_2O, 1 μl forward primer, 1 μl reverse primer (0.5 μM), and 1 μl cDNA template. Quantitative RT-PCR was performed using a CFX96TM real-time detection system (Bio-Rad, California, USA) with the following procedure: initial denaturation at 94 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 58 °C for 15 s, and 72 °C for 10 s. Levels of ApCREB mRNA were calculated relative to the *A. pernyi* 18SrDNA gene, according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Primers for qRT-PCR (Table 1) were designed with the Primer3 online tool (http://primer3.ut.ee/), and data are presented as the mean ± SD. Differences were considered statistically significant when *p*-values were less than .05.

2.3. Western blot analysis

Total protein from fat body tissue challenged with pathogenic microorganisms was extracted using RIPA lysis buffer (Aidlab Biotech, Beijing, China) supplemented with 1 mM phenylmethanesulfonyl fluoride (PMSF). Protein was collected by centrifuging the lysate at 12,000*g* for 10 min at 4 °C, and the concentration was determined using an EasyII Protein Quantitative Kit (TransGen, Beijing, China). Equal amounts of protein (30 μg) were subjected to 12% SDS-PAGE and transferred to methanol-activated polyvinylidene fluoride (PVDF) membranes (Millipore, Massachusetts, USA) which were blocked in 5% non-fat milk in TBST buffer (20 mM Tris-HCl, 150 mM Nacl, 0.05% (V/V) Tween 20) overnight at 4 °C, then incubated with primary antibody against CREB (1:3000, diluted with 3% non-fat milk in TBST) for 2 h at room temperature. Peroxidase-conjugated goat anti-rabbit IgG (Biosharp, Hefei, Anhui) was used as a secondary antibody at a 1:4000 dilution for 1 h at RT. Bands were detected with a HRP-DAB Detection Kit (Tiangen, Beijing, China). β-actin was used as an internal control.

2.4. Expression and purification of recombinant ApCREB and antibody preparation

Total RNA was extracted from fat body tissue of *A. pernyi* pupae using TRIzol reagent (Takara), then reverse-transcribed into first-strand cDNA using a HiFiScript 1st Strand cDNA Synthesis Kit (CWBIO, Beijing, China) following the manufacturer's instructions. Sequences of CREB from various animal species were aligned by Clustal W (http://www.ebi.ac.uk/Tools/ClustalW). Specific primers ApCREB-F and ApCREB-R (Table 1) designed with Primer premier 5.0 software and used for amplification of the ApCREB open reading frame (ORF). PCR amplification was carried out as follows: 4 min at 94 °C, followed by 38 cycles of 94 °C for 30 s, 58 °C for 40 s, 72 °C for 1 min, and a final elongation step of 72 °C for 10 min. Generated PCR products were analysed on 1% agarose gels and purified using a DNA Gel Extraction Kit (Axygen, Hangzhou, China). Purified PCR products were cloned into the pMD19-T vector and sequenced. The insert was then subcloned into the pET28a(+) vector using *Eco*RI and *Bam*HI restriction sites. *E. coli* Transetta (DE3) competent cells (TransGen) were used for plasmid transformation. Positive clones were further analysed by restriction enzyme digestion and PCR. Bacteria were grown until the absorbance at 600 nm (OD_{600}) reached 0.8, expression was induced with isopropyl-β-d-thiogalactoside (IPTG, 1 mM), and culturing continued at 37 °C for 4 h. Recombinant fusion protein was purified using a Ni-NTA agarose column (QIAGEN, China) following the manufacturer's protocol. Protein concentration was measured using an EasyII Protein Quantitative Kit (TransGen). Purified protein was sent to Hua'an Company (Hangzhou, China) for the preparation of ApCREB polyclonal antibodies.

Table 1
Primers used in this study.

Primers	Sequence (5′–3′)	Purpose
ApCREB-F	CGCGGATCCGCGATGGAGGGCTACTTCGACATCTCCA	RT-PCR
ApCREB-R	CCGGAATTCCGGTCACTTACGTGCGCCGCGC	RT-PCR
qApCREB-F	CCGCTCAAAGACCTCGACTA	qRT-PCR
qApCREB-R	CCTGAAGTACCGGAGCTTGA	qRT-PCR
dsApCREB-F	GGATCCTAATACGACTCACTATAGGCTTGTCCACCGTCACCCA	RNAi
dsApCREB-R	GGATCCTAATACGACTCACTATAGGAAATTGCCCGTCGAACCT	RNAi
Attacin-1-F	GGGTGGGAAACTGAAT	qRT-PCR
Attacin-1-R	CCAAGAGGTCCTAAAGTG	qRT-PCR
Cecropin B-F	TGCCTTCGTCAACAGT	qRT-PCR
Cecropin B-R	GCTTTGGCTTCTCCTA	qRT-PCR
Defensin-1-F	CGACGACTCATTTCAT	qRT-PCR
Defensin-1-R	GGTACGGAGTGTTTCTA	qRT-PCR
Lebocin-2-F	GTGTTCACCCGGAGCATC	qRT-PCR
Lebocin-2-R	AGTTATAGGCGTAAATTGGA	qRT-PCR
Gloverin-F	GACGGGTTATTCGGTAAA	qRT-PCR
Gloverin-R	GAGATCAAGAGCGGCATT	qRT-PCR

Note: Enzyme restriction sites are underlined.